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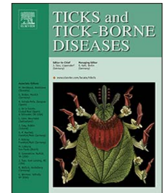

Original article

Lyme borreliae prevalence and genospecies distribution in ticks removed from humans

Patrick Waindok[a], Sabine Schicht[a], Volker Fingerle[b], Christina Strube[a,]*

[a] Institute for Parasitology, Centre for Infection Medicine, University of Veterinary Medicine Hannover, Buenteweg 17, 30559 Hanover, Germany
[b] German National Reference Centre for Borrelia, Veterinaerstraße 2, 85764 Oberschleissheim, Germany

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A B S T R A C T

Lyme borreliosis (LB) is the most important human tick-borne disease, but *Borrelia* genospecies cause different clinical manifestations. Ticks of the genus *Ixodes* removed from humans between 2006 and 2012 were analysed for *Borrelia burgdorferi* sensu lato (sl) infections. The majority of ticks originated from the Greater Hanover region in Northern Germany. The engorgement status varied over the entire spectrum from unengorged (no evidence of started blood feeding) to fully engorged. In the present study, prevalence data for *B. burgdorferi* sl 2011 and 2012 were obtained by quantitative real-time PCR and compared to those from a former study including years 2006–2010 (Strube et al., 2011) to evaluate *B. burgdorferi* sl infections in ticks affecting humans over a 7-year period. In 2011, 34.2% (70/205) of adult ticks, 22.2% (94/423) of nymphs, 8.3% of larvae (1/12) as well 3 of 6 not differentiated ticks were *Borrelia* positive. In 2012, 31.8% (41/129) of adult ticks, 20.4% of nymphs (69/337) as well as 1 of 4 of the not differentiated ticks were determined positive. Total *Borrelia* infection rates decreased significantly from 23.1% in 2006 to 17.1% in 2010, followed by a significant increase to 26.0% in 2011 and 23.4% in 2012. Furthermore, *B. burgdorferi* sl genospecies distribution in 2006–2012 was determined in the present study by applying Reverse Line Blot technique. *Borrelia* genospecies differentiation was successful in 641 (67.3%) out of 953 positive tick samples. The most frequently occurring genospecies was *B. afzelii* (40.5% of infected ticks), followed by *B. garinii*/*B. bavariensis* (12.4%). Amongst the 641 ticks analysed for their genospecies, 74 (11.5%) carried more than one genospecies, of which 69 (10.7%) were double-infected and five (0.8%) were triple-infected. Comparison of genospecies distribution in ticks removed from humans with those from questing ticks flagged in the same geographical area revealed that ticks removed from humans were significantly more frequently infected with *B. afzelii* ($p = 0.0004$), but significantly less infected with *B. burgdorferi* sensu stricto ($p = 0.0001$).

1. Introduction

In Central Europe, the castor bean tick *Ixodes* (*I.*) *ricinus* is a common vector for zoonotic diseases with Lyme borreliosis (LB) being the most important one. Due to the widespread distribution of *I. ricinus*, LB represents the most prevalent tick-borne disease in humans (Hubálek, 2009). An early infection may include the development of an erythema migrans at the site of the tick bite. At later stages, the disease can manifest and may affect the nervous system or result in arthritis or acrodermatits chronic atrophicans (ACA) (Stanek et al., 2011). Between 2009 and 2012, a total of 18,894 cases of LB were reported for the eastern federal states of Germany, corresponding to a mean incidence between 34.9 cases per 100,000 inhabitants in 2009 and 19.54 cases per 100,000 inhabitants in 2012 (Wilking and Stark, 2014). The causative agents of LB are spirochetes belonging to the *Borrelia burgdorferi* sensu lato (sl) complex which currently includes 22 genospecies. Eleven of them are reported from Europe: *B. burgdorferi* sensu stricto (ss), *B. garinii*, *B. bavariensis*, *B. afzelii*, *B. lusitaniae*, *B. spielmanii*, *B. bissettiae* [formerly known as *B. bissettii* (Margos et al., 2016)], *B. valaisiana* were detected in humans and ticks and *B. carolinensis* and *B. finlandensis* in ticks only (Casjens et al., 2011; Cotté et al., 2010; Fingerle et al., 2008; Hildebrandt et al., 2003; Margos et al., 2016), whereas *B. kurtenbachii,* formerly known as *B. bissettii* strain 25015, has been detected in humans (Margos et al., 2010;

