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Original article

High prevalence of *Rickettsia helvetica* in wild small mammal populations in Germany

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ABSTRACT

Since the beginning of the 21st century, spotted fever rickettsioses are known as emerging diseases worldwide. Rickettsiae are obligately intracellular bacteria transmitted by arthropod vectors. The ecology of *Rickettsia* species has not been investigated in detail, but small mammals are considered to play a role as reservoirs.

Aim of this study was to monitor rickettsiae in wild small mammals over a period of five years in four federal states of Germany. Initial screening of ear pinna tissues of 3939 animals by Pan-Rick real-time PCR targeting the citrate synthase (*gltA)* gene revealed 296 rodents of seven species and 19 shrews of two species positive for rickettsial DNA. Outer membrane protein gene (*ompB, ompAIV*) PCRs based typing resulted in the identification of three species: *Rickettsia helvetica* (90.9%) was found as the dominantly occurring species in the four investigated federal states, but *Rickettsia felis* (7.8%) and *Rickettsia raoultii* (1.3%) were also detected. The prevalence of *Rickettsia* spp. in rodents of the genus *Apodemus* was found to be higher (approximately 14%) than in all other rodent and shrew species at all investigated sites. General linear mixed model analyses indicated that heavier (older) individuals of yellow-necked mice and male common voles seem to contain more often rickettsial DNA than younger ones. Furthermore, rodents generally collected in forests in summer and autumn more often carried rickettsial DNA. In conclusion, this study indicated a high prevalence of *R. helvetica* in small mammal populations and suggests an age-dependent increase of the DNA prevalence in some of the species and in animals originating from forest habitats. The finding of *R. helvetica* and *R. felis* DNA in multiple small mammal species may indicate frequent *trans*-species transmission by feeding of vectors on different species. Further investigations should target the reason for the discrepancy between the high rickettsial DNA prevalence in rodents and the so far almost absence of clinical apparent human infections.

1. Introduction

Vector-borne diseases are of worldwide public health importance. Rickettsioses are vector-borne diseases that are still neglected in Europe (Merhej and Raoult, 2011). In general, murine typhus caused by *Rickettsia prowazekii,* and spotted fever caused by rickettsiae of the spotted fever group (SFG) are of major medical importance (Fournier and Raoult, 2009). Recent phylogenetic analyses suggested changes in the grouping of *Rickettsia* species into five taxa*:* the SFG, the transitional group including e.g. *Rickettsia felis*, the typhus group, and as own groups *Rickettsia helvetica*, *Rickettsia bellii* and *Rickettsia canadensis,* respectively (Merhej and Raoult, 2011; Murray et al., 2016). Worldwide, more than twenty *Rickettsia* species were identified by multi locus sequence typing and isolation in cell culture with growing numbers of newly described “Candidatus” species (Merhej and Raoult, 2011; Eremeeva, 2012). In Central Europe, several rickettsiae are endemic, i.e. representatives of the transitional group (*R. felis*), *R. helvetica*, and rickettsiae of the SFG. Examples of the latter are *Rickettsia conorii* in the

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Mediterranean region or *Rickettsia slovaca* in central Europe (Parola and Raoult, 2001; Parola et al., 2005; Dobler et al., 2009; Dobler and Wölfel, 2009). Depending on the etiological *Rickettsia* species the induced disease pattern can vary from mild to severe courses. Examples reported in Europe are uneruptive fever for infections with *R. helvetica* (Fournier et al., 2004) and tick-borne lymphadenopathy for infections with *R. slovaca* (Rieg et al., 2011) and *Rickettsia raoultii* (Switaj et al., 2012). In addition, more severe diseases include the "Mediterranean spotted fever" with rash and fever (e.g. caused by *R. conorii* (Parola et al., 2013)), or myocarditis and meningitis (e.g. *R. helvetica* infections in Sweden; Nilsson et al., 1999b, 2010). In Germany, rickettsioses are often not diagnosed due to occurring mild symptoms or lacking differential diagnosis (Dobler and Wölfel, 2009; Rumer et al., 2011; Wölfel et al., 2016).

Rickettsiae are transmitted by numerous types of arthropods, including ticks, fleas, mites and lice. These various arthropods act as reservoirs and/or vectors to humans and animals (Fournier and Raoult, 2009; Parola et al., 2013). The geographical distribution of different rickettsiae of the tick-borne SFG and *R. helvetica* in different arthropod vectors was in the focus of many studies (Marquez et al., 2002; Pichon et al., 2006; Wölfel et al., 2006; Silaghi et al., 2008; Mediannikov et al., 2008; Gilles et al., 2008b; Dobler et al., 2009; Dobler and Wölfel, 2009). Some of these *Rickettsia* species are associated with specific arthropods. *R. helvetica* is mostly found in the castor bean tick (*Ixodes ricinus;* Dobler and Wölfel, 2009; Sprong et al., 2009), whereas *R. raoultii* is mainly associated with *Dermacentor* ticks (e.g. *Dermacentor reticulatus*), and only in rare cases detected in *I. ricinus* (Mediannikov et al., 2008; Dobec et al., 2009; Chmielewski et al., 2009; Silaghi et al., 2011; Spitalska et al., 2012; Speck et al., 2013; Wojcik-Fatla et al., 2013; Wachter et al., 2015; Duscher et al., 2016; Karbowiak et al., 2016; Liesner et al., 2016; Obiegala et al., 2017). The cat flea (*Ctenocephalides felis*) is the most frequent arthropod vector for *R. felis* (Hawley et al., 2007; Capelli et al., 2009; Reif and Macaluso, 2009). In Germany, so far *R. raoultii, R. helvetica, R. felis, R. slovaca, Rickettsia monacensis,* and *Rickettsia massiliae* have been detected in arthropod vectors (Dobler and Wölfel, 2009; Wölfel et al., 2016).

