The first detection of *Leishmania major* in naturally infected *Sergentomyia minuta* in Portugal

Lenea Campino[1,2]/+, Sofia Cortes[1,3], Lídia Dionísio[4], Luís Neto[4], Maria Odete Afonso[1,5], Carla Maia[1,3,6]

[1]Grupo Leishmanioses [3]Centro de Malária e Outras Doenças Tropicais [5]Unidade de Parasitologia e Microbiologia Médicas, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal [2]Departamento de Ciências Biomédicas e Medicina [4]Departamento de Biologia e Bioengenharia, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Faro, Portugal [6]Faculdade de Medicina Veterinária, Universidade Lusófona de Humanidades e Tecnologias, Lisboa, Portugal

Phlebotomine sandflies of the genus Sergentomyia *are widely distributed throughout the Old World. It has been suggested that* Sergentomyia *spp are involved in the transmission of* Leishmania *in India and Africa, whereas* Phlebotomus *spp are thought to be the sole vectors of* Leishmania *in the Old World. In this study,* Leishmania major *DNA was detected in one* Sergentomyia minuta *specimen that was collected in the southern region of Portugal. This study challenges the dogma that* Leishmania *is exclusively transmitted by species of the genus* Phlebotomus *in the Old World.*

Key words: *Leishmania major* - *Sergentomyia minuta* - vector

Leishmaniases are parasitic diseases caused by protozoans of the genus *Leishmania*. These parasites, which infect various wild and domestic mammals, are transmitted by the bite of phlebotomine sandflies. Species of the genus *Sergentomyia* are widely distributed throughout the Old World and are known to feed on reptiles as well as other vertebrates, including humans (Bates 2007, Berdjane-Brouk et al. 2012). Although these sandflies are considered vectors of lizard *Sauroleishmania*, it has been recently suggested that certain species of the genus *Sergentomyia* are involved in the transmission of *Leishmania infantum* among dogs in Senegal (Senghor et al. 2011) and *Leishmania major* DNA was detected in *Sergentomyia darlingi* in a cutaneous leishmaniasis (CL) focus in Mali (Berdjane-Brouk et al. 2012). Earlier, Mukherjee et al. (1997) detected *Leishmania donovani* DNA in *Sergentomyia* spp in India.

In Portugal, 213 parasite strains have been isolated from sandflies and human and canine leishmaniasis cases have been identified as *L. infantum,* with *Phlebotomus perniciosus* and *Phlebotomus ariasi* as the proven vectors (Campino et al. 2006). However, *L. major/L. infantum* hybrids have also been isolated from four autochthonous human leishmaniasis cases, two of which were described elsewhere (Ravel et al. 2006). The identification of these multiple hybrids led to the hypothesis that *L. major* circulates in the country, most likely in infected sandflies. The risk of introduction of new *Leishmania* species in Portugal from travellers or immigrants from North Africa and the Indian subcontinent is a real concern, especially in the southern part of the country (Algarve region). Portuguese military missions in the Middle East pose an additional introduction risk.

An entomological surveillance study of phlebotomine species was performed during the last five years. During *Leishmania* transmission season, sandflies were captured by CDC miniature light traps and identified using entomological keys. Subsequently, kinetoplastid DNA-polymerase chain reaction (PCR) using primers Uni21 and Lmj4 (Anders et al. 2002) and nuclear ribosomal internal transcribed spacer (ITS)-1 PCR analyses were used to screen female sandflies for *Leishmania* infection (Maia et al. 2009). After ITS1-PCR, *Hae*III digestion was performed on the positive PCR products to differentiate *Leishmania* species (Schonian et al. 2003).

