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Original article

Are *Apodemus* spp. mice and *Myodes glareolus* reservoirs for *Borrelia miyamotoi*, *Candidatus* Neoehrlichia mikurensis, *Rickettsia helvetica*, *R. monacensis* and *Anaplasma phagocytophilum*?

C. Burri*, O. Schumann, C. Schumann, L. Gern

Institute of Biology, Laboratory of Eco-Epidemiology of Parasites, Emile-Argand 11, 2000 Neuchâtel, Switzerland

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ABSTRACT

In Europe, in addition to *Borrelia burgdorferi* sensu lato and tick-borne encephalitis (TBE) virus, other zoonotic pathogens, like *B. miyamotoi*, a species related to the relapsing fever spirochaetes, *Candidatus* Neoehrlichia mikurensis (*N. mikurensis*), *Rickettsia helvetica*, *Rickettsia monacensis*, and *Anaplasma phagocytophilum* have been reported in the ixodid tick *Ixodes ricinus*. No study was conducted to identify reservoir hosts for these pathogens. Here, we investigated the role played by wild rodents in the natural transmission cycle of *B. miyamotoi*, *N. mikurensis*, *R. helvetica*, *R. monacensis*, and *A. phagocytophilum* in Switzerland. In 2011 and 2012, small mammals were captured in an area where these pathogens occur in questing ticks. *Ixodes ricinus* ticks infesting captured small mammals were analysed after their moult by PCR followed by reverse line blot to detect the different pathogens. Xenodiagnostic larvae were used to evaluate the role of rodents as reservoirs and analysed after their moult. Most of the 108 captured rodents (95.4%) were infested by *I. ricinus* ticks; 4.9%, 3.9%, 24.0%, and 0% of the rodents were infested by *Borrelia*, *N. mikurensis*, *Rickettsia* spp., and *A. phagocytophilum*-infected larvae, respectively. *Borrelia afzelii*, *B. miyamotoi*, *N. mikurensis*, *Rickettsia* spp., and *A. phagocytophilum* were detected in 2.8%, 0.17%, 2.6%, 6.8%, and 0% of the ticks attached to rodents, respectively. *Borrelia afzelii* was transmitted by 4 rodents to 41.2% of the xenodiagnostic ticks, *B. miyamotoi* by 3 rodents to 23.8%, and *N. mikurensis* was transmitted by 6 rodents to 41.0% of the xenodiagnostic ticks. None of the tested rodent transmitted *Rickettsia* spp. or *A. phagocytophilum* to *I. ricinus* xenodiagnostic larvae. This study showed that rodents are reservoir hosts for *B. miyamotoi* and *N. mikurensis* in Europe.

Introduction

In Europe, the hard tick *Ixodes ricinus* is the vector of pathogens such as viruses, protozoa, and bacteria. During the last decades, new molecular tools revealed an even larger spectrum of tick-borne pathogens in questing ticks, ticks collected from various hosts and in host organs.

The *Borrelia burgdorferi* sensu lato complex is an important group of bacteria transmitted by *I. ricinus*. At least 10 *B. burgdorferi* sensu lato species have been described in *I. ricinus* (*B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. bavariensis*, *B. spielmanii*, *B. valaisiana*, *B. finlandensis*, *B. bissettii*, *B. carolinensis*, *B. lusitaniae*). Most of them are known to cause Lyme borreliosis (Rudenko et al., 2011) and have known reservoir hosts (Piesman and Gern, 2004). Recently, another *Borrelia* species, *B. miyamotoi* belonging to the relapsing fever group usually transmitted by soft ticks (Argasidae) has also been reported in hard ticks (Ixodidae). First recognized in *Ixodes persulcatus* in Japan (Fukunaga et al., 1995), *B. miyamotoi* was then described in *Ixodes scapularis* (Scoles et al., 2001), *Ixodes dentatus* (Scoles et al., 2001; Hamer et al., 2012), and *Ixodes pacificus* (Mun et al., 2006) in the USA, and in *I. ricinus* in Europe (Fraenkel et al., 2002; Richter et al., 2003; Gern et al., 2010). *Borrelia miyamotoi* was detected in one tick removed from human in Sweden (Wilhelmsson et al., 2010), and clinical cases associated with *B. miyamotoi* have been recently reported in Russia (Karan et al., 2010; Platonov et al., 2011) and in North America (Gugliotta et al., 2013). *Borrelia miyamotoi* was reported in the mouse *Peromyscus leucopus* in North America (Bunikis and Barbour, 2005; Hamer et al., 2010), in *Apodemus argenteus* (Fukunaga et al., 1995) and *Apodemus speciosus*, *Myodes rufocanus*, *Myodes rutilus* (Taylor et al., 2013) in Japan, and in wild turkey, *Meleagris gallopavo* (Scott et al., 2010), and in ticks removed from the Eastern chipmunk, *Tamias striatus* (Hamer et al., 2010) in North America. In Europe, *B. miyamotoi* was observed in *Cervus elaphus* (Wodecka, 2007) and

