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Original article

Association of *Borrelia* and *Rickettsia* spp. and bacterial loads in *Ixodes ricinus* ticks

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ABSTRACT

In recent years, awareness of coinfections has increased as synergistic or antagonistic effects on interacting bacteria have been observed. To date, several reports on coinfections of ticks with *Rickettsia* and *Borrelia* spp. are available. However, associations are rarely described and studies are based on rather low sample sizes. In the present study, coinfections of *Ixodes ricinus* with these pathogens were investigated by determining their association in a *meta*-analysis. A total of 5079 tick samples examined for *Rickettsia* and *Borrelia* spp. via probe-based quantitative real-time PCR in previous prevalence studies or as submitted diagnostic material were included. In *Borrelia*-positive ticks, genospecies were determined by Reverse Line Blot. Determination of bacterial loads resulted in an increase between developmental tick stages with highest mean bacterial loads in female ticks (7.96×10^4 in *Borrelia* single-infected, 4.87×10^5 in *Rickettsia* single-infected and 3.22×10^5 in *Borrelia-Rickettsia* coinfected females). The determined *Borrelia-Rickettsia* tick coinfection rate was 12.3% (626/5079) with a significant difference to the expected coinfection rate of 9.0% (457/5079). A significant slight association as well as correlation between *Borrelia* and *Rickettsia* were determined. In addition, a significant interrelation of the bacterial load in coinfected ticks was shown. At the level of *Borrelia* genospecies, significant weak associations with *Rickettsia* spp. were detected for *B. afzelii*, *B. garinii/bavariensis*, *B. valaisiana* and *B. lusitaniae*. The positive association provides evidence for interactions between *Borrelia* and *Rickettsia* spp. in the tick vector, presumably resulting in higher bacterial replication rates in the tick vector and possibly the reservoir host. However, coinfection may impact the vector negatively as indicated by an absent increase in coinfection rates from nymphs to adults. Future studies are needed to investigate the underlying mechanisms of the positive association in ticks and possible associations in the vertebrate host as well as the potential influence of environmental factors.

1. Introduction

The hard tick *Ixodes ricinus* (Ixodida: Ixodidae) is the most abundant tick species in Central Europe and serves as vector for a wide variety of pathogens. These include amongst others *Borrelia* (Spriochaetales: Spirochaetaceae) as well as *Rickettsia* species (Rickettsiales: Rickettsiaceae). The *Borrelia burgdorferi* sensu lato (s.l.) complex comprises the causative agents of Lyme borreliosis, which is the most frequent arthropod-borne disease in the Northern Hemisphere (Stanek et al., 2012). Most common manifestations of borreliosis include joints, the heart and the central nervous system. To date, the *B. burgdorferi* s.l. complex comprises 22 recognised genospecies, 11 of which were detected in Europe, namely *B. afzelii*, *B. bavariensis*, *B. bissettiae* [formerly known as *B. bissettii* (Margos et al., 2016)], *B. burgdorferi* sensu stricto (s.s.), *B. carolinensis*, *B. finlandensis*, *B. garinii*, *B. lusitaniae*, *B. kurtenbachii*, *B. spielmanii* and *B. valaisiana*. All genospecies were detected in humans, except for *B. carolinensis* and *B. finlandensis* (Casjens et al., 2011; Cotté et al., 2010; Fingerle et al., 2008; Hildebrandt et al., 2003; Margos et al., 2016; Rudenko et al., 2011). Besides the *B. burgdorferi* s.l. complex, relapsing fever-*Borrelia* spp. are described. This group includes *B. miyamotoi*, which was recently shown to be human-pathogenic (Platonov et al., 2011).

