Borrelia hispanica in *Ornithodoros erraticus*, Portugal

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Abstract

Tick-borne relapsing fever (TBRF) is a spirochetal infection caused by the genus *Borrelia*. The disease is distributed in the Old and New World with many different species reported. In Europe, TBRF is caused by *B. hispanica* transmitted to man by *Ornithodoros erraticus*, a soft tick usually found in old premises to shelter pig herds. In Portugal, the first human case of TBRF was reported in 1942 but since the beginning of the 1960s, the disease has rarely been described and seems to either have disappeared or have been undiagnosed. Therefore, in 2009 a survey was undertaken to evaluate the presence of the tick in this type of premises and to evaluate its role as a reservoir of *Borrelia*. The work was carried out where the ticks were previously reported in the Alentejo and Algarve regions. Of 63 pigpens surveyed, *O. erraticus* was collected from 19% (n = 12) of these pigpens using CO_2 traps. To evaluate potential *Borrelia* hosts, both pigs (n = 25) and small rodents (n = 10) inhabiting these pigpens were surveyed for *Borrelia* presence, by whole blood PCR and/or tissue culture, respectively. All results for pigs and rodents were negative for the presence of *B. hispanica*. PCR assays targeting the 16S rRNA gene and intergenic spacer region of *Borrelia* were used. Sequence analysis of the positive samples confirmed the presence of *B. hispanica* in 2.2% (n = 5) of ticks from a pigpen in Alentejo. These results confirm natural, but albeit low, persistence of this agent in Portugal.

Keywords: *Borrelia hispanica*, *Ornithodoros erraticus*, pigpen, Portugal, tick-borne relapsing fever
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Introduction

Tick-borne relapsing fever (TBRF) is a spirochetal infection caused by *Borrelia* species transmitted by infected soft ticks of the genus *Ornithodoros*. TBRF is characterized by episodes of recurring fever and unspecific symptoms such as headache, myalgia, arthralgia, chills and abdominal pains [1,2], and is highly endemic in some African countries [3,4]. In Eurasia, TBRF is sporadic, affecting mainly humans who enter caves, ruins or animal shelters infested with soft ticks [5]. In Portugal, the disease was confirmed for the first time in 1942, probably introduced from southern Spain, where it occurs more frequently [1]. Within the Iberian Peninsula the aetiological agent of TBRF is *Borrelia hispanica*, whose vector is *Ornithodoros erraticus* (Lucas, 1849). No human cases of relapsing fever have been recorded since 1961 in Portugal, although the disease may remain undiagnosed or mistaken with other febrile infections [1]. Tick-borne relapsing fever can be diagnosed, during the febrile periods, directly by dark-field microscopy or Giemsa-staining of blood smears; however, this may not be suitably sensitive for all cases. Molecular diagnosis by PCR is very sensitive and can discriminate between the different *Borrelia* species, using an intragenic spacer (IGS) and 16S rRNA genes [1,6,7].

Ornithodoros erraticus is a soft tick from the Argasidae family, which is 'leathery', nonscutate and a haematophagous nocturnal feeder [8–10]. Attachment to the host is only brief, about 20 min, with ticks residing within animal premises [11]. Argasid ticks may live up to 7 years without blood meals [12]. The biological cycle comprises larvae, nymphs (I–V) and adults of both genders [13]. Typically in

the Iberian Peninsula, this tick is associated with swine or rodents and their surroundings, where ticks remain buried in soil or within crevices. *Ornithodoros* can feed from multiple warm-blooded vertebrates, including humans [9,10,14]. Besides the transmission of *Borrelia*, the bite of *Ornithodoros* ticks can cause itch, skin irritation and local oedema [3,15]. Activity decreases during winter and resumes in spring and summer, being influenced by increasing temperatures [16]. Previous surveys of *O. erraticus* reported its presence in southern regions of Alentejo and the Algarve [11], which was only found in old buildings made of stone and clay with soil floors used to shelter Alentejano breed pigs (Fig. 1) [1,16]. Distribution of this breed correlates usually with an extensive production system, using cork oak forests whose acorns provide a preferred diet [11].