* Corresponding author. Tel.: +41 32 718 3070; fax: +41 32 718 3001.
E-mail address: caroline.burri@unine.ch (C. Burri).

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domestic ruminants (Richter and Matuschka, 2010). Ticks infected by *B. miyamotoi* were also collected on 4 species of passerine birds (Fukunaga et al., 1995; Hamer et al., 2012). *Borrelia miyamotoi* is known to be transovarially transmitted from *I. scapularis* females to their progeny, and *P. leucopus* has been identified as a reservoir host in North America (Scoles et al., 2001).

Rickettsiae of the spotted fever group (SFG) are known as obligate intracellular Gram-negative bacteria. Two *Rickettsia* species that belong to the SFG group, *R. helvetica* (Beati et al., 1993) and *R. monacensis* (Simser et al., 2002) are frequently reported in questing *I. ricinus* (e.g., Sprong et al., 2009; Lommano et al., 2012; Overzier et al., 2013), in ticks feeding on various hosts (e.g., Burri et al., 2011; De Sousa et al., 2012; Speck et al., 2013; Overzier et al., 2013) and in host tissues (Sprong et al., 2009; Schex et al., 2011; De Sousa et al., 2012). Whether *R. helvetica* is pathogenic in humans remains unclear, although cardiac and neurologic problems have been reported (Nilsson et al., 1999, 2010). Transovarial transmission of *R. helvetica* in *I. ricinus* has been reported to be very frequent and effective (Burgdorfer et al., 1979), whereas nothing is known on the transovarial transmission of *R. monacensis*.

In the late 1990s a new bacteria, *Candidatus* Neoehrlichia mikurensis (*N. mikurensis*), first named *Ehrlichia*-like belonging to the family of Anaplasmatacae was described in *Ixodes ovatus* in Japan (Kawahara et al., 2004). Thereafter, *N. mikurensis* was detected in field-collected *I. ricinus* ticks first in the Netherlands (Schouls et al., 1999), followed by reports from other European countries (Sanogo et al., 2003; Lommano et al., 2012; Silaghi et al., 2012). *Candidatus* Neoehrlichia *mikurensis* was also isolated from *I. frontalis* ticks fed on one migratory bird (Movila et al., 2013). *Candidatus* Neoehrlichia *mikurensis* appeared as pathogenic for humans and was identified in the blood of febrile patients in different countries in Europe (Fehr et al., 2010; von Loewenich et al., 2010; Welinder-Olsson et al., 2010; Maurer et al., 2013) and in China (Li et al., 2012) as well as from one dog in Germany (Diniz et al., 2011). *Candidatus* Neoehrlichia *mikurensis* was detected in tissues of wild rodents in Japan (Kawahara et al., 2004; Naitou et al., 2006) and in Europe (Beninati et al., 2006; Andersson and Raberg, 2011; Jahfari et al., 2012; Lommano et al., 2012; Silaghi et al., 2012; Vayssier-Taussat et al., 2012). Few investigations on the transovarial transmission of *N. mikurensis* have been undertaken (Jahfari et al., 2012).

Initially known to infect livestock, *Anaplasma phagocytophilum* was later recognized to be pathogenic for humans (Stuen et al., 2013). *Anaplasma phagocytophilum* is genetically diverse, has a broad host range, and has been detected in several tick species (Bown et al., 2003, 2007; Rar and Golovljova, 2011; Baráková, I., Derdáková, M., Carpi, G., Collini, M., Rosso, F., Tagliapietra, V., Hauffe, H. C., Rizzoli, A. (personal communication)). Studies on the reservoir competence of rodents showed that their role remains unclear (Stuen et al., 2013). Transstadial transmission of the bacterium can occur, but transovarial transmission has not been demonstrated (Rar and Golovljova, 2011; Stuen et al., 2013).