L. major DNA was detected in one *Sergentomyia minuta* specimen (sample sm3), which was collected in a rural area of the municipality of Albufeira, Algarve region. Furthermore, a characteristic *L. major* PCR product of 650 base pairs (bp) was obtained using the Uni21 and Lmj4 kinetoplastid primers as determined by agarose gel electrophoresis. After PCR amplification and *Hae*III digestion of the product, restriction fragments characteristic of *L. major* (203 bp and 132 bp) were observed. The amplified fragment obtained by ITS1-PCR was sequenced, aligned and compared with ITS-1 *Leishmania* sequences that were available in the GenBank database. A neighbour-joining tree (Figure) was constructed using SplitsTree4 (Huson & Bryant 2006). The *L. major* sequence from sample sm3 had a high level of identity with *L. major* sequences from strains recovered in the Middle East and Northern Africa (Egypt, accession FJ460456.1, Iran, accessions FN677357.1, AY550178.1, AY260965.1, Israel, accessions EU326229.1, DQ300195.1 and Tunisia, accession FN677342.1).

The foci of CL caused by *L. major* occur in 14 countries of the World Health Organization-Eastern Mediterranean Region (EMR), extending from Morocco to

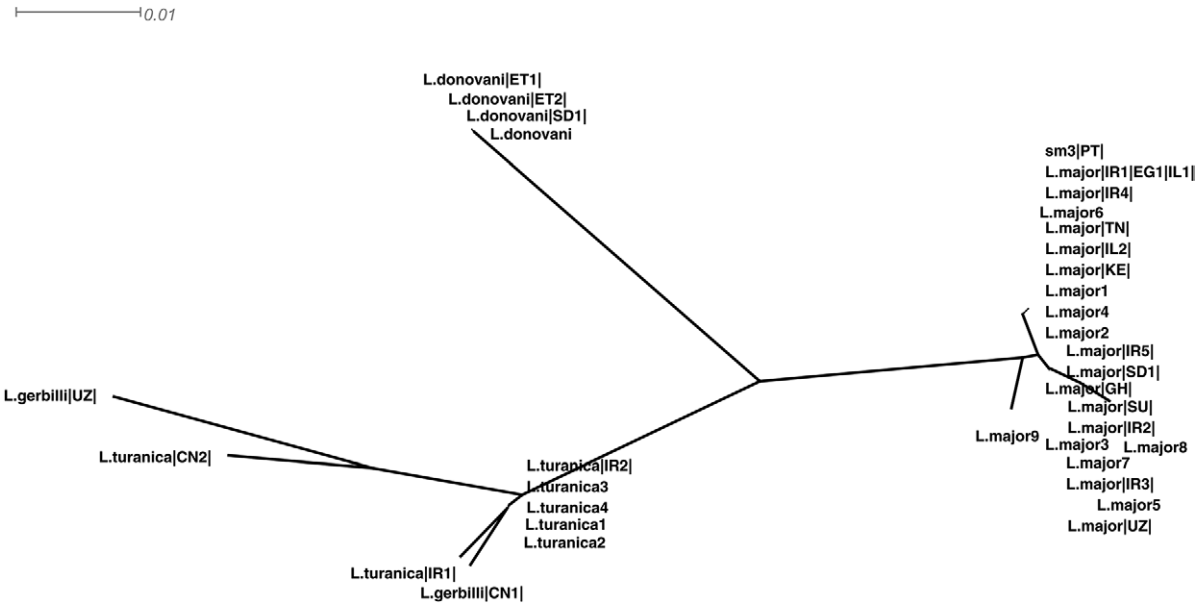
doi: 10.1590/0074-0276108042013020
Financial support: EU/FEDER (PTDC/CVT/112371/2009) (FCT/MMCTES), EU (FP7-261504 EDENext) catalogued by the EDENext Steering Committee as EDENext093.
The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.
CM (SFRH/BPD/44082/2008) and SC (SFRH/BPD/44450/2008) are FCT/MCTES fellows.
+ Corresponding author: campino@ihmt.unl.pt
Received 31 July 2012
Accepted 4 April 2013

Afghanistan (Postigo 2010). Although approximately 100,000 new CL cases were reported in the EMR in 2008, these were rarely caused by *L. infantum*. In contrast, *L. infantum* is the causative agent of CL in Western Europe. To date, no autochthonous human CL due to *L. major* has been identified in Portugal. However, unlike visceral leishmaniasis, the cutaneous clinical form is not under compulsory notification in the country and the misdiagnosis of cutaneous cases is frequent. Until now, the parasites were identified in only six CL cases: four strains were identified as zymodeme MON-1 (3) and MON-29 (1) (Campino et al. 2006) based on isoenzyme typing and two were identified by molecular typing. All of these cases were identified as *L. infantum*. It is imperative to know the causative agent, as the outcome and treatment may differ for *L. infantum* and *L. major* infections.

