Ticks and Tick-borne Diseases 4 (2013) 432–438

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Anaplasma phagocytophilum and *Rickettsia* spp. infections in hard ticks (*Ixodes ricinus*) in the city of Hanover (Germany): Revisited

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ARTICLE INFO

Article history:
Received 23 January 2013
Received in revised form 19 April 2013
Accepted 24 April 2013
Available online 6 July 2013

Keywords:
Anaplasma phagocytophilum
Rickettsia helvetica
Ixodes ricinus
Vector-borne diseases
Tick-borne diseases

ABSTRACT

The present study aimed to determine the prevalence of Rickettsiales (*A. phagocytophilum* and *Rickettsia* spp.) in 2100 *I. ricinus* ticks collected at 10 different sampling sites every month during the tick season 2010 in the city of Hanover, northern Germany. At the same time, the results served as a fifth-year-follow-up study to monitor whether changes or stagnation of tick infection rates – possibly due to climate change – were obvious or not. To detect infections with *A. phagocytophilum* and/or *Rickettsia* spp., tick samples were analysed by quantitative real-time PCR. Differentiation of *Rickettsia* species was accomplished using real-time pyrosequencing technology. Overall, 4.5% (94/2100) of the collected ticks were tested positive for *A. phagocytophilum* and 26.2% (551/2100) were positive for *Rickettsia* spp. infections. Species differentiation of *Rickettsia*-positive ticks via real-time pyrosequencing was possible in 48.6% (268/551) of samples, which were all identified as *R. helvetica*. Coinfections with both pathogens were found in 1.0% (20/2100) of ticks. Statistically significant seasonal fluctuations between sampling months as well as local differences between sampling sites were detected for *Rickettsia* spp. infection rates. For A. *phagocytophilum* infections, only significant seasonal variations were found. When comparing infection rates of Hanoverian ticks in 2010 to those in 2005, infection rates of *A. phagocytophilum*-infected nymphs increased statistically significant ($P = 0.008$, power: 0.762) from 2.3% in 2005 (Schicht et al., 2011) to 4.5% in 2010. *Rickettsia* spp. infections in female ticks decreased significantly ($P = 0.049$, power: 0.491) from 41.8% in 2005 (Schicht et al., 2012) to 32.4% in 2010. Comparison of the remaining tick stages showed no statistically significant differences.

Introduction

The hard tick *Ixodes ricinus* (Linneé 1758) is known as a vector for several pathogenic organisms. In central Europe, this most abundant tick species transmits *Anaplasma phagocytophilum* and different *Rickettsia* species amongst other pathogens. Both pathogens belong to the group of Gram-negative, obligate intracellular α-proteobacteria and are subsumed in the order Rickettsiales. *A. phagocytophilum* causes human granulocytic anaplasmosis (HGA), a disease that is accompanied by unspecific symptoms such as fever, myalgia, headache, and malaise. In ruminants, the agent causes tick-borne fever leading to economical losses due to reduced milk production, decreased weight gain, and abortion (Hudson, 1950; Stamp et al., 1950; Stuen et al., 2002). In horses and dogs, anaplasmosis has also been described with symptoms like fever, apathy, anaemia, lameness, thrombocytopenia, and leucopenia (Gribble, 1969; Jahn et al., 2010; Procajlo et al., 2011). In general, small mammals, sheep, and deer serve as reservoir hosts for *A. phagocytophilum* (Liz et al., 2000; Woldehiwet, 2006; de la Fuente et al., 2008; Silaghi et al., 2012a,b; Thomas et al., 2012).

Rickettsia species are the causative agent for various forms of spotted fever. In Germany, *I. ricinus* serves as a vector for *R. helvetica* and *R. monacenis*, however, *R. massiliae* and *R. felis* could also be identified in ticks in one case each (Simser et al., 2002; Dobler and Wölfel, 2009). Infection with *R. helvetica* in humans may lead to the so-called 'summer flu', a feverish infection that may be accompanied by headache and myalgia, but it is also implicated with meningitis and perimyocarditis (Nilsson et al., 1999, 2010, 2011). In contrast to the classic spotted fever illness, most clinical cases due to *R. helvetica* are presented without rash (Fournier et al., 2000; Dobler and Wölfel, 2009). *R. monacensis* and *R. massiliae* cause the classic form of spotted fever with symptoms like fever, headache, rash, and inoculation eschar. *R. felis* is associated with flea-borne spotted fever, a general infection accompanied by raised temperature and a measles-like rash (Fournier et al., 2000; Richter et al., 2002; Simser et al., 2002; Hartelt et al., 2004; Dobler and Wölfel, 2009). Ticks serve as the main host, but also as a reservoir for *Rickettsia* species (Burgdorfer et al., 1979; Parola et al., 2005). However, the importance of small rodents as reservoir hosts is additionally being discussed (Dobler et al., 2008).

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http://dx.doi.org/10.1016/j.ttbdis.2013.04.009

