Ticks and Tick-borne Diseases 6 (2015) 607–610

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Short communication

Ticks on passerines from the Archipelago of the Azores as hosts of borreliae and rickettsiae

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ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form 29 April 2015
Accepted 1 May 2015
Available online 11 May 2015

Keywords:
Azores
Birds
Borrelia turdi
Haemaphysalis punctata
Ixodes frontalis
Ticks

ABSTRACT

We examined the presence of borreliae and rickettsiae bacteria in ticks from wild passerine birds on three islands of the Archipelago of the Azores, the westernmost region of Palearctic. A total of 266 birds belonging to eight species from seven families were examined on São Miguel, Santa Maria and Graciosa islands in 2013. Ticks collected from these birds consisted of 55 *Ixodes frontalis* (22 larvae, 32 nymphs, 1 adult female) and 16 *Haemaphysalis punctata* nymphs. *Turdus merula* and *Erithacus rubecula* were the birds most infested with both tick species. Three *T. merula* in Santa Maria were infested with 4 *I. frontalis* infected with *Borrelia turdi*. No rickettsiae were found in the ticks. We report for the first time the presence of *I. frontalis* and *B. turdi* on the Azores islands and we showed that the spatial distribution reaches further west than previously thought.

Introduction

Tick infestation on birds and their role as reservoirs and vectors for tick-borne pathogens have been studied extensively throughout the world. Several recent studies have assessed patterns of tick infestation on birds in mainland Portugal and some emerging tick-borne pathogens have been detected in those ticks, including borreliae and rickettsiae (Norte et al., 2012, 2013; Santos-Silva et al., 2011). Tick species parasitizing passerine birds in Portugal include *Haemaphysalis punctata, Hyalomma marginatum, Hyalomma lusitanicum, Ixodes acuminatus, Ixodes arboricola, Ixodes frontalis*, and *Ixodes ricinus* (Norte et al., 2012). Such ground- and shrub-dwelling bird species as *Turdus merula*, *Erithacus rubecula* and *Sylvia melanocephala* have been found to be most heavily parasitized by ticks (Norte et al., 2012). According to our knowledge, there has been no information heretofore about ticks on birds from the Portuguese Atlantic islands, including the Azores, the westernmost region of Palearctic. A few studies have surveyed *Borrelia burgdorferi* s.l. genospecies and *Rickettsia* sp. infection in ticks and hosts on the Atlantic island of Madeira (De Sousa et al., 2012; Lopes de Carvalho et al., 2008). Although cases of Lyme borreliosis have been reported from the Azores (Lopes de Carvalho and Núncio,

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http://dx.doi.org/10.1016/j.ttbdis.2015.05.003
1877-959X/

2006), and notwithstanding published findings indicative of contact with borreliae and rickettsiae in small wild mammals on the Azores (Collares-Pereira et al., 1997), no studies on the prevalence of its etiologic agent in ticks had previously been made.

We examined tick infestation in wild sedentary passerine birds on three islands of the Archipelago of the Azores in 2013. The aims of these investigations were to characterize the tick assemblages on birds from the Azores and to assess their infection status for *Borrelia* and *Rickettsia* spp.

Materials and methods

The Macaronesian archipelago of the Azores is situated in the Atlantic between 36°55′ and 39°43′N and 25°01′ and 31°07′W, and it comprises nine islands. Their total surface area is about 2250 km^2 and the islands stretch more than 600 km from east to west. The nearest point on the mainland is Cabo Roca in Portugal, which is 1408 km east of Santa Maria.

In 2013, a total of 266 sedentary birds were mist-netted on the Azores. Birds were captured at various sites on each of the three islands explored. Call playback was used to bring birds to the net. The birds were identified using the field guide by Clark (2006) and ringed. Totals of 107, 84, and 75 birds from São Miguel (14–19 April 2013), Santa Maria (18–21 September 2013), and Graciosa (21–24 September 2013), respectively, were examined. Ticks were collected through naked eye examination with tweezers performed only by the senior author. The birds were released after examination. The ticks were stored in 96% ethanol and identified to species level in the laboratory.

Ticks were separated by instars and processed individually. To identify ticks from São Miguel, we used molecular tools as described previously (Ogrzewalska et al., 2014). Each tick was submitted to DNA extraction by the guanidine isothiocyanate-phenol technique (Sangioni et al., 2005), and tested by polymerase chain reaction (PCR) following Mangold et al. (1998). The primers used for the amplification of a 460-bp fragment of the 16S rRNA gene were forward 16S + 1 (5′-CCGGTCTGAACTCAGATCAAGT-3′); reverse 16S-1 (5′-GCTCAATGATTTTTTAAATTGCTGT-3′). PCRs (25 μl) were performed in an Applied Biosystems Thermocycler (Gene Amp PCR System 2700) by adding 2.5 μl of the DNA template to 12.5 μl of DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific Inc.), 1.0 μl of each primer at 20 μM, and 8.0 μl of nuclease-free water. Conditions included an initial denaturation step at 94 °C for 2 min followed by 35 cycles for 30 s at 94 °C, 30 s for primer annealing, and 45 s for primer extension at 72 °C. The annealing temperature of the first seven cycles was increased by 0.3 °C every second cycle from 47 to 48.8 °C, followed by 28 cycles using an annealing temperature of 50 °C. A final extension step was carried out for 7 min at 72 °C. A negative control (water) and a positive control (extracted DNA from *Amblyomma ovale*) were used. Ten microliters of the PCR product was separated by electrophoresis in a 1.5% agarose gel, stained with SYBR® Safe DNA gel stain (Life Technologies) and examined by UV transillumination. Amplified products were purified with ExoSAP-IT reagent (Affymetrix) according to manufacture's protocol and submitted to DNA sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit in ABI 3730 DNA Analyser (LifeTechnologies–Applied Biosystems) as previously described (Labruna et al., 2004). Sequences were edited and assembled using Geneious Basic 5.5.6 and compared with those available in GenBank by NCBI BLAST (Altschul et al., 1990). Ticks from Santa Maria and Graciosa were identified to species level by conventional keys and descriptions using a stereomicroscope (Estrada-Peña et al., 2004; Pérez-Eid, 2007).