Abbreviations: LB, Lyme borreliosis; sl, sensu lato; ss, sensu stricto; ACA, acrodermatits chronic atrophicans; *B.*, *Borrelia*; *I.*, *Ixodes*; SNP, Single nucleotide polymorphism; RLB, Reverse Line Blot; OspA, outer surface protein A; IGS, intergenic spacer; qPCR, quantitative real-time PCR; ITS2, internal transcribed spacer 2; Baf, *B. afzelii*; Bva, *B. valaisiana*; Bss, *B. burgdorferi* sensu stricto; Bga/Bba, *B. garinii*/*B. bavariensis*; Bsp, *B. spielmanii*; Blu, *B. lusitaniae*; Bbi, *B. bissettiae*

* Corresponding author.
E-mail addresses: patrick.waindok@tiho-hannover.de (P. Waindok), sabine.schicht@gmx.de (S. Schicht), volker.fingerle@lgl.bayern.de (V. Fingerle), christina.strube@tiho-hannover.de (C. Strube).

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Picken et al., 1996). Besides, the genospecies *B. burgdorferi* ss, *B. garinii*, *B. bavariensis*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae, B. spielmanii*, and *B. bissettiae* have also been detected in humans (Rudenko et al., 2011). Several studies revealed a correlation between the presence of *B. burgdorferi* ss and arthritis (Eiffert et al., 1998; Nocton et al., 1994), *B. afzelii* and ACA as well as *B. garinii* and neuroborreliosis (Balmelli and Piffaretti, 1995; van Dam et al., 1993). *B. bavariensis*, formerly known as *B. garinii* OspA type 4 is correlated with neuroborreliosis (Coipan et al., 2016).

The relapsing fever group spirochete *Borrelia miyamotoi*, which may cause febrile illness in humans, was first reported in German *I. ricinus* ticks (Richter et al., 2003). However, reported prevalence of *B. miyamotoi* ranged between 1.8% and 3.5% (Eshoo et al., 2014; Richter et al., 2003; Venczel et al., 2016), and seems to be much lower when compared to *B. burgdorferi* sl exhibiting prevalences between 3.1% in *I. ricinus* at the Baltic coast to 36.2% in Bavaria (Fingerle et al., 1999; Franke et al., 2011).

As knowledge of *Borrelia* prevalence and distribution of genospecies of the *B. burgdorferi* sl complex is crucial to understand epidemiology, prevention and diagnosis of LB, the aim of this study was to determine the *B. burgdorferi* sl infection rate in diagnostic tick material delivered to the Institute for Parasitology, University of Veterinary Medicine Hannover, as a follow-up to evaluate long-term development of annual *B. burgdorferi* sl prevalence data in ticks removed from humans. Furthermore, the genospecies diversity of *B. burgdorferi* sl-positive ticks from humans was analysed for a period of 7 years.

2. Material and methods

2.1. Tick material and determination of Borrelia infection rates

Ticks removed in 2011–2012 from human patients by the physician or the patients themselves were delivered as diagnostic material to the Institute for Parasitology, University of Veterinary Medicine Hannover, Germany. The majority of ticks originated from the Greater Hanover region and their engorgement status varied over the entire spectrum from unengorged (no evidence of started blood feeding) to fully engorged. As being a veterinary laboratory, ticks were examined for *Borrelia* infection, but no medical data from the patients were requested. If the tick was tested positive, the sender was informed that *Borrelia*-DNA was detected. No medical advice was given, but consultation with the family physician as competent contact person for further questions and potential approaches was suggested. In case of a negative diagnostic result, the sender was informed that no *Borrelia*-DNA was detected. Submitted tick samples were microscopically differentiated for species and developmental stage by morphological characteristics. Tick-DNA was isolated as described previously (Strube et al., 2010; Strube et al., 2011) immediately after arrival or after storage of ticks in individual sterile tubes at room temperature for a maximum of three days. Tick samples were investigated for the presence of *Borrelia*-DNA by probe-based duplex quantitative real-time PCR (qPCR) targeting the *B. burgdorferi* sl 5S-23S intergenic spacer (IGS) region as well as the *Ixodes* ITS2 region to ensure successful DNA isolation (Strube et al., 2010; Strube et al., 2011). All qPCR runs were performed as duplicates and included a no-template control as well as a plasmid DNA positive control containing 10^4 target copies. *Borrelia* positive tick samples were stored at −20 °C until subsequent determination of the *B. burgdorferi* sl genospecies.

