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Molecular detection and characterization of *Leishmania infantum* in free-ranging Egyptian mongoose (*Herpestes ichneumon*)

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ABSTRACT

Wild mammals are susceptible to infection by *Leishmania* parasites. Although canine leishmaniasis is widely distributed in mainland Portugal, the sylvatic cycle of the parasite remains poorly understood. In this study, the occurrence of *L. infantum* in wild carnivores from Portugal was assessed by molecular screening of 132 hunted or accidentally road-killed animals. Spleen samples from Egyptian mongoose, red fox, stone marten, common genet and European badger were tested by amplification of *Leishmania* kinetoplastid DNA and ITS1. Five egyptian mongoose were confirmed *Leishmania* DNA-positive by kDNA-PCR. Phylogenetic analysis of a kDNA amplicon sequence clustered the strain with *L. infantum* sequences from Portugal. These results may suggest that *L. infantum* strains circulating in wild animals are genetically related with strains from more humanized settings. Exposure of wild carnivores to *Leishmania infantum* emphasizes the need of systematic studies to clarify the role of several taxa in the eco-epidemiology of leishmaniasis in Portugal, particularly in areas of carnivore species synanthropy and wherein disease control in the domestic population is inefficient or insufficient.

1. Introduction

Leishmaniasis is a complex of diseases that affects humans, domestic and wild mammals worldwide. It is caused by parasitic protozoans classified as *Leishmania* species (order Kinetoplastida, family Trypanosomatidae). Natural transmission may be zoonotic with the involvement of reservoir hosts such as rodents, marsupials, edentates, monkeys, domestic dogs, and wild canids, usually by the bite of a phlebotomine sandfly species of the genera *Phlebotomus* or *Lutzomyia*. In a few cases and for particular *Leishmania* species, the transmission is strictly anthroponotic, i.e., transmitted from human to human (Ready, 2010; Millán, 2014; Quinnnell and Courtenay, 2009). *Leishmania infantum* infection is endemic in southern Europe but animal cases and vector sand flies have also been detected in central Europe (Maroli et al. 2008; Poeppl et al. 2013). This species is the agent of both cutaneous and visceral forms of human and viscero-cutaneous canine leishmaniasis in Europe. Dogs are considered the main reservoir of *L. infantum* infection in Mediterranean countries, with apparent prevalence rates ranging from 5% to 30%, depending on the region (Millán, 2014). Wildlife monitoring programs and the increasing use of molecular techniques such as PCR have enabled the identification of previously unreported infected species in Spain, Italy and France, specially among carnivores. Serological or direct evidence of *L. infantum* infection in animals from the Canidae, Felidae, Mustelidae, Viverridae and Herpestidae families have been reported (reviewed in Maia et al., 2018; Millán, 2014; Franco et al., 2011). Although there is no official strategy for the control of canine leishmaniasis, an increasing access to vaccination, awareness of dog owners, use of repellent collars and the treatment of animals, have jointly contributed to decrease the number of clinical cases. Despite the prophylactic measures provided to the canine population, leishmaniasis remains widely distributed in Portugal and highly endemic in certain areas. Notwithstanding the major burden

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of leishmaniasis in the dog population, it is also important to understand the contribution of wild carnivores to the epidemiology of this disease. This study is thus a preliminary work aiming to detect natural infection of wild mesocarnivores from Portugal with *Leishmania* spp., particularly focusing on a species that has been under expansion in the mainland territory for the last decades, the Egyptian mongoose (*Herpestes ichneumon*). Although not considered threatened, some wild species included in this study are widely distributed in Portugal (Bencatel et al., 2017) and thus are candidates for further studies to confirm them as *Leishmania* spp. suitable hosts.