The ability of *O. erraticus* to feed on multiple hosts raises the possibility of an extensive list of potential species acting as a natural reservoir for *B. hispanica*. Some relapsing fever *Borrelia* are perpetuated by rats and other small rodents, porcupines, bats and birds, which can be potential hosts. Pigs are considered either as reservoir hosts or refractory to infection [1,17].

Beyond human health, tick infestation represents an important problem for pig herds, as *O. erraticus* is a competent vector of African swine fever virus. Furthermore, farmers complain that tick-infested herds are associated with decreased productivity, increased piglet mortality and lesions in both the ham and skin [11].

To evaluate the prevalence of *B. hispanica* in Portugal, we undertook an investigation to determine its presence among *O. erraticus* collected from pigpens. Moreover, both pigs and small rodents inhabiting these pigpens were evaluated for their potential as reservoirs for *B. hispanica*.

FIG. 1. Traditional pigpen in the south Alentejo region (Castro Verde) infested by *Ornithodoros erraticus* ticks.

Materials and Methods

Tick collection and identification

The study was carried out between June and October 2009 in Alentejo and the Algarve, southern Portugal. Pigpens were selected according to their physical features, such as stone and clay walls and soil floor (Fig. 1), together with the results from previous questionnaires to veterinary surgeons on Alentejano pig herds [18].

A total of 63 pigpens were investigated for *Ornithodoros* ticks using manual collection and overnight CO_2 traps [19]. Collected ticks were stored in the dark with circulating air for up to 3 months at room temperature and 63% mean relative humidity until gender and developmental stages were determined as reported before [8,18]. Nymphs are grouped as small nymphs (stage I–III) and large nymphs (stage IV–V).

Preparation of DNA extracts from ticks

Ticks were washed with distilled water, 10% hydrogen peroxide and 70% ethanol, each for 5 min. Tick DNA was extracted individually using the ammonia method as previously described [20].

PCR amplification

Ticks were initially screened by PCR using primers REC4 and REC9 against the 16S rRNA gene [7]. All PCR mixtures were prepared with FastStart PCR Mastermix according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany) using a final reaction volume of 25 μL. PCR was performed as previously described [21] with the following modifications: denaturation step at 95°C for 10 s, annealing at 50°C for 10 s, and extension at 72°C for 1 min. Results were confirmed using a nested-PCR, targeting an intergenic spacer (IGS), as previously described [6]. Positive controls, *B. hispanica* and *B. recurrentis* DNA (kindly provided by Lise Gern and Danièle Póstic) are included in every PCR assay. To avoid contamination, different steps of the PCR and nested-PCR were conducted in separate laboratories and non-template controls were included in every assay. The resulting amplicons were resolved by electrophoresis on 1.5% agarose gel stained with GelRed according to the manufacturer's instructions (Biotium, Hayward, CA, USA).

Sequencing and phylogenetic analysis

All positive amplicons were purified using the Jetquick Purification PCR Product Spin kit (Genomed Inc., Lohne, Germany) and sequenced with the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 377 DNA sequencer. Sequencing reactions used

the same primers as for the PCR but in 3 pmol/μL, in both directions, for 16S rRNA and IGS genes. In IGS sequencing, the second round PCR primers were used. The sequences were analysed using Lasergene software (DNASTAR v7, Madison, WI, USA). All sequencing procedures were performed in the Molecular Biology Laboratory of National Health Institute Doutor Ricardo Jorge (Lisbon).

Sequence results were aligned and trimmed and a neighbour-joining phylogenetic tree was constructed by using MEGA5 [22]. A neighbour-joining phylogenetic tree was constructed using sequences published in GenBank to compare with our sequence [4,23].

Rodent collection

Sylvatic rodents were collected using Sherman and Tomahawk traps from the pigpens with ticks, during summer, when both ticks and rodents are more abundant and active. A total of 299 trap nights were carried out in those pigpens infested with *Ornithodoros*. Traps are placed in the shelter's interior and in nearby surroundings. Captured rodents were returned to the laboratory. After euthanasia by CO_2 gas exposure, whole blood, heart, bladder, kidney and ear skin were collected. DNA extraction of 200 μL whole blood samples was performed with QIAmp DNA Mini kits (Qiagen, Hilden, Germany) prior to PCR.