Rickettsia helvetica is the most prevalent *Rickettsia* species in Germany. In humans, *R. helvetica* infections may cause mild flu-like symptoms such as headache, myalgia or rash (Nilsson, 2009; Parola et al., 2005; Sekeyova et al., 2012). However, *R. helvetica* has also been isolated from cerebrospinal fluid of patients with meningitis of uncertain aetiology (Nilsson et al., 2010; Nilsson et al., 2011). Several

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studies on *Borrelia* and *Rickettsia* prevalence in ticks were conducted to elucidate the potential health risk to humans (Bingsohn et al., 2013; Hildebrandt et al., 2010; Schorn et al., 2011; Schwarz et al., 2012; Silaghi et al., 2008; Voegerl et al., 2012). In northern Germany, for example, prevalences in *I. ricinus* range from 20% to 34% for *Borrelia* spp. and 26% to 54% for *Rickettsia* spp. (May et al., 2015; May and Strube, 2014; Schicht et al., 2012; Strube et al., 2010; Tappe et al., 2014; Tappe and Strube, 2013). However, most prevalence studies consider mentioned pathogens individually, resulting in limited knowledge of coinfections and associations of those species. It has been speculated that synergistic as well as antagonistic effects on interacting bacteria may result in a higher transmission rate of pathogens or influence the severity of disease in humans. Those synergistic or antagonistic effects may be based on a direct or indirect relationship within a pathogen community (Belongia, 2002; Levin, 2007; Rojas et al., 2014). Exemplarily, an association of *B. burgdorferi* s.s. and *Anaplasma phagocytophilum* has been demonstrated in case studies with prolonged clinical manifestation of Lyme borreliosis and human granulocytic anaplasmosis as well as increased morbidity, bacterial load and severity of symptoms (Holden et al., 2005; Krause et al., 2002; Thomas et al., 2001). Regarding *Borrelia* and *Rickettsia* coinfections, few studies showed a positive interrelation of these pathogens when comparing observed and expected coinfection (May et al., 2015) or generalised linear mixed models (Václav et al., 2011); however, sample sizes were rather low. Therefore, the present study presents a *meta*-analysis on *Borrelia* and *Rickettsia* associations including more than 5000 *Ixodes* ticks, aiming to provide further data on the impact of pathogen coinfections on the vector as well as vertebrate host.

1.1. Material and methods

1.1.1. Meta-analysis

A total of 5079 *I. ricinus* ticks were included in a *meta*-analysis. Of these, 4589 ticks were questing ticks previously analysed for *Rickettsia* and *Borrelia* infection. These ticks were collected with the flagging method in 10 different recreational areas in the city of Hanover, Germany, in 2005 (Strube et al., 2010; Schicht et al., 2012) and 2010 (Tappe and Strube, 2013; Tappe et al., 2014) as well as in the city of Hamburg, Germany, in 2011 (May and Strube, 2014; May et al., 2015). In addition to the infection status, the infecting *Rickettsia* spp. and *Borrelia* genospecies were determined (May and Strube, 2014; Schicht et al., 2012; Tappe and Strube, 2013). Results of the *Borrelia* genospecies and *Rickettsia* species differentiation (May et al., 2015; Strube et al., 2010; Tappe et al., 2014) are included in the respective results section below.

The remaining 490 tick samples included in the *meta*-analysis were sent to the Institute for Parasitology, University of Veterinary Medicine Hannover, Germany, in 2014 as diagnostic tick samples covering the entire engorgement spectrum from unengorged to fully engorged. For those, tick differentiation as well as genomic DNA isolation were performed as previously described (Tappe and Strube, 2013). Detailed sample size divided into studies and developmental stages is provided in Table 1.

1.1.2. Determination of Borrelia and Rickettsia spp. infection rates

Borrelia and *Rickettsia* infection rates of the 4589 questing ticks were already determined in previous studies (May et al., 2015; May and Strube, 2014; Schicht et al., 2012; Strube et al., 2010; Tappe et al., 2014; Tappe and Strube, 2013). For the 490 diagnostic tick samples, respective infection rates were determined via probe-based quantitative real-time PCR (qPCR) as previously applied for the questing ticks. Concerning *Borrelia* infection rates, qPCR was performed in a duplex reaction for simultaneous detection of a 67 bp amplicon of the 5S-23S intergenic spacer (IGS) of *B. burgdorferi* s.l., and a 77 bp amplicon of the *I. ricinus* ITS2 region to confirm successful DNA isolation (Strube et al., 2010). As the qPCR has been proven to detect *Borrelia burgdorferi* s.l. as well as *B. miyamotoi* (unpublished results), both species contribute to obtained *Borrelia* prevalences. For *Rickettsia* detection, qPCR was performed in a singleplex reaction for amplifying of a 76 bp sequence of the citrate synthase (*gltA*) gene of *Rickettsia* species (Stenos et al., 2005). Reaction set-ups and thermal cycling were conducted as previously described (Tappe et al., 2014; Tappe and Strube, 2013).

