Ticks and Tick-borne Diseases 7 (2016) 1109–1115

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Tick species, tick-borne pathogens and symbionts in an insular environment off the coast of Western France

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ARTICLE INFO

Article history:
Received 14 January 2016
Received in revised form 3 August 2016
Accepted 28 August 2016
Available online 29 August 2016

Keywords:
Tick-borne pathogens
Dermacentor
Haemaphysalis
Insular environment

ABSTRACT

Insular environments provide ideal natural conditions to study disease ecology, especially emerging diseases, due to clear differentiation between local and long-distance transmission. Such environments are of particular interest regarding tick-borne pathogens (TBP), since animal exchange with the mainland (along with any ticks they carry) is limited, and because such locations could lie on migratory routes for birds carrying ticks. Therefore both tick species and TBP may display different prevalence than those observed on the continent. As such, an epidemiological survey was performed on Belle-Ile-en-Mer, an island off the coast of Western France, in order to estimate the prevalence of tick species and the microorganisms they carried. Three tick species, *Dermacentor marginatus*, *D. reticulatus*, and *Haemaphysalis punctata* were collected at five different sites in 2010 and 2011. All ticks were tested for pathogen's and symbiont's DNA by (i) PCR for *Anaplasma* spp., *Borrelia* spp., *Rickettsia* spp.; (ii) real-time PCR for *Francisella tularensis*, *Francisella*-like endosymbionts (FLE) and *Coxiella* spp. and (iii) PCR-RLB for *Babesia-Theileria* spp. Pathogen DNA detected in *D. marginatus* including *Borrelia* spp. (18%), *Rickettsia* spp. (13%) which was identified as *R. slovaca, Babesia* spp. (8%), and *Theileria* spp. (1%). Pathogens detected in *D. reticulatus* including *Rickettsia* spp. (31%) identified as *R. raoulti, Francisella*-like endosymbiont (86%), and *Babesia* spp (21%). Pathogens detected in *H. punctata* including *Rickettsia* spp. (1%) identified as *R. aeschlimannii*, FLE (0.4%), *Babesia* spp. (18%), and *Theileria* spp. (7%). *Anaplasma* spp., *F. tularensis*, or *Coxiella* spp. were not detected in any of the collected ticks. This study represents the first epidemiological survey of the insular Belle-Ile-en-Mer environment. It demonstrated the presence of expected pathogens, consistent with reports from island veterinarians or physicians, as well as unexpected pathogens, raising questions about their potential introduction through infected animals and/or their dispersion by migratory birds.
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1. Introduction

Ticks are the most common worldwide vectors of infectious animal diseases, and the second most common vector for human diseases, after mosquitoes (Colwell et al., 2011). They also transmit the largest variety of pathogens, including viruses, parasites, and bacteria (Rizzoli et al., 2014). Tick-borne diseases (TBD) are then extremely important in terms of economic impact and public health. Despite the documented presence of at least 5 different tick genera involved in human and livestock pathogen transmission, the most important tick species in Europe in terms of human and animal health (number of cases and variety of transmitted pathogens) are *Ixodes ricinus*, *Dermacentor marginatus* and *Dermacentor reticulatus* (Reis et al., 2011; Bonnet et al., 2013; Rubel et al., 2016). The abundances of *I. ricinus* and *Dermacentor* spp. ticks peak in spring and autumn, and they share the same habitat including woodland, pastures and urban parks (Estrada-Pena and Jongejan, 1999).