Cultivation of *Borrelia* spirochetes

Rodent tissues were disinfected by successive immersion in iodine, 70% ethanol and distilled water prior to inoculation into 8 mL of complete Barbour-Stoenner-Kelly (BSK-H) medium (Sigma, St. Louis, MO, USA). Cultures were maintained at 34°C for 12 weeks and examined weekly by dark-field microscopy to monitor the presence of spirochetes. After 12 weeks, 1 mL of each culture was centrifuged for 15 min at 7000 *g*, and DNA extraction was performed using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's recommendations. Extracted DNA was subjected to *Borrelia*-specific PCR as described before in this section.

Porcine blood collection

Blood samples were collected from 25 adult Alentejano pigs (*Sus scrofa domesticus*) from three pigpens infested with ticks. Whole blood (5–10 mL) was collected from superior vena cava, using heparin as anticoagulant. DNA from 200 μL of whole blood was extracted with QIAmp DNA Mini kit and subjected to PCR as described above.

Results

Collected ticks

Of the 63 pigpens investigated, 12 (19%) (E1–E12) were infested with *O. erraticus*. All were located in the south Alentejo region (Fig. 2). From the 63 total pigpens, 44.4% had the optimal described characteristics to hold the argasid, while the other 55.6% had some structural modifications that could have compromised the tick presence. The numbers of ticks collected (Fig. 3) are detailed in Table 1, and varied between six in pigpen E9 and 24 983 in pigpen E12. Engorged ticks were found in all pigpens, including those with no pigs for more than a year (E2 and E8). All pigpens from the Algarve (n = 11) and north Alentejo (n = 15) were negative for this parasite.

Detection of *Borrelia* within *Ornithodoros erraticus*

Collected ticks from each pigpen were tested for *B. hispanica* infection (Table 1), with between 1% in E5, E8, E11 and E12

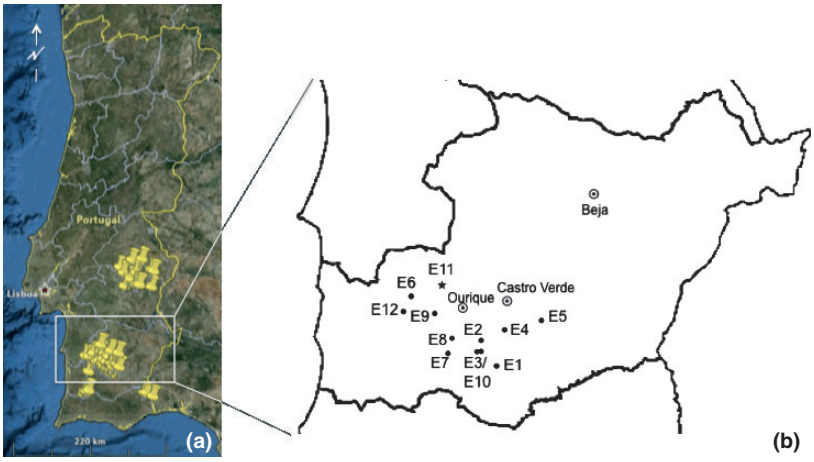

FIG. 2. (a) Location of pigpens investigated for *Ornithodoros erraticus* in Portugal (image from Google Earth © 2011 Google). (b) South Alentejo region: •, pigpens where ticks were found; ★, pigpen where *Borrelia hispanica* was identified.

FIG. 3. *Ornithodoros erraticus* collected using a dry ice trap, including some engorged individuals.

to 100% in E9 of total ticks collected being analysed. *Borrelia* DNA was detected within five ticks from a single pigpen, corresponding to 2.2% of the infections from this pigpen and 0.4% when considering the total ticks tested. All the infected ticks were nymphal large stage.

Sequencing and phylogenetic analysis

All the positive DNA sequences (n = 5) were identical, thus only one was used for phylogenetic tree construction. Analysis of 16S rRNA confirmed the gene of a relapsing fever spirochete that was probably *B. hispanica*, but was not highly discriminatory, including our sequence in a clade with *B. duttoni*, *B. crocidurae* and other *B. hispanica* sequences. Phylogenetic analysis of IGS proved more discriminatory with the sequences falling within a clade with *B. hispanica* identified in Morrocco [4], and more distantly associated with *B. crocidurae* and *B. duttonii*/*B. recurrentis*. The intergenic spacer and 16S sequence of the *B. hispanica* (strain PoTiBh1) have been assigned GenBank accession numbers JF440988 and JF440989, respectively (Fig. 4).