2. Materials and methods

2.1. Sample collection

One hundred and thirty-two wild carnivores belonging to Canidae, Mustelidae, Viverridae and Herpestidae taxonomic families (order Carnivora) were collected during 2011 and 2012, namely Egyptian mongoose (*Herpestes ichneumon*, n = 106), red fox (*Vulpes vulpes*, n = 18), stone marten (*Martes foina*, n = 2), European badger (*Meles meles*, n = 3) and common genet (*Genetta genetta*, n = 3). Animal carcasses were collected in all five regions from continental Portugal, originated from accidental road-kills or legal predator control actions (Egyptian mongoose and red fox) and were donated for scientific purposes (Fig. 1). All species listed as game species, or targeted by predator control actions (the case of red fox and mongoose), by Portuguese legislation (Portaria n.° 142/2015 - Diário da República n.° 98/2015, Série I), can be legally hunted by the hunting associations and fellow credentiated hunters authorized by the National Institute for Nature Conservation and Forest (ICNF). After collection, all animals were preserved in sealed plastic bags, refrigerated and transported to a collection centre, under a license from ICNF – Licence no. 222/2010/TRANS, where they were kept at −20 °C. The sex and age [determined by dental analysis- Bandeira et al. (2016)] of the specimens and the geocoordinates of the collection sites were registered. Necropsy and subsequent spleen collection were performed at the national reference laboratories for animal health, the National Institute for Agrarian and Veterinary Research (INIAV IP), or at University of Aveiro in appropriate facilities. Spleen fragments were collected and stored at −20 °C until further use.

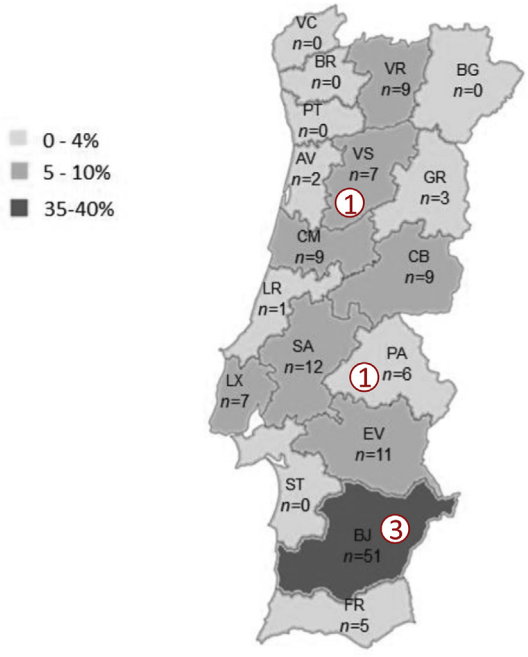

Fig. 1. Spatial distribution of wild carnivore samples in mainland Portugal. Administrative regions at the district level are indicated. The overall proportion of samples per district is indicated by the grey scale.
The abbreviatures of districts are as follows: Viana do Castelo (VC), Braga (BR), Vila Real (VR), Bragança (BG), Porto (PT), Aveiro (AV) Viseu (VS), Guarda (GR), Coimbra (CM), Castelo Branco (CB), Leiria (LR), Santarém (SA), Portalegre (PA), Lisboa (Lx), Setúbal (ST), Évora (EV), Beja (BJ) and Faro (FR). White circles with numbers in red specify the number and location of Egyptian mongooses that were kDNA-positive by PCR. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.2. DNA extraction and molecular analysis

The study samples were screened for the presence of *Leishmania* spp. using molecular methods. Total DNA was extracted from 25 mg of spleen tissue using the High Pure PCR Template Preparation kit (Roche), according to the manufacturer' instructions. *Leishmania* kinetoplast DNA (kDNA) minicircle was selected as the molecular target using primers MC1 (5′-GTTAGCCGATGGTGGTCTTG-3′) and MC2 (5′-CACCCATTTTTCCGATTTTG-3′), which hybridize with a hypervariable portion of kDNA minicircles sequence (Cortes et al., 2004). Reaction and PCR conditions were as described by Cortes and collaborators (2006) and, for positive samples, a 447 bp fragment amplicon was expected. As additional confirmation, amplification of internal transcribed spacer 1 (ITS1) region of the ribosomal RNA (rRNA) encoding gene was performed. This second PCR enables the amplification of a 300–350 bp fragment corresponding to the non-coding spacer region ITS1 found in the ribosomal operon, located between the 18S rRNA and the 5.8S rRNA coding regions. Primers used were LITSR (5′-TGATACC ACTTATCGCACTT-3′) and L5.8S (5′-CTGGATCATTTTCCGATG-3′) and reactions carried out according to El Tail et al. (2000). No-template (water) and positive controls (*Leishmania infantum* DNA from a smear-positive dog isolate) were used in each PCR batch. Amplification reaction products were analysed by electrophoresis on 2.0% agarose gels stained with ethidium bromide.

