Carolin Rauter

Detection of Borrelia infection in ticks and humans

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Carolin Rauter

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Abbreviations

ACA	acrodermatitis chronica athrophicans
B.b.	*Borrelia burgdorferi*
bp	base pair
BSK	Barbour-Stoenner-Kelly
CDC	Center of Disease Control
cp	circular plasmid
CSF	cerebrospinal fluid
ct	threshold cycle number
DFM	dark field microscopy
ELISA	enzyme-linked immunosorbent assay
EM	Erythema migrans
FRET	fluorescence resonance energy transfer
GS	Giemsa-stained smears
HybProbe	hybridization probe
I.	Ixodes
IB	immunoblotting
IFA	immunofluorescence assay
LB	Lyme Borreliosis
lp	linear plasmid
n.g.	not given
n.t.	not typable
osp	outer surface protein
PCM	phase contrast microscopy
PCR	polymerase chain reaction
RFLP	reverse fragment length polymorphism
RLB	reverse line blotting
rRNA	ribosomal RNA
RT	room temperature
s.c.	subcutaneous
s.l.	sensu lato
s.s.	sensu stricto
TBEV	tick-born encephalitis virus
TE	Tris/EDTA
T_m	melting temperature
vs	versus

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1 Introduction

Lyme Borreliosis (LB) was first described in the mid-seventies [218]. Since then, this disease has become recognized as the most frequent tick-borne infectious disease in the northern hemisphere. In endemic regions in southern Germany the incidence is about 50 – 600 per 100,000 inhabitants per year [14, 80] with a seroprevalence of up to 52% of highly exposed individuals [161].

1.1 Historical notes

The characteristic skin lesion – Erythema migrans (EM) – was already described at the beginning of the 20th century by Afzelius [2] and Lipschütz [135]. In the same period, the so called Bannwarth syndrome (early lymphocytic meningoradiculitis) and Acrodermatits chronica athrophicans (ACA, a chronic skin disease) were also known [5]. However, the association between these symptoms and a previous tick bite was only realized many years later.

In 1975, several cases of rheumatoid arthritis among children were reported in Lyme, Connecticut, USA. This resulted in an epidemiological and clinical investigation which led to the identification of a new disease entity called "Lyme disease" [218]. Soon this condition was suspected to be caused by an infectious agent transmitted by ticks.

The etiological agent was identified in 1981, when spirochetes were isolated and cultured from the midgut of *Ixodes dammini* ticks. These spirochetes were found to belong to the genus of Borrelia. In honour of Willy Burgdorfer, who first discovered the spirochete, the name *Borrelia burgdorferi* was proposed in 1984.

1.2 Borrelia burgdorferi - the pathogen

Borrelia burgdorferi sensu lato (*B. burgdorferi* s.l.), the causative agent of Lyme Borreliosis, is a motile, corkscrew-shaped, Gram-negative bacterium, with a length of 10 to 30 µm and a thickness of 0.2 to 0.3 µm. It is a slowly growing, fastidious, micro-aerophilic organism with a generation time of 8 to 12 hours and with an optimal growing temperature of 30 to 34°C in a complex medium (Barbour-Stoenner-Kelly medium) [7, 8, 105].

The structure of *B. burgdorferi* s.l. is similar to that of all spirochetes: a protoplasmic cylinder covered by a cell membrane is surrounded by 7 to 11 periplasmatic flagellae which are in turn covered by an loosely associated outer membrane [8, 105]. The main structural component of the flagellae is flagellin, a 41 kDa protein. The outer membrane of *B. burgdorferi* s.l. contains an abundance of outer surface proteins (Osp A – F) [125, 160, 194].

The genome of *B. burgdorferi* s.l. (strain B31) has been completely sequenced some years ago [57]. It is small (about 1.5 megabases) and consists of an unusual linear chromosome of 950 kilobases as well as 9 circular (varying from 9 to 32 kbp) and 12 linear (varying from 5 to 55 kbp) plasmids that constitute 40% of its DNA [26, 57]. The linear plasmids of *B. burgdorferi* s.l. are double-stranded linear DNA molecules that have terminal hairpin loops and short inverted terminal repeats. The organism shows a low G+C content of about 30% [57].

The ribosomal RNA (rRNA) genes of *B. burgdorferi* s.l. have an unique organization with a single 16S rRNA gene and two copies of the 23S and 5S rRNA genes. The two latter genes are tightly linked and tandemly dublicated [59, 207]. rRNA and flagellin genes are located on the chromosome.

Borrelia are not capable doing *de novo* synthesis of amino acids, lipids, nucleotides and enzyme co-factors. It is therefore assumed, that they must obtain these essential molecules from their arthropod or vertebrate hosts [25]. Despite being a Gram-negative bacterium, *B. burgdorferi* s.l. contains no enzymes necessary for the production of lipopolysaccharide. Instead the genome has approximately 130 genes coding for lipoproteins. A unique feature of Borrelia species is, that the genes encoding the major outer surface proteins (Osp) are located mainly on plasmids. The ospC gene is localized on a 26 to 28 kbp circular plasmid (cp26) and the ospA/B genes on a 49 to 55 kbp linear plasmid (lp54). The role of Osps during infection is not conclusively known, but as different osp genes are expressed at different time points during transmission, they seem to help the spirochete to survive in the extremly different mammalian and arthropod environments. For example in the gut of ticks, spirochetes abundantly synthesize OspA, but when ticks engorge, *B. burgdorferi* s.l. begin to express OspC and down-regulate OspA [53, 54, 183, 206]. Many other genes of *B. burgdorferi* s.l. are known to be differently expressed within ticks, during the process of engorgement and in the host [183], e.g., bbk32. BBK32 is a fibronectin-binding protein and seems to play an important role in the attachment of spirochetes to the extracellular matrix. In addition it functions as an antigen for the immune system of the host, similarly as OspC.

Based on their phylogenetic relationship of DNA, *B. burgdorferi* s.l. can be divided into at least 11 different subspecies: *B. burgdorferi* sensu stricto (s.s.), present in Europe and in the USA but rare in Russia and apparently absent from Asia; *B. garinii, B. afzelii*, *B. valaisiana* and *B. lusitaniae* in Eurasia [6, 18, 234, 237]; *B. japonica*, *B. tanukii* and *B. turdae* restricted to Japan, *B. sinica* to China, *B. andersonii* is found in the USA and *B. bissettii* mainly in the USA [48, 176, 241]. Among these, *B. burgdorferi* s.s., *B. garinii*, *B. afzelii* are known to be pathogenic for humans. For *B. valaisiana*, *B. lusitaniae* and *B. bissettii*, the pathogenicity is not entirely clear. *B. valaisiana* and recently also *B. lusitaniae* has been detected by PCR in skin biopsies of a few patients with EM and ACA [34, 188, 189]. The other genospecies named above have not been isolated from human cases of Lyme disease and are only known from isolates obtained from ticks or animals.

1.3 Ticks

All ticks are obligate blood sucking parasites of vertebrates. Their classification originates from the publications of Carl von Linné in 1746. Ticks are arthropods belonging, together with spiders and scorpions, to the class of Arachnida. Together with mites, they belong to the suborder of Acari. There are two major families of ticks: the soft or argasid ticks (family of Argasidae) and the hard or ixodid ticks (family of Ixodidae). The soft ticks are mainly found in warm climates. The hard ticks are more widely distributed and can be found even in subarctic areas.
Ticks have three basic life stages: the larval, nymphal and adult (male and female) stage (Fig. 1). During each stage, a blood meal is required for completing the life cycle. Ticks lack a distinct head. Instead they have mouthparts composed of sharp, paired chelicerae, acting as powerful cutting organs to penetrate the skin of the host and a midline, barbed hypostome for firm attachment to the host as well as for blood sucking.

FIG. 1. The four stages of unfed *I. ricinus*, in proportion to a pinhead. Clockwise: larva, nymph, male and female.

1.4 Ixodes ricinus

The most common tick in Europe is the hard tick *Ixodes ricinus* (*I. ricinus*). Typically, ixodid ticks have a 3-host life cycle (Fig. 2), with each feeding stage of the tick having a single host [166]. The development from egg to adult is usually completed within 2 to 3 years, but it may take up to 6 years, depending on environmental conditions, including temperature, relative humidity and photoperiod and access to suitable vertebrate hosts.

I. ricinus spends most of its life on the ground or among the vegetation. After the final blood meal, the adult females drop off the host. In the subsequent month they lay up to 2000 eggs and die. After hatching from the egg, the six-legged larvae, measuring about 0.5 mm, take their first blood meal usually on a mouse or vole. After 2 to 3 days feeding during which they increase their weight 10 to 20 times, they drop off into the vegetation and moult to 8-legged nymphs (1.5 to 2 mm in size). The nymph usually feeds in the following year for 4 to 5 days on a larger animal such as a bird or squirrel and finally develops into an adult tick (imago). Only a small proportion of all eggs succeed to complete the whole life cycle and reach the adult stage [180].

An adult female tick is 3 to 4 mm in size when unfed, while a male tick is 2 to 3 mm long. Copulation usually takes place on a host prior to the final blood meal. A male tick can fertilize several females and may take sporadic small meals but dies shortly afterwards. Adult female ticks parasitize large animals such as deers or livestock on which they feed for about 7 days. During this time they may increase their body weight up to 120fold. *I. ricinus* ticks in all stages are able to hibernate in the top layer of the soil or under leaf litter. They become active when the soil temperature rises to 5 to 7°C. *I. ricinus* ticks are usually seasonally active, seeking their hosts when environmental conditions are most suitable. In general, activity of questing ticks will begin in spring and last several months. In most areas a second, less intense phase of questing activity occurs in late summer or autumn [91, 149, 236].

As questing *I. ricinus* ticks require high humidity (above 80%) they are especially common in grass- and woodlands. The need for questing ticks to maintain a stable water balance is an important factor in determining the location and duration of activity. Host-seeking larvae quest in the leaf litter and very near the ground (up to 10 cm), nymphs are often found on grasses in a height up to 50 cm above the ground and adults quest mostly in positions up to 80 cm or even higher [94, 148]. Ticks show only little horizontal movement (up to 1 m for adults). Distribution of ticks over long distances occurs by means of hosts, mainly of birds during their northern and southern migration in the spring and fall, respectively [164].

I. ricinus are known to parasitize on more than 300 different animal species, including mammals and birds [60]. Larvae and nymphs can feed on almost any warm-blooded animal (as well as some reptiles) including large animals, but they tend to make most contact with hosts that move within the vegetation cover, while adults will only feed successfully on larger animals such as deer, sheep, cows and dogs. Men are bitten by all three stages, but nymphs are the most commonly involved [190].

Before feeding, a tick may wander around on its host for several hours. It inserts only its hypostome into the skin. Various substances produced by the salivary glands enter the host during this penetration, creating a feeding pool. During the first 24 to 36 hours of attachment, there is no or only little ingestion of blood. Ixodid ticks feed for long periods, and 2 to 15 days are required for a complete blood meal to be ingested, depending on the feeding stage, the type of host, and the site of attachment [166].

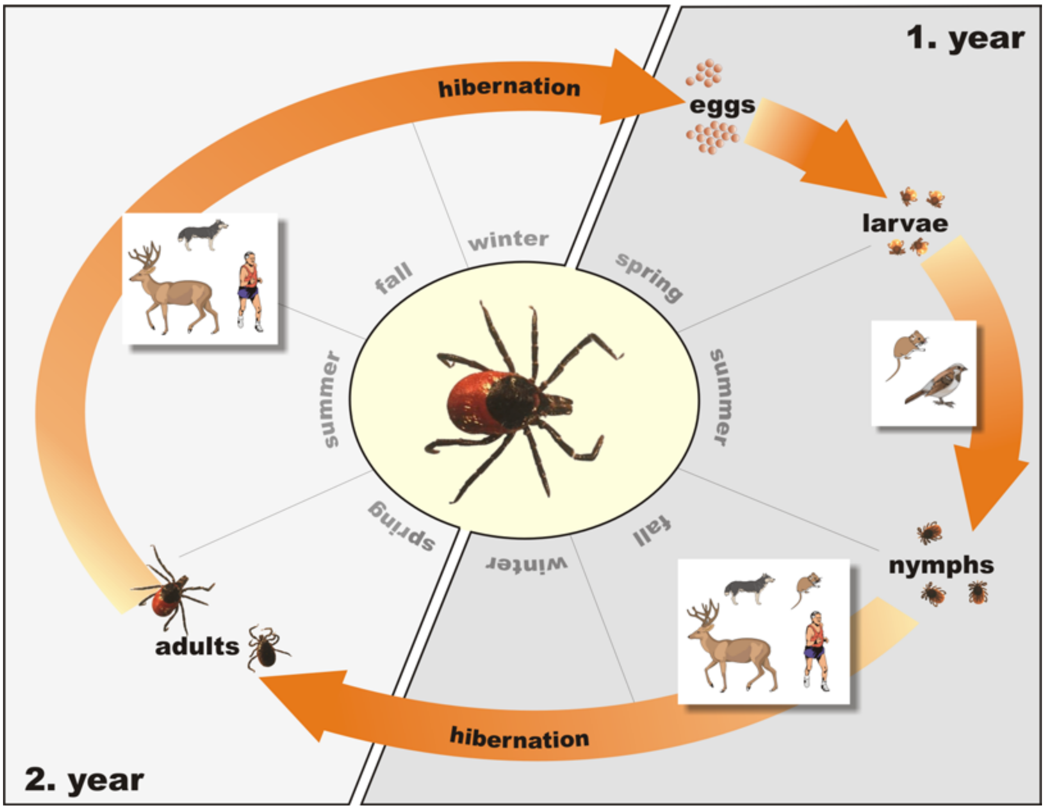

FIG. 2. Live cycle of *I. ricinus*.

1.5 Borrelia in ticks and hosts

Ticks may act not only as vectors, but also as reservoirs of tick-transmitted bacteria. The most important human pathogens transmitted by *I. ricinus* are *B. burgdorferi* s.l., tick-borne encephalitis virus (TBEV), *Rickettsia spp.*, *Ehrlichia spp.*, *Bartonella spp.* and *Babesia spp.*, among them *B. burgdorferi* s.l. is the most frequent. *I. ricinus* ticks acquire *B. burgdorferi* s.l. infection during a blood meal on a reservoir host, i.e., host species that maintain the Borrelia infection for a long period of time even during nontransmission periods [60]. Once infected with *B. burgdorferi*, *I. ricinus* transmit the infection to the next stage, a process known as transstadial transmission (e.g. transmission from larva to nymph). Larvae are rarely infected (< 5%) so it is evident that transovarial transmission is uncommon [85, 141, 146]. The mean infection rate of nymphs and adults in Europe is 10% and 20%, respectively [85].
To examine the reservoir status of a host, different procedures are currently used: (i) xenodiagnosis, i.e., the infestation of hosts by uninfected ticks in the laboratory and the subsequent demonstration of the pathogen in the ticks [37, 96], (ii) determination of the infection

status of engorged larval ticks collected from wild animals [78, 79, 123, 164], or (iii) detection of infection in unfed host-seeking ticks collected in nature combined with a test, which enables the identification of the host species that unfed ticks have fed on in their previous stage [116].

I. ricinus parasitize a large number of different species of animals among them many serve as reservoirs for *B. burgdorferi* s.l.. The majority of these are rodents, the most important probably being mice, voles and squirrels. Several insectivores are also involved including shrews and hedgehogs. Recently it became apparent that many birds play an important role as reservoir hosts for *B. burgdorferi* s.l. [60]. It is now known that e.g. pheasants and blackbirds are reservoir competent. Larger animals, such as deer, sheep, cattle or horses seem to be incompetent as reservoir hosts [100]. However (i) they are necessary for the maintenance of the tick population, since adult ticks mainly engorge successfully on large hosts [227], (ii) copulation takes place on these animals, and (iii) the skin of those hosts can serve as an interface for cofeeding transmission, i.e., the transmission of spirochetes from infected to uninfected ticks feeding together on a host [63].

In unfed ticks, the spirochetes stay mainly in the midgut, however, in a proportion of infected ticks systemic infections affecting different organs may occur [131]. In about 1% of infected female ticks the systemic infection reaches the ovaries, leading to a transovarial transmission of spirochetes to the eggs. If an infected tick is attached to the host, the spirochetes in the midgut of the tick migrate through the midgut wall, reach the salivary glands and are inoculated with the tick saliva into the host 2 to 3 days after attachment [111, 131]. Once in the host's skin, spirochetes may remain at the inoculation site for a few days until they disseminate.

1.6 Transmission of Borrelia to patients

Humans accidentally enter the life cycle of competent tick vectors and vertebrate reservoirs, and represent dead-end hosts for the pathogen. The risk of infection in a given area depends largely (i) on the density of ticks, (ii) on their feeding habits, and (ii) the abundance of reservoir hosts. In areas with sufficient humidity at the base of the vegetation unfed ticks can survive for several weeks. Therefore, disease transmission can occur at any time during the warmer months of the year. Nevertheless, most early manifestations of LB, such as EM and acute neuroborreliosis occur in mid- to late summer, which can be explained by the higher occurrence of ticks on vegetation, higher leisure activity of people and the incubation time of the disease. Late manifestations of LB do not show any typical seasonal prevalence pattern. The tick stage responsible for most cases of Borrelia infection seems to be the nymph, since nymphs are about 10fold more numerous than adults [180] and are much smaller than adults, so they are often overlooked when attached.

Ixodid ticks need up to 24 to 48 hours to transmit Borrelia to humans and animals [111]. This is probably because the migration of the spirochete from midgut to salivary glands usually takes 2 to 3 days. However, since a small proportion of unfed ticks already carry spirochetes in their salivary glands, in some cases a much earlier transmission may occur.

1.7 Associations of Borrelia genospecies with ticks and hosts

A variety of methods allowing a differentiation of *B. burgdorferi* s.l. into genomic groups have been described. The most common methods are those which use PCR to amplify species-specific DNA sequences, since isolation of the organism is not necessary: PCR with genomic group-specific primers [39, 133], analysis of the PCR product by DNA sequencing [46], restriction fragment length polymorphism (RFLP) [137, 181], or hybridization to species-specific probes (reverse line blotting, RLB) [187]. More recently melting point analysis of the PCR product was used to distinguish between the different genospecies [154, 174, 182].

In Europe studies of the occurrence of Borrelia species in hosts show clearly a differential infection pattern: *B. afzelii* is mainly associated with small rodents [78], *B. afzelii* and *B. burgdorferi* s.s. with squirrels and *B. garinii* and *B. valaisiana* with birds [79, 121, 123, 164]. The specific mechanisms underlying differential transmission of *B. burgdorferi* s.l. may be explained by different effects of the complement system in the host serum on each genospecies of *B. burgdorferi* s.l. as it was shown recently *in vitro* [124]. This effect may occur as well in the host, which will not accept all Borrelia species, as in the tick which fed on an inadequate host by complement taken up during the blood meal.

A small proportion of ticks is infected simultaneously by several genospecies of *B. burgdorferi* s.l. [117, 120, 161, 182]. The presence of multiple genomic groups in *I. ricinus* may be explained in several ways: (i) Transovarial transmission of Borrelia in ticks. Therefore, larvae may already be infected with one or more genomic groups of *B. burgdoferi* s.l.. (ii) Blood meals are taken from various hosts during the tick life cycle. (iii) The tick may parasitize a competent reservoir host, which is infected with several genomic groups. (iv) Cofeeding transmission of various spirochetes may occur between *I. ricinus* ticks which feed on the same host and are infected by different *B. burgdorferi* s.l. species, allowing exchange of spirochetes among ticks [63].

Beside taxonomical, ecological and epidemiological considerations there are also clinical reasons indicating the need for *B. burgdorferi* s.l. differentiation. There is considerably evidence resulting from isolation from patients biopsies, PCR and serological data that the different clinical manifestations are caused by distinct species of *B. burgdorferi* s.l. [193, 228]. ACA was mostly associated with *B. afzelii* [24, 163, 192] and neuroborreliosis is frequently caused by *B. garinii* [39]. *B. burgdorferi* s.s. is most often associated with Lyme arthritis [4], however, all three species have been deteceted in synovial fluid samples from patients with Lyme arthritis [230]. Overlap between species in relation to clinical manifestations occurs [192] and all cause the pathognomic symptom Erythema migrans (EM) [163, 173]. However, there is evidence that in Europe this early sign occurs more frequently in *B. afzelii* infections than in those caused by *B. garinii* [192]. In European neuroborreliosis patients and patients with an EM or ACA, mixed infections of 2 or 3 different species have been detected [39, 188]. The clinical significance of human infection caused by more than one species of Borrelia remains to be investigated.

B. afzelii, *B. garinii* and *B. valaisiana* are the most abundant genospecies in central Europe [120]. At present there are not many data available about the distribution of the different genospecies within Europe. *B. burgdorferi* s.s. seems to be relatively rare in any European region. One possible explanation could be a relatively recent introduction of *B. burgdorferi* s.s. into Europe.

1.8 Lyme Borreliosis

LB is a multisystemic disorder that can affect several organ systems, primarily skin, joints, nervous system and heart. The disease has been divided into three stages: the early localized, the early disseminated and the late chronic stage (for review see [113, 216]). The intervals between these stages can vary considerably in their duration. Furthermore, some patients present late manifestations without having experienced, or noticed early stage symptoms. LB is not fatal, however the damages which occur during an infection are often irreversible and can be disabling.

1.8.1 Stage I: localized infection

The most common and typical manifestation of early localized LB is Erythema migrans (EM), appearing within days or weeks after the tick bite as an expanding erythematious macule or papule, often with central clearing. The cause for the rash is the infiltraton of plasma and immune cells, which are recruited to attack the pathogen migrating through the skin [195]. It is recognized in about 60% of the patients [136] and may be accompanied by systemic clinical features such as fever, malaise, headache, myalgia, or arthralgia.

Borrelia lymphocytoma, an uncommon form of early localized LB, is a painless intense bluish-red nodule that typically appears on the earlobe (especially in children), nipple or scrotum. Microscopic examination shows a very dense infiltrate of lymphocytes.

1.8.2 Stage II: disseminated infection

Stage two is characterized by a bacteremia, i.e., the systemic dissemination of the pathogen. Few weeks to over a year after the initial infection the spirochetes can spread through the bloodstream and lymphatic system to multiple tissues, including heart, nervous and musculoskeletal system or other parts of the skin, resulting in a wide variation of clinical features.

Clinical manifestations of early disseminated LB may include multiple EM or systemic symptoms such as myalgia, arthralgia, headache, and fatigue. Although rare, carditis may occur at this stage. It is usually manifested by partial atrioventricular block. Early neurological symptoms of LB also occur within weeks after the infection. Neuroborreliosis is the most frequent manifestation of stage II, particularly in its appearance as lymphocytic meningoradiculitis, which usually reveals typical clinical symptoms as the radicular pain syndrome and/or cranial nerve palsy. Other early neurological symptoms include lymphocytic meningitis, mild encephaltits and mononeuritis multiplex.

1.8.3 Stage III: chronic infection

If left untreated, the initial Borrelia infection may progress after several years to a chronic infection possibly involving the joints (Lyme arthritis), skin (ACA) or, rarely, result in chronic neurological symptoms. Lyme arthritis typically shows intermittent attacks of monoarticular or oligoarticular arthritis of the large joints, particularly the knee. It usually disappears after several years, but may leave some joint damage.

Chronic neurological symptoms such as Lyme encephalopathy can occur after months or years of latent infection but are rare events. Parapareses and tetrapareses are the most common symptoms, probably caused by direct infection of the nervous system. Acrodermatitis chronica atrophicans, probably the most common manifestation of chronic LB in Europe is a chronic, slowly progressive fibrosing skin lesion in which the skin develop a thin shiny, papery appearance. It usually occurs in the lower limbs of elderly people. *B. burgdorferi* s.l. may persist in the host for many years. Live spirochetes have been cultured from an ACA lesion as long as 10 years after the onset of disease [3].