All ticks were individually tested for the presence of *Rickettsia* by PCR following protocol described by Labruna et al. (2004) using primers forward CS-78 (5′-GCAAGTATCGGTGAGGATGTAAT-3′) and reverse CS-323 (5′-GCTTCCTTAAAATTCAATAAATCAGGAT-3′) which amplify a 401-bp fragment of the citrate synthase gene *gltA* for all *Rickettsia* species. PCRs (25 μl) were performed in an Eppendorf Mastercycler Thermal Cycler by adding 2.5 μl of the DNA template to 12.5 μl of DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific Inc.), 1.0 μl of each primer at 20 μM, and 8.0 μl of nuclease-free water. PCR cycling conditions were as follows: 1 initial cycle at 95 °C for 3 min; 40 cycles of 15 s at 95 °C, 30 s at 48 °C, and 30 s at 72 °C; and 1 final cycle at 72 °C for 7 min. For each reaction, a negative control (water) and a positive control (Extracted DNA from *R. parkeri*-infected Vero cells) were used.

Extracted DNA from ticks from Santa Maria and Graciosa was also analyzed in a nested PCR targeting the 5S–23S rDNA intergenic spacer region of *Borrelia* spp. (Rijpkema et al., 1995). *B. japonica* was used in positive controls and UltraPure DNase/RNase-Free Distilled Water was used in negative controls. Amplified products of the nested PCR reactions were visualized on 1.5% agarose gels (Roche diagnostics GmbH, Mannheim, Germany). After purification of the amplicons in a JETquick system (Genomed, Inc.), genospecies were determined by DNA sequencing using a Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems) on a ABI 377 DNA sequencer, and BLASTn analysis (NCBI). Positive 5S–23S samples which did not produce quality sequences for genospecies determination were further tested with primers targeting the *flaB* gene (Johnson et al., 1992).

Genospecies were determined by DNA sequencing and BLASTn analysis (NCBI) after purifying the amplified products of nested PCR using the JETquick system (Genomed, Inc.). *B. burgdorferi* s.l. sequencing procedures were performed at the Molecular Biology Laboratory of National Institute of Health Dr. Ricardo Jorge in Lisbon.

Results

A total of 266 passerines belonging to eight species were captured and screened for ticks (Tables 1 and 2). Ticks ($n = 71$) collected from these birds consisted of 55 *I. frontalis* (22 larvae, 32 nymphs, one adult female) and 16 *H. punctata* nymphs. *T. merula* (*I. frontalis* prevalence = 39.4%, mean infestation intensity 3.4 ± 0.9; *H. punctata* prevalence = 9.1%, mean infestation intensity 3.3 ± 1.9) and *E. rubecula* (*I. frontalis* prevalence = 22.2%, mean infestation intensity 1.5 ± 0.3; *H. punctata* prevalence = 5.6%, with only one bird was parasitized with one tick) were the birds most infested with both tick species. *I. frontalis* from São Miguel were identified using molecular tools. The highest 16S rDNA similarity in GenBank was with *Ixodes frontalis* (KJ414454) (100%, 433/433 bp for 13 specimens; 97%, 423/437 bp for 2 specimens, and 96% 424/440 bp for 2 specimens) (GenBank accession numbers KP769863, KP769862, and KP769861, respectively).

Only ticks from Santa Maria and Graciosa were screened for *Borrelia* spp. Three out of 18 (prevalence 17%) *T. merula* from Santa Maria carried 4 (2 larvae and 2 nymphs) *I. frontalis* infected with *B. turdi*. The 5S–23S rRNA gene amplified sequence showed 97% similarity to Ya501 *B. turdi* reference sequence in Genbank, and the *flaB* sequence was identical to a previously detected sequence from a *T. merula* biopsy (PoAnB9 – GenBank accession number JQ765378; Norte et al., 2013).

No rickettsiae were found in the ticks (all ticks were examined).

Discussion

Ticks collected from birds on the Azores consisted of *I. frontalis* and *H. punctata*. *Ixodes frontalis* is an ornithophilic tick infesting mainly passerines from various parts of Europe, Asia and North