2.3. Sequencing and phylogenetic analysis

Amplicons from kDNA positive samples were excised from agarose gel, purified using a commercial kit (QIAquick gel extraction kit, Qiagen or Gel Band Purification, GE Healthcare) and sequenced at a commercial company (StabVida, Portugal). The same pairs of primers used in the amplification of each fragment were used in sequencing. Original chromatogram files were inspected and manually reviewed using the ChromasPro® software (version 2.6.5). Edited sequences were compared with similar reference sequences available in public databases (http://www.ncbi.nlm.nih.gov) by BLASTn analysis and submitted to GenBank (http://www.ncbi.nlm.nih.gov).

The phylogenetic relationships between the *L. infantum* sequence obtained during this study with other available sequences in GenBank were investigated by Maximum Likelihood (ML) using MEGA7 software (Kumar et al., 2016). The same software was used to perform the multiple sequence nucleotide alignments (msa) and determine the appropriate substitution model, using the model selection analysis. Robustness of the tree nodes was assessed by bootstrapping 1000 times. The graphical edition of the phylogenetic tree was performed resourcing tree explorer, MEGA7 software (Kumar et al., 2016).

3. Results

Detection of *Leishmania* kDNA in the spleen samples of 18 red fox, two stone marten, three European badger, three common genet and 101 of the surveyed Egyptian mongoose was negative. However, a kDNA fragment of the expected size (approximately, 447 bp) was obtained in five Egyptian mongoose samples among the 132 DNA spleen extracts analysed. A second, independent PCR directed towards the ITS1

intergenic region was employed to test in parallel the 132 carnivore samples. Confirmation of the presence of *Leishmania* DNA by both techniques was attained in only one spleen sample. This specimen belonged to a female adult Egyptian mongoose collected in Beja district during 2011, southern Portugal. A kDNA sequence with 402 bp obtained from the amplicon of this positive Egyptian mongoose was deposited in GenBank under accession number MH799321. Despite several attempts, it was not possible to sequence the amplicon obtained from amplification of the ITS1 fragment.

Positive animals were detected in Beja (n = 3), Portalegre (n = 1) and Viseu (n = 1) administrative units (Fig. 1).

Using partial kinetoplastid minicircle nucleotide sequences, the relationship between this strain and other *Leishmania infantum* strains from nine countries, mostly from the Mediterranean basin (mainland Portugal, Spain, Morocco, Tunisia, Algeria and Italy) was investigated by Maximum Likelihood (ML) phylogenetic inference. The Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) with gamma distributed rate variation among sites (+G) (HKY + G) showed the lowest BIC (2456.521) and AICc (2147.195) values and was subsequently used to infer phylogenetic relationships using ML analysis. It is noteworthy that the T92+G model also displayed similar BIC (2456.564) and AICc (2161.279) values.

Phylogenetic analysis suggests a higher resemblance of the *L. infantum* strain obtained from the Egyptian mongoose (MH799321) with other *L. infantum* strains obtained in mainland Portugal from 1970 to 2003, originating from dogs (*Canis lupus familiaris*) and humans (*Homo sapiens*), although the Bootstrap (Bs) values supporting those nodes are < 70 (Fig. 2). This closer genetic relatedness is also suggested by the high genetic similarity, with homologies ranging from 97,51% to 98,01% (Table 1, supplementary material), and low genetic distances, ranging from 0,01282 to 0,01807 (Table 2, supplementary material), found between strain MH799321 and the *L. infantum* strains from mainland Portugal.

4. Discussion

Leishmaniasis is a parasitic disease of great importance in veterinary medicine, considering the high number of infected dogs, but also with a major impact on Public Health. Canine leishmaniasis is virtually present throughout Portugal, although prevalence rates vary across regions. In the present study, the confirmation of *Leishmania* infection in wild carnivores was based on the detection of *Leishmania infantum* nucleic acids in spleen samples. Using an end-point PCR for the detection of kinetoplastid DNA, it was possible to identify five positive animals among Egyptian mongoose. The kinetoplast DNA minicircle was selected as the molecular target as it is present in hundreds of copies in *Leishmania*, increasing the probability of detecting this protozoan in frozen samples in which microscopic analysis for detection of amastigotes or protozoan isolation could not be performed. The animal from whom kDNA was sequenced was an adult female and probably an asymptomatic carrier, since during the necropsy no evidence of clinical disease nor cutaneous or visceral lesions were apparent. This specimen was collected in Beja district, one of the southern regions with higher seroprevalence of canine leishmaniasis (12.12%) (Cortes et al., 2012). Sequencing analysis of the kinetoplastid DNA allowed the identification of *L. infantum* and phylogenetic inference by ML suggested a closer genetic relationship of this strain with other *L. infantum* strains from mainland Portugal, originating from dogs and humans. This geographic relationship of *Leishmania* sp. isolates and, particularly, the genetic relatedness between strains circulating in the wild and in more humanized environments emphasize that the interface between these two compartments at a local scale may be attenuated by the vector-borne nature of *Leishmania* transmission.