2.2. B. burgdorferi sl genospecies identification

Borrelia-positive tick samples of the present as well as a previous study on ticks removed from humans between 2006 and 2010 (Strube et al., 2011) were subjected to Reverse Line Blot (RLB) hybridization for *Borrelia* genospecies determination as described (Tappe et al., 2014). Used oligonucleotide probes detected *B. afzelii*, *B. garinii*, *B. burgdorferi* ss (Burri et al., 2007), *B. bissettiae*, *B. lusitaniae*, *B. spielmanii* (Gern et al., 2010) and *B. valaisiana* (Poupon et al., 2006). As the *B. garinii*-probe cannot discriminate between *B. garinii* and *B. bavariensis*, additional custom Sanger sequencing (SEQLAB Sequence Laboratories, Germany) was performed in case of *B. garinii* positivity. Nucleotide sequences were aligned and compared to published sequences of *B. garinii* and *B. bavariensis* to distinguish between genospecies by identification of single nucleotide polymorphisms (SNPs) as described by Tappe et al. (2014).

2.3. Statistical analysis

To test for statistically significant differences of *Borrelia* infection rates in diagnostic ticks delivered between 2011 and 2012, results were analysed using χ^2-test (SigmaStat® version 3.11, Systat Software, Germany). Additionally, long term prevalence data were compared against each other by including data obtained between 2006 and 2010 (Strube et al., 2011) by χ^2-test and subsequent Bonferroni-Holm correction. Data for 2010 were adjusted for additional ticks that had not been considered in the previous report. Furthermore, *Borrelia* genospecies distribution in adult diagnostic ticks was compared to those of nymphs. Finally, the distribution of genospecies in diagnostic ticks removed from humans was compared to those from questing ticks collected at ten different sampling sites and defined time points in the city of Hanover (Tappe et al., 2014) using χ^2-test (GraphPad Prism™ version 6.03, GraphPad Software, USA). This comparison was reliable as from sender information the majority of diagnostic ticks originated from Greater Hanover and both, diagnostic and questing ticks were processed by the same techniques.

3. Results

3.1. Tick material and determination of tick species

In 2011 and 2012 a total of 1121 ticks removed from humans were delivered to the Institute for Parasitology, University of Veterinary Medicine Hannover, Germany. Microscopical examination revealed 203 adults, 423 nymphs and 12 larvae in 2011 and 125 adults, 333 nymphs and 5 larvae in 2012. Ticks were identified as *I. ricinus* except two *I. hexagonus* adults in 2011 and four *I. hexagonus* adults and nymphs, respectively, in 2012. Six ticks delivered in 2011 and four in 2012 were damaged in essential morphological characteristics, hampering differentiation at species level.

3.2. Borrelia infections in ticks

In total, 26.0% of the ticks delivered in 2011 and 23.4% in 2012 were tested positive for *Borrelia* infections (Table 1). All positive ticks were identified as *I. ricinus*, except one *I.hexagonus* nymph in 2012. An overview of infection rates in different developmental stages is given in Table 2. The reported percentage of 12.8% positive ticks in 2010 (Strube et al., 2011) was adjusted to 17.1% due to additional ticks not considered in the previous report.

In comparison to prevalence rates determined in 2009 and 2010, the *B. burgdorferi* sl infection rate increased in 2011 and 2012 (Table 1, Fig. 1). Statistical analysis of the long term period 2006–2012 revealed significant differences between 2006 vs. 2010, 2007 vs. 2009 and 2010, 2008 vs. 2010, 2009 vs. 2011, as well as 2010 vs. 2011 and 2012. P-values and adjusted α-values are shown in Fig. 1.

3.3. B. burgdorferi sl genospecies identification

Genospecies differentiation by RLB technique of *B. burgdorferi* sl-infected ticks was successful in 641 out of 953 positive tick samples (67.3%), divided in 232 of 330 adults (70.3%), 400 of 607 nymphs (65.8%), 2 of 4 larvae (50%) as well as 7 of 12 (58.3%) unidentifiable

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