1.9 Diagnosis

Diagnosis of LB is usually based on the recognition of a characteristic clinical picture, together with a history of tick exposure in areas where LB is endemic, while serological tests confirm the diagnosis. The diagnosis of EM in locations endemic for LB is purely clinical because under these circumstances, laboratory testing is neither necessary nor recommended.

At present diagnosis of LB still represents a major problem since the commercially available and in-house tests still vary considerably in their specificity and sensitivity and therefore do not meet the desirable standardized performance. False-positive tests, resulting for instance from cross-reactivity, lead to misdiagnosis and inappropriate treatment. False-negative results, originating from the lack of sensitivity, have more serious effects for the patients since the disease might develop into a chronic stage, which is more difficult to treat. Although clinical manifestations of the illness are variable and rarely exclusive for *B. burgdorferi* infection, diagnosis must be made in the light of careful evaluation of the patient's clinical history, physical findings, laboratory evidence and exposure risk evaluation. On the other hand, infection with *B. burgdorferi* should not be excluded if awareness or recollection of a tick bite are not present as this is not always the case.

1.9.1 Serological methods

The development of an antibody response to *B. burgdorferi* s.l. infection in untreated patients is well described. IgM antibodies against *B. burgdorferi* s.l. first become detectable 3 to 4 weeks after the infection, peak after 6 to 8 weeks, and usually decline subsequently. Therefore serodiagnostic tests are insensitive during the first weeks of infection. IgG antibodies appear 6 to 8 weeks after the infection and remain detectable for many years. It is important to note that both IgG and IgM responses may persist for years even after successful antibiotic treatment [51]. Therefore serology is insufficient to measure the success of treatment.

A two-step serological approach is currently recommended when LB is suspected: a positive enzyme-linked immunosorbent assay (ELISA) or an indirect immunofluorescence assay (IFA) is followed by an immunoblot (Western blot) to confirm the first result. The immunoblot is a suitable procedure to distinguish differentiated immune responses, but the interpretation of the number and intensity of bands must still be standardized. If the Western blot is negative, the reactive ELISA very probably was a false-positive result, which can be caused by cross-reactive bacterial antigens such as heat-shock proteins or with treponema in syphilis. In the course of herpes virus infections (especially Epstein-Barr virus infections) a polyclonal activation of B cells may lead to the production of Borrelia-reactive antibodies especially of the IgM type.

ELISAs of first generation are based on sonicated antigens. Second or third generation tests use either semi-purified antigens, one or several purified antigens, recombinant antigens (e.g., VlsE) or synthetic peptides (e.g., C6 peptide derived from VlsE) and should be preferred to first generation tests because of a higher degree of specificity.

As a confirmatory assay the immunoblot should have high specificity. Depending on the species used as source of antigen the interpretation criteria of an immunoblot vary considerably [81]. Due to the different Borrelia strains occuring in Europe, the serological response in European

LB patients differs from that in their American counterparts. Consequently, in Europe it is not possible to use the immunoblot criteria recommended by the Center of Disease Control (CDC) in the United States. First efforts have been made to define a standardization of immunoblotting protocols in Europe, too [239].

1.9.2 Microbiological detection methods

The culture of *B. burgdorferi* s.l. from patients material permits a definitive diagnosis of LB and allows the conclusion that the infection is current. However, it is insensitive, time consuming and difficult to perform and therefore rarely attempted for diagnostic purposes. Although culture of skin biopsy specimens from patients with early EM and biopsies from ACA patients may be useful [172], *B. burgdorferi* s.l. is rarely cultivated from either cerebrospinal fluid (CSF) samples or synovial fluid.

1.9.3 Molecular detection methods

Although there can be up to 40,000 spirochetes per ml of synovial fluid [204] and up to 10,000 organisms were found in a 2-mm biopsy of a patient with EM [138], the number of Borrelia genomes in human body fluids seems to be generally low (< 50 organisms per ml), especially in late, chronic disease stages [65, 67]. Methods using the amplification of DNA by PCR, are able to identify the small numbers of *B. burgdorferi* s.l. that may be present in various specimens. These assays have been applied to samples derived from multiple sources, including skin, blood, plasma, serum, synovial fluid and tissue, CSF, and urine [66, 67, 129, 138, 204]. For optimizing the amount of *B. burgdorferi* s.l. DNA, different procedures have been developed, depending on the specimen taken and a variety of gene targets for amplification have been tested, such as plasmid targets (e.g. ospA, ospC, vlsE) or chromosomal targets (e.g. 16SrRNA, 23SrRNA, fla, recA, p66) (for review see [200]).
Despite all efforts that have been made in the last years, PCR is currently not widely accepted for laboratory diagnosis of LB. This is mainly because of the following limitations: (i) Variable sensitivity for different clinical specimens, e.g., PCR is highly sensitive for detection of *B. burgdorferi* s.l. DNA in skin biopsies of patients with EM, but as clinical diagnosis can be easily made if a typical EM is seen in a patient such a test may not be necessary. For patients with chronic LB, PCR is informative, but it is usually less sensitive. (ii) Possibility of a false-positive result due to sample contamination. (iii) No possibility to distinguish between a current or resolving infection since PCR (in contrast to culture) detects DNA from both live and dead organisms. However, a rapid decay of dead Borrelia in tissue can be assumed. A true-positive PCR is only an indicator for the presence of *B. burgdorferi* s.l. DNA.

As recently summarized in a metaanalysis, the sensitivity of PCR varies considerably, particularly with regard to the specimens examined. It is in the range of 60 – 90% for detection of *B. burgdorferi* s.l. DNA in skin biopsies from patients with EM or ACA, 6 – 90% in CSF, 23 – 100% in synovial fluid, 0 – 50% in plasma/serum and 13 – 100% in urine [44].

1.10 Treatment

All manifestations of LB should be treated with antibiotics as early as possible. Amoxicillin, doxycycline and third-generation cephalosporins are the drugs of choice. The duration of treatment ranges from 2 to 4 weeks. Most people treated for LB have an excellent prognosis. In multicentre studies of patients with EM, more than 90% of patients had satisfactory outcomes [157]. However, a small percentage of patients do not respond sufficiently even to repeated courses of antibiotic treatment. Different hypotheses were put forward to explain the reason for these treatment resistant cases as it was recently discussed by Diterich et al. [42]. Preventive antibiotic treatment following a tick bite is not recommended, since only a minority of ticks are infected with spirochetes, the majority of infections pass asymptomatically and the frequency of LB after a recognized tick-bite is only about 1% if an attached tick is quickly removed.

2 Aims of the study

Lyme Borreliosis (LB), caused by the spirochete *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.), is the most common tick borne disease in Europe and the United States. In Europe at least three Borrelia species are known to be pathogenic for humans. In the last years an association between these species and different manifestations of LB became more and more evident. Therefore, knowledge of the geographic distribution of different genospecies of *B. burgdorferi* s.l. within their tick vector has not only ecological and epidemiological but also clinical relevance. Methods which enable a typing of Borrelia species such as reverse line blotting have been described in the literature, but they are laborious and time-consuming.

- The aim of the first part of this thesis was the establishment of a method which allows a simple, rapid and sensitive differentiation of the Borrelia species, employing real-time PCR which distinguishes the three species by different numbers of mismatches with a fluorescent probe.

For humans the risk to be infected with Borrelia depends on (outdoor) lifestyle, the density of tick populations as well as on the infestation of the ticks with Borrelia. Therefore, data describing the prevalence of Borrelia in ticks can be used to assess the risk of LB to public health.

- The established real-time PCR was employed in an epidemiological study on the prevalence rate of Borrelia and the distribution of genospecies in *I. ricinus* ticks in the region of Konstanz.
- In the second part of the present thesis, the gained data were discussed in the context of the situation in Europe by metaanalysis.

Diagnosis of LB is currently based on clinical criteria confirmed by serological tests. However, serology has a number of limitations. Hence, new diagnostic tools which enable a direct detection of Borrelia are important. An attractive approach is the use of urine as sample material for PCR. Many efforts have been made in recent years to establish urine PCR as routine diagnostic tool in LB, but results are not satisfying and controversial. In the third part of the thesis a study with spiked urine samples was conducted to clarify the situation. In particular the following issues were addressed:

- A nested real-time PCR based on the real-time PCR established in the first part of the thesis was developed, allowing a highly sensitive quantification of Borrelia in spiked urine samples.
- Urine sample preparation was optimized in a quantitative way.
- The optimized protocol was applied to urine of LB patients.

3 Distribution of clinically relevant Borrelia genospecies in ticks assessed by a novel single-run real-time PCR

Carolin Rauter[1], Rainer Oehme[2], Isabel Diterich[1], Matthias Engele[1] and Thomas Hartung[1]*

[1]Biochemical Pharmacology, Department of Biology, University of Konstanz, and [2] Landesgesundheitsamt Stuttgart, Germany

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3.1 Abstract

A LightCycler-based PCR protocol was developed which targets the ospA gene for the identification and quantification of the different *Borrelia burgdorferi* sensu lato species in culture and in ticks, based on the use of a fluorescently labeled probe (HybProbe) and an internally labeled primer. The detection limit of the PCR was 1-10 spirochetes. A melting temperature determined from the melting curve of the amplified product immediately after thermal cycling, allowed the differentiation of the three different *Borrelia burgdorferi* s.l. genospecies (*B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*) that are clinically relevant in Europe in a single PCR run. This method represents a simplified approach to study the association of different Borrelia species in ticks, the risk of Lyme borreliosis and the putatively species-specific clinical sequelae.

To determine the reliability of the real-time PCR protocol, we studied the prevalence of *Borrelia burgdorferi* sensu lato infection in *Ixodes ricinus* ticks. A total of 1,055 ticks were collected by flagging vegetation in five different sites in the region of Konstanz (south Germany) and were examined for the distribution of *B. burgdorferi* species by real-time PCR. The mean infection rate was 35%. Of 548 adult ticks, 40 % were positive, and of 507 nymphs, 30% were positive. The predominant genospecies (with 18% mixed infections) in the examined areas was *B. afzelii* (53%), followed by *B. garinii* (18%), *B. burgdorferi* senso stricto (11%); 0.8% of the infecting *Borrelia* could not be identified.

3.2 Introduction

Lyme borreliosis (LB) – the most common arthropod-borne infection in Europe [215] and the United States [217] – is a complex multisystem disorder caused by *Borrelia burgdorferi* sensu lato (s.l.), a group of genetically diverse spirochetes. The principal vectors of these spirochetes are ticks belonging to the genus *Ixodes* [22].

The development of an Erythema migrans rash at the site of the tick bite often characterizes the onset of LB. If left untreated, the infection can persist for years and may result in a range of clinical symptoms, which vary depending on the duration of the infection and the organs affected.

Isolates of *B. burgdorferi* s.l. can be classified into different genomic species [6, 106]. Only one of them, *B. burgdorferi* sensu stricto (s.s.), has been implicated as the cause of disease in North America, but in Europe three genospecies, *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. are known to be pathogenic, and still others, such as *B. valaisiana* and *B. lusitaniae* have been identified but are of unknown pathogenicity [47]. Coinfections by two or more genomic groups of *B. burgdorferi* s.l. have been found in ticks [133, 153] and patients with LB [39].

There is strong evidence that different species are involved in distinct clinical manifestations of the disease [228]. Different studies have presented indirect evidence for the association of *B. garinii* with predominantly neurological symptoms [39], while *B. burgdorferi* s.s. and *B. afzelii* tend to lead to arthritic symptoms [229] and cutaneous manifestations [23], respectively. New rapid and sensitive methods are therefore required for differentiating the three pathogenic Borrelia species to test the strengths of these associations.

PCR is increasingly employed for the detection of Borrelia [39, 165, 187, 207]. Recently, the LightCycler PCR, which assesses the amount of amplified DNA after each PCR cycle, was introduced. Beside nonspecific DNA measurement by intercalating dyes (such as SYBR Green), specific gene probes labeled with fluorescent dyes allow the quantification of formed amplicon. Furthermore, calculation of the melting point of the DNA-probe adduct enables identification of the PCR product. This method can be exploited to distinguish sequence deviations, e.g., polymorphisms of different bacterial strains.

Recently, two LightCycler PCR based assays for the differentiation of Borrelia species were described [154, 174]. The latter method, an amendment of the first, could distinguish among all three Borrelia species known to be pathogenic for humans. However, the method requires two LightCycler PCRs targeting the recA gene and the p66 gene, respectively. Melting curve analysis of the recA gene amplicon allows the separation of *B. garinii* from *B. burgdorferi* s.s. and *B. afzelii*, and melting curve analysis of the p66 gene amplicon is employed for the separation of *B. burgdorferi* s.s. from *B. afzelii* and *B. garinii*.

The LightCycler PCR described here allows rapid genotyping of the three *Borrelia burgdorferi* species in a single PCR run. Therefore, a sequence of the ospA gene which results in zero, two, or three mismatches in the three species was chosen as probe, allowing differentiation by melting point analysis. In contrast to the above-mentioned method of Mommert et al., a fluorescently labeled hybridization probe and an internally labeled primer were used rather than the fluorescent dye SYBR Green. This combination allows a rapid genotyping of the three *B. burgdorferi* species in a single PCR run.

In Europe, where all three genospecies of Borrelia known to be pathogenic for humans are found, the identification of the genospecies in patient specimens and ticks from patients is necessary to make a distinction between their respective roles in the pathophysiology of LB manifestations. Furthermore, field studies in areas of endemicity to assess the prevalence of *B. burgdorferi* s.l. species in ticks will aid risk assessment and allow a determination of the infectivity of different species.

In the present study, we used real-time PCR to characterize the distribution of species of *B. burgdorferi* s.l. in ticks collected in the field. We show that the three genomic groups were present in 1,055 *Ixodes ricinus* (*I. ricinus*) ticks collected in the southern part of Germany during 1999 and 2000 and that coinfections with two or three genomic groups of *B. burgdorferi* s.l. occur among these ticks.

3.3 Materials and Methods

3.3.1 <u>Study area and tick collection</u>

A total of 1,478 *I. ricinus* ticks were collected by the flagging method in five locations (A, B, H, L, M) in the region of Konstanz (South Germany) in 1999 and 2000. The distances between the different locations were less than 10 km. At site A and L, ticks were collected from the edges of roads and trails with mixed woodland on one side and meadows on the other side. Site B was a barbecue site in a forest situated nearby a marsh. M was a kindergarten in a forest and at site H, ticks were collected from within the woodland near a path. Ticks were separated into nymphs and mature females and males, and stored at –80°C until use. At least 200 ticks (100 nymphs, 50 males and 50 females) from each location were examined by PCR.

3.3.2 <u>Bacterial isolates and culture conditions</u>

The Borrelia strains used in this sudy (*B. burgdorferi* s.s. [N40], *B. garinii* [PSTH, A218] and *B. afzelii* [VS461]) were cultured in modified BSK-H medium (Sigma, Deisenhofen, Germany) at 33°C as described previously [42]. All Borrelia strains were kindly provided by Dr. T. Kamradt (Berlin, Germany), with the exception of *B. garinii* A218, which was a kind gift from H. Martilla.

Culture density was determined by microscopy using a modified Thoma counting chamber (Merck Eurolab, Ismaning, Germany).

3.3.3 DNA extraction

DNA from each Borrelia culture was extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions or by a Chelex-based method [232], which is a faster DNA extraction procedure. The bacterial culture was diluted 1:10 with 20% Chelex 100 (Bio-Rad Laboratories, Munich, Germany). After being thoroughly mixed, the sample was incubated at 56°C for 30 min. Then, the suspension was boiled for 10 min, and the debris was removed by centrifugation (13,800 x g, 3 min). The supernatant was either used directly for amplification or stored at –20°C until use. To extract the DNA from the ticks, they were mechanically crushed with sterile pestles, and nucleic acid extractions were performed with the Chelex-based method using 40 µl and 45 µl Chelex 100 for nymphs and adults, respectively.

3.3.4 Real-time PCR

Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics GmbH, Mannheim, Germany). A 296-bp fragment from the gene encoding OspA was amplified. The reverse primer used was published previously by Demaerschalck et al. [39]. The forward primer, serving as the acceptor probe of the HybProbe detection system, was labeled internally with the fluorescent dye LC Red 640. Thus, first-strand DNA already contained fluorescent dye. For detection and differentiation of the species, we used a fluorescein-labeled probe. The forward primer was located further downstream than the originally published forward primer so that the fluorescein-labeled probe could bind the first-strand DNA close enough to the LC Red 640 for electron transfer to occur. The probe and primers were designed as shown in Fig. 3 and Table 1. The forward primer and probe were both designed by TIB Molbiol (DNA Synthesis Service, Roche Diagnostics, Berlin, Germany), who also synthesized all primers and the probe.

Primer or probe	Sequence
Reverse primer………	5′- CTA gTg TTT TgC CAT CTT CTT TgA AAA –3′
Forward primer………	5′- AgC CTT AAT AgC ATg $^{C}/_{T}$AA gCA AAA #X′TG –3′
Hybridization probe …	5′- gCg CTg TTT TTT TCA TCA Agg CTg CTA AC§X –3′

TABLE 1: Oligonucleotide primer and probe sequences used in PCR amplification and detection protocol of the *Borrelia burgdorferi* s.l. strains. §X = fluorescein-labeled base; #X' = LC Red 640-labeled base.

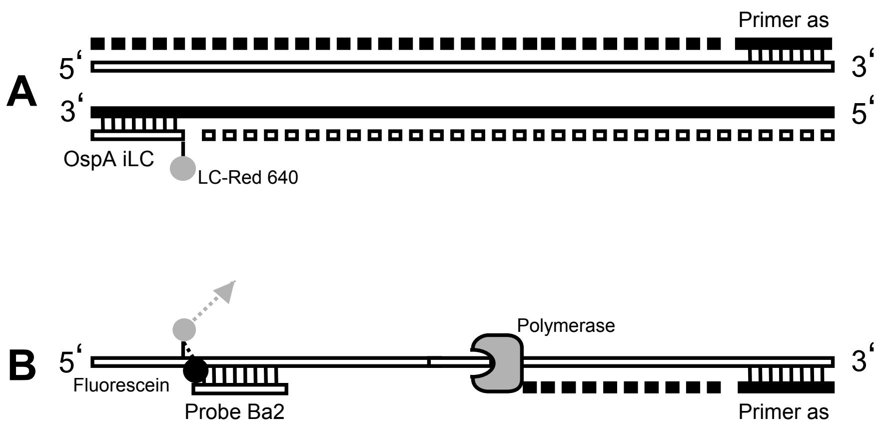

FIG. 3. Design of forward primer and probe for the identification of Borrelia species in the LightCycler. The forward primer (OspA iLC) labeled with LC Red 640 is incorporated into the first strand of DNA amplicon (A), and the fluorescein-labeled probe (Probe Ba2) binds to this DNA strand (B). The proximity of the two fluorochromes allows induction of LC Red 640 fluorescence by FRET. Due to the surplus of probes, the FRET signal depends directly on the amount of amplicon formed.

The 10-µl (final volume) PCR reaction included 1 µl of a commercial ready-to-use reaction mixture for PCR (LightCycler-DNA Fast Start master hybridization probes; Roche Diagnostics) that contains Hot Start *Taq* DNA polymerase, desoxynucleoside triphosphate mixture, reaction buffer and 1 mM $MgCl_2$. $MgCl_2$ was added to a final concentration of 5 mM. The final concentrations of the probe and the primers were 0.1 and 0.5 µM, respectively. Finally, 1 µl of template DNA was added.

The reaction mixture was loaded into glass capillary tubes (Roche) which were snap sealed with plastic caps. The conditions for thermal cycling were as follows: initial denaturation for 10 min (to activate the fast-start *Taq* polymerase), followed by the amplification program, which included a denaturation step at 95°C for 10 s, an annealing step at 57°C for 10 s, and extension at 72°C for 13 s. Fluorescence was measured at the end of each annealing phase. The amplification was followed by a melting program, which started at 54°C for 45 s and then increased to 95°C at 0.1°C/s, with the fluorescence signal continuously monitored.

3.3.5 Agarose gel electrophoresis

PCR amplification products were resolved on 1.5% agarose gels by electrophoresis and visualized under UV light with ethidium bromide. As marker, a 100-bp ladder was used (Gibco BRL, Karlsruhe, Germany). The expected amplification product was 296 bp.

3.3.6 Nested PCR and sequencing

Nested PCR targeting 5S-23S rRNA was performed by following the protocol of Rijpkema et al. [187].

The nucleotide sequences of PCR-amplified fragments were determined by the dideoxy chain termination technique [196] with the Prism Big Dye Terminator Cycle-Sequencing Ready-Reaction kit (Applied Biosystems) using the ABI Prism System 310 DNA sequencer.

3.3.7 Statistics

The Fisher test, an option of GraphPad (San Diego, Calif.) Instat, was used to determine statistical significance. A P value of ≤ 0.05 was considered significant.

3.4 Results

3.4.1 Real-time PCR of Borrelia in culture samples

A total of 55 amplification cycles were performed with genomic DNA of each Borrelia genotype and a template-free control. The forward primer (internally labeled with LC Red 640) was incorporated into the first-strand DNA. After being annealed, the 3' fluorescein-labeled probe bound to the first-strand DNA so that the fluorophores were separated by one base. This close proximity of the two dyes during hybridization allowed fluorescence resonance energy transfer (FRET) between the fluorophores. The resulting light emission of LC Red 640 was detected, providing real-time monitoring of the amplification process.

The fluorescence signal was measured at the end of each annealing phase and increased as the product accumulated in an exponential manner. No increase in fluorescence signal was observed in the absence of template. Figure 4 shows the progress of a PCR with Qiagen-extracted Borrelia DNA as the template. The DNA templates prepared by the Chelex method gave similar results (data not shown). The detection limit of the PCR was tested with serially diluted DNA templates of the three Borrelia strains from 10^8 Borrelia organisms/µl down to 10^0 Borrelia organisms/µl. It was found that a template DNA amount corresponding to 1 to 10 spirochetes (from strain N40 [data not shown], PSth [data not shown] or VS461 [Fig. 4]) was sufficient for detection. When uninfected ticks were spiked with known amounts of Borrelia, the recovery limit was also 1 to 10 spirochetes, despite attenuated PCR efficacy (slope of amplification), indicating the presence of inhibitors.

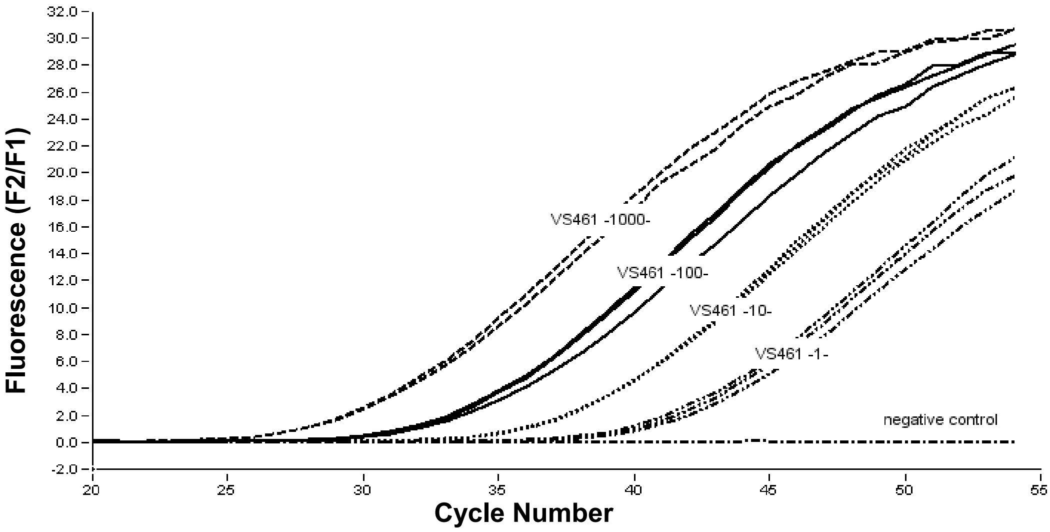

FIG. 4. Detection limit of the PCR assay for the detection OspA determined with serial dilutions of VS461 genomic DNA. The dilutions were utilized in doublets or triplets. All samples were amplified simultaneously. DNA in the sample corresponded to 1,000, 100, 10 and 1 spirochete of *B. afzelii* (VS461). As a negative control, water was added to the reaction mixture instead of template.