The historical process that led to Egyptian mongoose colonization of Iberia is an issue that is still under debate. While Gaubert et al. (2011) proposed that mongooses reached Iberia through the Strait of Gibraltar during the Middle to Late Pleistocene, more recently Detry et al. (2018) collected data that suggested that this herpestid might have been introduced by the Romans, during their presence in Hispania. This African origin of mongoose could provide additional clues for an ancient *Leishmania*/mongoose relationship.

Mongoose have underwent a tremendous natural expansion process in the last decades from southern to northern Portugal. And recently, the species also invaded the North-eastern areas of the country, from where it was absent in the beginning of twentieth century (Barros et al., 2015, 2016). The recent abandon of croplands, rural depopulation, prodigious bio-ecological adaptability, generalist opportunistic behaviour and lack of natural predators, possibly cumulatively led to this expansion success (Barros et al., 2015). Some population biology aspects of this mesocarnivore species are still largely unknown, namely its health status, contribution to pathogen cross-species transmission and competence as *Leishmania* spp. reservoir. Although mongooses have distributions across several African regions and some SW European areas, *Leishmania* infection has only been reported in two mongooses from Sudan (Elnaiem et al., 2001). In the report from Elnaiem and collaborators (2001), DNA sequences were not publicaly available for phylogenetic comparison, but the authors reported that the species *L. donovani* was responsible for infection in mongoose.

A recent report identified *L. infantum* DNA in rodents, lagomorphs and wild carnivores from Southeast Spain, including stone marten, common genet and red foxes (Risueño et al., 2018). The red fox was the second most tested species [13.6% (18/132)] in our study. Although it is frequently reported as an *L. infantum* carrier and a previous study performed in the 80's in the Arrábida region (mainland Portugal) identified positive animals (Abranches et al., 1983), no kDNA-positive red fox could be confirmed among the population we surveyed. Other species, such as the stone marten, common genet and European badger have occasionally been found positive in other regions of the world, but we did not find any evidence of positive cases, which could also be related with the limited number of samples examined from these species.

This study enabled the detection of *L. infantum* infection in wildlife from Western Iberia. Such data contributes to consolidate knowledge on the epidemiology of this pathogen in the Mediterranean basin and identifying, for the first time, *Herpestes ichneumon* as a possible carrier host in Portugal. Although the results obtained suggest that most mesocarnivore species are not particularly exposed to this parasite in Portugal, it is necessary to carry out further studies to better clarify the role of wild carnivores and taxa, such as leporids and rodents, in the enzootic *Leishmania* transmission cycle and the potential consequences thereof. The limited number of positive cases in this study may also suggest that tissue tropism of *Leishmania* sp. may vary according to host species. To confirm this hypothesis, future studies should consider testing skin and other organs besides spleen. Furthermore, xenodiagnostic analyses could also bring some light into the role of this species as a reservoir or as dead-end host. Anthropogenic changes of ecosystems resulting in the expansion or overabundance of particular wild species, such as the mongoose, may provide more opportunities for direct or indirect contact with domestic animals and humans, significantly affecting protozoan distribution and risk.

The surveillance programs directed towards wildlife or focused on particular pathogens should thus be adapted and extended to other animal taxa and regions of the national territory. Such programs should be a priority in areas where interaction between wild animals, domestic animals, and humans is pronounced, the prevalence in dogs is high and the control strategy is insufficient. Scrutiny in areas wherein natural and anthroponotic conditions propitiate proliferation of phlebotomine vectors should also be granted.