Recent climate change has raised concerns about increasing emergence of infectious diseases, particularly vector-borne diseases and there is increasing evidence that vectors, including ticks, and vector-borne diseases worldwide have expanded their geographical distribution (Leger et al., 2013; Hernandez-Delgado, 2015). The spread of zoonotic diseases may be influenced by migratory birds which can facilitate widespread transmission of TBP, as Lyme Borreliosis spirochaetes, *Anaplasma* spp., *Rickettsia* spp., *Babesia* spp. (Lindgren et al., 2012; Capligina et al., 2014), espe-

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http://dx.doi.org/10.1016/j.ttbdis.2016.08.014
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cially along islands proximate to bird migratory routes. Such insular environments represent then ideal areas in which to study emerging TBD, which can still be influenced by both anthropogenic and non-anthropogenic factors, and where exchanges between migratory birds, and humans or animals from a neighboring continent, are limited (Tortosa et al., 2012). Only one study was conducted in an insular environment in France, in Corsica, which is a large island of 8680 km2 (Grech-Angelini et al., 2016). In this publication, the authors identified different tick species already known to be well established in the south of France on the continent but they also identified *Hyalomma scupense* in different area of the island with a high abundance, whereas this species was known to be absent or sporadically present on other islands around Corsica and in the south of France. Nevertheless in this study, TBP present in ticks from this island were not compared to ticks present from the nearest continent.

Belle-Ile-en-Mer, the largest island off the coast of Brittany, France, has never been the object of a comprehensive survey of the island's tick fauna and potential pathogens they carry. This insular environment represents a particularly interesting location for such studies as the wild and domestic fauna is limited, interactions with animals from the continent are rare (Gélinaud, 2015; Lavollee and Vermesse, 2015), and several local cases of TBD have been reported by veterinarian practitioners or physicians. Bovine theileriosis was observed in the 1990s, and physicians have reported human cases of both Lyme borreliosis and TIck-BOrne LymphAdenopathy (TIBOLA). Even though Belle-Ile-en-Mer hosts a large hare population (*Lepus europaeus* and *Lepus capensis*), no cases of tularemia have been reported. Nor have any cases been reported of Q fever or anaplasmosis, albeit those diseases are under surveillance in Brittany (Lavollee and Vermesse, 2015).

A study realized at only 100 km from Belle-Ile-en-Mer on the continent showed that French ticks (*Ixodes ricinus*) could carry a high number of microorganisms (pathogens or symbionts) (Cotte et al., 2010). Nevertheless, tick species present on an island (and the microorganisms they carried) could be different from those present in the continent. Therefore the aim of our study was to have a large overview regarding the prevalence of tick species and microorganisms they carried on Belle-Ile-en-Mer, an island closed to one of the major route of migratory birds in Europe, focusing on pathogen of medical and veterinary importance including *Borrelia* spp., *Anaplasma* spp., *Rickettsia* spp., *Francisella* spp., *Coxiella* spp., *Theileria* spp. and *Babesia* spp.

2. Material and methods

2.1. Study area and tick collection

Belle-Ile-en-Mer is an island off the Brittany coast of Western France, in the department of Morbihan, and is the largest of Brittany's islands. It is located 14 km from the Quiberon peninsula, and measures 17 km by 9 km (about 84 km^2), with an average altitude of 40 m. Livestock mainly consists of bovines (*Bos taurus*), ovines (*Ovis aries*), caprines (*Capra aegagrus hircus*) and horses (*Equus caballus*) (Lavollee and Vermesse, 2015). There are no large wild mammals such as deer (*Capreolus capreolus*) or foxes (*Vulpes vulpes*), but there are many birds (seabirds and terrestrial birds), reptiles (*Podarcis muralis*, *Lacerta viridis*, *Anguis fragilis*, *Natrix natrix*), lagomorphs (*Oryctolagus cuniculus*, *Lepus europaeus*, *Lepus capensis*), rodents (*Sorex minutus*, *Crocidura russula*, *Myodes glareolus*, *Apodemus sylvaticus*, *Mus musculus*, *Rattus rattus*), hedgehogs (*Erinacus europaeus*) and mole (*Talpa europaea*). An ornithological reserve shelters many colonies of seagulls (*Larus argentatus, L. fuscus, L. marinus, L. cachinnans, Rissa tridactyla, Fulmarus glacialis*), cormorants (*Phalacrocorax aristotelis*), and other birds (*Pyrrhocorax pyrrhocorax*, *Corvus monedula*, *Corvus monedula*, *Corvus corax*, *Columbia livia*, *Falco peregrinus*, *Falco tinnunculus*). Pheasants and partridges (*Phasianus colchicus, Alectoris rufa*) are present in high density on the island and are often exported towards the continent (Gélinaud, 2015).