A cutoff criterion was defined based on the fluorescence variations of 60 uninfected ticks in 10 different LightCycler runs: the distribution of absolute fluorescence maxima of these 60 uninfected ticks was assessed, and the cutoff was defined as mean ± 3 standard deviations, resulting in 0.5 F2/F1, i.e., the quotient of LC Red 640 to fluorescein signal. Ticks which did not reach 0.5 F2/F1 until cycle 55 were considered negative.

Real-time PCR allows the relative quantification of the amount of DNA template by using the cycle number at which the fluorescence signal starts to rise above a defined threshold (termed the ct value). In order to test the reproducibility of this measure, a Borrelia DNA standard was included in 55 independent LightCycler runs. The standard deviation was 1.32 cycles, showing excellent reproducibility of the method. The specificity of the PCR reaction was confirmed by agarose (1.5%) gel electrophoresis (Fig. 5).

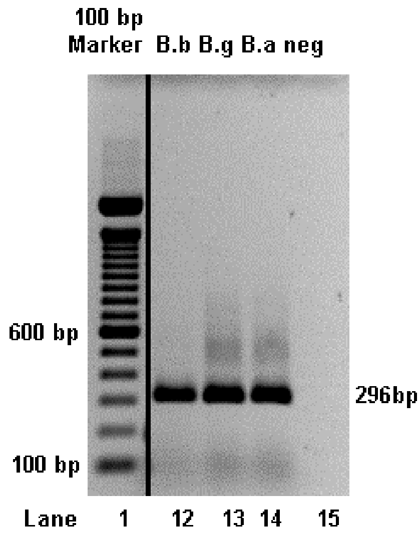

FIG. 5. Gel electrophoresis of PCR products from three *B. burgdorferi* s.l. strains. The predicted lengths of the products were confirmed on a 1.5% agarose gel. A 100-bp ladder was used as a size marker (first lane). The other lanes show PCR products obtained from *B. burgdorferi* s.s. (B.b; lane 12), *B. garinii* (B.g; lane 13), and *B. afzelii* (B.a; lane 14) and a water control (neg; lane 15).

Since no reference method ("gold standard") is available and extraction of DNA from whole ticks excludes testing by methods other than PCR, sensitivity and specificity of the assay cannot be assessed. In order to challenge the test and estimate these parameters, borderline positive-negative ticks were tested several times. Ticks (159) which were borderline in the first assay were analyzed repeatedly (a total of 410 measurements) until a definite classification was possible, i.e., the first result was either reproduced or falsified by repeated measurements. These data showed 3 false-negative (137 true-negative) and 8 false-positive (262 true-positive) measurements. From these data, a sensitivity of 98% and a specificity of 97% was estimated for this method.

3.4.2 Melting point analysis

After amplification, a melting curve was generated for genotyping, i.e., fluorescence was monitored continuously while the temperature was raised from 54 to 95°C. This resulted in a sharp decrease in LC Red 640 fluorescence at the temperature at which the probe dissociates from the template. When the strand and the probe are perfectly matched, strong hybridization results and the melting temperature (T_m) is high. A mismatch, even of a single base, results in a lower T_m, due to the decreased hybridization stability that is reflected in the peak of the first derivative of the melting curve (-dF2/dT). Here, the probe was designed to match the sequence of the Borrelia species *B. afzelii* perfectly. The sequences of *B. garinii* and *B. burgdorferi* s.s. contained two and three mismatches with the probe, respectively. Therefore, a characteristic melting profile for each genotype was obtained.

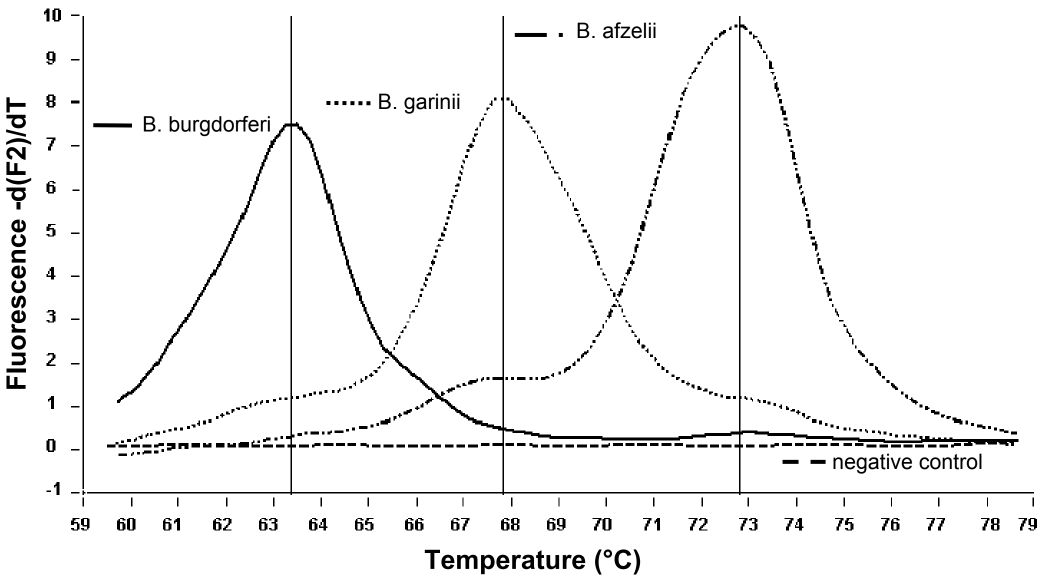

FIG. 6. Identification of T_ms for the three *B. burgdorferi* s.l. species. The melting peak analysis which followed each PCR run showed that the T_ms were 63°C for *B. burgdorferi* s.s. (N40), 68°C for *B. garinii* (PSth) and 72.5°C for *B. afzelii* (VS461).

The temperatures at which the probes detached from PCR products during the melting program were calculated using the LightCycler software. The T_ms of the three Borrelia species differed, as shown in Figure 6. The average T_ms of the reference strains of *B. burgdorferi* s.s. (N40), *B. garinii* (PSth) and *B. afzelii* (VS461) were 63°C, 68°C and 72.5°C, respectively. Thus, the difference in the T_ms of N40 and PSth was 5°C and between those of PSth and VS461 was more than 4°C. As with this method each T_m corresponded specifically to one Borrelia species, we were also able to detect the different species in mixtures of two or three Borrelia genospecies (Fig. 7). Two criteria were used to define a positive signal in T_m analysis: (i) a relative maximum (i.e., a peak, to be distinguished from a plateau) of fluorescence at the characteristic temperature and (ii) a height of this peak of at least 10% of the peak of the control (representing about 100 Borrelia organisms). This procedure resulted in a cutoff around 0.3 -dF2/dT.

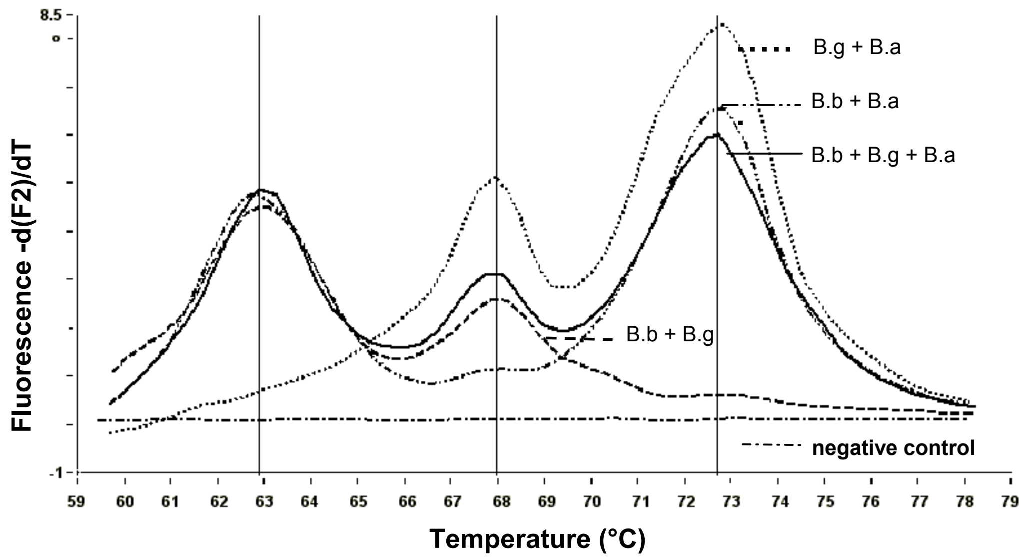

FIG. 7. Melting point analysis of mixtures of Borrelia species. Differentiation of species in coinfections with two (*B. afzelii* [B.a.] VS461 plus *B. garinii* [B.g.] PSth; VS461 plus *B. burgdorferi* s.s. [B.b.] N40 or N40 plus PSth) or three (N40 plus PSth plus VS461) Borrelia species. In the negative control, water was used as a template.

3.4.3 Detection of *B. burgdorferi* s.l. in ticks by real-time PCR

The reliability of the real-time PCR protocol for the amplification of *B. burgdorferi* ospA was tested by assessing the infection rate in ticks collected in the field (Table 2). A total of 1,055 *I. ricinus* ticks collected at five different sampling sites in the region of Konstanz were investigated by real-time PCR. As summarized in Table 2, the infection rate for the ticks examined at the different sites varied significantly from 20 to 57% ($p < 0.001$). The overall prevalence of *B. burgdorferi* s.l. in ticks was 35%. The highest infection rate (57%) was found in location B. Further, the overall infection rates in nymphs were significantly ($p < 0.001$) lower (30%, i.e., 152 out of 507), than those in the adult ticks (40%, i.e., 219 out of 548).

Tick	Infection rate											
	A[a]		B		H		L		M		∑	
	No. +	%	No. +	%	No. +	%	No. +	%	No. +	%	No. +	%
females	15/50	30	50/83	60	10/40	25	17/50	34	11/52	21	103/275	37
males	18/50	36	43/67	64	16/56	29	18/50	36	21/50	42	116/273	42
adults	33/100	33	93/150	62	26/96	27	35/100	35	32/102	31	219/548	40
nymphs	27/100	27	53/106	50	14/100	14	36/100	36	22/101	22	152/507	30
∑	60/200	30	146/256	57	40/196	20	71/200	36	54/203	27	371/1055	35

TABLE 2: Summary of Borrelia infection rates in different maturation stages of *I. ricinus* ticks from the different collection sites. [a] 5 different collection sites in Konstanz. +, positive.

An estimation of Borrelia numbers in ticks was carried out employing the ct values of the individual runs. In spike experiments, 1,000 Borrelia genome equivalents corresponded to a ct value of 30, 100 genome equivalents corresponded to 34, 10 genome equivalents corresponded to 38, and 1 genome equivalent corresponded to more than 40. The distribution of ct values of all 371 positive ticks is shown in Figure 8, with a median of 34, i.e., about 4,000 Borrelia organisms per tick.

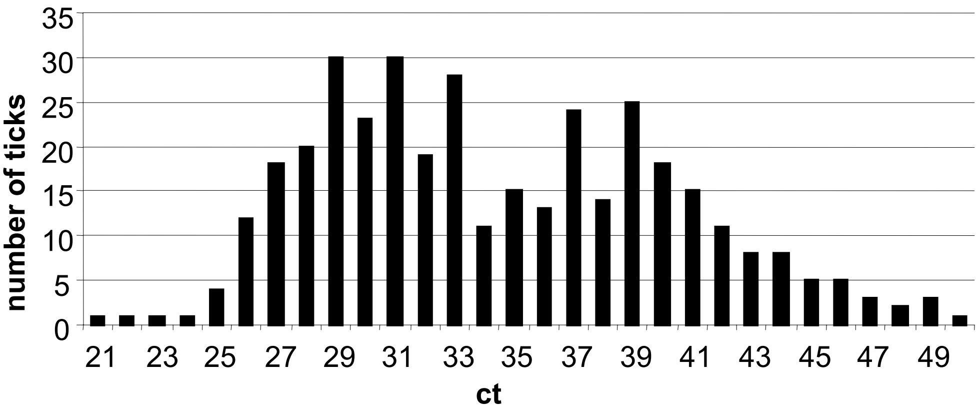

FIG. 8. Distribution of Borrelia counts in all 371 positive ticks. The cycle number at which the PCR fluorescence commences (ct value) allows the relative quantification of the number of genome equivalents present in the probe. Dilution series of Borrelia DNA showed that 1,000 Borrelia genome equivalents corresponded to a ct value of 30, 100 genome equivalents corresponded to 34, 10 genome equivalents corresponded to 38 and 1 genome equivalent corresponded to more than 40 cycles.

Genotyping (Table 3) indicated that *B. afzelii* was the predominant species in all of the areas studied. *B. afzelii* was detected in 70% of the infected ticks, followed by *B. garinii* (34%) and *B. burgdorferi* s.s. (12%). The Borrelia species infecting three ticks (0.8%) could not be identified, since they showed a T_m of 58.6°C, which did not correspond to any T_m of the other species. Mixed infections by two or three species were detected in 18% of the ticks characterized as positive by PCR. We detected double infections of *B. afzelii* with *B. garinii* (88%) or *B. burgdorferi* s.s. (9%), respectively. In one case, we could detect a mixed infection of all three species. A combination of *B. garinii* and *B. burgdorferi* s.s. alone was not found.

The PCR products of 24 Borrelia isolates from ticks were sequenced to determine genospecies. All 10 *B. afzelii*, 10 *B. garinii* and 4 *B. burgdorferi* s.s. isolates showed the expected number of mismatches with the probe, stressing the validity of genotyping by melting point analysis. However, two well-characterized strains of *B. garinii* (B29, A218) resulted in two peaks at 68°C and 72.5°C in the melting point analysis, which would falsely indicate a double infection with *B. garinii* and *B. afzelii*. Sequencing of these strains did not clarify the occurence of the two peaks, since both strains displayed the two mismatches only with the probe characteristic for *B. garinii*. Nevertheless, this finding might indicate that the frequency of double infection (5.6% of all ticks) with *B. garinii* and *B. afzelii* is overestimated. However, assuming independence of both infections, a very similar frequency of double infections of 3% can be calculated from the frequencies of either infection alone.

Species[a]	Infection rate											
	A		B		H		L		M		Σ	
	No. +	%	No. +	%	No. +	%	No. +	%	No. +	%	No. +	%
B.b.	7	12	16	11	7	18	0	0	9	17	39	11
B.g.	20	33	20	14	7	18	13	18	6	11	66	18
B.a.	23	38	80	55	18	45	46	65	28	52	195	53
Unknown	0	0	2	1	0	0	2	3	0	0	4	1
B.b. + B.g.	0	0	0	0	0	0	0	0	0	0	0	0
B.b. + B.a.	0	0	2	1	0	0	1	1	3	6	6	2
B.g. + B.a.	9	15	26	18	8	20	9	13	7	13	59	16
B.a .+ unknown	0	0	0	0	0	0	0	0	1	2	1	0,3
B.b. + B.g. + B.a.	1	2	0	0	0	0	0	0	0	0	1	0,3
Total positive	60	30	146	57	40	20	71	36	54	27	371	35
Total measured	200		256		196		200		203		1055	

TABLE 3: Identification of different *Borrelia burgdorferi* s.l. species in *I. ricinus* ticks collected at five different sites (A, B, H, L, M) in Konstanz. [a] B.b., *B. burgdorferi* s.s.; B.g., *B. garinii*; B.a., *B. afzelii*. +, positive.

3.5 Discussion

The aim of our study was the development of a new PCR method for the detection and differentiation of the *B. burgdorferi* s.l. species *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s.. The use of the LightCycler system allowed the simultaneous differentiation of these species in one PCR run in a single tube, representing a much faster, less laborious and less expensive method for genotype identification than the commonly used methods, including species-specific PCR, randomly amplified polymorphic DNA analysis, PCR-based sequencing, and restriction fragment length polymorphism.

The ospA gene was used as the target of real-time PCR. This gene is located on a linear 49-kb plasmid. The sequences of the ospA genes of the three major Borrelia species are different [238]: ospA genotypes 1 and 2 correspond to *B. burgdorferi* s.s. and *B. afzelii*, respectively, and ospA genotype 3 to 7 correspond to *B. garinii*. These genotypes correspond to the OspA serotypes 1 to 7 [240]. Due to the hypothesized multiplicity of plasmid genes in clinical probes [167], portions of the ospA gene were frequently chosen as templates for PCR [39, 58, 155, 230].

A study by Will et al. [238] showed highly conserved ospA genes within the *B. burgdorferi* s.s. group as well as within the *B. afzelii* group but heterogeneity within the ospA genes of *B. garinii*-type strains. In line with this notion, a BLAST search in the National Center for Biotechnology

Information (GenBank) databases (22 *B. garinii* sequences) for the *B. garinii* ospA sequence binding to the probe showed two distinct groups of *B. garinii*, each characterized by two mismatches with our probe (at positions 1 and 6 and 1 and 12, respectively). All of 10 *B. burgdorferi* s.s. and 16 *B.afzelii* sequences showed zero or three mismatches, respectively. Since the method is extremely sensitive to changes in the sequence binding to the probe, misclassifications cannot be completely excluded.

The PCR assay described here is sensitive enough to detect fewer than 10 spirochetes of each of the three clinically relevant genospecies of *B. burgdorferi* s.l. in a sample. Therefore, the detection limit is comparable to conventional nested PCRs. However, the real-time PCR is less laborious and considerably faster. The entire assay can be completed in approximately one hour. Amplification, hybridization, and analysis are performed in one closed capillary tube, decreasing the risk of cross contamination. The melting curve analysis allows the differentiation of Borrelia species even in a mixture of all three species. Thus, the method presented here appears to be the first LightCycler-based PCR which allows the differentiation of the three Borrelia species *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* in one PCR run.

Since any naturally occuring tick can only be analyzed by a single type of procedure and no gold standard for Borrelia detection is available, it was only possible to compare the new method to an established nested PCR in a block cycler targeting 5S-23S rRNA [187]. Of 100 positive ticks, 86 were also positive in nested PCR, while all 50 negative ticks were negative in both assays. Taking nested PCR as the reference method, this would indicate a sensitivity of 100%, specificity of 86%, and accuracy of 91%.

The observation that 14 ticks were positive only by real-time PCR might indicate either a higher sensitivity of the LightCycler or false-positive results. Employing serial dilutions of Borrelia DNA, no major difference in limit of detection was observed. However, the nested PCR might contain higher concentrations of tick-borne inhibitors due to the larger amounts of tick extract used per tube (10% versus 20% of total volume).

There is an ongoing discussion about the clinical relevance of *B. valaisiana* and *B. lusitaniae*, which are occasionally found in ticks and birds [47]. Due to sequence similarity, *B. valaisiana* yields the same melting point as *B. afzelii* in our system and can thus not be distinguished. A total of 39 tick samples classified as *B. afzelii* infected were therefore subjected to Southern blot analysis according to the method described [187]. Only a single case of *B. valaisiana* infection was found (data not shown), indicating that the prevalence of this genotype is very low in the investigated area.

The sensitivity and specificity of our method were estimated to be 98% and 97%, respectively, based on 159 ticks, which were analyzed two to six time. It is worth to noting that ticks with low and borderline bacterial burdens were selected to challenge the method, thus actually underestimating the reproducibility in practice.

To test the feasibility and reliability of the real-time PCR protocol for the amplification of *B. burgdorferi* ospA, we investigated the distribution of *B. burgdorferi* s.l. in ticks collected in Konstanz and determined the genomic groups present. Information about the prevalence of Borrelia infection in ticks in areas of endemicity is necessary for risk assessment. It has been shown that most habitats where ticks carrying Borrelia have been found are recreational sites [70]. Therefore, we selected four sites with recreational function within the five collection sites. Significant variability was observed in the prevalence of *B. burgdorferi* s.l. among the sites examined, with infection rates ranging from 20 to 57%. These considerable differences between the prevalence of infected ticks even in habitats in close proximity indicates that the occurrence of Borrelia species in nature is affected by many ecological factors.

A very high infection rate (57%) was found at one of the five sites. Similar results showing high infection rates in particular sites have also been described by others [32, 108, 185]. One possible explanation for this phenomenon could be that the transmission of the spirochetes to ticks is amplified by cofeeding on a vertebrate host, as has been proposed [63].

By using melting point analysis, we were able to differentiate the three genospecies of Borrelia known to be pathogenic for humans in the ticks collected. All three genomic groups (*B. burgdorferi* s.s., *B. garinii* and *B. afzelii*) were found in tick isolates from the investigated sites. Our findings indicate that *B. afzelii* (70%) was present most abundantly, followed by *B. garinii* (34%). *B. burgdorferi* s.s. (12%) appears to be less common here. Similar findings were made in Slovenia, where out of 60 Borrelia-positive ticks, 53% were identified as infected by *B. afzelii*, 33% were identified as infected by *B. garinii*, and only 13% were identified as infected by *B. burgdorferi* s.s. [221].

Interestingly, three of the 1,055 ticks examined were positive for *B. burgdorferi* s.l. but the isolates could not be classified into one of the three species, as their T_m (58.6°C) differed from those of the others: *B. burgdorferi* s.s. (63°C), *B. garinii* (68°C) and *B. afzelii* (72.5°C). A DNA sequence analysis (performed by MWG Biotech, Ebersberg, Germany) indicated that these spirochetes belong to the recently described Borrelia species A14S [233]. A14S is phenotypically and genetically different from all other *B. burgdorferi* s.l. species described and therefore most likely represents a new Borrelia genotype. Since it was cultured from a skin biopsy specimen of a patient with Erythema migrans it seems to be pathogenic for humans.

Mixed infections were found in 18% of the Borrelia-positive ticks – mainly double infections by *B. afzelii* and *B. garinii*. Double infections of *B. garinii* and *B. burgdorferi* s.s. were not found.

The quantification of Borrelia species in ticks showed a very heterogeneous distribution ranging from 1 to more than 1,000 Borrelia equivalents. Similar numbers were found by Stünzner et al. by microscopically counting Borrelia organisms in tick guts [222]. This finding illustrates that a

method with a low detection limit is required to assess all infected ticks. To our knowledge, the impact of different Borrelia burdens on infectivity for mammals has not yet been studied.
In conclusion, we have demonstrated that the use of the new real-time PCR method provides a rapid and sensitive tool for differentiating *B. burgdorferi* s.l. species known to be pathogenic for humans. We have shown that the method can be applied for the detection and differentiation of Borrelia genospecies in ticks collected in the field and ticks removed from humans. The novel method appears to represent a versatile tool to assess the roles of different genospecies in the pathophysiology of Lyme disease in Europe.

