First detection of Leishmania major in dogs living in an endemic area of Zoonotic Cutaneous Leishmaniasis in Tunisia

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First detection of *Leishmania major* in dogs living in an endemic area of Zoonotic Cutaneous Leishmaniasis in Tunisia

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Abstract

Background: Dogs are considered the main domestic animals that may be reservoir for *Leishmania infantum*, the agent of Zoonotic Visceral Leishmaniasis (ZVL) in several countries of the world. Dog may host other *Leishmania* species but its epidemiological role in the maintaining and spreading of these parasites is not completely elucidated. Zoonotic Cutaneous Leishmaniasis (ZCL), caused by *Leishmania major*, affect thousands of people every year and is particularly diffused in many countries of North Africa and Middle East Asia. In ZCL endemic countries few reports of *L. major* positive dogs have been reported, probably because most human cases occur in poor rural areas where the social role of the dog and its medical management is not well considered. The aim of the present study is to better understand the possible involvement of domestic dogs in the epidemiology of ZCL. **Methods:** Our research focused on a well-established endemic focus of ZCL, in the area of Echrarda, Kairouan Governorate, central Tunisia. Fifty-one dogs with no apparent clinical signs of vector borne diseases, were selected in small villages where human cases of ZCL are yearly present. All dogs were sampled for the *Leishmania* spp. diagnosis, by using the following procedures: blood sample for serology and

buffy coat qPCR, popliteal fine needle aspiration and cutaneous biopsy punch for lymph node and skin qPCR.

Results: The results demonstrated a high percentage (21.6%) of dogs positive at least at one or more test, the most sensitive technique was the lymph node qPCR that detected 8/11 positive dogs. Nine, out of the eleven positive dogs, resulted infected by *Leishmania infantum*; ITS1-PCR-sequencing allowed *Leishmania major* identification in the remaining two cases, both from the popliteal lymph node samples, that can suggest a possible visceral spread of a cutaneous *Leishmania* species in dog. Interestingly, one of the two *L. major* positive dogs was living in the same house where 6-year-old children showed cutaneous lesions referred to ZCL.

Conclusions: To our knowledge, this is the first report of *L. major* positive dogs in Tunisia, the epidemiological role of which remains under investigation.

Keywords: *Leishmania major*, dogs, leishmaniosis

Background

Tunisia represents a perfect example of a Mediterranean Country in which different *Leishmania* species may express their infectivity. Visceral leishmaniasis (VL) caused by *Leishmania* (*L.*) *infantum* and Zoonotic cutaneous leishmaniasis (ZCL) caused by *L. major* are endemic, while Chronic cutaneous leishmaniasis (CCL) caused by *L. tropica* is considered confined in specific foci of interest involving the South-Est and South-West of the country. Interestingly, the two most important cutaneous *Leishmania* species, *L. major* and *L. tropica* may overlap in some areas of the Tunisia [1, 2]. Likewise visceral and ZCL endemic foci overlap was identified in some northern central districts also if they remain geographically distinct [3, 4]. The role of different animals in the epidemiological transmission of these *Leishmania* species is continually under revision. *L. major* is transmitted by the sand fly vector *Phlebotomus papatasi* [5], with *Psammomys obesus* and *Meriones shawi* considered as the principal reservoir hosts [6]. *Mustela nivalis*, *Paraechinus aethiopicus*, *Atelerix algirus*,