In contrast, studies on the ecology of *Rickettsia* spp. in small mammal populations are rare in comparison to the large number of studies dealing with *Rickettsia* spp. in vectors. In Germany, small-sized surveys investigated host animals in small geographic areas (Pluta et al., 2010; Schex et al., 2011; Obiegala et al., 2016, 2017). The objective of this study was to determine the occurrence and prevalence of *Rickettsia* species in small mammal populations at four trapping areas with two different habitats each over a period of five years in Germany. To obtain better knowledge of the ecology of *Rickettsia* species in the small mammal populations throughout Germany, we evaluated the impact of habitat, seasonality and small mammal demography on the probability to detect rickettsial DNA.

2. Materials and methods

2.1. Rodent trapping and sample collection

Small mammals were collected by snap trapping three times per year from 2010 to 2014 in four federal states and two different habitats (forest and grassland) with three plots per habitat. The design is described in more detail by (Fischer et al., 2018). Collection of samples was performed according to relevant legislation and permission of the federal authorities (permits Regierungspräsidium Stuttgart, Baden-Württtemberg, 35-9185.82/0261; Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen 8.87-51.05.20.09.210, Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern 7221.3-030/09; Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz 22-2684-04-15-107/09).

The molecular species and sex confirmation for selected rodents and shrews followed previously described standard protocols (Fischer et al., 2018). Sex, reproductive status (male = scrotal testes; female = vaginal opening, pregnant or lactating) and weight (measured to the nearest gram with a spring scale as a proxy for age) were documented.

2.2. DNA isolation

DNA was isolated from ear pinna tissue using Lysing Matrix A (MP Biomedicals, Santa Ana, CA, United States) and adding a volume of 800 µl minimal essential Medium (MEM, Life Technologies, Darmstadt, Germany). The samples were homogenized using a MP Fast Prep 24 instrument (MP Biomedicals) at 6.5 m/s for 90 s and at 4 °C. DNA isolation was carried out with a 200 µl aliquot of homogenized ear pinna tissue in the Magna Pure LC instrument with the Total NA variable elution volume blk program (Roche, Basel, Switzerland (Wölfel et al., 2008; Schex et al., 2011)). A volume of 50 µl DNA was aliquoted and stored at −20 °C until further use.

2.3. Detection and typing of rickettsial DNA

DNA samples were initially screened by a SFG rickettsia citrate synthase gene (*gltA*) –specific *Pan-Rick* TaqMan real-time PCR (rtPCR) (Schex et al., 2011; Wölfel et al., 2008). Thereafter, *gltA* positive samples were tested in a partial outer membrane protein B gene (*ompB*) –PCR as described in detail by Roux and Raoult (2000) and modified by Schex et al. (2011). For selected *gltA*-positive samples, results were confirmed by sequencing of fragment IV of the outer membrane protein A gene (*ompA*) as described by Fournier et al. (1998). Both conventional typing PCRs were performed on a GeneAmp® PCR System 9700 (Applied Biosystems, Darmstadt, Germany). Direct sequencing was performed by GATC sequencing service (Konstanz, Germany). The received sequences were analyzed with the freeware program Chromas LITE 2.01 (Technelysium PTY Ltd, South Brisbane, Australia; http://technelysium.com.au/), as well with the CLUSTAL W program from European Bioinformatics Institute© (EMBL-EBI, Cambridgeshire, United Kingdom; Larkin et al., 2007). Obtained sequences were compared to existing sequences using NucleotideBLAST of NCBI (National Center for Biotechnology Information, Bethesda, USA, http://www.ncbi.nlm.nih.gov/). All sequences were submitted to GenBank (Accession numbers: MG242260 - MG242313, MG266431-MG266436, MG266689).

2.4. Statistics

Predicted host and habitat association of *Rickettsia* spp. as well as the relative effect of seasonal, geographical and demographic factors on the individual probability of carrying rickettsial DNA were statistically analysed using a generalized linear mixed model (GLMM) with binomial error distribution and a logit link function and a random factor that accounted for the nested spatial design of the study (study site nested in federal state) as described in detail in Fischer et al., 2018. Briefly, individual probabilities for carrying rickettsial DNA depending on species were used to estimate host specificity using the lsmeans-package. The influence of demographic factors (sex, reproductive activity and weight) as well as their respective two-way interactions was analyzed from global species-specific models with backwards model simplification using likelihood ratio test. All analyses were performed in R version 3.3.2 (R Core Team, 2015, Vienna, Austria).

3. Results

3.1. Molecular detection of Rickettsia spp.

For 3939 of the 4023 (97.9%) trapped small mammals ear pinna tissue was available for rickettsia PCR analysis (Table 1). The screening *pan-Rick* PCR of ear pinna tissue samples revealed 315 (8.0%) small mammals positive for rickettsial DNA, from which 188 samples had a