Declaration of competing interest

On behalf of all authors, the corresponding author states that there

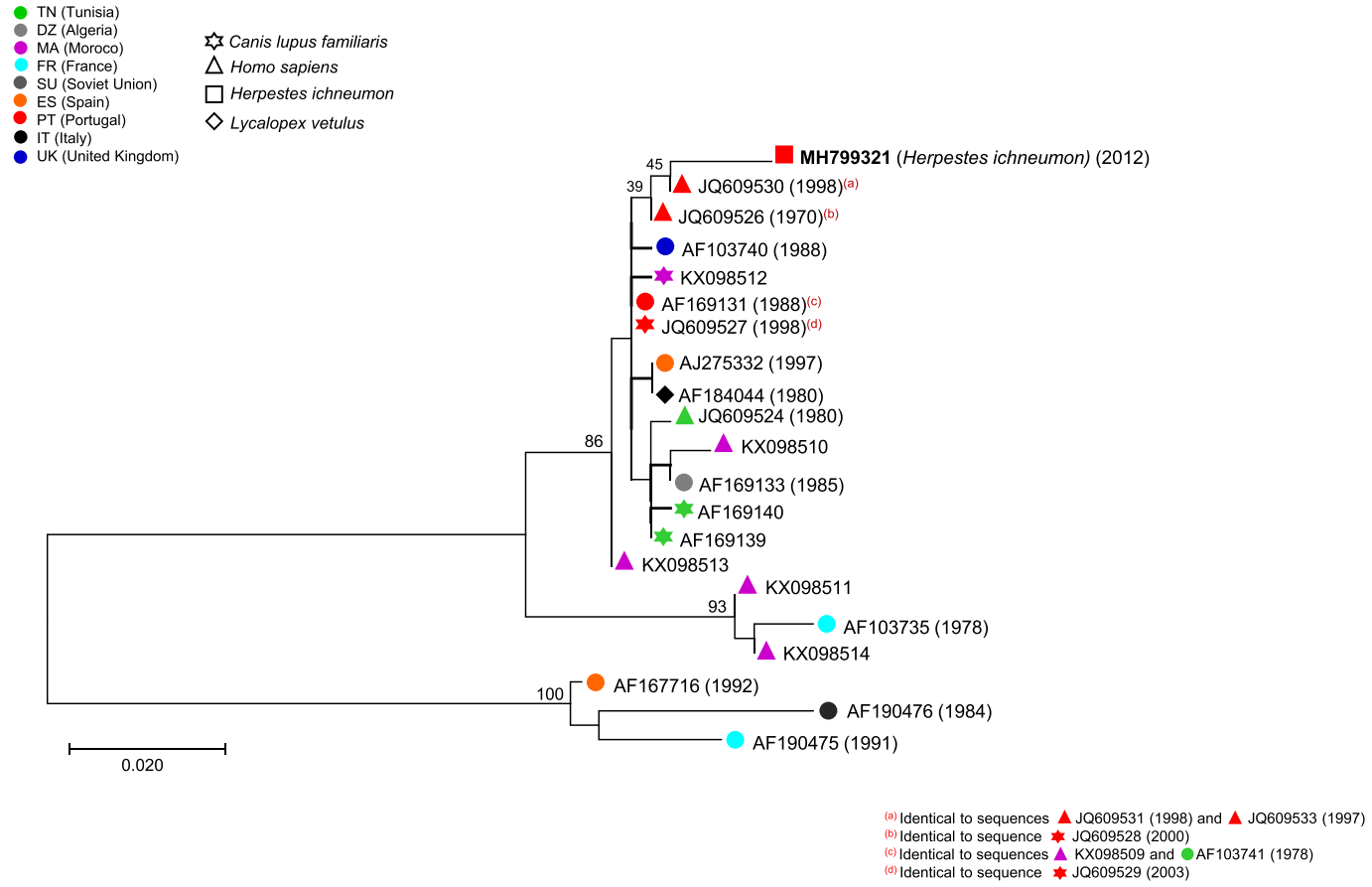

Fig. 2. Maximum Likelihood (ML) phylogenetic tree of 27 *L. infantum* nucleotide sequences (410 nt long in the final dataset, including gaps), obtained during this study (MH799321) and others available in GenBank, based on the Hasegawa-Kishino-Yano model (HKY) (Hasegawa et al., 1985). The tree with the highest log likelihood (−1026.87) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3289) (HKY + G). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.
Robustness of the tree nodes was assessed by bootstrapping 1000 times. The graphical edition of the phylogenetic tree was performed with tree explorer, MEGA7 software (Kumar et al., 2016).
Only bootstrap (BS) values equal or greater than 70 are shown on the tree, with the exception of the MH799321 cluster wherein the Bs values, although < 70, are displayed for the reader.
A 2-letter code and a specific colour (Top left) was attributed to each country for better identification of the origin of each strain. Whenever possible, the host was identified by a specific shape (Top left), namely dog (*Canis lupus familiaris*, star), human (*Homo sapiens*, triangle), Egyptian mongoose (*Herpestes ichneumon*, square) and hoary fox (*Lycalopex vetulus*, diamond). Sampling dates are indicated, whenever available. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.02.001.

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