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First detection of *Leishmania major* DNA in *Sergentomyia* (*Sintonius*) *clydei* (Sinton, 1928, Psychodidae: Phlebotominae), from an outbreak area of cutaneous leishmaniasis in Tunisia

Chiraz Ayari [a,e], Souad Ben Othman [a], Jomaa Chemkhi [b], Ahmed Tabbabi [a], Roser Fisa [c], Afif Ben Salah [d], Souha BenAbderrazak [a,*]

[a] Laboratoire de Parasitologie Médiale, Biotechnologies et Biomolécules (LR11IPT06), Institut Pasteur de Tunis, 13, Place Pasteur-BP74, 1002 Tunis, Tunisia
[b] Laboratoire d'Epidémiologie Moléculaire et Pathologie Expérimentale Appliquée aux Maladies Infectieuses (LR11IPT04), Institut Pasteur de Tunis, 13, Place Pasteur-BP74, 1002 Tunis, Tunisia
[c] Laboratoire de Parasitologie, Faculté de Pharmacie, Université de Barcelone, Av. Joan XXIII s/n, 08028 Barcelone, Spain
[d] Laboratoire de Transmission, Contrôle et Immunologie des Infections (LR11IPT02), Institut Pasteur de Tunis, 13, Place Pasteur-BP74, 1002 Tunis, Tunisia
[e] Faculté des Sciences de Bizerte, Université de Carthage, Tunis, Tunisia

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ABSTRACT

In recent years there has been growing interest in *Sergentomyia* species. Their role in the spread of mammalian leishmaniasis appears repeatedly in the literature and the possibility of its implication in *Leishmania* transmission to humans remains controversial. *Sergentomyia* (*Sintonius*) *clydei* is one of several cryptic species sharing therefore common morphologic criteria with others species of the subgenera *Sintonius*. Little is known about this specie in Tunisia.

We sampled and identified different specimens including four specimens of *S. clydei* collected from Sidi Bouzid and Kairouan areas (center of Tunisia) using morphological tools. Male *Sergentomyia clydei* and *Sergentomyia christophersi* are known to share several morphological characters and can be mistaken for. Consequently we took advantage of 5 male *S. christophersi* available in our collection (Tataouin, South of Tunisia). In our study morphological tools were completed by molecular study of cytochrome b gene to identify *S. clydei*.

For the detection of *Leishmania* spp. that might infect our specimens, *Leishmania* DNA was analyzed by amplification of kinetoplast minicircle DNA using real-time PCR and nested-PCR. Obtained result was confirmed by restriction analysis of the amplified ribosomal internal transcribed spacer 1 (ITS1).

We provide in our study, the first molecular identification of *S. clydei*, in Tunisia. Our Neighbor Joining tree based on mitochondrial cytochrome b gene shows two different clusters. The first includes the Tunisians *S. clydei* and other specimens from Africa, Middle East and the Arabic peninsula, and the second cluster containing the specimens from Seychelle.

Unexpectedly, we also demonstrate the infection of one anthropophilic female *S. clydei* by *Leishmania major* DNA. This finding shows that more attention should be paid when identifying parasites by molecular tools within sandfly vector.

1. Introduction

Leishmaniasis in their various forms appears to be globally emerging in the world (Desjeux, 2001). It is generally seen as one of the neglected tropical diseases and has strong links with poverty (Alvar et al., 2006). Cutaneous leishmaniasis (CL) are generally characterized by large and/or multiple cutaneous lesions with a variable tendency to self-cure. Most lesions occur on the face and the naked part of arms and legs and often leave permanent scars with deformation of the infected area (Aoun and Bouratbine, 2014).

In Tunisia the epidemic CL emerged in central part since 1982 and expanded to the whole central and southern parts of the country (15/23 governorates are considered as endemic in 2002). The epidemics are cyclic and annual incidence ranges from 2000 to 10,000 cases.

In the recent decade, several new foci and several forms of the disease have emerged increasing the spread of the disease. This has been reinforced by the co-transmission of more than one form of the parasite in the same foci (Salah et al., 2007). CL cases may be caused by three *Leishmania* (*L*) species: *Leishmania major*, *Leishmania tropica* and *Leishmania infantum*. These three species prevail under different bioclimates and differ by the nature of their vectors and reservoir hosts.

