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Research paper

First detection of *Leishmania* DNA in *Psammomys obesus* and *Psammomys vexillaris*: Their potential involvement in the epidemiology of leishmaniasis in Tunisia

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ABSTRACT

Leishmaniasis, a public health problem in Tunisia, are diseases caused by different *Leishmania* species. Cutaneous leishmaniasis is present from the North to the South under different forms, due to *Leishmania* (*L.*) *major*, *L. infantum* or *L. tropica*. Whereas, *Psammomys* (*P.*) *obesus* is the confirmed reservoir host of *L. major*, those of *L. tropica* and dermotropic *L. infantum* wait to be identified. Importantly, *P. vexillaris* species have been recently highlighted; however, no studies have been carried out to explore its potential role in leishmaniasis epidemiology.

Seventy two rodents were collected from Central and South-West of Tunisia between 2007 and 2010. Using several methods, 43 animals were identified as *P. obesus* and 29 as *P. vexillaris*. *Leishmania* kinetoplast DNA was detected in liver samples by real-time PCR in 18 *P. obesus* and in 8 *P. vexillaris*. Then, the direct sequencing of the amplified internal transcribed spacer 1, allowed the identification of *L. infantum* DNA in five *P. obesus* and in three *P. vexillaris*, as well as *L. tropica* DNA in three other *P. vexillaris*. Whereas, PCR fluorescent fragment length analysis of the 7 spliced leaders, allowed identifying *L. major* among infected *P. obesus* and *P. vexillaris*, and interestingly co-infection (*L. major*/*L. infantum*) among two *P. obesus*.

We report here for the first time, the infection of *P. obesus*, from Central Tunisia, by *L. infantum*. Suggesting that *P. obesus* the known reservoir host of *L. major*, may also serve as reservoir host for *L. infantum* and thus play a role in the spread of sporadic cutaneous or visceral leishmaniasis in this region. Of equal importance, this work establish for the first time, the natural infection of *P. vexillaris* by different *Leishmania* species, suggesting its potential epidemiological role as reservoir host.

1. Introduction

Leishmaniasis are parasitic diseases caused by Protozoa of the genus *Leishmania*, transmitted between mammals by an infected bite of a female sandflies. These are a broad range of diseases with a large spectrum of clinical manifestations ranging from cutaneous lesion(s) to visceral infection, caused by several species of *Leishmania* genus. In Tunisia, it's considered as a threat to public health, with an incidence exceeding thousands of cases each year (Aoun and Bouratbine, 2014).

The epidemic cutaneous leishmaniasis (CL) has appeared in the Central part of the country since 1982, following a wave of rodent's outbreaks. In the recent decades, several new foci have emerged indicating the spread of the disease to the whole country (Salah et al., 2007). Tunisia lists the three clinico-epidemiological forms of CL:

a) The zoonotic CL (ZCL) due to *Leishmania* (*L.*) *major* known to be a major public health problem in the Center and the South of the country, and more recently in the North (Ben Abda et al., 2009;

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Haouas et al., 2012; Mili et al., 2012). The zoonotic cutaneous leishmaniasis occurs as seasonal epidemics with an annual incidence ranging from 2 to 10 thousand cases (Bettaieb et al., 2014). The parasite is transmitted by *Phlebotomus* (*Ph.*) *papatasi*, which is present in all the territories (Chelbi et al., 2009; Ghrab et al., 2006), and other fly species (Ben-Ismail et al., 1987; Ben Ismail et al., 1987; Ghrab et al., 2006). The rodents *Psammomys* (*P.*) *obesus* (Cretzschmar, 1828) and *Meriones* (*M.*) *shawi* (Duvernoy, 1842) are the main reservoir hosts identified (Belazzoug, 1983; Belazzoug, 1986; Ben-Ismail et al., 1987). However, the nocturnal rodent *M. libycus* (Lichtenstein 1823) has been reported to act as secondary reservoir contributing to the propagation and increasing the distribution of the parasite between the *P. obesus* colonies (Ben-Ismail et al., 1989; Bouratbine-Balma, 1988; Fichet-Calvet et al., 2000; Helal et al., 1987). More recently, some small mammals were described as infected by *Leishmania* parasites in Tunisia, such as the least weasel *Mustela nivalis* (Ghawar et al., 2011a) and hedgehogs *Atelerix algirus* (Chemkhi et al., 2015). Their potential roles as reservoir hosts request further confirmation.

b) The chronic CL (CCL) caused by *L. tropica* MON-8, first described at 2005, in a microfocus of Tataouine in the South Eastern part of the country (Rioux et al., 1986a). Following that, new *L. tropica* foci have emerged in the Central and the South Western parts of the country (Ben Abda et al., 2009; Bouratbine et al., 2005; Bousslimi et al., 2010; Haouas et al., 2005). The annual incidence of CCL disease was estimated to 10 cases per year (Haouas and Babba, 2017). This variant is transmitted by *Ph. sergenti*, described in Central and Southern regions (Tabbabi et al., 2011). However, the reservoir is still controversial, even if the wild rodent *Ctenodactylus gundi* has been recently found infected with *L. tropica* (Bousslimi et al., 2012; Jaouadi et al., 2011).

c) The sporadic CL (SCL) caused by dermotropic *L. infantum* MON-24 prevails in Northern Tunisia with an extension to the Center. It has been sporadically reported with an annual incidence of approximately between 50 and 100 cases (Aoun et al., 2008; BenSaid et al., 2006; Haouas et al., 2012; Kallel et al., 2008). The extension of SCL to the Center corresponds to the spread of sandflies of the subgenus *Larroussius* incriminated in its transmission. This spread occurred following intense irrigation applied to the arid bio-geographical areas (Barhoumi et al., 2016). *Larroussius* subgenus is represented in this region by *Ph. perfiliewi* and *Ph. langeroni*. *Leishmania infantum* MON-24 was rarely isolated from dogs (Aoun and Bouratbine, 2014; Benikhlef et al., 2004), despite that, its reservoir remains unidentified.