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4 Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe – a Metaanalysis

Carolin Rauter and Thomas Hartung

Biochemical Pharmacology, Faculty of Biology, University of Konstanz

Submitted to *Applied and Environmental Microbiology*

4.1 Abstract

In Europe, *Borrelia burgdorferi* sensu lato, the causative agent of Lyme Borreliosis, is mainly transmitted by the tick *Ixodes ricinus*. Since its discovery, *B. burgdorferi* has been the subject of many epidemiological studies concerning its prevalence and the distribution of the different genospecies in ticks. The current study systematically reviews the literature on epidemiological studies of *Ixodes ricinus*-ticks infected with *B. burgdorferi* sensu lato. 1,186 abstracts in English and German published from 1984 to 2003 were identified by a PubMed® keyword search and from the compiled article references. A multi-step filter process was used to select relevant articles. 111 articles from 24 countries contained data on the infection rates of *Ixodes ricinus* with Borrelia in Europe (113,685 ticks) and 46 articles from 20 countries included species-specific analyses (4,367 positive ticks). These data were used to evaluate the overall infection rate of *Ixodes ricinus* with Borrelia genospecies, regional distributions within Europe and changes over time, as well as the influence of different detection methods on the infection rate.
While the infection rate was significantly higher in adults (18.5%) compared to nymphs (10.1%), no effect of detection method, tick gender or collection period (1986 – 1993 vs. 1994 – 2002) was found. The highest infection rates of *Ixodes ricinus* were found in countries of Central Europe. *B. afzelii* and *B. garinii* are the most common Borrelia species, but the distribution of genospecies seems to vary in different regions in Europe. The most frequent coinfection of Borrelia species occurred between *B. garinii* and *B. valaisiana*.

4.2 Introduction

Lyme Borreliosis (LB), the most frequent tick-borne disease in the northern hemisphere, is a multisystemic disorder caused by the spirochete *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.). In Europe, the principal vector of Borrelia is the tick *Ixodes ricinus* (*I. ricinus*). The risk to humans of infection with Borrelia depends on (outdoor) lifestyle, the density of tick populations as well as on the infestation of the ticks with Borrelia. Therefore, data describing the prevalence of Borrelia in ticks can be used to assess the risk of LB to public health.

B. burgdorferi s.l. is a genetically diverse group of spirochetes. In Europe, six different genospecies of *B. burgdorferi* s.l. are described: *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto (s.s.), *B. valaisiana*, *B. lusitaniae* and *B. bissettii* [6, 38, 61, 79, 84, 120]. Different reservoir hosts seem to harbor different genospecies of *B. burgdorferi* s.l., which is explained by differential properties of the host's complement system, favoring certain genospecies [124].

At least three Borrelia species (*B. afzelii*, *B. garinii* and *B. burgdorferi* s.s.) are known to be pathogenic for humans. The pathogenicity of the other species is still unclear, although *B. valaisiana* and *B. lusitaniae* have been detected in skin biopsies of some LB patients [34, 189]. There is strong evidence that infections with different genospecies of Borrelia correlate with different clinical symptoms of LB. Lyme arthritis is mainly attributed to *B. burgdorferi* s.s., *B. garinii* infection is preferentially associated with neuroborreliosis and skin manifestations are mainly associated with *B. afzelii* [4, 39, 228]. Therefore, knowledge of the geographic distribution of different genospecies of *B. burgdorferi* s.l. within their tick vector has not only ecological and epidemiological but also clinical relevance.

Individual ticks can be infected with more than one genospecies of *B. burgdorferi* s.l. [133, 153, 161, 182]. Information on the patterns of such mixed infections may reveal important biological and ecological principles of *B. burgdorferi* s.l. and also have clinical relevance, since such mixed infections have also been detected in patients [39, 188]. However, mixed infections in ticks appear to be rather rare, thus studies which further specify the mixed infections usually do not contain enough data to draw valid conclusions. A metaanalysis which merges the available data could provide more insight also on mixed infections.

This paper presents a metaanalysis of epidemiological studies on the prevalence and the distribution of genospecies of *B. burgdorferi* s.l. in host-seeking ticks in various European countries based on a systematic literature review, describing (A) the distribution of *B. burgdorferi* s.l. and (B) the occurrence of different Borrelia genospecies in *I. ricinus* tick populations in Europe. The following questions were examined:

(A) (i) What is the mean infection rate of *I. ricinus* ticks with Borrelia in Europe? (ii) How do the infection rates of ticks differ across Europe? (iii) Has tick infection changed during recent years? Do the method used for the detection of Borrelia in ticks influence the infection rate?

(B) (i) What is the predominant Borrelia species in Europe? (ii) Are there differences in the distribution of the Borrelia species in different parts of Europe? (iii) Is there a difference in Borrelia species distribution in tick nymphs and adults or in females and males? (iv) Which mixed infections of different Borrelia species occur most often?

4.3 Materials and Methods

We performed a computerized literature search using PubMed® to identify all citations concerning tick infection rates with Borrelia published from 1984 to 2003 using the keyword search terms "Ixodes" and "Borrelia". A copy of the abstract of each identified English- or German-language citation was obtained. A multistage assessment was used to determine which articles contain relevant data. In a first step we reviewed the abstracts to determine which articles reported epidemiological data on (i) the infection rate of *I. ricinus* with *B. burgdorferi* s.l. or (ii) the distribution of the different Borrelia genospecies in *I. ricinus*. Of these, only articles with the following criteria were included: (i) the area of tick collection was located in Europe (ii) the ticks examined were *I. ricinus* and (iii) the ticks were unfed, host-seeking ticks. In a second step, these articles were retrieved and their bibliographies were screened for missed citations. In a third step of assessment, papers with incomprehensible, inaccurate or already published data were excluded. Data on larvae were excluded. If this was not possible, the entire paper was excluded. Every paper was checked twice.

4.3.1 Data Abstraction

Every variable referring to area and period of tick collection, stage and gender, infection with Borrelia and Borrelia species in the selected articles was documented. Specific variable analysis was limited to articles containing that variable, thereby eliminating the need to address missing data. These variables formed the foundation of the final database. Difficulties in abstracting data came from non-reported information or reported data accounting for only a subset of the database. For data abstraction the following steps were carried out:

- The infection rate (p) of ticks examined in pools (at most 5 ticks per pool) were recalculated where possible, using the following formula: $p=1-\sqrt[k]{1-f}$, (k = number of specimens in each pool; f = proportion of infected pools) [37].
- If the number of positive ticks in the study was not given, it was calculated, if possible, based on the number of ticks examined and the reported infection rate.
- Papers were divided into separate records if they examined (i) ticks collected in different years, (ii) different ticks examined by different methods, (iii) ticks from different countries or

(iv) ticks from collection areas larger than 1° latitude or 2° longitude. This division was only carried out if the number of ticks examined per record did not fall below 100.

- From papers, which examined the same ticks with different methods leading to different results, only data obtained with polymerase chain reaction (PCR) or immunofluorescence assay (IFA) were included.
- If not reported, longitude and latitude of the sampling site of every study was approximated. If more than one collection point was given, the collection area was determined and the coordinates were averaged.
- Each genospecies found within a mixed infection was added to the respective single infection tally.
- Records on species-specific analyses of pooled ticks were excluded.

4.3.2 Literature Description

1,186 English and German language abstracts published from 1984 to 2003 were identified in PubMed®. After examining the abstracts, 191 papers proceeded to the second stage and their reference sections were scrutinized for missed publications, identifying another 26 articles. For analysis of infection rates 111 articles and for species-specific analysis, 46 articles progressed through the third stage and to data extraction. 155 and 53 records were extracted for analysis of infection rates and species-specific analysis, respectively.

4.3.3 Statistics

Statistical analyses were done using GraphPad Prism 3.0 (GraphPad Software, San Diego, USA). The data expressed in the bar charts represent the mean ± SEM; the mean is indicated in scatter blots by a horizontal line. An unpaired t-test with Welch's correction was performed when comparing two groups. For the comparison of more than two groups, analysis of variance (One-way ANOVA) followed by Bonferroni's multiple comparison test was used. P-values < 0.05, < 0.01 and < 0.001 were considered significant and depicted as *, ** and *** or §, §§ and §§§, respectively. Linear regression was performed to analyze the relationship between two variables.

4.4 Results

4.4.1 A. Infection rates

4.4.1.1 Overall infection rates

Table 4 lists all records extracted for the analysis of infection rates of *B. burgdorferi* s.l. in *I. ricinus* ticks in Europe. The overall mean prevalence of Borrelia in ticks was 13.6% (15,516 of 113,685 ticks). Compared to nymphs (10.1%; 6,435 of 63,894 ticks), adults (18.5%; 7,974 of 43,126) showed a considerably higher infection rate. No difference was seen between the infection rates of females and males (18.2% and 16.3%, respectively). Of studies which examined both nymphs and adults in parallel and at least 100 of each, 28 of 64 records found at least twice as many infected adults than infected nymphs, 26 between one and two times more infected adults, 7 reports found as many infected adults as nymphs and the infection rate in adults was lower than in nymphs in only 3 records.

country	reference	year	method	inf. rate [%]	total	nymph	adult
Austria	[222]	1997	DFM	20.8	1,163	853	310
	[93]	1989-02	DFM	23.2	422	211	211
Belgium	[153]	1996	PCR	23	489	444	45
Bulgaria	[28]	2000	PCR	32.7	202	90	112
	[27]	n.g.	BSK	17	47	–	47
Croatia	[185]	1995	PCR	45	124	34	90
Czech Republic	[10]	1995-98	PCR	4.5	779	572	207
	[87]	1988-96	DFM	20.7	8,339	3,546	4,793
	[245]	1987-91	IFA	15.3	5,104	4,417	687
	[89]	1992	DFM	27	163	34	129
	[40]	2000	PCR	20.3	370	261	109
	[88]	1995	DFM	24.3	350	150	200
	[88]	1996	DFM	22	350	150	200
	[88]	1997	DFM	26	350	150	200
	[88]	1998	DFM	24.9	350	150	200
	[88]	1999	DFM	17.7	350	150	200
	[88]	2000	DFM	17.7	350	150	200
	[88]	2001	DFM	24	350	150	200
	[93]	1989-02	DFM	21.8	1,094	586	508
	[90]	1991	DFM	15.2	350	150	200
	[90]	1992	DFM	23	350	150	200
	[90]	1993	DFM	22.3	350	150	200
	[90]	1994	DFM	19.8	350	150	200
	[92]	1988	GS	8.5	378	–	378
	[86]	1988-89	DFM	20.2	460	209	251
	[119]	n.g.	DFM	7.9	570	n.g.	n.g.
	[219]	1997	DFM	5.4	555	145	410
	[219]	1998	DFM	11	163	13	150
Denmark	[126]	1990-91	IFA	18.6	317	202	115
	[103]	1995	IFA	5.3	396	396	–
	[103]	1996	IFA	3.1	829	829	–
	[103]	1997	IFA	2.4	327	327	–
	[104]	1996	IFA	5.1	1,151	1,045	106
Estonia	[144]	2000	PCR	15	100	–	100
Finland	[108]	1996	PCR, DFM, BSK	32.2	726	303	423
	[109]	1992	BSK	5.5	1,210	392	818
	[144]	2000	PCR	5.1	454	343	111
France	[170]	1994	PCR	12	249	249	–
	[171]	1995	PCR	8.2	461	461	–

	[64]	1992	IFA	9.8	1,556	1,014	542
	[64]	1992	IFA	6.4	1,822	1,422	400
	[64]	1992	IFA	4.7	1,295	811	484
	[246]	1991	IFA	10.7	383	314	69
	[179]	1994-95+98	PCR	14.3	638	419	219
Germany	[52]	1985-86	IFA	14.6	2,369	1,157	1,212
	[53]	1994	IFA	19.1	472	157	315
	[55]	1997	PCR	36.2	492	91	401
	[182]	1999-00	PCR	35.2	1,055	507	548
	[73]	1992-93	DFM	10.4	279	68	211
	[73]	1992-92	DFM	4.7	1,185	1,185	–
	[73]	1992-93	BSK	8.4	598	485	113
	[73]	1992-93	IFA	12.4	194	147	47
	[110]	1991	IFA	18.4	414	414	–
	[243]	1992	DFM	16	100	–	100
	[243]	1993	BSK	18	100	–	100
	[243]	1994	PCR	22	100	–	100
	[212]	1996	PCR	9	3,926	3,520	406
	[120]	n.g.	PCR	18.1	226	–	226
	[122]	1987-88	IFA	6.3	1,954	1,954	–
	[161]	1998-00	PCR	15.8	3,138	n.g.	n.g.
	[82]	1999-00	PCR	11.1	305	243	62
	[112]	1986	BSK	5.8	173	49	124
	[184]	1994	PCR	9	112	112	–
	[163]	n.g.	PCR	31.5	165	n.g.	n.g.
	[242]	1993-94	BSK	16.3	559	–	559
	[147]	1991	DFM, IFA	17.9	756	531	225
	[74]	1992	DFM	8.6	758	660	98
Ireland	[71]	1997-98	PCR	12	183	164	19
	[72]	n.g.	IFA	12	100	100	–
	[117]	1995	PCR	14.9	915	686	229
	[69]	1992-93	IFA	2.6	2,587	2,518	69
	[68]	1990-91	IFA	6.5	612	509	103
	[118]	1995	PCR	18.4	512	411	101
Italy	[32]	1994	PCR	40	227	218	9
	[30]	n.g.	PCR	4.4	420	n.g.	n.g.
	[197]	1997-98	PCR	2.9	1,503	1,475	28
	[31]	n.g.	PCR	19.8	86	84	2
	[50]	1998	PCR	11.4	55	55	–
	[198]	n.g.	PCR	4.3	141	125	16
	[145]	1995	BSK	0	80	80	–
	[145]	1995	PCR	0	110	110	–
Latvia	[120]	n.g.	PCR	31.3	300	–	300
	[49]	1999	PCR	31.2	205	55	150
	[49]	2000	PCR	25.7	300	150	150
Lithuania	[156]	1988-91	DFM	10.1	3,820	287	3,533
Netherlands	[228]	n.g.	BSK	10.5	248	176	72
	[187]	1994	PCR	15.6	96	39	57
	[187]	1994	IFA	19.5	210	120	90
	[186]	1988-93	IFA	24.6	463	341	122
	[37]	1991	DFM	12.5	522	476	46
Norway	[102]	1999	PCR	15.8	341	185	156
Poland	[211]	1997-98	PCR	21.6	616	48	568
	[214]	2000	PCR	11.6	424	–	424
	[209]	1999	PCR	7.8	1,710	1,160	550
	[235]	1993	IFA	11.5	1,666	1,070	596
	[21]	2000	IFA	11.3	1,387	1,264	123
	[21]	2001	IFA	9.6	993	931	62
	[169]	1996	DFM, BSK	20.7	92	6	86
	[169]	1996	DFM, BSK	12.8	815	110	705
	[151]	1998	IFA	18.6	587	538	49
	[151]	1999	IFA	13.6	536	464	72
	[208]	1999	PCR	18.9	514	234	280
	[244]	1998	PCR	4	1,262	835	427
	[244]	1999	PCR	5.7	1,379	1,006	373
	[244]	2000	PCR	4.8	1,356	955	401
	[244]	2001	PCR	3.3	1,439	894	545
	[33]	2000	DFM	8.8	114	50	64
	[33]	2001	PCR	5.3	549	265	284
	[213]	1996	IFA	8.1	1,333	554	779

	[236]	1994	IFA	8.8	3,958	2,395	1,563
Portugal	[38]	1998	PCR	75	55	–	55
	[120]	n.g.	PCR	14.7	217	–	217
Slovakia	[43]	1991	DFM	18.5	2,857	–	2,857
	[61]	1998	IFA	49.1	114	–	114
	[220]	1994	DFM	4.8	809	149	660
	[220]	1995	DFM	17.2	805	215	590
	[220]	1996	DFM	15.6	805	155	650
	[220]	1996	DFM	14.2	399	113	286
	[120]	n.g.	PCR	40.5	585	–	585
	[41]	2002	PCR	43.3	60	20	40
	[78]	2000	PCR	28.2	177	93	84
	[79]	1999	PCR	32.2	966	382	584
	[79]	2000	PCR	33.2	328	174	154
Slovenia	[191]	1990	IFA	7.7	496	411	85
	[221]	n.g.	BSK	19	363	206	157
Spain	[9]	1992-93	PCR	1.5	134	–	134
Sweden	[76]	1988-91	IFA	14.4	2,974	397	2,577
	[225]	1994-95	PCM	13	1,908	1908	–
	[56]	1999	PCR	11	301	–	301
	[226]	1991	PCM	15.4	408	408	–
	[226]	1992	PCM	20.6	471	471	–
	[226]	1993	PCM	24.1	551	551	–
	[149]	1988	PCM	12.8	539	459	80
	[149]	1989	PCM	8.6	1005	946	59
	[15]	1991	IFA	20.8	149	92	57
	[100]	1991	PCM	9.1	396	396	–
	[17]	1989	PCM	2	42	42	–
	[101]	1992	PCM	12.6	381	381	–
	[101]	1993	PCM	11	581	581	–
	[101]	1994	PCM	14.2	604	604	–
	[75]	n.g.	IFA	29.6	54	16	38
Switzerland	[168]	1987-93	IFA, DFM, BSK	30.4	727	–	727
	[95]	1993	BSK	13.7	95	22	73
	[152]	1987	IFA	30.8	182	94	88
	[131]	1991	BSK	16.5	133	–	133
	[132]	n.g.	PCR	49	100	–	100
	[107]	1999-01	IFA	10.9	460	415	45
	[96]	1993-94	BSK	19.1	235	93	142
	[210]	n.g.	BSK	13.6	118	n.g.	n.g.
	[62]	1990	DFM	21.4	56	56	–
UK	[36]	1997	BSK	8.5	128	n.g.	n.g.
	[123]	1995-96	PCR	4.6	680	580	100
	[77]	n.g.	PCR	7.7	65	–	65
	[162]	1994	IFA or PCR	8.3	109	73	36
	[162]	1995	IFA or PCR	3.9	333	221	112
				13.6 (average)	**113,685 (total)**		

TABLE 4. List of records with data on infection rates of *I. ricinus* ticks with *B. burgdorferi* s.l. in Europe. The total infection rate (inf. rate) is given. The number of examined nymphs and adults shows the stage composition of all examined ticks (total) per record.
BSK, cultivation in BSK medium; DFM, dark-field microscopy; GS, Giemsa-stained smears; IFA, immunofluorescence assay; PCR, polymerase chain reaction; PCM, phase contrast microscopy; n.g. = not given

Correlating the infection rates of nymphs with that of adults enabled a linear regression (Fig. 9) resulting in the following formula: $I_N = 0,97 * I_A – 7.35$ (I_N and I_A is the mean infection rate of nymphs and adults, respectively). By means of this formula, missing data of studies which examined only nymphs or only adults can be added reciprocally, thus completing the data points. In all of the following results it was tested whether such correction impacted the analysis.

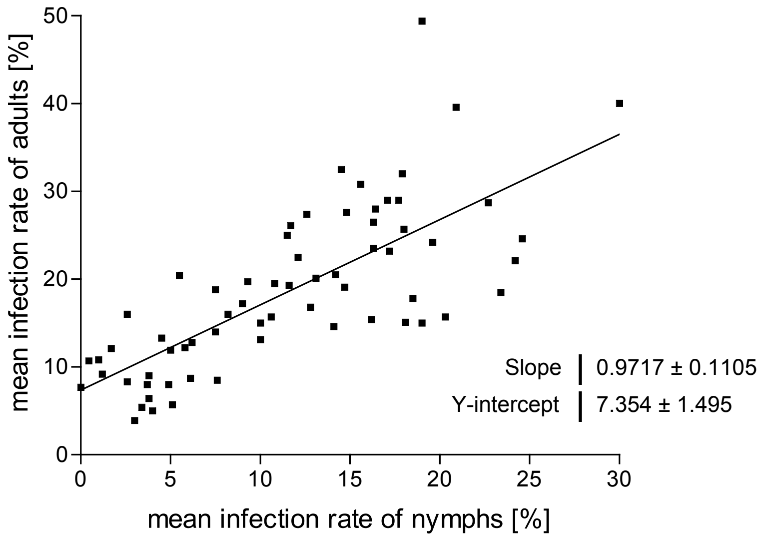


FIG. 9. Regression of mean infection rates of Borrelia in nymphs and adults. Only studies which examined both nymphs and adults and at least 100 of each were included. Each data point represents one record.

4.4.1.2 Influence of detection methods

Methods generally used for the detection of Borrelia in ticks are cultivation in BSK medium, dark-field microscopy (DFM), immunofluorescence assay (IFA) and polymerase chain reaction (PCR). A comparison of the mean infection rates of studies which used at least one of these methods for the detection of Borrelia in ticks, revealed no significant difference in either nymphs or adults (Fig. 10). Noteworthy, the highest infection rates (nymphs > 30% and adults > 35%) were reported almost exclusively with PCR in Bulgaria, Croatia, southern Germany, Latvia and Slovakia. In the following, no distinction of the detection methods was made.

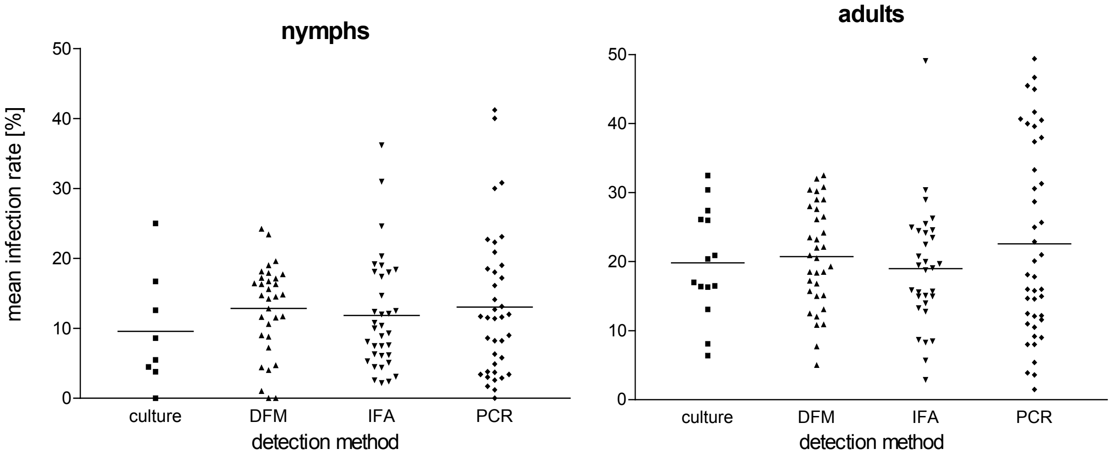


FIG. 10. Influence of detection methods on infection rates.

4.4.1.3 Infection rates in different regions of Europe

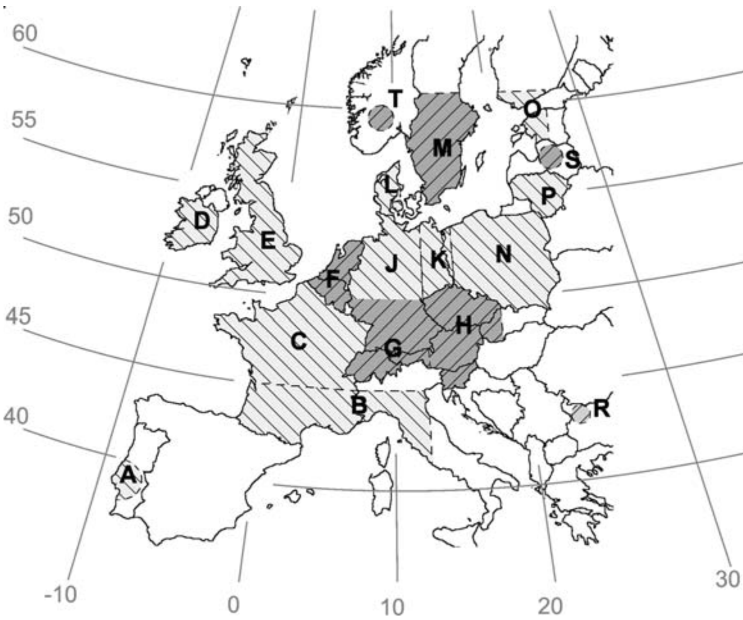
The infection rates of ticks with Borrelia were correlated with the latitude or longitude of the sampling site of every study. For this purpose, the coordinates of the sites of tick collection were determined. In studies with a large area of collection the mean of latitude and longitude, respectively, were calculated. Coordinates were transformed to decimal values. Negative longitudes represent the zone west, positive the zone east of Greenwich.