Usually in the old world, the proven vectors for *Leishmania* parasite are the sandflies belonging to the subgenus *Phlebotomus*

* Corresponding author.
E-mail address: souha.benabderrazak@pasteur.rns.tn (S. BenAbderrazak).

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(Killick-Kendrick, 1990). Nevertheless, the detection of *Leishmania* DNA in *Sergentomyia* species caught in different areas reported by several studies, suggests their probable role in *Leishmania* transmission. Indeed, studies conducted in different foci in Iran, Mali and Portugal have reported the detection of *L. major* DNA in *Sergentomyia sintoni* (Parvizi and Assmar, 2007), *Sergentomyia darlingi* (Berdjane-Brouk et al., 2012) and *Sergentomyia minuta* (Campino et al., 2013), respectively. Earlier, *L. major* has been also isolated from *Sergentomyia garnhami* in Kenya (Mutinga et al., 1994). Other reports have also detected *Leishmania donovani* DNA in *Sergentomyia babu* in India (Mukherjee et al., 1997) and more recently, *Leishmania siamensis* DNA has been detected in *Sergentomyia gemmea* in Thailand (Kanjanopas et al., 2013) and *L. tropica* DNA in *Sergentomyia ingrami* and *Sergentomyia hamoni* in Ghana (Nzelu et al., 2014).

In Tunisia six *Sergentomyia* species have been reported, among them is *Sergentomyia* (*Sintonius*) *clydei* (Sinton, 1928). This specie has been described, in Africa, Middle East and Asia. *Sergentomyia clydei* is a cryptic specie, as it shares different morphologic criteria with other species from the subgenera *Sintonius*. Male of *S. clydei* captured in Tataouine (southeast of Tunisia) (Rioux et al., 1969), seems to be closely related to *Sergentomyia* (*Sintonius*) *christophersi* Sinton, 1927. It was thus agreed, to temporarily exclude *S. clydei* from the list of sandflies fauna in Tunisia (Rioux et al., 1971). However, recently, Chelbi and Zhioua (2012) confirmed by morphological tools, the presence of *S. clydei* in Sidi Bouzid area in the centre of Tunisia.

Cytochrome b (cyt b) a mitochondrial marker and thus a gene of maternal inheritance, was successfully used to highlight molecular variation within *Phlebotomine* species from a wide geographical range (Esseghir et al., 1997; Pesson et al., 2004; Yahia et al., 2004; Perrotey et al., 2005; Depaquit et al., 2014).

The cyt b gene together with the cacophony (cac) gene has also been used in a recent study that has explored the genetic variability within *S. clydei* from 12 different countries (Depaquit et al., 2014).

In view of the poor description of *S. clydei* in Tunisia, we thought to extend and deepen *S. clydei* identification to the molecular level. Doing this, we bring the first molecular detection and identification of *Leishmania* spp. in *S. clydei* in a focus of cutaneous leishmaniasis in Tunisia.

2. Materials and methods

2.1. Study area and sample collection

Using CDC miniature light traps (John W. Hock, USA) and sticky paper we have captured a set of 1742 sandlies in various habitats from the central and southern part of Tunisia. Capture was done during four summer seasons (July–September): 2008 to 2011. The traps were set before sunset near animal shelter and inside house were cases of leishmaniasis have been reported. All specimens were preserved in 95% ethanol or/and in nitrogen liquid.

Using morphological tools and based on external and internal characters of the head and genitalia (according to the keys of Lewis (1967, 1974) and Artemiev (1978)), we were able to identify 15 different species among which *S. clydei*.

We focus our study on the four specimens belonging to this specie that were collected in three neighboring localities (Faidh, Ouled Haffouz and Nasrallah), from Sidi Bouzid and Kairouan governorates (Fig. 1). Those villages are located at moderate altitude; in higher arid bioclimatic floor characterized by sparse vegetation dominated by Chenopodiacae. *Psammomys* sp. and *Meriones* sp. are the most prevalent wild rodents in these areas (Fichet-Calvet et al., 2000).