Five collection sites at different locations on the island were selected, with variable vegetation and/or a natural or suburban environments: (A) a great coastal heathland where goats, cattle, and draft horses graze (47.29N, 3.14W); (B) a sheep and cattle pasture (47.33N, 3.20W); (C) a suburban area with cottontail rabbits (*Oryctolagus cuniculus*), pheasants (*Phasianus colchicus*) and small rodents (*Sorex*, *Apodemus*, etc.) (47.34N, 3.18W); (D) a suburban pasture with a cattle river crossing (47.34N, 3.17W); and (E) a maritime pine wood (47.35N, 3.25) (Fig. 1).

Adult questing ticks were collected using the flagging technique (Vassallo et al., 2000) between April 2010 and May 2011. Ticks were identified at the species level by morphologic examination (Pérez-Eid, 2007) and preserved in ethanol. After washing once in 70% ethanol for 5 min and twice in distilled water for 5 min, adults were individually crushed in 300 μl of Dulbecco's Modified Eagle Medium (DMEM) with six steel balls using the Precellys®24 Dual homogenizer (Bertin, France) at 5500 rpm for 20 s.

2.2. DNA extraction

DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Charbonnières, France) following the manufacturer's instructions. Total DNA was eluted in 80 μl of rehydration solution per sample. DNA samples were stored at −20 °C until further use.

2.3. Pathogen detection

2.3.1. *PCR:* Borrelia *spp.,* Anaplasma *spp., and* Rickettsia *spp.*

For *Borrelia* spp. and *Rickettsia* spp., the *16S* RNA gene or the *gltA* gene (Table 1) were amplified, respectively, using the Thermo Scientific Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific, MA, USA) (Regnery et al., 1991; Marconi and Garon, 1992). Each reaction was carried out in 20 μl reaction volume containing 5 μl tick DNA, 4 μl of 5X PCR buffer, 200 μM each dNTP, 0.5 μM each primer and 0.02 U/μl Phusion DNA polymerase. Thermal conditions were 98 °C for 30 s followed by 35 cycles at 98 °C for 10 s, 51 °C (for *B. burgdorferi* s.l.) or 56 °C (for *Rickettsia* spp.) for 30 s, and 72 °C for 30 s, with a final elongation step at 72 °C for 10 min.

For *Anaplasma* spp., the *16S* RNA gene (Table 1) was amplified as previously described (Hornok et al., 2008). Each reaction was carried out in a 25 μl volume containing 2 μl tick DNA, 0.8 μM primer, 200 μM each dNTP, 2.5 μl of 10X PCR buffer, and 1U of Taq DNA polymerase (5U/μl Takara Biomedical Group, Shiga, Japan). For the first cycle, DNA was denatured at 95 °C for 8 min, followed by 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 54 °C for 1 min, and elongation at 72 °C for 1 min, plus one extension cycle at 72 °C for 10 min.

Both negative (water) and positive (DNA from cultured strains or field samples) controls were included in each PCR.

Amplicons were sequenced twice (with both forward and reverse primers) by Eurofins MWG Operon (Germany). Generated sequences were assembled using BioEdit software (Hall, 1999), and compared to sequences from the NCBI database.

2.3.2. *PCR-Reverse-line blot:* Babesia *and* Theileria *spp.*

PCR was performed to amplify the *Babesia* and *Theileria 18S* rRNA V4 hypervariable region (Nagore et al., 2004). Reactions were carried out in 50 μl with 5 μl tick DNA using a thermocycler 2720 (Applied Biosystems, California, USA). PCR products were then used for reverse-line blot (RLB) hybridization, as previously described (Schnittger et al., 2004). For each piroplasm,

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