Ctenodactylus gundi, and *Psammomys vexillaris* are considered as potential reservoirs for *L. major* [7]. Dogs undebatable play a pivotal role in many situations where the transmission cycle of *L. infantum* occurs while its contribution to the life cycle of *L. major* is considered not relevant despite this species has been detected in very few cases in dogs [8, 9]. *L. major* was firstly identified from an ear ulcer of a dog in Saudi Arabia [10] and from the spleen and blood of two dogs from Egypt [11]. The first clinical report was described in Israel [8], but because of the scarcity of clinical demonstrations, there are no definitive indication for the clinical features nor for the treatment. The infection has been also reported during some epidemiological studies performed in Middle East Countries, like Saudi Arabia, Iran, Iraq, Turkey and recently in Burkina Faso [12, 13], while a previous study performed in a neighboring area of Tunisia did not allow to identify this species in dog [6]. The role of the dog in sustaining the transmission of *L. major* to humans remain under investigation. The aim of the present study is to assess the presence of *L. major* infected dogs in Tunisia to add information on the potential involvement of this domestic animal in the ZCL epidemiology, by focusing the research in a restricted area considered a stable endemic focus of ZCL.

Methods

The study was performed in small built-up areas belonging to the district of Echrarda (35° 07’ 08’’ Nord, 10° 01’ 49’’ Est), South part of Kairouan Governorate, Tunisia. The selection of small cluster of houses hosted dogs was based on the knowledge of recent ZCL confirmed diagnosis, while in the whole district and incidence of 96.7 cases/100,000 Ha was reported [14]. The area is characterized by a rural, semi-arid environment. The registered population is 27,518 (2014) on 330 km^2, while there is scarce information on the dogs’ population. Fifty-one dogs, 31 males, 20 females, with an estimated age ranging between 1-12 years were recruited, based on the compliance of the owners to join the anti-rabies vaccination campaign. After the vaccine administration, the owners were informed about the possibility to submit the dogs to further sampling for *Leishmania* spp. diagnosis, informed consent was obtained from all participants. Dogs were submitted to clinical examination by filling a clinical form, and sampled for blood, popliteal lymph node (LN) aspirate, skin punch

biopsy and conjunctival swab. Blood samples (3 mL) were obtained by peripheral veins, then divided in two aliquots respectively in empty and EDTA coated tubes, for serum, plasma and buffy coat (BC) collection. Lymph node aspiration was performed by one of the two popliteal lymph node, skin biopsy was obtained by punching the surface between neck and ear, with a 1 mm punch biopsy. Conjunctival swabs were performed by both lower eyelids but processed together. The collected materials were stored in 1.5 ml tubes. Samples collection was performed following the Good Clinical Practice medical procedures, in accordance with the international guidelines for animal welfare. Tubes were kept at 4°C until the arrival at laboratory. Blood was centrifuged 10 min at 2500 rpm, to separate red cells, BC and plasma. Plasmas were stored at − 20° until serological analysis; BC, LN aspirates, skin biopsy samples and conjunctival swabs were stored at − 20° until DNA extraction. Serological diagnosis was performed by ELISA (Enzyme linked immunosorbent assay) ID Screen Leishmaniasis Indirect Test® kit (ID vet, Innovative diagnostics, France), the same used in a previous study [6]. Briefly, Optical densities were read at 450 nm (ELISA plate reader Anthos®, Bristol, England). Results were expressed as percentages plasmas whose results were between 40% and 50% and designated by manufacturer as “doubtful” were tested by an indirect immunofluorescence antibodies test (IFAT) using spot slides sensitized by L. *infantum* promastigotes; a threshold of 1:100 defining seropositivity. Additional serological test was performed on *Leishmania* spp. Samples, to detect possible co-infections with other canine vector borne diseases (CVBDs). Antibodies to *Anaplasma phagocytophilum/Anaplasma platys*, *Borrelia burgdorferi*, *Ehrlichia canis/Erlichia ewingii*, and antigens to *Dirofilaria immitis* were detected by SNAP 4Dx Plus test (IDEXX Laboratories, Westbrook, Maine, US).