Regression analyses of the mean infection rates with their corresponding longitude or latitude, showed a significant increase in infection rates in adult ticks from western to eastern Europe (14 to 24%; calculated from the regression, $p < 0.05$), whereas no such trend was seen for nymphs (data not shown). Latitude had no effect on tick infection prevalence either in nymphs or adults. The effect of longitude on the infection rates did not change, (i) if only studies which examined at least 100 nymphs or adults were included, or (ii) if retrospectively missing data of nymphs or adults were added using the regression analysis, described above. However, both corrections led to a significant increase of infection rates in adult ticks from northern to southern Europe (12 to 23%; calculated from the regression, $p < 0.01$).

To calculate the infection rates of several regions in Europe, the means of the infection rates of all studies per region were formed for nymphs and adults, respectively. For the definition of the regions, several criteria were taken into account: (i) The regions were defined large enough to include the studies with large collection areas. (ii) Geographic conditions were taken into consideration. (iii) For valid conclusions to be drawn, a region should include at least 5 records. Unfortunately, this was not possible in some cases. The available data, are summarized and mapped in Figure 11. Using the means of the infection rates, one can classify regions with low (nymphs $\leq$ 11%; of adults $\leq$ 20%) and high (of nymphs > 11%; of adults > 20%) infection rates. In 5 of the 18 regions adult ticks were infected at least twice as often, in 10 regions between once and twice as often as nymphs. Only in two regions (“T” and “F”) nymphs and adults seem to be infected equally often, however, the infection rate of region “T” was based on a single study. In three regions (“A”, “R” and “S”) the mean infection rate of adults was extremely high (>30%, Fig. 11.b).

Limitation to studies which examined at least 100 adults or 100 nymphs led to considerably different results in four of the regions: In region “A” and “M” the infection rate of adults decreased from 30 to 8% and 21 to 11%, respectively. In region “R” the infection rate of adults increased from 31 to 46% and for nymphs no data could be used. In region “S”, an increase from 17 to 23% in the infection rate of nymphs could be seen.

a)



b)

region	Inf. N [%]	# records	Inf. A [%]	# records
A	–	0	30.4 §	3
B	2.1	3	8.1	2
C	8.3	8	10.8	6
D	10.,6	6	15.7	5
E	1.5	3	16.3	3
F	20.0	5	22.1	5
G	17.5	10	25.0	10
H	16.3	29	27.5	33
J	8.0	4	17.9	4
K	8.3	17	14.5	16
L	7.2	5	10.7	2
M	15.6	12	21.2	5
N	9.0	14	15.8	14
O	3.8	2	10.1	3
P	1.0	1	10.8	1
R	17.2	1	31.3 §	2
S	17.7	2	32.7 §	3
T	16.2	1	15.4	1

FIG 11. a) Map of the defined regions. Areas with low infection rates (nymphs ≤ 11%; adults ≤ 20%) are marked in light gray, areas with higher infection rates are dark gray. b) Table of regions with the means of infection rates of nymphs (N) and adults (A). The number of records used for every region is given.
§ Extremely high infection rates (> 30%).

4.4.1.4 Years of tick collection

To compare the course of infection rates over the years, studies with a collection period longer than one year were excluded unless the data could be separated into years. As the infection rates of ticks in several regions in Europe vary significantly (Fig. 11), a comparison of infection rates per year requires a representative profile of regions with high and low infection rates in each year. Since this was not given, data from ranges of years (1986 to 1993 and 1994 to 2001) were merged. Data of regions with extremely high infection rates (“A”, “R”, “S”), were excluded to attain a similar proportion of areas with high and low infection rates in both periods. A comparison of the two periods showed no significant difference in the mean of infection rates of nymphs or adults (Fig. 12).

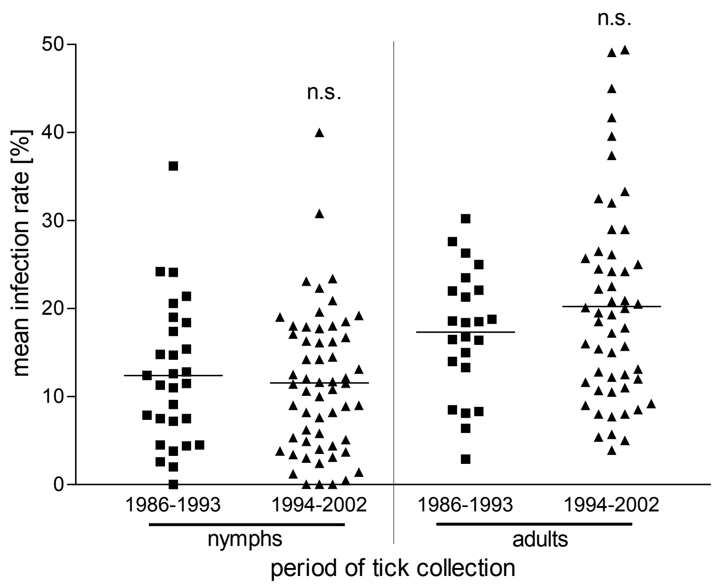


FIG. 12. Comparison of infection rates of nymphs and adults in two collecting periods. Each data point represents one record. To attain a similar proportion of areas with high and low infection rates in both periods, data of extremely high infection rates (> 30%) were excluded.

4.4.2 B. Species-specific analysis

4.4.2.1 Overall ratio of Borrelia species in Europe

The data from studies with species-specific analyses of *B. burgdorferi* s.l. in *I. ricinus* ticks are summarized in Table 5 and 6. The following problems were noticed: (i) The number of ticks examined per study was extremely low in some cases (5 of 53 records included less than 10 ticks). Therefore, it was first checked in every analysis whether these studies distorted the results. If this was the case, they were excluded as mentioned in the respective results. (ii) The stage and gender of the examined ticks was not stated in every study, reducing the number of records considerably. In Table 5 each species found in a mixed infection reported separately was added to the respective single infection tally in order to harmonize results (therefore the sum of the percentages may result in values greater than 100%).

country	reference	total	n.t.	B.a.	B.g.	B.b.	B.v.	B.l.
Austria	[222]	29	0	11	17	2	–	–
	[223]	50	0	25	21	5	–	–
Belgium	[153]	114	17	14	79	56	–	–
Bulgaria	[28]	24	1	18	2	5	2	1
	[27]	8	0	0	4	4	–	–
	[29]	46	6	26	3	15	3	1
	[29]	22	2	11	5	0	5	0
Croatia	[185]	56	8	37	5	2	15	–
Czech Republic	[10]	34	0	15	21	0	–	–
	[40]	76	1	45	16	8	9	–
	[219]	9	0	2	3	7	–	–
Estonia	[144]	15	0	12	3	0	0	0
	[108]	142	5	101	38	0	–	–
	[144]	23	0	12	2	9	0	0
France	[170]	25	0	19	9	3	–	–
	[171]	38	12	17	9	3	–	–
	[246]	3	0	0	1	2	–	–
	[179]	91	5	36	29	5	27	0
Germany	[182]	371	5	261	126	46	–	–
	[120]	41	3	18	15	0	6	0
	[241]	67	0	6	26	27	8	–
	[139]	17	1	3	13	0	–	–
	[82]	34	0	6	19	11	2	–
	[184]	10	0	4	5	1	0	0
	[163]	52	0	13	31	3	0	0
	[161]	1,106	116 [a]	463 [a]	286 [a]	144 [a]	186 [a]	–
Ireland	[71]	22	4	1	10	0	8	–
	[117]	136	8	9	47	27	64	–
	[118]	94	2	16	26	21	47	–
Italy	[32]	91	21	8	42	56	1	–
Latvia	[120]	94	1	39	34	5	19	0
	[49]	64	1	20	29	3	15	0
	[49]	77	8	35	20	10	10	0
Netherlands	[228]	26	0	26	0	0	–	–
	[187]	15	0	6	7	0	5	–
	[159]	63	13	2	19	29	–	–
	[203]	4	0	2	1	0	2	–
Norway	[102]	54	0	49	1	14	–	–
Poland	[211]	121	14	57	45	48	–	–
Portugal	[38]	41	0	0	0	0	0	41
	[120]	32	5	1	14	0	18	0
Slovakia	[61]	37	0	25	5	0	5	2
	[120]	237	26	129	59	5	42	0
	[78]	50	0	21	5	0	23	1
	[79]	311	0	186	79	7	68	6
	[79]	109	0	45	8	0	53	3
Slovenia	[221]	60	0	32	20	8	–	–
Sweden	[56]	32	2	14	10	4	2	–
Switzerland	[168]	50	0	3	19	26	2	–
	[107]	20	2	0	13	0	3	3
	[96]	41	2	14	8	21	0	0
	[210]	28	0	6	12	8	2	–
UK	[123]	23	2	0	15	2	7	–

TABLE 5. List of records extracted for the analysis of *B. burgdorferi* s.l. genospecies in *I. ricinus* ticks in Europe. The number of *B. afzelii* (B.a.), *B. garinii* (B.g.), *B. burgdorferi* s.s. (B.b.), *B. valaisiana* (B.v.) and *B. lusitaniae* (B.l.) positive ticks and the number of totally examined ticks (total) is given. Each species found in a coinfection reported separately was added to the respective single infection tally. [a]personal communication.

country	reference	total	B.a. B.g.	B.a. B.b.	B.g. B.b.	B.a. B.v.	B.g. B.v.	B.b. B.v.	triple infection
Bulgaria	[28]	5	1	2	0	1	0	0	0
Croatia	[185]	11	0	1	0	10	0	0	0
Czech Republic	[10]	2	2	0	0	–	–	–	0
	[40]	4	0	2	0	0	2	0	0
	[219]	3	2	0	1	–	–	–	0
Finland	[108]	2	2	0	0	–	–	–	0
France	[170]	6	3	1	2	–	–	–	0
	[171]	3	2	1	0	–	–	–	0
	[179]	11	2	1	0	2	6	0	0
Germany	[182]	66	59	6	0	–	–	–	1
	[82]	4	0	0	4	0	0	0	0
	[161]	80	16 [a]	20 [a]	5 [a]	14 [a]	14 [a]	2 [a]	9 [a]
Ireland	[117]	18	0	1	0	2	14	0	1
	[118]	17	0	3	0	3	10	0	1
Italy	[32]	31	0	1	23	0	1	0	6
Latvia	[120]	4	0	2	0	0	2	0	0
	[49]	4	0	1	0	0	2	1	0
	[49]	6	0	3	0	0	3	0	0
Netherlands	[187]	3	0	0	0	3	0	0	0
Norway	[102]	10	0	10	0	–	–	–	0
Poland	[211]	41	11	17	11	–	–	–	2
Portugal	[120]	6	0	0	0	0	6	0	0
Slovakia	[120]	24	2	2	0	0	20	0	0
	[79]	34	2	2	0	2	26	0	2
Switzerland	[96]	3	0	2	0	0	0	0	1
UK	[123]	3	0	0	0	0	3	0	0

TABLE 6. Summary of records of mixed infection of the *B. burgdorferi* s.l. genospecies *B. afzelii* (B.a.), *B. garinii* (B.g.), *B. burgdorferi* s.s. (B.b.) and *B. valaisiana* (B.v.). The number of ticks infected with various combinations of these species is given. [a]personal communication.

Figure 13 shows the overall ratio of Borrelia species in Europe. No distinction was made here between nymphs and adults. The mean percentages of *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana* and *B. lusitaniae* positive ticks, were 37, 34, 17, 20 and 7%, respectively. 5% of the ticks were untypable and 13% showed a mixed infection. No significant difference was seen between *B. afzelii* and *B. garinii*, but the means of both species were significantly different from the means of the other three species.

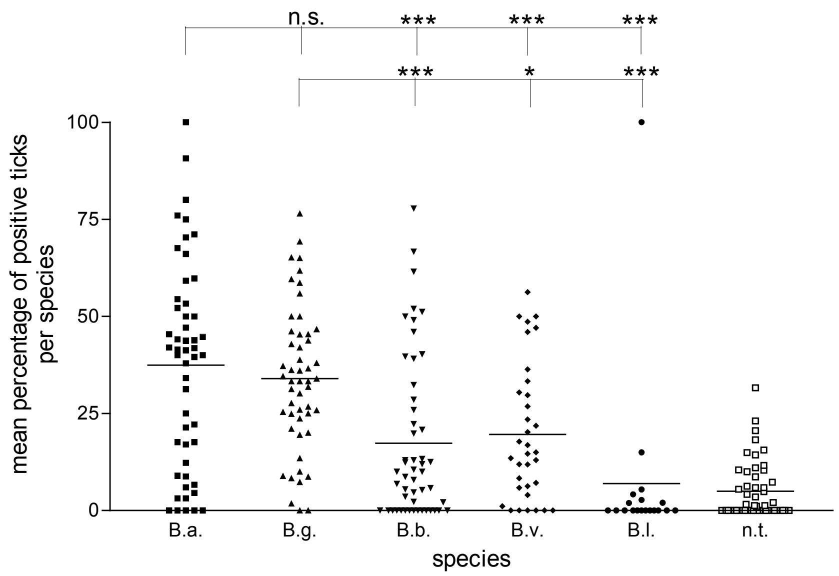


FIG. 13. Overall ratio of the Borrelia species *B. afzelii* (B.a.), *B. garinii* (B.g.), *B. burgdorferi* s.s. (B.b.), *B. valaisiana* (B.v.) and *B. lusitaniae* (B.l.) in *I. ricinus* ticks in Europe. 53 records with 4,367 positive ticks were included. n.t., not typable ticks.

Since all the studes distinguished between the three genospecies *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. (53 records), but not every study tested for *B. valaisiana* (34 records) and only a few for *B. lusitaniae* (19 records), the data on the latter two species are weaker. Also, in records which did not determine *B. valaisiana* and *B. lusitaniae*, these species might be recognized as "not typable" or be falsely recognized as one of the other species. For example, we reported this problem in a study employing real-time PCR which distinguished species by different numbers of mismatches with a fluorescent probe. The PCR product of *B. valaisiana* had the same melting point as that of *B. afzelii* and was therefore recognized as *B. afzelii* [182].
To check if studies containing *B. lusitaniae* and not typable positive ticks influenced the results, we repeated the analysis after excluding these populations. The mean percentages of *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s. and *B. valaisiana* positive ticks, remained similar to those in Fig. 13 (36, 34, 15 and 21%, respectively).

4.4.2.2 <u>Stage and gender dependent distribution of Borrelia species in Europe</u>

No significant difference was seen when the prevalence of each Borrelia species in nymphs was compared to that in adults (Fig. 14). This result did not change when we included only studies which distinguished between the 4 genospecies, *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s. and *B. valaisiana*.

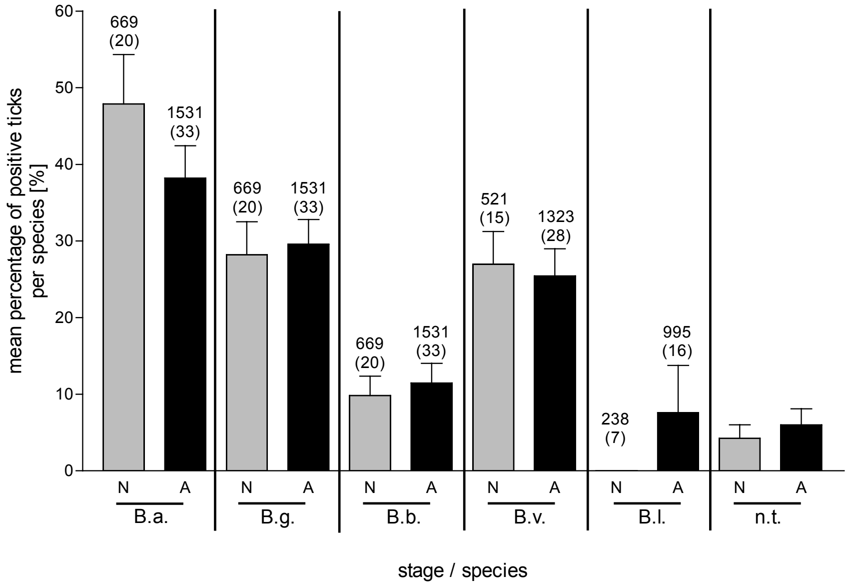


FIG. 14. Distribution of the Borrelia genospecies B.a., B.g., B.b., B.v. and B.l. in nymphs (N) and adults (A). The number of ticks examined is stated above the bars with the number of records in parenthesis.

Only 11 records on 156 female and 97 male ticks in total were available for a direct gender based comparison of Borrelia species distribution. Since no information on the gender was given concerning the ticks with mixed infections, we were not able to add each species of a mixed infection to the respective single infection tally. Hence, for gender-specific comparison, only infections with a single species were included. Data were extracted from 11 records (156 females and 97 males). No significant difference in the mean percentage was seen between females and males for any species (data not shown). Insufficient data were available for analysis with *B. valaisiana* and *B. lusitaniae*.

4.4.2.3 Mixed infections

The occurrence of mixed infections in nymphs (12.1%) was not statistically different from that in adults (13.6%). The distribution of combinations of mixed infections is shown in Figure 15. Only studies which distinguished between *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s. and *B. valaisiana* were included (24 records). The combination of *B. garinii* and *B. valaisiana* occurred significantly more often than all other species combinations. A mixed infection with *B. lusitaniae* was described only once in combination with *B. garinii* [28] (data not shown). Combinations of three species occurred only rarely.

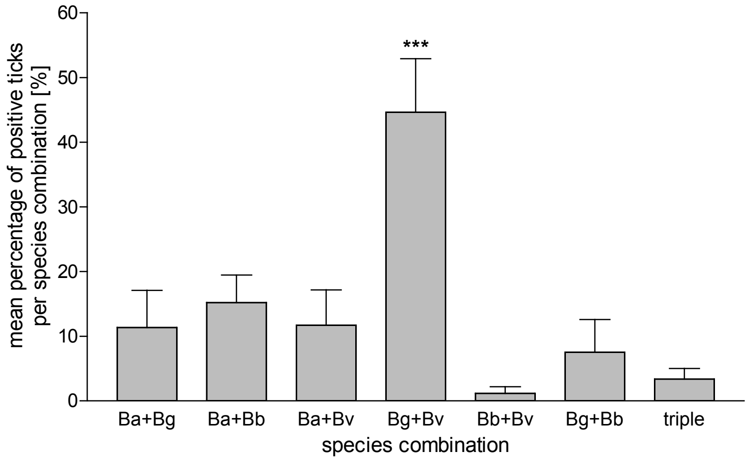


FIG. 15. Distribution of mixed infections in ticks. Only records which examined B.a., B.b., B.b. and B.v. were included (24 records).

A direct comparison of species combinations between nymphs and adults showed no significant differences (data not shown) though only few records were available (10 records for nymphs, 14 records for adults).

4.4.2.4 Borrelia genospecies distribution in different parts of Europe

To compare the distribution of Borrelia species in various parts of Europe, we merged the data from different countries. For this purpose, the following inclusion criteria were taken into account: (i) The countries had to be adjacent (except group 3). (ii) The ratio of genospecies in the respective records was similar. (iii) At least four records had to be included per group. Based on these criteria, seven groups were defined (Table 7). Because of the low number of records, studies which examined less than 10 ticks were excluded to avoid distortion. Since only few data are available for *B. lusitaniae*, it was also excluded from this analysis. The results clearly show diverse patterns of species distribution in the different areas of Europe. Group 1, 2 and 3 show significantly more ticks infected with *B. afzelii* than with *B. garinii*, while in group 4 and 5 *B. garinii* seems to predominate. In group 4 ticks seemed to be equally frequently infected with *B. valaisiana* and *B. garinii*. Group 6 and 7 revealed no significant difference between *B. afzelii* and *B. garinii*.

group	countries	B.a.	B.g.	B.b.	B.v.	n.t.	# Rec agb	# Rec v
1	southern Germany Czech Republic Slovakia	53 ± 4	25 ± 5***	5 ± 2*** §§	25 ± 6***	3 ± 2	9	7
2	Norway Sweden Finland Estonia	68 ± 9	18 ± 5***	16 ± 8***	2 ± 2	2 ± 1	5	3
3	Bulgaria Croatia Slovenia	60 ± 5	16 ± 5***	14 ± 6***	16 ± 5***	8 ± 3	5	4
4	UK Ireland	7 ± 4 §§	43 ± 8	13 ± 5 §§	41 ± 5	9 ± 3	4	4
5	central and northern Germany	26 ± 6§§	53 ± 6	15 ± 7§§§	7 ± 3§§§	2 ± 1	6	5
6	Austria Switzerland	25 ± 8	44 ± 7	25 ± 9	7 ± 3	3 ± 2	6	4
7	Netherlands Belgium northern France	46 ± 15	34 ± 9	19 ± 9	33	11 ± 5	6	1

TABLE 7. Distribution of Borrelia genospecies in different parts of Europe. 7 different groups were defined as described in the text. The mean percentages ± SEM of positive ticks per species are given. Statistics refer to the species with the highest mean percentage (group 1-3: B.a., * and group 4,5: B.g., §). # Rec agb, number of records for B.a., B.g., B.b. and n.t.; # Rec v, number of records for B.v.

As mentioned above, some studies only distinguished between the three genospecies *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s.. Since *B. valaisiana* and untypable ticks were also included in Table 7, this may result in a disproportion in the ratio of the first three species. Due to the low number of records it was not possible to compare only studies which examined all 4 species. Therefore, we repeated the analysis and excluded untypable and *B. valaisiana* positive ticks, allowing a direct comparison of *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s.. No significant difference in the results was seen compared to the results in Table 7 (data not shown).

4.5 Discussion

A metaanalysis provides a versatile alternative to the more traditional review methods, and allows quantitative conclusions to be drawn. The prevalence of Borrelia infection in ticks is one of the most essential components of risk assessment in LB. In recent years, many studies concerning the infection rate of ticks with Borrelia have been reported. The analysis of data from 155 records of studies conducted in Europe showed an overall mean infection rate of 13.6%, with a significantly higher infection rate of adult ticks than of nymphs, which was seen in the majority of studies, and is explained by host-seeking adult ticks having had two blood meals on different hosts. These data are in line with those reviewed by others [70, 85].

The correlation of infection rates of adult ticks with the coordinates of sampling sites showed a significant trend, increasing from western to eastern Europe. No effect was seen for nymphal ticks. The effect of longitude on *B. burgdorferi* s.l. infection rates of unfed *I. ricinus* ticks was also examined by Gray et al. in 1998. They reported that infection rates for both, nymphal and adult *I. ricinus* ticks were significantly higher in eastern Europe than in the west. [70]. In contrast to our analyses they restricted the area to the coordinates from 5° W to 20° E. Indeed a restriction of our data to this area also led to a significant increase in infection rates of nymphs (7 to 15%, calculated from regression, $p < 0.05$) from western to eastern Europe. The map in Figure 11 shows that the regions with the highest infection rates are located in Central Europe (Austria, Czech Republic, Southern Germany, Switzerland, Slovakia and Slovenia). These high infection rates in Central Europe combined with the large number of studies carried out in these regions, probably led to the overall trend from western to eastern regions of Europe.

Including only studies which examined at least 100 adults and/or nymphs led to different results in only four of the regions, indicating that most of the values are robust. Since in these four regions only few records were available, they are vulnerable to outliers. In region “A” for example the decrease of infection rate is based on the loss of a single record with an infection rate of 75%. Therefore, more studies with a higher number of ticks examined are required in such regions to come to more robust results.