Moreover, *Ph. perniciosus*, vector of visceral *L. infantum* MON-1, is also present in the Center and the South of the country (Ghrab et al., 2006; Guerbouj et al., 2007).

Wild rodents constitute a very large biomass of potential reservoirs for *Leishmania* (Ashford, 1996; Quinnell and Courtenay, 2009). On the basis of morphometric and karyotypic differences (Cockrum et al., 1977) two species of *Psammomys* namely *P. obesus* Cretzschmar, 1828 and *P. vexillaris* Thomas, 1925, have been reported. However, this dichotomy has been criticized for a long time and only a single species, *P. obesus*, has long been recognized until our lab revealed the existence of these two distinct species in Tunisia (Mostafa et al., 2006). The identification was proven by morphometric, biochemical, cytogenetic and molecular analysis. The existence of those two different evolutionary units raises the question of their played role in leishmaniasis epidemiology. Indeed, the susceptibility to *Leishmania* could be different between the two species, as reported for other closely related rodent species showing a different capacity to maintain pathogens (Gora et al., 2000; Korobitsyna, 1974; Mostafa et al., 2006). An understanding of the reservoir system is important in the design of rational control measures (Ashford, 1996; Quinnell and Courtenay, 2009). For this purpose, we have explored the potential role of *P. vexillaris* as reservoir of *Leishmania* spp. and its implication in the leishmaniasis epidemiology and investigated the possible involvement of *P. obesus* in other cycles of cutaneous leishmaniasis in Tunisia.

2. Materials and methods

2.1. Study area and rodents collection

Seventy two rodents were collected from Central and Southern parts of Tunisia. Forty three animals trapped from Sidi Bouzid, were randomly taken from a larger collection, during a study conducted between 2008 and 2010 (Ghawar et al., 2011b). Trapping was carried at three sites belonging to a ZCL endemic area: El Khbina, El Mnara and Ouled Mhamed. These sites are situated on a salt flat of halophytic vegetation. Predominantly, plants of the family *Chenopodiaceae* (*Salsola*, *Suaeda*, and *Arthrocnemum* sp., with occasional *Atriplex* sp.) represented the much disturbed remnants of the edge of the sebkha (Ozenda, 1991), classic biotope of *P. obesus* species. During another study, which was conducted from 2007 to 2009 in the South-West of Tunisia, twenty nine specimens were collected in three locations of Kebili city: Chemata, Fagoussi and Mohalhel; a typical Saharian region where the average annual rainfall does not exceed 100 mm. The vegetation is composed of *Arthrophytum* sp. and *Retama* sp., where *P. vexillaris* species was suspected to be.

These two species live closely, and are both present in the desert bioclimatic stage, while *P. obesus* is also found in the semi-arid/arid area.

All rodents were live-trapped using mesh cage-traps (Besançon Technique Service, France) baited with fresh food-plants and placed close to burrow entrances (Fichet-Calvet et al., 1999). Both *P. obesus* and *P. vexillaris* are not protected species. Trapped rodents were collected from the field and transported to the laboratory for examination, where they were sacrificed after anesthesia without producing any stress to the animals.

2.2. Rodent's identification

Taxonomic differentiation between two species belonging to the same genus needs the combination of morphometric, biochemical, molecular and cytogenetic analysis. In our case we used the first three methods.

2.2.1. Body measurements and age determination

Each specimen was weighted (Wt) to the nearest 0.1 g on a pan balance. Then, four external measurements were recorded to the nearest 1 mm: head and body length (HB), tail length (TL), hind feet length (HFL) and ear length (EL).

The weight of the desiccated eye lens (ELW) was used as an indirect measure for age determination of rodents caught in Sidi Bouzid as described by (Fichet-Calvet et al., 2003). Indeed, the age of the trapped specimens allows us knowing the transmission period of the disease, to determine if each individual whether it has lived a transmission season of *Leishmania* infection. For the rodents caught in Kebili area, the age determination was not possible due to the deterioration of their eye lens.

2.2.2. Biochemical study: Multilocus enzyme electrophoresis (MLEE)

Pieces of liver or spleen tissue were homogenized in grinding buffer (Pasteur et al., 1987) using a Heidolf homogeniser on ice. Homogenates were centrifuged and supernatants were removed and used for electrophoresis on cellulose acetate plates (Helena Laboratories, Texas). Enzyme staining was adapted from (Ben Abderrazak et al., 1993; Pasteur et al., 1987). Eleven allozymic systems were studied. For the purpose of the present paper, only two enzyme systems are presented because they appear to constitute two diagnostic loci, namely Glutamate Oxaloacetate Transaminase (GOT, E.C. 2.6.1.1) and 6-