DNA was extracted from 51 buffy coat, lymph node and skin biopsy samples, using the DNeasy Blood & Tissue kit (Qiagen, Leipzig, Germany) according to the manufacturer’s instructions. Moreover, DNA was extracted from 51 conjunctival swabs, using the Leishmania Screen Glow (Avantech Group, Angri, Italy) following the protocol described by Maurelli et al. [15]. Three different PCR protocols were used for amplification of DNA samples: i) qPCR to amplify a region of the minicircle kinetoplast DNA (kDNA) [16] was used to analyze all the DNA extracted (total= 200 DNA samples); ii) nested-PCR to amplify the

small subunit ribosomal RNA (SSUrRNA)[17] ; iii) end-point PCR to amplify the Internal Transcribed Spacer 1 (ITS-1) region [18]. These last two protocols (endpoint and nested were used in order to confirm positive results obtained by qPCR and to characterize the *Leishmania* species. Briefly, for qPCR a PCR mix was prepared in a final volume of 20 µL, containing 1X Bio-Rad Universal Master Mix (Bio-Rad, USA), 0.3 mM of each specific primer (LEISH-1 5'-GGCGTTCTGCGAAAACCG-3'; LEISH-2 5'-AAAATGGCATTTTCGGGCC-3'), 0.25 mM of probe (5'-FAM-TGGGTGCAGAAATCCCGTTCA-3'-BHQ1) and 2 µL of extracted DNA was prepared. Each amplification was performed in duplicate. To prepare a standard curve, a serial dilution of a positive sample, provided by the National Reference Center for Leishmaniosis (CReNaL), consisting of equivalents of DNA from 1 × 10^6 cells to 1 cell per amplified sample, was prepared. A negative control was added for each run to verify contaminations. The thermal cycling conditions included a 10 min denaturation at 95 °C and 40 cycles of 95 °C for 15 sec and 60 °C for 35 sec. The reactions were performed in a CFX96 (Bio-Rad, USA). To quantify parasite burdens, cycle threshold (Ct) values obtained for each test sample were compared with those obtained for the corresponding standard curve. For SSUrRNA amplification, a first PCR mix was prepared in a final volume of 50 µL, containing 1X EmeraldAmp® GT PCR mix (Takara, France), 25 pmol/ µL of each specific primer (R221 5'-GGTTCCTTTCCTGATTTACG-3'; R332 5'-GGCCGGTAAAGGCCGAATAG-3') and 5 µL of extracted DNA. DNA samples of *Leishmania* was used as positive controls. The thermal cycling conditions included 5 min denaturation at 94°C and 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 5 min. The reaction was performed in a T100 (Bio-Rad, USA). The nested-PCR was prepared in a final volume of 50 µL, containing 1X EmeraldAmp® GT PCR mix (Takara, France), 25 pmol/µL of each specific primer (R223 5'-TCCCATCGCAACCTCGGTT- 3'; R333 5'-AAAGCGGGCGCGGTGCTG-3') and 5 µL of DNA amplified with the first PCR. DNA samples of *Leishmania* was used as positive controls. The thermal cycling conditions included 5 min denaturation at 94°C and 35 cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 5 min. The reaction was performed in a T100 (Bio-Rad, USA). The PCR products obtained from the nested-PCR were detected on a 2% ethidium bromide-stained

low melting agarose gel (Bio-Rad, USA). Bands were cut from the gel under UV exposure, and the amplified DNAs were purified by QIAquick Gel Extraction KIT (Qiagen, Germany). The purified PCR products were sequenced, and the obtained sequences, in both forward and reverse directions, were analysed using the Chromas version 2.6.6 software and compared with sequences present in GenBank, using BLASTn system and ClustalW. For ITS-1 amplification, a PCR mix was prepared in a final volume of 50 µL, containing 1X EmeraldAmp® GT PCR mix (Takara, France), 0.50 mM of each specific primer (LITSR 5'-CTGGATCATTTTCCGATG-3'; L5.8S 5'-TGATACCACTTATCGCACTT-3') and 5 µL of extracted DNA. DNA samples of *Leishmania* was used as positive controls. The thermal cycling conditions included 4 min denaturation at 95°C and 36 cycles of 95°C for 40 sec, 53°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 6 min. The reaction was performed in a T100 (Bio-Rad, USA). The PCR products were detected on a 2% ethidium bromide-stained low melting agarose gel (Bio-Rad, USA). Bands were cut from the gel under UV exposure, and the amplified DNAs were purified by QIAquick Gel Extraction KIT (Qiagen, Germany). The purified PCR products were sequenced, and the obtained sequences, in both forward and reverse directions, were analysed using the Chromas version 2.6.6 software and compared with sequences present in GenBank, using BLASTn system and ClustalW.