To our knowledge, this study presents the first detection of *L. major* DNA in a sandfly from the *Sergentomyia* genus in Europe. This finding challenges the dogma that *Leishmania* is exclusively transmitted by species of the genus *Phlebotomus* in the Old World and that *Sergentomyia* is exclusively a *Sauroleishmania* vector. Unfortunately, little prior work has been conducted regarding the development of *Leishmania* sp. in *Sergentomyia* sandflies. To determine the possible role of *S. minuta* in the transmission of *L. major*, it should be demonstrated that the species feeds on humans and that it supports the complete development of the parasite in natural conditions after the infectious blood meal has been digested.

L. major typically uses rodents as reservoir hosts and Jaouadi et al. (2013) have found *Mus musculus* DNA in *S. minuta*. These findings have prompted us to continue to examine *Leishmania* infection in rodents, namely in *M. musculus*, as it is common and widely distributed in the country. Few studies in rodents have been performed by our research group, although *Leishmania* infection has not been detected. However, Charrel et al. (2006) found Toscana virus, a virus responsible for human meningitis and encephalitis in Mediterranean countries, in *S. minuta* (Charrel et al. 2006); thus, the blood meal preferences of this species should also be studied to better understand if *S. minuta* can play a role in the transmission of human pathogens or vector borne diseases.

Further surveillance with extensive and systematic epidemiological surveys on *Leishmania* hosts and vectors are crucial given that increased migration, military deployments and other travel increase the risk of the introduction and spread of *Leishmania* species to non-endemic regions. Furthermore, the "new" parasites could be transmitted by sandfly species that are normally considered non-permissive to *Leishmania* infection. Eco-epidemiological and phylogenetic studies should be performed to clarify the introduction or evolution of *L. major* and potential reservoirs and vectors in Portugal. In addition, the national health services should be encouraged to improve CL diagnosis and parasite identification.



Neighbour-joining unrooted tree built up from internal transcribed spacer (ITS)-1 sequence data of 195 characters from 37 sequences using Kimura-2P model with equal rates variation. Tree generated by SplitsTree4. Sequences used for these analysis (species code and accessions) were as follows: *Leishmania major* |IR1|,|IR2|,|IR3|,|IR4|,|IR5| (FN677357.1, AY550178.1, AY283793.1, AY260965.1, EF653269.1), *L. major* |EG1| (FJ460456.1), *L. major* |IL1|,|IL2| (EU326229.1, DQ300195.1), *L. major* |TN| (FN677342.1), *L. major* |UZ| (FN677357.1), *L. major* |SU| (AJ000310.1), *L. major* |KE| (AJ300482.1), *L. major* |GH| (DQ295825.1), *L. major* |SD| (AJ300481.1), *L. major* 1, 2, 3, 4, 5, 6, 7, 8, 9 (FJ753394.1, FJ753393.1, FJ753392.1, FJ753391.1, JF831924.1, FJ753395.1, EF413075.1, GQ402544.1, GQ402543.1), *Leishmania turanica* |IR1|,|IR2| (EU395712.1, JN860744.1), *L. turanica* |CN2| (HQ830350.1), *L. turanica* 1, 2, 3, 4 (AJ272380.1, AJ272379.1, AJ272378.1, HM130607.1), *Leishmania gerbilli* |CN1| (HQ830351.1), *L. gerbilli* |UZ| (AJ300486.1), *Leishmania donovani* |ET|,|ET2| (FN182209.1, FN182207.1), *L. donovani* |SD| (FN677362.1), *L. donovani* (AJ249620.1), sm3, Portuguese ITS-1 sequence. CN: China; EG: Egypt; ET: Ethiopia, GH: Ghana; IL: Israel; IR: Iran; KE: Kenya; PT: Portugal; SD: Sudan; SU: ex-URSS; TN: Tunisia; UZ: Uzbakistan.