The available data did not allow analysis of the tick infection rate by years. Therefore, we compared two time periods (1986 to 1993 vs. 1994 to 2001). In the first of the two periods, ticks were examined by IFA, DFM or culture. From 1994 on, PCR – which is generally considered to be the most sensitive method – became the most commonly used detection method. Nevertheless, this did not lead to any differences of reported infection rates between the two periods. In the following, no distinction of the detection methods was made. Noteworthy, the highest infection rates reported for nymphs (> 25%) and adults (> 30%), respectively, were found only in the period of 1997 to 2001 and were all detected with PCR. Taken together, there is no indication for an increase in tick infection over time or by introduction of the more sensitive method of PCR.

In Europe there are at least three species known to be pathogenic for humans, i.e., *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s., the latter being the only species found in the United States [6]. As the different Borrelia species appear to be associated with different clinical manifestations, an accurate knowledge of the distribution of these species in Europe might be helpful. Analyses of data from 53 records showed a distinct pattern of Borrelia species distribution in Europe: *B. afzelii* and *B. garinii* are the most common species, followed by *B. valaisiana* and *B. burgdorferi* s.s.. *B. lusitaniae* was tested only in a few studies, but seems to be rather rare or nonexistent in most regions. The occurrence of *B. bissettii* was described once in combination with B. afzelii and *B. garinii* [79] in Slovakia. The prevalence of Borrelia genospecies in *I. ricinus* ticks seems to vary in different parts of Europe. This could be explained by the prevalence of different reservoir hosts, although considerably more data will be required before robust patterns can be determined and a correlation between ecological data and clinical treatment manifestations can be carried out.

Various studies showed, that specific associations exist between Borrelia species and reservoir hosts: *B. afzelii* is preferentially transmitted by small rodents and *B. valaisiana* and *B. garinii* are mainly associated with birds, though the latter genospecies is very heterogeneous and some strains preferentially infect ticks via rodents. *B. burgdorferi* s.s. seems to occur in both birds and rodents [78, 79, 96, 121, 123, 164]. This host-association can be explained by the specific effect of the host's complement system on each *B. burgdorferi* s.l. genospecies [124]. Direct comparison of questing nymphs and adults showed no significant difference in the Borrelia species distribution, suggesting similar hosts for larvae and nymphs.

13% of *I. ricinus* Borrelia positive ticks showed an infection with multiple genospecies. Such mixed infections in individual ticks can be explained by (i) superinfection of ticks, already infected transovarially, (ii) cotransmission of multiple Borrelia species from an infected to an uninfected tick feeding on the same host, (iii) cotransmission of several strains from a host infected by more than one Borrelia species, or (iv) consecutive infectious blood meals. In contrast to a questing nymph, which has only had one previous blood meal as larva, an adult has fed on two different hosts. Therefore, it appeared likely that adults would contain more mixed infections than nymphs, though this was not the case. A possible explanation could be that the effect of complement may occur not only in the host, which selects for Borrelia species, but also in the midgut of the tick by complement taken up during the blood meal, as discussed by Kurtenbach et al. [120, 124]. In fact, the most frequent combination of Borrelia species found in Europe occurred between *B. garinii* and *B. valaisiana*, both occuring most commonly in birds.

In conclusion, the prevalence of different *B. burgdorferi* s.l. genospecies was deduced for various regions in Europe. While the infection rate was about two fold higher in adult ticks compared to nymphs, no effect of detection method, tick gender or collection period (1986-1993 vs. 1994-2002) was found. This analysis should enable a correlation of the distribution of Borrelia species with the prevalence of certain clinical manifestations, e.g., for selective antibiotic treatment.

4.6 Acknowledgments

We thank Isabel Diterich and Markus Müller for helpful discussion, Sebastian Hoffmann for statistical help and Sonja von Aulock for revision of the manuscript.

5 Critical evaluation of urine PCR for Lyme Borreliosis

Carolin Rauter, Markus Mueller, Isabel Diterich, Sabine Zeller, Thomas Meergans and Thomas Hartung

Biochemical Pharmacology, Faculty of Biology, University of Konstanz

submitted to *Journal of Clinical Microbiology*

5.1 Abstract

Many approaches were made in recent years to establish urine PCR as a diagnostic tool for Lyme Borreliosis, but results are contradictory. In the present study, a standardized protocol spiking urine from healthy donors with a defined amount of whole Borrelia or Borrelia DNA was established. The development of a nested real-time PCR targeting ospA enabled a highly sensitive and quantitative analysis of these samples.

We could show that (i) storage of spiked urine samples for up to 6 months at -20°C had no negative effect on spike recovery, and (ii) centrifugation of 10 ml urine at 40,000 x g for 30 min resulted in a concentration of both spikes, i.e., whole Borrelia and DNA. (iii) The inhibition of DNA spike recovery in 48% (11/23) of urine samples tested could be attributed to nuclease activity. This was abrogated by alkalising the urine or by working on ice.

Despite optimized conditions, analysis of urine samples of 12 patients with Erythema migrans, the clinical stage considered to be associated with the highest bacterial load, revealed a positive result in only one sample. All 12 samples were negative using an alternative PCR targeting flagellin. The results of our study support doubts that urine is a suitable material for diagnosis of Lyme Borreliosis.

5.2 Introduction

Diagnosis of Lyme Borreliosis (LB) is currently based on clinical criteria confirmed by serological tests. However, serology has a number of limitations such as serological cross-reactivity, the delayed production of antibodies in early stages of LB and the inability to distinguish between ongoing and previous infections due to the persistence of antibodies. In patients with ongoing or recurring symptoms after antibiotic therapy it is often impossible to definitely attribute clinical symptoms to a persisting infection with *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.), the causative agent of LB. A way out of this dilemma might be the direct detection of this pathogen in clinical samples, such as the detection of Borrelia DNA by polymerase chain reaction (PCR). The use of urine as a sample material is an attractive approach, since urine is obtained without invasive procedures.

Since 1991, when the detection of Borrelia DNA in urine was shown for the first time by Goodman et al. [67], many studies have been carried out (for review see [44, 127, 200]). A comparison between the different reports is nearly impossible because of significant differences in patient selection, study design, DNA extraction and PCR methods. Nevertheless, a metaanalysis of urine PCR assays for diagnosis of LB showed an overall sensitivity of 68% (range 13 to 100%) and a specificity of 99% (range 95 to 100%) [44].

Even when comparing only reports on urine of patients with EM, who clearly have an active infection, detection of Borrelia DNA in urine ranged between 13% and more than 90% [1, 16, 129, 150, 199, 201]. A caution was issued by Brettschneider et al. who were not able to confirm the presence of Borrelia DNA of the 27% PCR-positive urine specimens by hybridization or sequencing [20]. Thus, despite all efforts the value of PCR as a diagnostic tool for LB and of urine as a sample material remains unclear and is discussed controversially [20, 45, 201].

Until now it is not clear whether urine of LB patients contains whole Borrelia or Borrelia DNA. There is accumulating evidence, that the urinary tract is a site of persistent infection in most animals, since various studies demonstrated the presence of spirochetes in the urinary bladder tissue in experimentally and naturally infected animals [66, 83, 130, 205]. However, culture of spirochetes from animal urine failed [11, 98, 205], or was successful only very rarely [66]. Maiwald et al. postulated that not whole Borrelia but rather soluble or protein-bound DNA is excreted in the urine [142] as the Geneclean®kit worked best for sample preparation. However, this conclusion was based on the false premise that this procedure involves no protein denaturing, but the chaotrophic salt NaI is added for sample preparation.

Only few studies quantified *Borrelia burgdorferi* s.l. DNA in clinical specimens: The number of spirochetes in a 2 mm skin biopsy of 50 patients with Erythema migrans (EM) ranged from 10 to 10,000, with a median of 1,450 spirochetes [138] and in 5 synovial fluid samples the number varied from 20 to 41,000 per ml [204]. For urine no proper quantification is available, but Goodman et al. calculated an equivalent of 50 to 5,000 organisms per ml urine by extrapolation of signal intensities [67]. Hence, the number of spirochetes in clinical specimens seems to be rather low and procedures that concentrate the number of organisms in a given sample and that display high sensitivity are imperative.

Furthermore, urine appears to contain inhibitory factors. Urea inhibits the PCR reaction in concentrations of 50 mM and higher [13, 115]. Given that the normal concentration of urea in adult urine is about 330 mM it is clear that the method chosen for DNA extraction plays an important role. Until now this inhibitory effect has only been analyzed on a qualitative, not quantitative basis. In the present study, we establish an optimized, quantitative, nested real-time PCR to determine the diagnostic potential of PCR with urine samples spiked with whole Borrelia and Borrelia DNA and apply this method to patient samples.

5.3 Materials and Methods

5.3.1.1 Isolation of DNA.

DNA extraction was performed as described elsewhere [16]. Briefly, 1 ml DNAzol (Invitrogen, Karlsruhe, Germany) and 6 µl of a polyacrylcarrier (Fermentas, St. Leon-Rot, Germany) were added to each sample. DNA was precipitated with ethanol, centrifuged at 17,500 x g for 20 min, and washed twice with 70% ethanol according to the manufacturer’s instructions. The final pellet was dissolved in 100 µl Tris/EDTA (TE) buffer. 10 µl were used as DNA template for PCR.

5.3.1.2 Nested real-time PCR

For amplification of *B. burgdorferi* s.l. DNA, a nested PCR system with primer pairs targeting the outer surface protein A (ospA) was established as a standard PCR, followed by a real-time PCR. The first PCR amplification was performed in a volume of 50 µl containing 2 mM $MgCl_2$, 0.2 µM of each primer, 1 x PCR-Buffer, 0.2 mM desoxynucleoside triphosphate (all PE Applied Biosystems, Weiterstadt, Germany), 1 U of *Taq* DNA polymerase (Eppendorf, Hamburg, Germany) and 10 µl extracted DNA. The sequence of the outer primer pair (Thermo Hybaid, Ulm, Germany) was 5’ ATg AAA AAA TAT TTA TTg gg and 3’ CTg TTg ATg ACT TgT CTT T, resulting in a 347-bp product. PCR was performed in a GeneAmp PCR System 2400 (PE Applied Biosystems) using the following protocol: initial denaturation at 95°C for 2 min followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 40 s, elongation at 72°C for 18 s and a final extension at 72°C for 10 min. After amplification 1 µl of the first PCR served as template for the second PCR, which was performed as real-time PCR in a LightCycler® rapid thermal cycler system (Roche Diagnostics GmbH, Mannheim, Germany). Real-time PCR was performed as described previously [182]. The use of the LightCycler® software 3.5 enabled quantification by import of an external standard curve. An alternative PCR was performed according to Schmidt et al., with primers targeting the *B. burgdorferi* s.l. flagellin gene [199].

In each step (DNA extraction, outer and inner PCR), negative controls containing water instead of DNA were included to exclude cross-contamination. The *B. afzelii* strain VS461 was used as a positive control. Positive controls contained 5 Borrelia per µl template in the first PCR and 100 Borrelia per µl template in the real-time PCR. To avoid cross-contamination and sample carry-over, pre- and post-PCR sample processing and PCR amplification were performed in separate rooms and cotton-plugged pipette tips were generally used.

5.3.2 Treatment of patient urine samples

Midstream urine samples from 12 patients with clinically diagnosed Erythema migrans (EM) were collected by a general practitioner and frozen immediately at –20°C. Urine samples were obtained before antibiotic therapy. The samples were transported frozen and stored at –20°C until use. Urine samples were thawed at 4°C over night and 10 ml were centrifuged at 40,000 x g and 4°C for 30 min. DNA extraction was performed with the pellet, as described above.

5.3.3 Spiked urine samples

The efficacy of the DNA preparation was evaluated by extraction and amplification of experimentally spiked urine or water. Midstream urine specimens were collected from healthy donors. If not explicitly mentioned, 500 whole Borrelia or 1 pg DNA (equivalent to 500 Borrelia) were added to 50 µl urine or water. The extracted DNA was eluted in a volume of 100 µl TE, resulting in a theoretical concentration of 5 Borrelia equivalents per µl eluate.
For spike experiments the *B. afzelii* strain VS461 was used. It was cultured as described previously [42] and was kindly provided by Dr. T. Kamradt (Berlin, Germany). Culture density was determined by microscopy using a modified Thoma counting chamber (Merck Eurolab, Ismaning, Germany). DNA from Borrelia culture was extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was quantified photometrically (BioPhotometer, Eppendorf). Two fg DNA were regarded equivalent to one Borrelia [129].

5.3.4 Detection of nuclease activity

For detection of nuclease activity in urine, 500 ng of the plasmid DNA (pJuFo [35]) was incubated at room temperature with urine or water. After different incubation times, the DNA was resolved on 1% agarose gels by electrophoresis and visualized under UV light with ethidium bromide.

5.3.5 Chemical characterization of urine

Urine samples were characterized using test strips (Multistix® 10SG, Bayer, Fernwald, Germany), urea kit (Urea Nitrogen, Sigma Diagnostics, Deisenhofen, Germany) and magnesium kit (Magnesium Merckotest®, Merck, Darmstadt, Germany) according to the manufacturer's instructions.

5.4 Results

5.4.1 Nested PCR of Borrelia

We previously described a real-time PCR, which offers the advantage of quantification and differentiation of Borrelia species [182]. As only low amounts of Borrelia DNA are expected in clinical samples, the real-time PCR was modified to a nested PCR, by preceeding it with a 20-cycle standard PCR. With this modification, we were still able (i) to distinguish between the different Borrelia species *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. by melting curve analysis (data not shown) and (ii) to carry out a quantitative analysis as shown in Figure 16. Starting with 31.6 Borrelia per µl, DNA was diluted in steps of 1:√10 in triplicates. The nested PCR decreased the detection limit of our original real-time PCR [182] by a factor of 10 to 0.1 Borrelia per µl (i.e., 1 Borrelia per 10 µl template employed in the standard PCR) (Fig. 16).

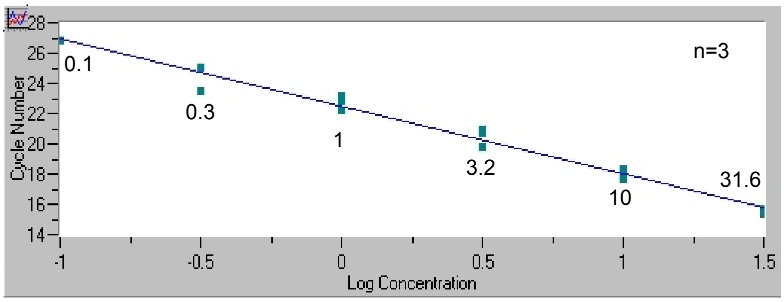


FIG. 16. Quantification of nested real-time PCR. Triplicates with concentrations of 0.1 to 31.6 Borrelia per µl were analyzed. 0.1 Borrelia per µl template was detected only once, 0.3 Borrelia per µl twice.

Since it is not clear whether urine of LB patients contains Borrelia DNA or whole Borrelia, most of the following experiments were carried out with both. To eliminate possible inhibitors of PCR from the urine, we performed DNA extraction using a guanidin-detergent lysing solution (DNAzol) combined with a polyacrylamide carrier, as recently described by Bergmann et al. as the only DNA extraction method resulting in positive results in urine of LB patients [16].

To determine the variance of our nested real-time PCR between different experiments we determined the standard deviation of the threshold cycle numbers (ct-values) of positive controls from 16 independent experiments. Positive controls of DNAzol extraction, which ran through the entire procedure, showed a standard deviation of ct-values of 0.81.

Variance within one experiment was determined by 5fold preparation of a positive control (containing 500 Borrelia). Before each step (DNAzol, block PCR and LC PCR, respectively) one of the 5 samples was split into 5 replicates and treated as separate samples in the next steps (Fig. 17). The coefficients of variation of the samples of each step were 40, 32 and 4% for DNA extraction, standard PCR and real-time PCR, respectively. These data suggest that DNA extraction contributes more to the variance of results than PCR.

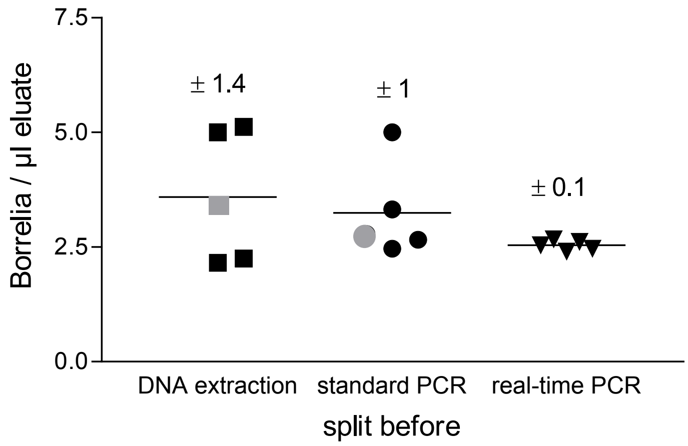


FIG. 17. Variance within one experiment. DNA of 5 identical samples, each containing 500 Borrelia equivalents, was extracted with DNAzol. After DNA extraction, one of the 5 samples (grey square) was split into 5 and the resulting 9 samples were inserted in the standard PCR. Before real-time PCR was performed, one of the samples (light grey sphere) was again split into 5. All 13 samples were quantified in the same real-time PCR run.

5.4.2 Spike experiments

To investigate whether there is inhibition of PCR by urine components, dilution series of Borrelia and Borrelia DNA in water were compared to dilution series in urine samples of two healthy donors (urine A and B). 50 µl water or urine were spiked with different amounts of DNA. DNAzol was added after an incubation time of 10 min at room temperature. After extraction with DNAzol, DNA was eluted in 100 µl TE. Nested real-time PCR was performed and the spike recovery was determined. No difference was seen between the detection limit of Borrelia DNA in water and urine A. In both cases, the recovery of a spike of 10 Borrelia equivalents (i.e., 0.1 Borrelia per µl eluate) was possible. However, in urine B, only the highest spike (i.e., 1,000 Borrelia equivalents or 10 Borrelia per µl eluate) could be detected (Fig. 18). This inhibitory effect was not seen when urine B was spiked with whole Borrelia instead of DNA (data not shown), indicating that inhibition was not due to direct inhibition of the PCR reaction by components of the urine transferred into the PCR.

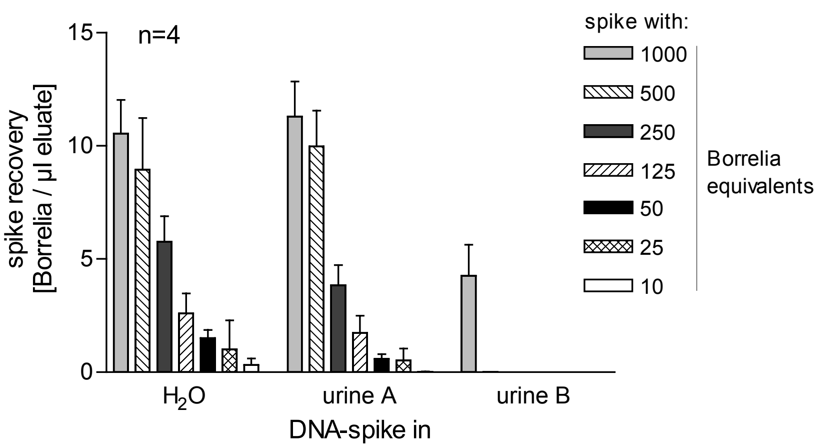


FIG. 18. Dilution series of Borrelia DNA in water or urine samples (A and B) from two healthy donors. DNA equivalents of 1,000, 500, 250, 100, 25 and 10 Borrelia were added to 50 µl water or urine, which should result in concentrations of 10 down to 0.1 Borrelia per µl eluate. Data are depicted as means +/- SEM from quadruplicates.

To investigate, whether the inhibitory effect on DNA recovery could also be seen in other urine samples, we spiked urine from healthy donors with 500 Borrelia equivalents of DNA each. The extracted DNA was eluted in a volume of 100 µl, resulting in a theoretical concentration of 5 Borrelia per µl eluate. Figure 19 shows the spike recovery in different urines in descending order. Definition of one Borrelia per µl eluate as the cutoff (corresponding to a 20% recovery of the spike), revealed inhibitory effects in 11 of 23 urines (48%).

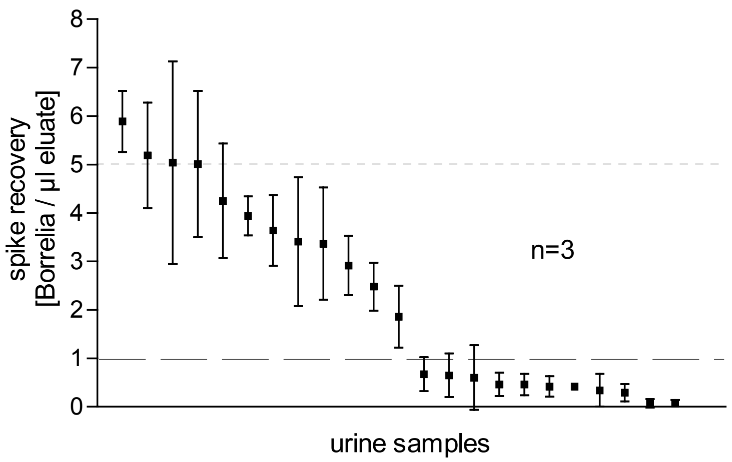


FIG. 19. Inhibitory effect of urines. Urine samples from 23 donors were spiked with 500 Borrelia equivalents each, which should lead to detection of 5 Borrelia per µl eluate (dotted line). Three aliquots of each urine sample were spiked and DNA was extracted independently. If the spike recovery was lower than one Borrelia per µl eluate (dashed line), the urine was defined as inhibitory. Values shown are means +/- SD.

Since it is well known, that one of the main inhibitors of PCR in urine is urea [13, 115], we measured the urea concentration of every urine sample and compared it to the respective spike recovery rate, but no correlation was evident (data not shown). This result was not surprising, as DNA extraction with DNAzol could eliminate even high concentrations of urea, as shown by spiking urea solutions (0 mM to 1 M) with Borrelia DNA. These solutions were either employed directly in the PCR or the DNA was extracted with DNAzol before PCR. Whereas in the first case an inhibitory effect of the urea was seen already at 50 mM, extraction with DNAzol enabled spike recovery even at 1 M urea (data not shown). In contrast to other characteristics of urine, such as high amounts of phosphate, magnesium, protein or a high osmolality, which did not correlate with disturbed spike recovery (data not shown), the inhibitory effects of urine could be correlated to a pH between 6 and 6.5 (Fig. 20).

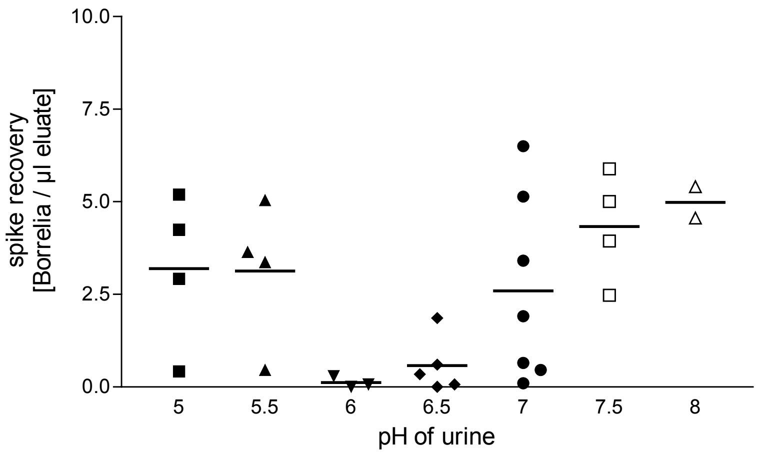


FIG. 20. pH dependence of spike recovery. 29 urine samples from 23 donors were spiked with DNA of 500 Borrelia and the spike recovery was correlated to the pH of the respective urine.

The inhibitory influence of pH 6 or 6.5 could be abrogated by adjusting its pH to 8 with NaOH before the spike was added. In each of the 11 samples tested this resulted in a positive spike recovery (Fig. 21). In addition, the inhibitory effect was also no longer observed, if urine samples were kept on ice until DNAzol was added (Fig. 22.a). Under these conditions the influence of pH disappeared (Fig. 22.b).