Results

The results (Tab.1) demonstrated a high percentage (21.6%) of dogs positive at least at one or more assay and matrix, four of them (36.3%) exhibited clinical signs. The most sensitive technique was the lymph node qPCR that identified 8/11 (72.7%) positive dogs, conjunctival swabs resulted always negative. Clinical, serological and molecular results are summarized in the table 1. By qPCR eight lymph node samples and one skin sample (correspondent to one positive lymph node sample) resulted positive to *Leishmania* spp., with values of amastigotes/ml from 3 to 2,379 for lymph nodes samples, while a value of 943,300 amastigotes/ml was obtained for skin sample. Positive samples obtained by qPCR were confirmed also by SSUrRNA and ITS-1 PCRs. By nested-PCR for amplification of SSUrRNA a band of 358 bp was obtained for each

positive sample. After purification of PCR products and sequencing, five lymph nodes and the positive skin sample showed an identity of 100% with *L. infantum* sequences (GenBank access number: MK495995.1), while two samples showed 99.72% of identity with *L. infantum/major/donovani* (GenBank access numbers: MK495995.1/MT560279.1/LR812647.1). By amplification of ITS-1 a band of 350 bp was obtained for each positive sample. After purification of PCR products and sequencing, five lymph nodes and the positive skin sample showed an identity of 100% with *L. infantum* sequence (GenBank access number: KM677128.1), while two samples showed an identity of 99.08% and 99.17% respectively with *L. major* sequences present in GenBank (Access numbers: FJ753395.1/MN604136.1). The new two sequences have been registered in GenBank with Access numbers: PP534960 and PP536550. Serology conventionally used to detect antibodies against *L. infantum* showed positive results in 6/11 dogs, while resulted negative in the two dogs infected by *L. major*.

IDEXX 4x rapid test identified 8 dogs co-infected with other CVBDs, the most frequent *Anaplasma* spp. that was present in both *L. major* positive dogs, followed by *Ehrlichia canis* and *Dirofilaria immitis.* One dog was infected by 4 different pathogens. Interestingly, one dog infected by *L. major* was living in the same house where a 6-year-old child showed cutaneous lesions referred to ZCL.

Discussion

Among Neglected Tropical Diseases (NTDs), Cutaneous Leishmaniases (CL) represent one of the most important sanitary problems in many Countries of the World, affecting each year millions of people, in which cutaneous disfiguring effects may result after the infection occur. ZCL causes thousands of new clinical cases in Tunisia, with the population of the governorates of Kairouan, Sidi Bouzid, and Gafsa, representing the 87% of the total population at risk [19]. Wild animal reservoirs are considered as a major source of parasite's transmission to maintain the zoonotic cycle of *L. major.* Sand fly and rodent reservoirs control programs have not reached the desirable result due to the