Five male *S. christophersi* specimens were also available in our collections. Those later were collected in the village of Ghomrassen from Tataouine governorate (Table 1). This village is located in a mountainous area with a moderate altitude (300 m). This village is built on mountain flanks and has an arid climate. The regional landscape is characterized by poor vegetation and rocky escarpment.

As male *S. clydei* and *S. christophersi* which share several morphological characters, could be mistaken for, we also include the *S. christophersi* specimen to our molecular study.

2.2. Molecular study

2.2.1. DNA extraction and PCR amplification

Genomic DNA of all specimens was extracted from the thorax and the attached anterior abdomen, as described by Cornel and Collins (1996). It was eluted in 20 μL of TE, following the manufacturer's instructions.

Polymerase chain reactions (PCRs) were carried out to amplify into the mitochondrial DNA 336 bp of 3′ end of cytochrome b gene along with 67 bp of complete tRNA for serine, 22 bp interval sequences (including stop codons), and 124 bp of 3′ end of NADH1 gene. Using 50 ng of genomic DNA in 25 μL PCR reaction containing 10 pmol of each of the two primers CB3-PDR(5′GGTA(C/T)(A/T)TTGCCTCGA(T/A)TTCG(T/A)TATGA-3′) and N1N-PDR(5′-CA(T/C)ATTCAACC(A/T)GAATGATA-3′) (Esseghir et al., 1997), 200 μmol DNTPs, 1 U Taq DNA polymerase (product number: D1806, SIGMA-ALDRICH, USA), 2.5 μL 10× buffer. The amplification was carried following this thermal profile: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 46 °C for 60 s and extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR products were analyzed using 1% agarose gel electrophoresis, followed by ethidium bromide staining and visualization using UV light.

2.2.2. Sequence analyses and tree construction

PCR products were directly sequenced using the primers used for DNA amplification. Sequences were edited using Clustal-X version 1.81 software (Larkin et al., 2007).

To study the genetic variability of the 3′ end of cyt b gene, an alignment of obtained cyt b sequences and other sequence reference in databases was performed with MEGA5 as described (Tamura et al., 2007). Neighbor-joining (Saitou and Nei, 1987) and UPGMA (Sneath and Sokal, 1973) distance analyses were performed using the Jukes–Cantor Model. All bootstrap support values are based on 1000 replicates. *Sergentomyia minuta* were used as an out group (accession number (KP968846)).

2.2.3. Real time PCR

In an attempt to determine the infection state of the *S. clydei* specimen, the DNA obtained from the latter were analyzed for the detection and quantification of *Leishmania* spp. DNA amplification of kinetoplast minicircle DNA sequence by real-time PCR (qPCR) (Tomas-Perez et al., 2014). Each amplification was performed in a 20 μL reaction mixture containing 1× iTaq supermix with Rox (Bio-Rad, Hercules, CA, USA), 15 pmol of direct primer (CTTTTCTGGTCCTCCGGGTAGG), 15 pmol of reverse primer (CCACCCGGCCCTATTTTACACCAA), 50 pmol of the labeled Taq Man probe (FAM-TTTTCGCAGAACGCCCCTACCCGC-TAMRA) and 5 μL of sample DNA. The ABI Prism 7700 system (Applied Biosystems) at 94 °C and 55 °C cycling over 40 cycles was used. A non-template control was used in each run as the qPCR negative control.

A 6-fold dilution series of DNA from promastigotes (MHOM/ES/04/BCN-61, *L. infantum* zymodeme MON-1) was used as calibrators (serial dilution from 10^5 parasites/mL to 1 parasites/mL), allowing plotting of a standard curve. The qPCR was considered positive for *Leishmania* when the threshold cycle (Ct) was <33 which corresponds to a parasitic concentration (Cc) > 1 promastigote per microliter, following the standard curve.

2.2.4. Nested PCR

For *Leishmania* species identification, nested PCR was performed with the external primers CSB2XF (C/GA/GTA/GCAGAAAC/TCCCGTTCA)