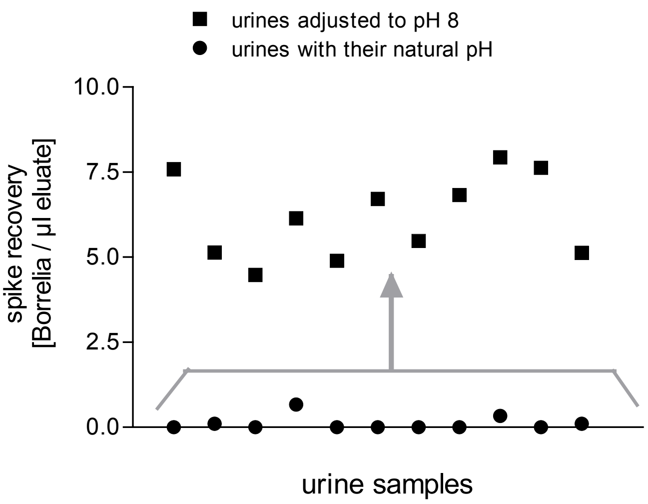


FIG. 21. pH influence on spike recovery. The spike recovery of 11 inhibitory urine samples was compared to the recovery in the same urine samples adjusted to a pH of 8.

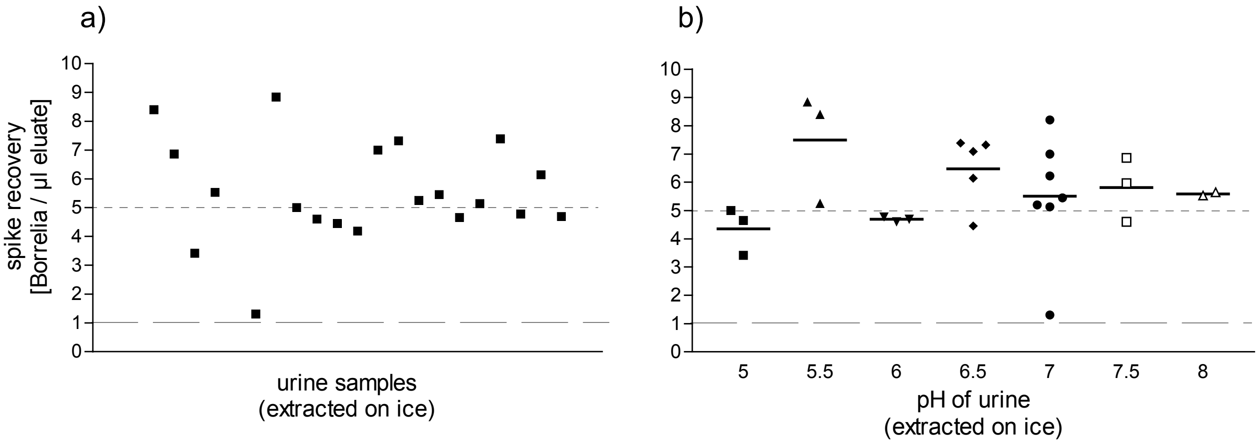


FIG. 22. a). Spike recovery in 20 of the 23 urine samples from Figure 19. The experiment was performed as in Figure 19, with the exception that urine was left on ice until DNAzol was added. Urine samples are plotted in the same order as in Figure 19.
b). Same approach as in Figure 20 with 27 urine samples and the exception that urine was kept on ice until DNAzol was added.

These results indicated that active nucleases, known to exist in urine [99], were responsible for the inhibitory effect on DNA spike recovery. To prove this, purified plasmid DNA was incubated with an inhibitory urine sample for one hour at room temperature. Complete degradation of the plasmid DNA occurred as seen by ethidium bromide staining, which did not occur if the pH of the urine was shifted to 8 or if bivalent ions were chelated with 10 mM EDTA before plasmid DNA was added (data not shown). Both experiments substantiate nuclease activity in the original urine sample. The intensity of degradation of plasmid DNA could be correlated with the level of spike recovery. This was demonstrated in three different inhibitory urines and three urines with good spike recovery (data not shown).
Figure 23 demonstrates the time (Fig. 23.a) and pH (Fig. 23.b) dependency of nuclease activity. In experiments shown in Figure 23.a, plasmid DNA was incubated with two urine samples (one urine with a good spike recovery and one strongly inhibitory urine) for different times at room temperature. The degradation of plasmid DNA, incubated with a urine sample with good spike recovery started after 25 minutes, whereas degradation of DNA incubated with the inhibitory urine sample could be seen immediately. To demonstrate pH dependency, the inhibitory urine sample was adjusted to pH values between 5 and 9 before plasmid DNA was added and incubated for 10 minutes at room temperature. The gel shows that the greatest degradation effect occured in urine with a pH between 6 and 7. No degradation was seen in urine with pH 5 or pH 8 and higher (Fig. 23.b).

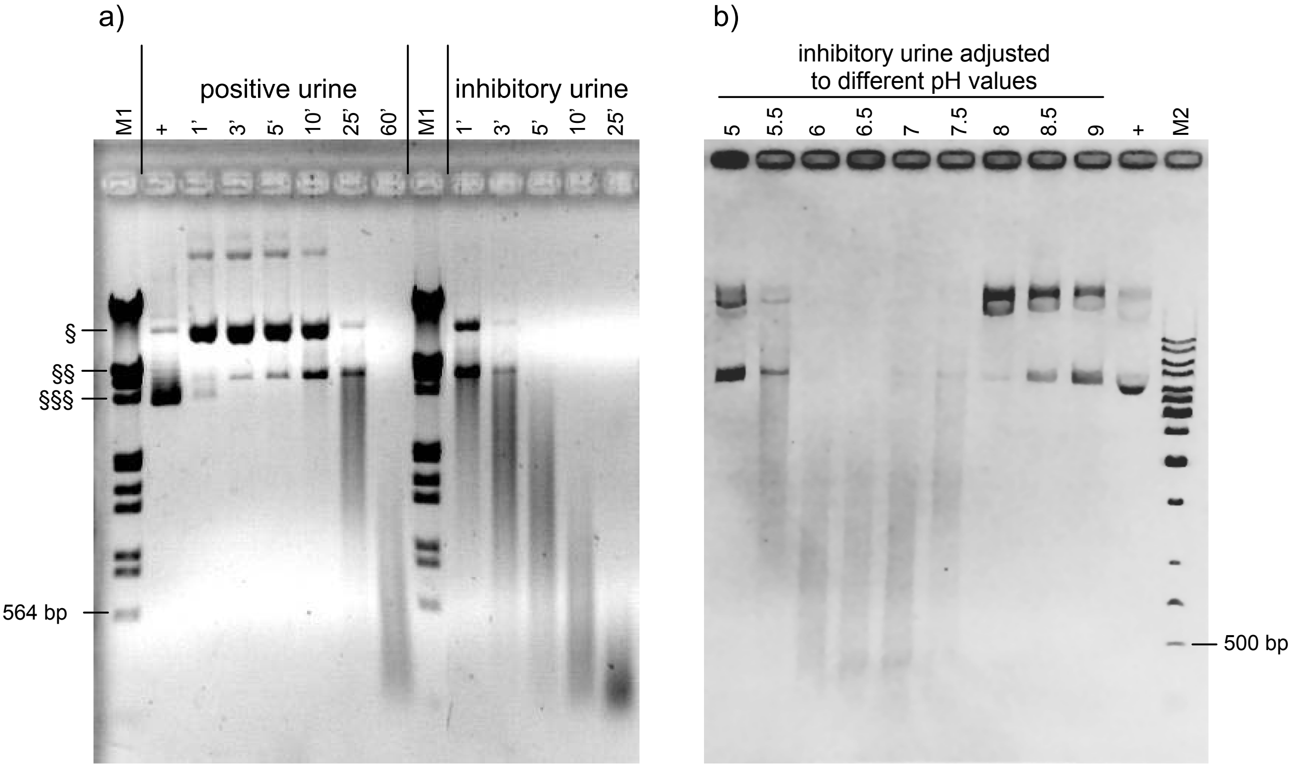


FIG. 23. a) Time dependency of nuclease activity. pJuFo plasmid was incubated with a previously tested urine with excellent spike recovery (positive urine) and an inhibitory urine sample for different times (given in minutes) at room temperature. EcoRI/HindIII digested Lambda DNA was used as marker (M1). b) pH dependency of nuclease activity. pJuFo plasmid was incubated for 10 min at room temperature with an inhibitory urine which was adjusted to different pH values. The marker is a 1kb ladder (M2).
As positive control (+), water instead of urine was spiked with plasmid. § = relaxed circular, §§ = linearized and §§§ = supercoiled form of plasmid.

5.4.3 Storage of urine

Bergmann et al. [16] reported, that storage of frozen urine samples for more than three months increased the inhibitory nature of the urine. We tried to assess this observation quantitatively. We froze triplicates of spiked urine samples (50 µl urine, which was inhibitory in previous tests, spiked with 500 Borrelia DNA or whole Borrelia) at different time points over a 6-month period. All aliquots were thawed on ice to avoid nuclease activity and extracted and amplified in parallel. No significant difference in spike recovery was seen (i) between the spiked water and urine, (ii) over the time, or (iii) between Borrelia DNA and whole Borrelia (Fig. 24). Thus, there is no indication for a decline in Borrelia- or Borrelia DNA-recovery within a 6-month observation period in our experimental setting.

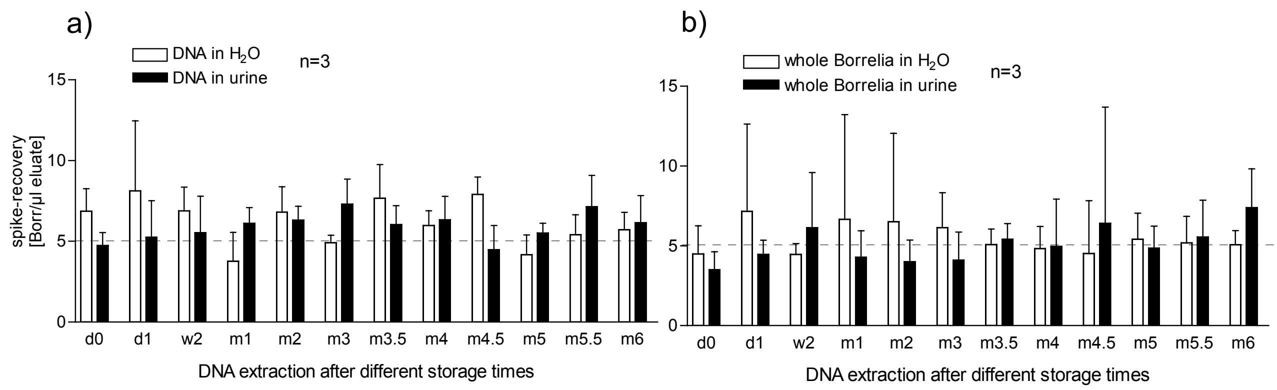


FIG. 24. Influence of storage time on spike recovery. 50 µl inhibitory urine from a healthy subject was spiked with DNA equivalent to 500 Borrelia (a) or with whole Borrelia (b) at different time points in triplicates and stored at –20°C. After 6 months DNA extraction of all the triplicates and all the time points was carried out. Bars correspond to triplicates ± SD and the dotted line to the expected value of 5 Borrelia per µl eluate. d = day, m = month, d0 = without freezing.

5.4.4 Effect of centrifugation

Many publications adressing urine PCR try to concentrate samples before DNA extraction by centrifugation of several ml of urine [16, 39, 140, 201], but so far it has not been proven whether centrifugation really improves the sensitivity. To examine a possible effect of centrifugation quantitatively, two 10 ml aliquots each of different urine samples or water were spiked with 5,000 Borrelia equivalents (Fig. 25.a) or 5,000 whole Borrelia (Fig. 25.b), respectively.

One of the two aliquots was centrifuged for 30 min at 40,000 x g and 4°C. The supernatant was discarded as completely as possible and the pellet was submitted to DNA extraction. The pellet was stored on ice until addition of DNAzol. Assuming a yield of 100% of the initial value, one should find 50 Borrelia per µl eluate after DNA extraction. From the second aliquot 50 µl were submitted directly to DNA extraction. These should contain 25 Borrelia, resulting in 0.25 Borrelia per µl eluate. Figure 25 shows the positive effect of centrifugation on all urine samples tested, both for detection of DNA and whole Borrelia. No such concentration effect was seen when water instead of urine was spiked with DNA. It is remarkable, that the concentration effect was highly different in the various urine samples. By repeating the experiment with similar results, we could show that this effect did not stem from the sample preparation but rather from variances in the different urine samples. Compared to samples spiked with DNA, centrifugation of samples spiked with whole Borrelia showed a greater concentration effect in all cases.

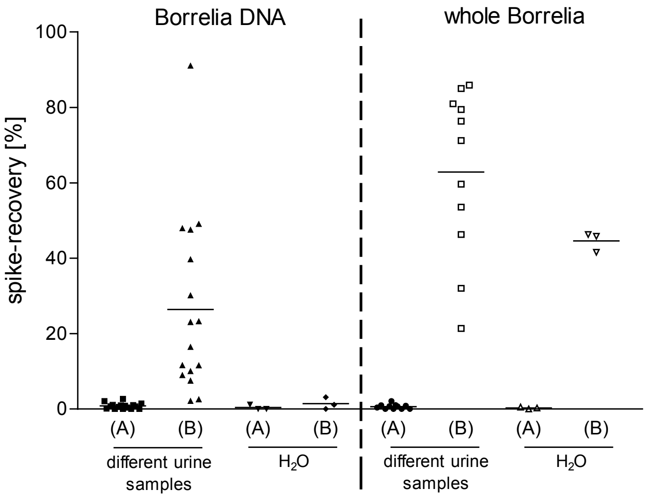


FIG. 25. Effect of urine centrifugation. 10 ml of urine or water were spiked with 5,000 equivalents of Borrelia DNA or 5,000 whole Borrelia. Either 50 µl of 10 ml spiked urine or water (A) were submitted to DNA extraction, or 10 ml urine or water were centrifuged at 40,000 x g for 30 min and the pellet was then submitted to DNA extraction (B).

Comparison of the spike recovery with the specific density of the respective urine showed a positive correlation (Fig. 26). No such correlation was seen for recovery of whole Borrelia (data not shown).

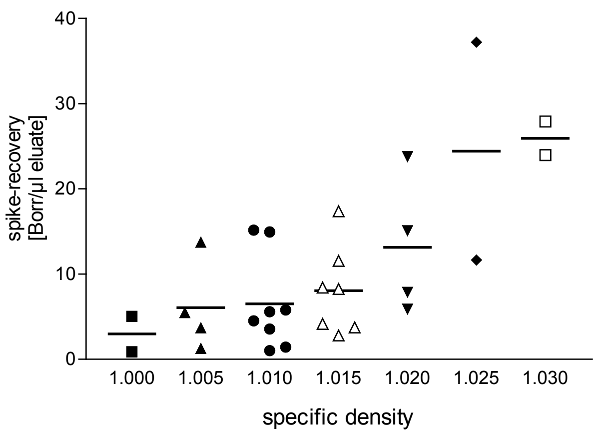


FIG. 26. 10 ml of 29 urine samples from 23 donors were spiked with DNA from 5,000 Borrelia and the spike recovery rate was set into relation to the respective specific density of each urine sample.

5.4.5 Urine samples from Borreliosis patients

Urine from 12 EM patients was collected by a practitioner and stored at –20°C. To prevent potential nuclease activity, urine was thawed on ice, and kept on ice until addition of DNAzol. DNA was extracted from 10 ml urine of each patient and tested in the real-time nested PCR. Borrelia DNA was only detected in one patient sample (the Borrelia genospecies was *B. afzelii*). Since Schmidt et al. were able to detect Borrelia DNA in the urine of more than 90% of patients with EM, we repeated DNA amplification using the nested PCR based on Borrelia flagellin according to Schmidt et al. [199]. With this PCR no Borrelia DNA was detected in any of the 12 urine samples, although the positive control was detected readily. The characterization of the urine samples from the patients resulted in pH values between 5.5 and 7.5 (3 x 5.5, 2 x 6, 3 x 6.5, 2 x 7, 2 x 7.5). The urine which was positive in the ospA PCR had a pH of 6.5.

5.5 Discussion

To evaluate urine PCR as a diagnostic tool in LB, we established a standardized protocol spiking urine samples from healthy donors with a defined amount of whole Borrelia or Borrelia DNA. The use of a nested real-time PCR enabled a quantitative analysis of these spiked samples with a sensitivity of 0.1 to 1 Borrelia per 10 µl template.

As it is still unclear, whether whole Borrelia or Borrelia DNA are present in the urine of LB patients, we performed spike experiments with either. The reported increase of inhibitory qualities of urine samples from healthy donors which were stored for a period longer than three months [16] could not be confirmed in our study, regardless whether we spiked urine with whole Borrelia or DNA. In contrast to Bergmann et al. we spiked urine samples before freezing and used the same urine sample for every time point. Since Bergmann et al. used different urine samples for the different storage times, it appears that the increase in inhibition was not due to long-term storage but rather variances of individual samples.

Centrifugation of spiked urine samples resulted in improved detection of both whole Borrelia and DNA and is therefore advisable. It was surprising that the concentration effect was also seen for the DNA spike, as soluble DNA does not sediment in aqueous solution at 40,000 x g. Probably, the DNA binds to or is dragged down by particles in the urine (e.g. crystals or cell debris). In line with this assumption, urine samples with a higher specific density also showed a higher concentration effect upon centrifugation, pointing to a co-precipitation effect. Therefore, centrifugation of greater volumes of urine with low specific density might increase the amount of Borrelia DNA recovered.

Spike recovery was inhibited in nearly 50% of urine samples from healthy donors spiked with Borrelia DNA. Addition of plasmid DNA and visualization on agarose gels demonstrated that the inhibitory effect was due to degradation of the DNA. This implied an enzymatic activity as no apparent degradation of the plasmid took place in water as expected. DNase I, which is the major nuclease present in urine, is a Ca^{2+} and Mg^{2+} dependent endonuclease, with an optimal activity at pH 6.5 [99]. By adjusting inhibitory urine samples to pH 8, adding EDTA or keeping them on ice, we could abrogate the degradation and demonstrate that it was due to nuclease activity in the urine.

We transferred the optimized protocol to the detection of Borrelia DNA in samples from patients with an EM. This patient group has an acute infection, therefore detection of Borrelia DNA in the urine should be most likely. The urine samples were frozen immediately and thawed on ice to prevent degradation of possible Borrelia DNA. To concentrate the amount of DNA or Borrelia, 10 ml urine were centrifuged and nested real-time PCR was performed to increase sensitivity. Despite these optimized conditions, only one of the urine samples from the 12 tested LB-patients revealed in a positive PCR result. The PCR based on flagellin was not even positive for this donor.

Assuming that whole Borrelia are present in the urine of patients, a negative result of urine PCR could be only due to an extremely low count or absence of Borrelia in the given specimen. With the nested real-time PCR, we were able to detect 1 Borrelia in 10 µl template. Hence, the concentration in the eluate was 10 Borrelia per 100 µl eluate. In our spike experiments with whole Borrelia the average yield by centrifugation of 10 ml urine was about 63% (Fig. 25). Therefore, 2 Borrelia per ml urine would be required to result in a positive PCR. This sensitivity thus exceeds the reported estimate of 50 to 5,000 Borrelia per ml urine [67].

An average of 1,450 spirochetes were detected in 2 mm biopsies [138]. Therefore the number of Borrelia in an EM with a diameter of 10 cm and an equal distribution of spirochetes would be around 4×10^6. Assuming (i) a stable number of Borrelia in the EM, (ii) a generation time of Borrelia of 12 hours [7] and (ii) a urine volume of 1.5 l per day, already 0.04% of these Borrelia passing into the urine, would lead to a positive PCR.

Since it is questionable whether whole Borrelia can pass through an intact kidney barrier, it would be likely that Borrelia which reach the urine only derive from a persistent infection of the urinary tract – an assumption which is not likely at least in such an early stage of LB as an EM.

Assuming that the urine of LB patients contains Borrelia DNA, negative results could be due to a low amount of DNA or the presence of nucleases, whereas the first point may result from the second. As in the case of DNA the average yield by centrifugation of 10 ml spiked urine was only about 26% (Fig. 25), 0.08% DNA equivalents reaching the urine of the assumed number of 4×10^6 Borrelia in an EM would result in a positive PCR.

The kidney plays a role in the clearance of plasma DNA and the human kidney barrier is permeable to DNA molecules [19, 224]. In an experiment on laboratory mice only 0.06% of s.c. injected DNA was excreted into the urine within 3 days in a polymeric form [19]. Thus it appears unlikely that sufficient Borrelia DNA for a positive PCR result woud be contained in 10 ml urine.

Addition of EDTA to the samples immediately after collection inhibit nuclease activity in human urine, but DNA destruction due to nuclease activity in the bladder could not be ruled out. The predominant fraction of DNA fragments found in human urine were 150 bp to 250 bp [19, 224]. This is in line with our results, where the nuclease activity on plasmid DNA led to no complete degradation (Fig. 22). Since the amplicon of our standard PCR is about 350 bp in length, most of the DNA fragments would be too short for amplification. But it could be that Borrelia DNA may be bound to proteins and may thus be protected from nucleases better than the naked DNA used in our experiments. If urine of LB patients did contain Borrelia DNA, improved detection by inhibition of nuclease activity already in the bladder might be attained by systematic adjustment of the pH in the urine to at least pH 8 by bicarbonate mediated alkalization of urine *in vivo*.

Taken together, we have developed an optimized, well-controlled and highly sensitive PCR protocol for the species-specific detection of Borrelia DNA. Extrapolations indicate that even with this protocol it is unlikely that Borrelia DNA would be detected in patient urine. In line we were only able to detect Borrelia DNA in the urine of one of twelve EM patients. The results of our study raise doubts that urine is a suitable material for diagnosis of LB.

5.6 Acknowledgments

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6 Summarizing discussion

Diagnosis of Lyme Borreliosis (LB) is currently based on clinical criteria confirmed by serological tests. However, serology still presents many problems since the commercially available and in-house tests vary considerably in their specificity and sensitivity and therefore do not meet the desirable standardized performance.

Limitations such as serological cross-reactivity, the delayed production of antibodies in early stages of LB and the inability to distinguish between ongoing and previous infections due to the persistence of antibodies lead to misdiagnosis and inappropriate treatment. In patients with ongoing or recurring symptoms after antibiotic therapy it is often impossible to definitely attribute clinical symptoms to a persisting infection with *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.). Therefore the development of new, reliable approaches in LB diagnostics is still a major challenge in this infectious disease. One valuable tool might be PCR as it enables a direct and sensitive detection of the pathogen.

In Europe there are at least three Borrelia species known to be pathogenic for humans, i.e., *B. afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto (s.s.), the latter one being the only species found in the United States [6]. The pathogenicity of other species is still unclear, although *B. valaisiana* and *B. lusitaniae* have been detected in skin biopsies of some LB patients [34, 188]. Increasing data indicate that particular clinical manifestations are associated with the different species of *B. burgdorferi* s.l. [4, 39, 228]. Therefore, detection of the genospecies in patients' specimens may allow a more systematic and accurate treatment of clinical manifestations. Another important point to address is the considerable polymorphism of antigen composition among the individual genospecies. Taking this into account, an interesting approach would be the use of different Borrelia antigens to detect antibodies in patient serum in the different endemic regions.

For humans, the risk to be infected with Borrelia depends on (outdoor) lifestyle, the density of tick populations as well as on the infestation of the ticks with Borrelia. Hence, data describing the prevalence of Borrelia in ticks can be used to assess the risk of LB to public health.