geographical context, semi-arid large environment very difficult to cover, and the animal behavior characterized by the digging of burrows with many entrances. Additionally, many factors may amplify the presence of the transmitting insects and rodents, the main the inadequate household garbage disposal, around the rural houses of this area. In this social context with low economical resources, the dogs are most breaded as guardian dogs living outdoor, exposed to the same sand fly biting risk as humans. Canine reservoirs well accepted as a main source of zoonotic transmission to maintain peri-urban and rural *L. infantum* infection [20]. Domestic dogs were found infected by *L. infantum* also in a neighboring ZCL endemic area, as assessed by a previous study that evidenced a lower prevalence of infection when compared with endemic well-established foci of canine and human infections [6]. Our results confirm that also in endemic ZCL areas, the presence of *L. infantum* parasite can result in high prevalence of infection in dogs, indicating the dog as the most sensible host to this *Leishmania* species. Many factors can contribute to the establishment and progression of *L. infantum* infection, the severity of late-stage disease is correlated with the decrease of the cellular immunity, high antibody levels and increasing parasite load. Several studies have demonstrated that dogs exposed tick-borne co-infections have a higher relative risk of progression to clinical leishmaniosis. Dogs with canine leishmaniosis (CanL) and co-infections with either *Ehrlichia canis, Babesia canis,* and *Rickettsia conorii* had shorter survival time [21]. Additionally, Toepp et al. [22] found that dogs with multiple tick-borne co-infections had statistically significant increased risk for progression of CanL and increased risk for mortality. The high prevalence of single or multiple tick-transmitted infections together with the low sanitary management of the *Leishmania* spp. infected dogs found in the present study, confirms the establishment of *Leishmania* infection as a contribution of many immune unbalancing factors. The detection of mild clinical picture of the infected dogs is probably due to the young average age of the enrolled dogs, however it was not possible to assess the definitive severity of the disease with additional haematological and biochemical parameters. The two dogs found infected with *L. major* exhibited mild clinical signs, both showed antibodies against tick-transmitted infection, *Anaplasma* and *Ehrlichia* spp. The contribution of these arthropod-borne bacterial infections for the establishment

of *L. major* infection has never been studied, but their role could be very similar to what happens in dogs infected with *L. infantum*. No cutaneous lesions were detected in these dogs, where negative skin PCR in both animals may suggest how the skin was not the most relevant infected tissue. Interestingly the parasite's DNA was found in popliteal lymph node of both dogs. The presence of *L. major* in lymph node has been yet demonstrated [8] in a six-month young dog with cutaneous manifestation on the muzzle. These findings can suggest a possible visceral spread of a cutaneous *Leishmania* species in dog. Interestingly, also one of the two positive dogs of the present study was young, with an estimated age of 1.5 year, similarly to another young puppy, found clinically sick in Israel [9]. Due to the limited number of cases, it is not possible to have a clear correlation between age and infection, in addition to the limited life expectation of dogs living in this difficult socio-economical context. Diagnosis of *L. major* infection in dogs is not easy due to the limited availability of serological test. ELISA and IFAT serology, that use the whole *Leishmania* promastigote antigen which do not allow to distinguish among different *Leishmania* species. In addition, dogs infected with *L. major* are negative to rK39 antigen kit, while dogs infected with *L. infantum* and *L. tropica* tested positive [9]. The definitive diagnosis of *L. major* infection in dog is performed by PCR with DNA sequencing, this finding which makes complicate the studies on large number of dogs.

Conclusions

The epidemiological role of dog for this *L. major* transmission remains under investigation, due to the limited number of diagnoses, and the absence of knowledge on the infectiousness of the healthy infected dogs, the epidemiological role of which is well assessed for *L. infantum*. Interestingly, one positive dog to *L. major* was living in the same house where a 6-year-old child showed cutaneous lesions referred to ZCL, however we had not the possibility to perform adjunctive epidemiological investigations The recent demonstration of eight *L. major* canine cases in a survey performed in Burkina Faso, where *L. major* is considered endemic for humans, amplifies the need of knowledge on

the role of dog as reservoir [13]. Undoubtable, the development of urbanized areas in endemic ZCL rural contexts may contribute to the change of rodents' natural habitats, with an increased possibility to have the dog as a major blood source for transmitting sand flies and to consider it a potential reservoir of *L. major* parasite.

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Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MMP, BFNEH and LR performed molecular analyses, ZL, FMV, BI, NH, BA, GM, AK, GO performed clinical examination, collection of samples and serological analyses. All authors contributed to data analysis and preparation of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from Animal Ethics Committee of the National School of Veterinary Medicine, Sidi Thabet (CEEA-ENMV 24/21).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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