Table 1
Tick samples from previous prevalence studies and diagnostic tick samples gathered in the *meta*-analysis. Tick samples from previous prevalence studies comprised 4589 questing ticks originating from (1) the city of Hanover collected in 2005 (1089 ticks) and 2010 (2100 ticks) and (2) the city of Hamburg collected in 2011 (1400 ticks). Diagnostic tick samples (490 ticks, unengorged to fully engorged) were sent to the Institute for Parasitology, University of Veterinary Medicine Hannover, in 2014. Bold values indicate total tick numbers of previous prevalence studies and developmental tick stages.

Developmental stage	Strube et al. (2010), Schicht et al. (2012)	Tappe and Strube (2013), Tappe et al. (2014)	May and Strube (2014), May et al. (2015)	Diagnostic tick samples	Total
Adult	669	372	141	183	**1365**
Male	341	196	81	0	**618**
Female	328	176	60	183	**747**
Nymph	332	1697	1259	284	**3572**
Larva	88	31	0	14	**133**
Not specified	0	0	0	9	**9**
Total	**1089**	**2100**	**1400**	**490**	**5079**

prevalence studies and developmental tick stages.

1.1.3. Borrelia genospecies differentiation

To identify infecting *B. burgdorferi* s.l. genospecies, Reverse Line Blot (RLB) was conducted. In the 4589 questing ticks, RLB was already performed in previous studies (May et al., 2015; May and Strube, 2014; Schicht et al., 2012; Strube et al., 2010; Tappe et al., 2014; Tappe and Strube, 2013). RLB of the 490 diagnostic tick samples was performed according to Tappe et al. (2014) with the following modifications: Qiagen *Taq* DNA Polymerase (5 U/µl, Qiagen, Hilden, Germany) and 10 µl of tick DNA template amplified by primers B-5SBor (biotinylated) and 23SBor (Alekseev et al., 2001) were used in conventional PCR. Furthermore, PCR was performed under following cycling conditions: Initial denaturation at 94 °C for 3 min, 45 cycles of 94 °C for 20 s, 52 °C for 30 s and 72 °C for 30 s with a final cycle of 72 °C for 7 min. Hybridisation of PCR amplification products to the probes was conducted at 52 °C.

1.1.4. Tick bacterial load

In the 4589 previously analysed questing ticks (May et al., 2015; May and Strube, 2014; Schicht et al., 2012; Strube et al., 2010; Tappe et al., 2014; Tappe and Strube, 2013), plasmid standards containing the detected *Borrelia* 5S-23S IGS and *Rickettsia gltA* sequence, respectively, were used as serial dilutions ranging from 10^0 to 10^6 gene copies to determine bacterial loads in individual questing ticks. Since the 5S-23S IGS region of the *Borrelia burgdorferi* s.l. complex is a double copy gene as indicated by sequences deposited in the GenBank database of the National Center for Biotechnology Information (NCBI), bacterial loads resemble half of the determined gene copy numbers. The *Rickettsia gltA* gene is a single copy gene; thus, bacterial loads equal determined gene copy numbers. Different amounts of tick DNA template [2 µl template used by Strube et al. (2010) and Schicht et al. (2012) vs. 10 µl template used by May and Strube (2014), May et al. (2015), Tappe and Strube (2013) and Tappe et al. (2014)] were considered in the mathematical calculations.

1.1.5. Statistical analysis

Differences between tick stages concerning infection rates and occurrence of various *Borrelia* genospecies were determined by the four-