Rodents and pig blood collection

A total of ten rodents were collected from the pigpens with ticks; four *Rattus rattus*, two *R. norvegicus*, three *Mus musculus* and one *M. spretus*. All porcine blood samples (n = 25) and mice blood (n = 10) and tissue (n = 40) samples were negative for *Borrelia* by PCR and culture.

Discussion

In Portugal, no human cases of TBRF have been reported since 1961, although the disease could remain under-diagnosed or mistaken for other febrile syndromes. There are no bibliographic reports of TBRF prevalence rates in Portugal, making the incidence difficult to compare between years; instead we are reliant only upon sporadic case reports. Reduction of human TBRF cases may have been an indirect consequence of African swine fever outbreaks in Portugal from 1960 until 1993, resulting in decreasing numbers of Alentejano pig herds and traditional pigpens in this region [11]. As a consequence, pig production housing was modified, with modern shelters being constructed with glass fibre or metal, which are unsuitable for tick survival. It became evident that traditional shelters constructed with stone and clay, often with cracks and crevices, were essential for *O. erraticus* infestation, with no infestation being found in pigpens with smooth walls and floor.

TABLE 1. Pigpen identification and respective pig inhabitation. Total *Ornithodoros erraticus* collected and development stage distribution. Description of tested samples (number, development stage and percentage) and *Borrelia hispanica* infection rate (number and percentage of infected ticks)

Pigpen (geographical coordinates)	Swines presence/ absence in the shelter	*O. erraticus* total (L/SN/LN/F/M)[a]	Tested ticks (SN/LN/F/M)[a] (% from total)	*O. erraticus* positive for *B. hispanica*/infection rate (%)
E1 (37°35′24.78″N, 08° 07′44.59″W)	+	2074 (0/5/1334/495/240)	62 (0/40/15/7) (3)	0/0
E2 (37°36′43.39″N, 08°11′11.19″W)	–	3170 (38/400/1356/874/502)	30 (4/13/8/5) (2)	0/0
E3 (37°35′42.68″N, 08°11′36.13″W)	+	23 (0/20/3/0/0)	13 (15/3/0/0) (30)	0/0
E4 (37°38′15.83″N, 08°05′23.79″W)	+	144 (0/66/66/6/6)	30 (13/13/2/2) (20)	0/0
E5 (37°39′55.68″N, 08°01′48.24″W)	+	8193 (1157/93/4652/1435/856)	163 (2/110/33/18) (1)	0/0
E6 (37°44′49.56″N, 08°23′44.88″W)	+	350 (0/85/242/19/4)	70 (17/48/4/1) (20)	0/0
E7 (37°37′36.00″N, 08°15′36.36″W)	+	20 (0/0/7/9/4)	9 (0/3/4/2) (50)	0/0
E8 (37°38′30.24″N, 08°14′06.18″W)	–	17 080 (31/220/14487/1996/346)	342 (5/290/40/7) (1)	0/0
E9 (37°42′40.14″N, 08°19′06.90″W)	+	6 (0/0/1/2/3)	6 (0/1/2/3) (100)	0/0
E10 (37°35′42.72″N, 08°11′34.68″W)	+	43 (0/11/23/8/1)	14 (3/7/3/1) (30)	0/0
E11 (37°43′30.66″N, 08°14′36.66″W)	+	7549 (0/226/7214/69/40)	226 (7/215/2/2) (3)	5[b]/2.2
E12 (37°42′01.74″N, 08°21′22.20″W)	+	24 983 (0/699/21534/792/1958)	249 (7/215/8/19) (1)	0/0
Total		63 635	1214	5/0.4

+, presence; –, absence (for one or more years).
[a]Larvae/small nymphs/large nymphs/females/males.
[b]All large nymphs.