ACKNOWLEDGEMENTS

To I Mauricio, for critical and English revision, and to JM Cristóvão, for technical support.

REFERENCES

Anders G, Eisenberger C, Jonas F, Greenblatt C 2002. Distinguishing *Leishmania tropica* and *Leishmania major* in the Middle East using the polymerase chain reaction with kinetoplast DNA-specific primers. *Trans R Soc Trop Med Hyg 96*: (Suppl. 1): S87-S92.

Bates P 2007. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. *Int J Parasitol 37*: 1097-1106.

Berdjane-Brouk Z, Koné AK, Djimdé AA, Charrel RN, Ravel C, Delaunay P, del Giudice P, Diarra AZ, Doumbo S, Goita S, Thera MA, Depaquit J, Marty P, Doumbo OK, Izri A 2012. First detection of *Leishmania major* DNA in *Sergentomyia (Spelaeomyia) darlingi* from cutaneous leishmaniasis foci in Mali. *PLoS ONE 7*: e28266.

Campino L, Pratlong F, Abranches P, Rioux J, Santos-Gomes G, Alves-Pires C, Cortes S, Ramada J, Cristovão JM, Afonso MO, Dedet JP 2006. Leishmaniasis in Portugal: enzyme polymorphism of *Leishmania infantum* based on the identification of 213 strains. *Trop Med Int Health 11*: 1708-1714.

Charrel RN, Izri A, Temmam S, de Lamballerie X, Parola P 2006. Toscana virus RNA in *Sergentomyia minuta* files. *Emerg Infect Dis 12*: 1299-1300.

Huson DH, Bryant D 2006. Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol 23*: 254-267.

Jaouadi K, Haouas N, Chaara D, Boudabous R, Gorcii M, Kidar A, Depaquit J, Pratlong F, Dedet JP, Babba H 2013. Phlebotomine (Diptera, Psychodidae) blood meal sources in Tunisian cutaneous leishmaniasis foci: could *Sergentomyia minuta*, which is not an exclusive herpetophilic species, be implicated in the transmission of pathogens? *Ann Entomol Soc Am 106*: 79-85.

Maia C, Afonso MO, Neto L, Dionísio L, Campino L 2009. Molecular detection of *Leishmania infantum* in naturally infected *Phlebotomus perniciosus* from Algarve region, Portugal. *J Vector Borne Dis 46*: 268-272.

Mukherjee S, Hassan MQ, Ghosh A, Ghosh KN, Bhattacharya A, Adhya S 1997. *Leishmania* DNA in *Phlebotomus* and *Sergentomyia* species during a kala-azar epidemic. *Am J Trop Med Hyg 57*: 423-425.

Postigo J 2010. Leishmaniasis in the World Health Organization Eastern Mediterranean Region. *Int J Antimicrob Agents 36*: 62-65.

Ravel C, Cortes S, Pratlong F, Morio F, Dedet J, Campino L 2006. First report of genetic hybrids between two very divergent *Leishmania* species: *Leishmania infantum* and *Leishmania major*. *Int J Parasitol 36*: 1383-1388.

Schonian G, Nazereddin A, Dinse N, Schweynoch C, Shallig H, Presber W, Jaffe C 2003. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis 47*: 349-358.

Senghor M, Niang A, Depaquit J, Faye M, Ferté H, Faye B, Gaye O, Elguero E, Alten B, Perktas U, Diarra K, Bañuls AL 2011. Canine leishmaniasis caused by *Leishmania infantum* transmitted by *Sergentomyia* species (Diptera: Psychodidae) in Senegal: ecological, parasitological and molecular evidences. Available from: isops7.org/ISOPS7_Abstract_book.pdf.