Different reservoir hosts seem to harbor different genospecies of *B. burgdorferi* s.l., which is explained by differential properties of the host's complement system, favoring certain genospecies [124]. Methods allowing a species-specific differentiation of *B. burgdorferi* s.l. could be used to receive an accurate knowledge of the geographic distribution of different genospecies within their tick vector. This may reveal not only clinical but also epidemiological and ecological impact.

6.1 Development of a real-time PCR

Methods for the detection and identification of genospecies of *B. burgdorferi* s.l. either use species-specific primers for PCR, or they analyze the PCR product by DNA sequencing, hybridization to species-specific probes, or by restriction endonuclease polymorphism. All these approaches are laborious and time-consuming. Therefore and for reasons discussed above a PCR which enables a simple and rapid differentiation of Borrelia species would be desirable.
The present work focused on the development of a real-time PCR based method which enabled (i) species differentiation in ticks and human specimens, and (ii) quantification of DNA amount for critical evaluation of urine PCR as diagnostic tool in LB. By using a HybProbe system we were able to distinguish Borrelia species by different numbers of mismatches in one single PCR run. The entire assay can be completed in approximately one hour and the software allows a quantification with an external standard curve.

6.1.1 Target selection and species-specific analysis

The ospA gene was used as the target of real-time PCR. This gene is located on a linear 49-kb plasmid and has been used as a PCR template in many studies [39, 58, 155, 230]. In synovial fluid, primer sets that amplify ospA plasmid DNA have generally been more sensitive than those that amplify chromosomal DNA, a phenomenon called target imbalance [158, 167]. To explain this, it has been postulated that *B. burgdorferi* s.l. in the synovium may shed blebs containing plasmids into joint fluid, but live spirochetes may not always be present there. Persing et al. concluded, that the most sensitive and reliable targets for PCR detection of *B. burgdorferi* s.l. lie on extrachromosomal elements and not on the genome itself [167].
The ospA gene of *B. burgdorferi* is genetically heterologous among the three major Borrelia species [238]. A study by Will et al. showed highly-conserved ospA within the *B. burgdorferi* s.s. group as well as within the *B. afzelii* group but heterogeneity within the ospA of *B. garinii* type strains [238]. Since the HybProbe system is extremely sensitive to changes in the sequence binding to the probe, misclassifications cannot be completely excluded.
The melting curve analysis allows the differentiation of Borrelia species even in a mixture of all three species. Thus, the method presented here appears to be the first LightCycler based PCR which allows the differentiation of the three Borrelia species *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* in one single PCR run.

6.1.2 Sensitivity

The real-time PCR assay described in the present work is sensitive enough to detect fewer than 10 spirochetes of each of the three clinically relevant genospecies of *B. burgdorferi* s.l. in a sample. Due to the low number of Borrelia in clinical specimens we decided to enhance sensitivity by development of a nested real-time PCR, preceding a standard PCR to the subsequent real-time PCR. To preserve the advantage of quantification, we performed the standard PCR with only 20 cycles not reaching the plateau of the reaction. Compared to the original real-time PCR we were able to decrease the detection limit to about one Borrelia per µl.

6.2 Epidemiological studies on ticks

To test the feasibility and reliability of the real-time PCR protocol for the amplification of *B. burgdorferi* ospA, we investigated the distribution of *B. burgdorferi* s.l. in ticks collected in Konstanz and determined the genomic groups. The results of this study were set in the context of data received from a metaanalysis of epidemiological studies on the prevalence and the distribution of genospecies of *B. burgdorferi* s.l. in host-seeking *I. ricinus* ticks in Europe based on a systematic literature review.

The analysis of data from 157 records of studies conducted in Europe showed an overall mean infection rate of 13.6%. Our study on ticks of 5 different regions around Konstanz showed, that they belong to the highest infected parts of Europe which seem to be located mainly in Central Europe.

In the region of Konstanz significant variability in the prevalence of *B. burgdorferi* s.l. among the sites examined was observed. Infection rates ranged from 20% to 57%. These considerable differences between the prevalence of infected ticks even in habitats in close proximity points out that the occurrence of *Borrelia* in nature is affected by many ecological factors. A very high infection rate (57%) was found at one of the five sites. Similar results of high infection rates in particular sites have also been described by others [32, 108, 185]. One possible explanation for this phenomenon could be that the transmission of the spirochetes to ticks is amplified by cofeeding on a vertebrate host, as has been proposed [63].

In line with the situation in european countries, a significantly higher infection rate of adult ticks than of nymphs was found. This can be explained by host-seeking adult ticks having had two blood meals on different hosts, in contrast to nymphs which have fed only once. These data are in line with those reviewed by others [70, 85].

A comparison of infection rates between two periods (1986-1993 vs. 1994-2002) by metaanalysis revealed no differences. Therefore, the reported increase of LB cases during the recent years can be rather explained by (i) a changed outdoor recreational activity of the population, (ii) an improvement of diagnostic methods, and (iii) better informed practitioners and public .

Analyses of data from 51 records with regard to Borrelia species distribution in Europe showed a distinct pattern: *B. afzelii* and *B. garinii* are the most common species, followed by *B.*

valaisiana and *B. burgdorferi* s.s.. *B. lusitaniae* was tested only in a few studies, but seems to be rather rare or nonexistent in most regions.

The prevalence of Borrelia genospecies in *I. ricinus* ticks seems to vary in different parts of Europe. This could be explained by the prevalence of different reservoir hosts, although considerably more data will be required before robust patterns can be determined and a correlation between ecological data and clinical trials can be carried out. By using melting point analysis, we were able to differentiate the three genospecies of Borrelia known to be pathogenic for humans in the ticks collected around Konstanz. All three genomic groups (*B. burgdorferi* s.s., *B. garinii* and *B. afzelii*) were found in ticks from the investigated sites. Our findings indicate that *B. afzelii* was present most abundantly, followed by *B. garinii*. *B. burgdorferi* s.s. appears to be less common. A similar distribution of Borrelia species is reported from the Czech Republic, Slovakia and further locations in South Germany.

Various studies showed, that specific associations exist between Borrelia species and reservoir hosts: *B. afzelii* is preferentially transmitted by small rodents and *B. valaisiana* and *B. garinii* are mainly associated with birds, though the latter genospecies is very heterogeneous and some strains preferentially infect ticks via rodents. *B. burgdorferi* s.s. seems to occur in both, birds and rodents [78, 79, 96, 121, 123, 164]. This host-association can be explained by the specific effect of the host's complement system on each *B. burgdorferi* s.l. genospecies [124]. Direct comparison of questing nymphs and adults in Europe showed no significant difference in the Borrelia species distribution, suggesting similar hosts for larvae and nymphs.

In Europe 13% of Borrelia positive *I. ricinus* ticks showed an infection with multiple genospecies. Such mixed infections in individual ticks can be explained by (i) superinfection of ticks, already infected transovarially, (ii) cotransmission of multiple Borrelia species from an infected to an uninfected tick feeding on the same host, (iii) cotransmission of several strains from a host infected by more than one Borrelia species, or (iv) consecutive infectious blood meals. In contrast to a questing nymph, which has only had one previous blood meal as a larva, an adult has fed on two different hosts. Therefore, it appears likely that adults would contain more mixed infections than nymphs, though this was not the case. A possible explanation could be that the effect of complement may occur not only in the host, which selects for Borrelia species, but also in the midgut of the tick by complement taken up during the blood meal, as discussed by Kurtenbach et al. [120, 124]. In fact, the most frequent combination of Borrelia species found in Europe occurred between *B. garinii* and *B. valaisiana*, both occuring most commonly in birds.

In Konstanz, mixed infections were found in 18% of the Borrelia positive ticks – mainly double infections by *B. afzelii* and *B. garinii*. Due to sequence similarity, *B. valaisiana* yields the same melting point as *B. afzelii* in our system and can thus not be distinguished. Therefore, it might be possible, that the seen double infections are rather combinations of *B. valaisiana* and *B. garinii*.

In conclusion, the prevalence of different *B. burgdorferi* s.l. genospecies was deduced for various regions in Europe performing a metaanalysis. While the infection rate was about two fold higher in adults compared to nymphs, no effect of detection method, tick gender or collection period (1986-93 vs. 1994-2002) was found. This analysis should enable a correlation of the distribution of Borrelia species with the prevalence of certain clinical manifestations, e.g., for selective antibiotic treatment.

We could demonstrate that the use of the new real-time PCR method provides a rapid and sensitive tool for differentiating *B. burgdorferi* s.l. species known to be pathogenic for humans and for their quantification. We have shown that the method can be applied for the detection and differentiation of Borrelia genospecies in ticks collected in the field. The novel method represents a versatile tool to assess the role of different genospecies in the pathophysiology of LB in Europe.

6.3 Urine PCR

The use of urine as a sample material for diagnostic is an attractive approach, since urine is easy to collect and is obtained without invasive procedures. Since 1991, when the detection of Borrelia DNA in urine was shown for the first time by Goodman et al. [67], many efforts have been made to establish urine PCR as a routine diagnostic tool in LB [1, 12, 16, 20, 39, 97, 114, 128, 129, 134, 140, 143, 150, 175, 177, 178, 199, 201, 202, 231]. But results are controversial and hence the value of PCR as a diagnostic tool for LB and of urine as a sample material remains unclear. Despite the ambiguous results in various studies and a lack of standardization, urine PCR is already used by many diagnostic laboratories.

By use of (i) a standardized protocol spiking urines from healthy donors with a defined amount of Borrelia or Borrelia DNA, (ii) the high sensitivity of the nested real-time PCR, and (iii) its ability of quantification, the usefulness of urine PCR in the diagnostic of Borreliosis was evaluated in the present thesis.

The urinary tract seems to be a site of persistent infection, as the presence of spirochetes in urinary bladder tissue has been shown in experimentally and naturally infected animals [66, 83, 130, 205]. In humans the situation is unclear. Borrelia antigens has been shown in the urine of a few patients with LB [98], but culture of spirochetes out of urine failed [11, 98, 205]. Maiwald et al. postulated that not whole Borrelia but rather soluble or protein-bound DNA is excreted in the urine [142]. However, their conclusion was based on a false premise. Consequently it is still unknown, whether whole Borrelia or Borrelia DNA are present in the urine of LB patients. Therefore, we performed spike experiments with both.

Urine is known to contain significant amounts of PCR inhibitors [13, 115]. For example, urea inhibits the PCR reaction already in concentrations of 50 mM [13, 115]. Given that the normal concentration of urea in adult urine is about 330 mM it is clear that the method chosen for DNA extraction plays an important role. A guanidin-detergent lysing solution (DNAzol) combined with a polyacrylamide carrier, was recently described by Bergmann et al. as the only DNA extraction method resulting in positive results in urine of LB patients [16]. We could show quantitatively, that extraction with DNAzol enabled purification of DNA even at 1 M urea.

The reported increase of inhibition in urine samples from healthy donors which were stored for a period longer than three months [16] could not be confirmed in our study (implying a correct handling of urine, e.g., thawing on ice), regardless if we spiked urine with whole Borrelia or DNA. In contrast to Bergmann et al. [16] we spiked urine samples before freezing and used the same urine for every time point. Since Bergmann et al. used different urines for the different storage times, it can be assumed that the increase of inhibition is not due to long-term storage but rather coincidence.

The number of spirochetes in clinical specimens seems to be rather low [67] and procedures that concentrate the number of organisms in a given sample and that display high sensitivity are imperative. We were able to demonstrate quantitatively that centrifugation of spiked urine samples resulted in a positive effect for both, whole Borrelia and DNA and is therefore advisable. Surprisingly, a concentration effect was seen for DNA, even though soluble DNA can not be centrifuged at 40,000 x g. A possible explanation would be that DNA is bound to particles or only dragged down by particles (like crystals or cell debris) in the urine. In line with this assumption, urines with a higher specific gravity showed a higher concentration effect by centrifugation. Therefore, centrifugation of greater volumes of urines with a low specific gravity might increase the amount of Borrelia DNA.

Despite the optimal extraction method, spike recovery was inhibited in nearly 50% of urine samples from healthy donors spiked with Borrelia DNA. We could demonstrate, that this inhibitory effect was due to nuclease activity in the urine. DNase I, which is the major nuclease present in urine, is a Ca^{2+} and Mg^{2+} dependent endonuclease, with an optimal activity at pH 6.5 [99]. By adjusting inhibitory urine samples to pH 8, adding EDTA or keeping them on ice, we could abrogate nuclease activity, resulting in a positive spike recovery.

Extrapolations indicate that even with our optimized protocol it is unlikely that Borrelia DNA would be detected in patient urine, regardless whether whole Borrelia or Borrelia DNA are present. In line we were only able to detect Borrelia DNA in the urine of one of twelve EM patients. Assuming that Borrelia DNA is present in the urine of LB patients, improved detection by inhibition of nuclease activity already in the bladder might be attained by systematic adjustment of the pH in the urine to at least pH 8 by intake of bicarbonate by the patient. Taken together, the results of our study enforce doubts that urine is a suitable material for diagnosis of LB.

7 Summary

In Europe at least three different Borrelia species are known to be pathogenic for humans. An interesting aspect of species classification is their correlation with epidemiological observations and clinical manifestations. Therefore, simple and rapid methods which enable a species-specific differentiation are more and more important for research as well as for diagnostic purposes.

In the first part of the thesis, the development of a method for quantification and simultaneous identification of different Borrelia species was carried out and its practical usefulness was tested by performing an epidemiological study in ticks.

- A real-time PCR based on a HybProbe system was developed, which enables differentiation of the three Borrelia species *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. in one single PCR-run. Species differentiation was also possible in a mixture of two and three Borrelia strains.
- A detection limit of 1 to 10 Borrelia per µl, sensitivity of 98% and specificty of 97% was reached. PCR results were highly reproducible.
- In an epidemiological study on 1,055 *I. ricinus* ticks, collected in the region around Konstanz a total infection rate of 35% was determined.
- Species-specific analysis of the positive ticks resulted in 70% *B. afzelii*, 34% *B. garinii* and 12% *B. burgdorferi* s.s.
- 18% of ticks showed a mixed infection with two or three species.
- Quantification of Borrelia DNA in ticks showed more than 100 Borrelia genome equivalents in 50% of the isolates.

The prevalence of Borrelia infection in ticks is an essential component of risk assessment. In the second part of the thesis (i) Borrelia infection and (ii) species distribution in *I. ricinus* ticks in Europe was determined performing a metaanalysis.

- Analyses of 155 records with data on infection rates of *B. burgdorferi* s.l. in *I. ricinus* ticks showed an overall mean of 13.6%, with a significantly higher infection rate of adult ticks (18.5%) than of nymphs (10.1%). No difference in infection rate was seen between females and males.
- Countries of Central Europe (Austria, Czech Republic, Southern Germany, Switzerland, Slovakia and Slovenia) showed the highest infection rates of *I. ricinus*.
- A comparison between (i) different detection methods and (ii) two time periods (1986 to 1993 and 1994 to 2001) did not show any differences of reported infection rates.
- Analyses of data from 53 records showed a distinct pattern of Borrelia species distribution in Europe: The mean percentages of *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana* and *B. lusitaniae* positive ticks, were 37%, 34%, 17%, 20% and 7%, respectively. 5% of the positive ticks were untypable.

- In 13% of the positive ticks a mixed infection was detected. The most frequent combination of Borrelia species occurred between *B. garinii* and *B. valaisiana*.
- Direct comparison of questing nymphs and adults showed no significant difference in the Borrelia species distribution.
- The prevalence of Borrelia genospecies in *I. ricinus* ticks seemed to vary in different parts of Europe.

Serology, which is the main diagnostic tool for Lyme Borreliosis to date, has various disadvantages. Therefore, many efforts have been attempted to develop alternative methods, e.g., PCR, which enables a direct detection of the pathogen. Despite the lack of standardization and ambiguous results in various studies, urine PCR, which is regarded as a desirable diagnostic tool, is already used by many diagnostic laboratories. To clarify this unsatisfactory situation, in the third part of the thesis the method of urine PCR was analyzed quantitatively by use of a highly standardized protocol.

- The already developed real-time PCR could be used as nested PCR, by preceding it with a standard PCR. Quantification and species differentiation was still possible. The detection limit could be decreased by a factor 10 to about 1 Borrelia per µl.
- Spike experiments in urine of healthy donors with whole Borrelia or Borrelia DNA showed (i) a concentration effect by centrifugation of several milliliter of spiked urine, as well as (ii) no negative effect of storage time at -20°C during a period of 6 months.
- Inhibitory effects of urine on DNA spikes could be correlated to the activity of nucleases in the urine. This inhibition could be abrogated by alkalinizing the urine or by working on ice.
- Despite optimized conditions, only one of 12 examined urines from patients with Erythema migrans was found to be Borrelia-positive. We conclude, that (i) no or too low amounts of whole Borrelia or DNA were present in the urine or (ii) that DNA was degraded by nucleases already in the bladder.

In summary, we have developed a highly sensitive real-time PCR which enables not only species-specific differentiation of Borrelia genotypes, but also their quantification. This method was successfully employed in an epidemiological study on ticks, which was itself set into relation to the situation in Europe performing a metaanalysis. A critical evaluation with help of our method revealed that urine PCR as diagnostic tool for Lyme Borreliosis is not advisable – at least for diagnosis of early stages of Lyme Borreliosis.

8 Zusammenfassung

In Europa sind mindestens drei verschiedene humanpathogene Borrelienspezies bekannt. Ein interessanter Aspekt der Speziesklassifizierung ist ihre Korrelation mit epidemiologischen Beobachtungen und klinischen Manifestationen. Daher werden einfache und schnell durchzuführende Methoden, die eine speziesspezifische Bestimmung ermöglichen, sowohl in der Forschung als auch in der medizinischen Diagnostik immer wichtiger.

Im ersten Teil der vorliegenden Arbeit stand die Entwicklung einer Methode, die eine Quantifizierung und gleichzeitige Identifizierung verschiedener Borrelienspezies erlaubt. Ihr praktischer Nutzen wurde mittels einer epidemiologischen Studie an Zecken getestet.

- Es wurde eine auf einem HybProbe System basierende real-time PCR entwickelt, die eine Differenzierung der drei Borrelienspezies *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. in einem einzigen PCR Lauf ermöglicht. Die Speziesunterscheidung war auch in einer Mischung aus zwei oder drei Borrelienstämmen möglich.
- Es wurde mit dieser Methode ein Detektionslimit von 1 bis 10 Borrelien, sowie eine Sensitivität von 98% und eine Spezifität von 97% erreicht. Die PCR Ergebnisse waren hoch reproduzierbar.
- Eine epidemiologische Studie im Raum Konstanz an 1,055 gesammelten Zecken ergab eine Gesamtinfektionsrate von 35% Borrelien-positiven Zecken
- Eine speziesspezifische Untersuchung dieser positiven Zecken ergab 70% *B. afzelii*, 34% *B. garinii* und 12% *B. burgdorferi* s.s.
- 18% der Zecken wiesen eine Mischinfektion mit zwei oder drei Spezies auf.
- Eine Quantifizierung der Borrelien DNA in den Zecken zeigte in 50% der Isolate mehr als 100 Borrelien Genomäquivalente .

Das Vorkommen von Borrelieninfektionen in Zecken ist eine essentielle Komponente in der Risikoabschätzung. Im zweiten Teil der vorliegenden Arbeit wurde die (i) Borrelieninfektion und (ii) Speziesverteilung in *I. ricinus* Zecken in Europa mit Hilfe einer Metaanalyse bestimmt.

- Die Analyse von 155 Datensätzen mit Angaben über Infektionsraten von *B. burgdorferi* s.l. in *I. ricinus* Zecken, ergab einen Gesamtdurchschnitt von 13.6%, wobei adulte Zecken (18.5%) signifikant höher infiziert waren als Nymphen (10.1%). Kein Unterschied zeigte sich in den Infektionsraten von Weibchen und Männchen.
- Länder aus Zentraleuropa (Österreich, Tschechische Republik, Süddeutschland, Schweiz, Slovakei und Slowenien) zeigten die höchsten Infektionsraten von *I. ricinus*.
- Ein Vergleich zwischen (i) unterschiedlichen Detektionsmethoden bzw. (ii) zwei Zeitperioden (1986 bis 1993 sowie 1994 bis 2001) zeigte keinen Unterschiede in den Infektionsraten.
- Die Analyse von 53 Datensätzen zeigte ein ausgeprägtes Muster in der Verteilung der Borrelienspezies in Europa: Die durchschnittlichen prozentualen Anteile von positiven

Zecken mit *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana* und *B. lusitaniae* lagen bei 37%, 34%, 17%, 20% bzw. 7%. 5% der positiven Zecken waren nicht typisierbar.

- In 13% der positiven Zecken wurde eine Mehrfachinfektion detektiert. Die häufigste Kombination von Borrelienspezies trat zwischen *B. garinii* und *B. valaisiana* auf.
- Ein direkter Vergleich zwischen Nymphen und Adulten zeigte keinen signifikanten Unterschied in der Verteilung der Borrelienspezies.
- Das Vorkommen von Borrelienspezies in *I. ricinus* scheint in den verschiedenen Teilen Europas zu variieren.

Die Serologie, zur Zeit das hauptsächliche diagnostische Werkzeug bei Lyme Borreliose, weist verschiedene Nachteile auf. Daher wurden viele Anstrengungen unternommen alternative Methoden zu entwickeln, wie z.B. die PCR, die einen direkten Erregernachweis ermöglicht. Trotz fehlender Standardisierung und ambivalenten Ergebnissen in verschiedenen Studien wird die als wünschenswertes diagnostisches Werkzeug betrachtete Urin PCR, bereits von vielen Diagnostiklabors eingesetzt. Um diese unbefriedigende Situation zu klären, wurde im dritten Teil der vorliegenden Arbeit die Methode der Urin PCR mit Hilfe eines hochstandardisierten Protokolls in quantitativer Weise analysiert.

- Die bereits entwickelte real-time PCR konnte, durch Voranschalten einer Standard-PCR, als nested PCR genutzt werden. Quantifizierung und Speziesdifferenzierung waren dabei immer noch möglich. Das Detektionslimit konnte um den Faktor 10 auf etwa 1 Borrelie pro µl gesenkt werden.
- Spike Experimente mit ganzen Borrelien oder Borrelien DNA im Urin gesunder Spender zeigten (i) einen Konzentrierungseffekt durch Zentrifugation mehrerer Milliliter gespikten Urins sowie (ii) keinen negativen Effekt durch Lagerung bei –20°C über einen Zeitraum von 6 Monaten.
- Inhibitorische Effekte von Urin auf DNA Spikes konnten mit der Aktivität von Nukleasen im Urin korreliert werden. Diese Inhibition konnte durch eine Alkalisierung des Urins oder durch das Arbeiten auf Eis aufgehoben werden.
- Trotz optimierter Bedingungen wurde nur einer von 12 untersuchten Urinen von Patienten mit Erythema migrans als Borrelien positiv identifiziert. Wir folgern, dass (i) keine oder zu geringe Mengen an ganzen Borrelien oder DNA im Urin waren oder (ii) dass die DNA bereits in der Blase durch Nukleasen degradiert wurde.

Zusammenfassend waren wir in der Lage, eine hochsensitive real-time PCR zu entwickeln, die nicht nur eine speziesspezifische Unterscheidung der Borrelien Genotypen erlaubt, sondern ebenso ihre Quantifizierung. Diese Methode wurde erfolgreich in einer epidemiologischen Studie an Zecken verwendet, welche ihrerseits wiederum durch eine Metaanalyse in Relation mit der Situation in Europa gesetzt wurde. Eine kritische Evaluierung mit Hilfe unserer Methode deckte auf, dass Urin PCR als diagnostisches Werkzeug in der Lyme Borreliose nicht empfehlenswert erscheint – zumindest nicht bei der Diagnostik von frühen Formen der Lyme Borreliose.

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