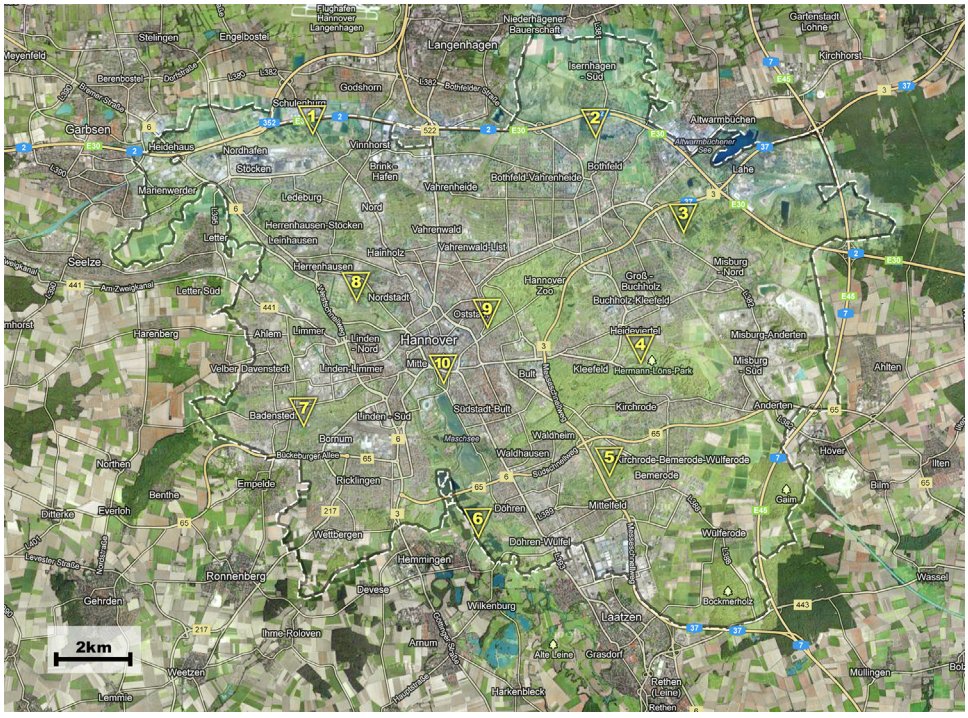

Fig. 1. Distribution of tick collection sites in the city of Hanover in 2010. 1, Mecklenheide (woodland); 2, Große Heide (woodland and meadow); 3, Misburger Wald (woodland); 4, Annateiche (woodland and meadow); 5, Seelhorster Wald (woodland); 6, Ricklinger Teiche (woodland); 7, Bornumer Holz (woodland); 8, Georgengarten (bush and meadow); 9, Eilenriede (woodland); 10, Maschpark (bush and meadow). The dashed line represents the city border (Map source: Google Earth).

Several studies deal with the prevalence of *A. phagocytophilum* and *Rickettsia* spp. in ticks (e.g. Schorn et al., 2011; Burri et al., 2011; Cotte et al., 2010; Jahn et al., 2010). In Germany, the maximum prevalence of *A. phagocytophilum* in *I. ricinus* ticks was found to be 8.7% (Silaghi et al., 2012a,b), and the highest prevalence for *Rickettsia* spp.-infected ticks was 34.2% (Strube et al., 2011). In previous studies, the prevalence of Rickettsiales in *I. ricinus* collected every month during the tick season 2005 in the city of Hanover, Germany, have been analysed and resulted in an *A. phagocytophilum* infection rate of 3.2% and a *Rickettsia* spp. infection rate of 33.3% (Schicht et al., 2011, 2012). To monitor changes or stagnation of tick infection rates with Rickettsiales, the presented fifth-year-follow-up study was aimed to obtain new data concerning infection rates of Hanoverian ticks in 2010. Again, ticks were collected in different recreational areas in the city of Hanover and analysed for *A. phagocytophilum* and *Rickettsia* spp. followed by data comparison with the former studies.

Material and methods

Sampling sites and biological material

Questing ticks were collected in the city of Hanover, the capital of the northern German federal state of Lower Saxony. With about 523,000 inhabitants, Hanover is one of the 15 largest cities in Germany. As it has numerous large-scale inner-city parks, gardens, and the 'Eilenriede', with about 640 hectare the largest continuous urban woodland in Europe, the city of Hanover was nicknamed 'The Green Metropolis'. In 2011, it was elected as the German Capital of Biodiversity.

Ticks were collected monthly from April to October 2010 in 10 of the city's recreation areas (Fig. 1) using the flagging method. Two collection sites were located in the north, in the south, in the east, in the west, and in the centre of the city, respectively. At each site, 30 ticks were collected per month. Tick species identification and determination of stage and sex was done microscopically based on morphological parameters. Tick samples were individually stored at −75 °C until DNA isolation.

Genomic DNA isolation

Isolation of genomic DNA was conducted by use of the NucleoSpin® 8 Blood Kit (Macherey-Nagel) according to the manufacturer's instructions. First, ticks were individually homogenised using polystyrene pistils (Roth) and incubated overnight in 180 μl lysis buffer supplemented with 20 μl Proteinase K. To obtain genomic DNA, 2 elution steps using 70 μl and 60 μl double-distilled water, respectively, were performed. Genomic DNA was stored at −20 °C until further use.

Real-time quantitative PCR (qPCR) detection of A. phagocytophilum and Rickettsia spp. in I. ricinus

For detection of *A. phagocytophilum*, a duplex qPCR targeting the *A. phagocytophilum* 16S rRNA gene as well as the *I. ricinus* ITS2 gene (as a positive control for successful DNA isolation) was carried out using the Mx3005 Multiplex Quantitative PCR System (Stratagene). qPCR was performed based on primer-probe combinations by Sirigireddy and Ganta (2005) and Strube et al. (2010). The reaction contained 12.5 μl Absolute Blue QPCR low row mix (Thermo Fisher), 0.15 μl of each primer (50 μM, Life Technologies), 0.06 μl of each probe (10 μM, Applied Biosystems), 9.78 μl double-distilled water, and 2 μl tick DNA template. Thermocycling conditions were as follows: initial denaturation at 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s.

For detection of *Rickettsia* spp., a qPCR targeting the *gltA* gene was used based on a primer-probe combination by Stenos et al. (2005). Oligonucleotide concentration and reaction set-up were as described above with the exception that 10 μl template-DNA was used, and the amount of double-distilled water was adjusted to