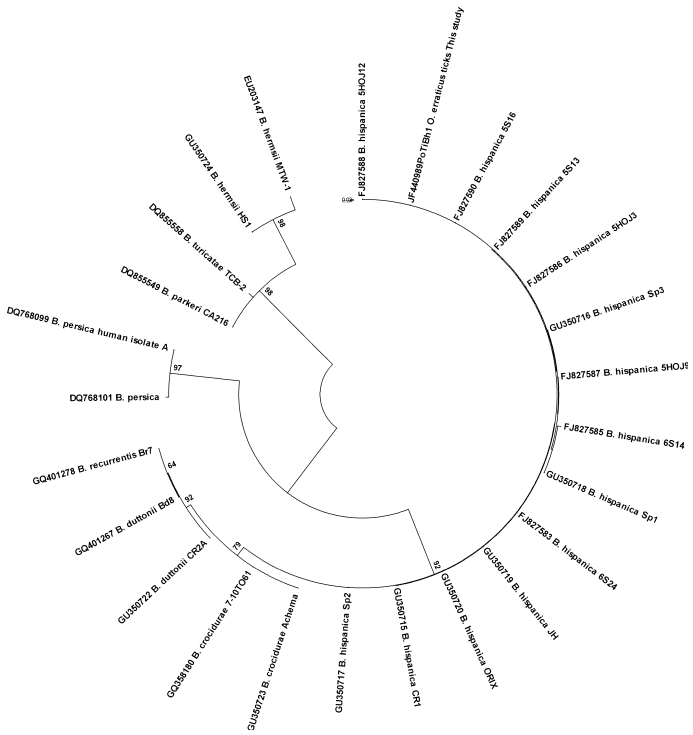

FIG. 4. Phylogenetic tree based on IGS sequences of *Borrelia hispanica* from *Ornithodoros erraticus* collected in Portugal. Tree constructed by using the unweighted pair group method with arithmetic mean, using MEGA5. The sequences used for comparison were obtained from the GenBank database.

Borrelia hispanica has not been detected in Portugal for 50 years, either in human or animal samples. However, a study reported the isolation of *B. hispanica* from three Spanish patients with relapsing fever, two of them having spent holidays in a rural area of Spain and the other being a pig farm worker. Infected *O. erraticus* with similar *Borrelia* were found in both locations and are alleged to have fed from these humans [22,24]. As the geographical area where these cases were detected is near the Portuguese border and has the same ecological characteristics as the southern region of Portugal, it can be expected that TBRF *Borrelia* will also be present in our country, albeit with a low prevalence, and may be under-diagnosed.

In the present study, we report the continued presence of *B. hispanica* in Portugal, albeit with a low prevalence (0.4%). Phylogenetic analysis confirmed its identity as *B. hispanica*, aligning it with an isolate from North Africa (Morocco) [22]. Although there are some minor differences, our sequences fall within a clade alongside Spanish sequences [23] and other Moroccan sequences, suggesting that these all evolved from a common origin. The more distant relationship with species from Asia and America further supports this hypothesis (Fig. 4). Our results demonstrate that, although at a low rate, *B. hispanica* is still present in Portugal and may be responsible for some cases of undiagnosed flu-like human infection detected each year, affecting especially farm workers, veterinarians and hunters [18].

Despite the presence of the tick in 12 pigpens, *B. hispanica* was only present in one. This highly focal distribution could reflect the limited movement range of these ticks [11]. Furthermore, reduction of traditional pigpens in recent years, coupled with regulatory rules and increased distance between premises undertaking pig production, has further added to the demise of these ticks.

The remaining high infestation rate of vacated traditional pigpens is a concern. Whether this is a result of tick longevity or due to as yet undisclosed alternative blood sources, remains to be substantiated. Engorged ticks were found in all pigpens, even in those with no pigs, suggesting the ability of these ticks to feed on multiple alternative hosts, such as swallows or small rodents, whose nests are often found in pigpen interiors. The results of porcine and rodent blood tests failed to yield *B. hispanica*, probably due to the low prevalence of the agent coupled with the small numbers of animals assessed. Low rodent capture could be due to food availability in these farms, which competes with trap baits. Usually producers store high quantities of cereal near pigpens, unwittingly allowing mice to feed. Despite the low

capture, the species collected are those expected for that region [25].

Borrelia maintenance can also be related to transovarial transmission, which occurs in some Argasids. Transovarial transmission can be very efficient, despite probable loss of infectivity [26]. Importantly, the risk of TBRF should be conveyed to those having contact with such pigpens.

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Transparency Declaration

The author declares no conflict of interest in relation to this article.

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