Despite the frequent reports of these new pathogens, the natural cycle of most of them remains unknown. More specifically, the identification of reservoir hosts responsible for their maintenance in nature has been neglected. Reservoir hosts for tick-borne pathogens must fulfil different criteria. One criterion is that it must be a source of infection for ticks and allow the pathogen to be transmitted to ticks feeding on them (Kahl et al., 2002). The fact that pathogens are detected in host organs determines carrier hosts and not reservoir hosts since it does not mean that these hosts are infective for ticks (Kahl et al., 2002). Similarly, the detection of pathogens in ticks attached on hosts does not demonstrate unambiguously that pathogens have been transmitted from the hosts to the ticks and hereby their reservoir role. The aim of the present study was to investigate whether wild rodents act as reservoir hosts for *B. miyamotoi*, *R. helvetica*, *R. monacensis*, *N. mikurensis*, and *A. phagocytophilum* in natural cycles.

Materials and methods

Study site

This study was carried out in a mixed deciduous forest named Bois de l'Hôpital, located at 600 m above sea level (Neuchâtel, Switzerland) (47°00′23.67″N; 6°56′50.00″E). *Borrelia miyamotoi*, *B. burgdorferi* s.l., *A. phagocytophilum*, *N. mikurensis*, and *Rickettsia* spp. were reported in questing *I. ricinus* ticks in this forest (Gern et al., 2010; Lommano et al., 2012).

Rodent trapping and tick collection

Rodents were trapped in September, October, and November 2011 and in April and May 2012. For each trapping session, 100 wooden box traps (Czech trap model, http://members.vienna.at/shrew/trapping.html) were used spaced at 5–10 m intervals and baited with seeds, pieces of apple, and hay. Traps were set at sunset and checked early the following morning. The Department of Agriculture and Nature from Canton of Neuchâtel approved the capture of rodents. For maintenance and all experimental procedures, the Department of Economy of Canton Neuchâtel delivered authorization 2/2009. Captured rodents were brought to the laboratory to be identified, sexed, and caged individually over a pan of water until the engorged ticks dropped off. Engorged ticks were collected from water, dried, identified (Cotty, 1985), and placed in tubes at 98% RH and room temperature until moult. One month after moult, ticks were washed in 70% ethanol and stored at −20 °C until investigation for *Borrelia* spp., *N. mikurensis*, *Rickettsia* spp., and *A. phagocytophilum* infection. A maximum of 10 nymphs per captured rodent was analysed.

Xenodiagnosis

After all feeding ticks had dropped off, a xenodiagnosis was performed on rodents to evaluate their reservoir capacity for *Borrelia* spp., *Rickettsia* spp., and *N. mikurensis*. Approximately 50 *I. ricinus* larvae, from our laboratory colony (Graf, 1978) free of pathogen infection, were placed on the head of each rodent and allowed to feed until repletion. Engorged ticks were maintained as described in the "rodent trapping and tick collection" section and were examined as moulted nymphs (maximum of 10 nymphs per rodents) for *Borrelia* spp., *Rickettsia* spp., *N. mikurensis*, and *A. phagocytophilum*. Rodents were then released at the trapping site. To confirm the absence of *Borrelia* spp. and *N. mikurensis* in the ticks used for xenodiagnoses we screened 200 larvae derived from 5 females from the laboratory colony. These larvae were tested in pools containing 20 individuals.

DNA isolation from blood

After tick drop-off, rodents were anaesthetized intramuscularly with 0.03 ml of Xylasol (GRAEUB, Bern, Switzerland) (0.02 ml) and Ketasol-100 (GRAEUB) (0.01 ml), and blood was obtained from the retro-orbital sinus using Pasteur pipettes (1.1 mm diameter). A volume of 125 μl of each blood sample was placed directly on FTA Classic Cards (Whatman®, Buckinghamshire, UK). Cards were left open for 24 h and stored as indicated by the manufacturer. To obtain DNA, 1.2 mm diameter discs were punched into FTA card blood spot by using a Harris Micro-Punch® (Whatman®, Buckinghamshire, UK). Between samples, the tip of the Harris Micro-Punch® was rinsed in bleach and 70% alcohol and dried with sterile wipe. Discs were washed 3 times in 200 μl FTA purification reagent