Leishmania Major Infection Among *Psammomys Obesus* and *Meriones Shawi*: Reservoirs of Zoonotic Cutaneous Leishmaniasis in Sidi Bouzid (Central Tunisia)

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Abstract

A study was undertaken between November 2008 and March 2010, in the focus of cutaneous leishmaniasis of Central Tunisia, to evaluate the role of *Psammomys obesus* ($n=472$) and *Meriones shawi* ($n=167$) as reservoir hosts for *Leishmania major* infection. Prevalence of *L. major* infection was 7% versus 5% for culture (p =not significant [NS]), 19% versus 16% for direct examination of smears (p =NS), and 20% versus 33% (p =NS) for Indirect Fluorescent Antibody Test among *P. obesus* and *M. shawi*, respectively. The peak of this infection was in winter and autumn and increased steadily with age for the both species of rodents. The clinical examination showed that depilation, hyper-pigmentation, ignition, and severe edema of the higher edge of the ears were the most frequent signs observed in the study sample (all signs combined: 47% for *P. obesus* versus 43% for *M. shawi*; p =NS). However, the lesions were bilateral and seem to be more destructive among *M. shawi* compared with *P. obesus*. Asymptomatic infection was ~40% for both rodents. This study demonstrated that *M. shawi* plays an important role in the transmission and the emergence of *Leishmania major* cutaneous leishmaniasis in Tunisia.

Key Words: *Leishmania major*—*Meriones shawi*—*Psammomys obesus*—reservoir host—Tunisia—zoonotic cutaneous leishmaniasis.

Introduction

ZOO NOTIC CUTANEOUS LEISHMANIASIS in Tunisia is caused by *Leishmania major* MON-25 and transmitted by the vector *Phlebotomus papatasi* Scopoli 1786 (Diptera: Psychodidae) (Ben Ismail et al. 1987b, 1988). This vector frequently used rodents' burrows for daytime resting and breeding (Helal et al. 1987, Essegir et al. 1993). Many ecological studies of the reservoir hosts identified three rodent species carrying *L. major*: *Psammomys obesus* Cretzschmar 1828 with a major part in amplifying the transmission, *Meriones shawi* Duvernoy 1842 and *Meriones libycus* Lichtenstein 1823 with a role to propagate the parasite between *P. obesus* colonies because of their common migration, thus increasing the distribution of the parasite (Bouratbine Balma 1988, Fichet-Calvet et al. 2000). *P. obesus*, the fat sand rat, is distributed through the semi-desert on the northern fringe of the Sahara, from Mauritania through Morocco, Algeria, Tunisia, Libya, Egypt, Palestine, Jordan, Saudi Arabia, and Syria. It is the main reservoir host of *L. major* and the source of epidemics in this region. Its local

distribution is governed by that of the halophytic *Chenopodiaceae* on which it depends for food (Ashford 1996, 2000). These saline ecological biotopes are discontinuous in distribution in the Center and South of Tunisia, thus leading to a fragmentation of the populations of sand rats.

On the other hand, *M. shawi* is the reservoir host of *L. major* in some parts of Tunisia, Algeria, and Morocco (WHO 1990, Wasserberg et al. 2002, Ben Salah et al. 2007). This desert rodent inhabits clay and sandy deserts, arid steppes, grasslands, and mountain valleys. It is seen essentially in fields in nonirrigated cereal crops, *Ziziphus* mounds and *Opuntia* hedges or in the bases of jujubes tufts (WHO 1992). It is a terrestrial rodent that spends most of time occupying underground burrows with several small chambers used for food storage (Nowak 1991).

According to Ashford and Jarry (1999), the *P. obesus*–*P. papatasi*–*L. major* association in North Africa and the Middle East constitute stable well-described zoonotic systems. On the other hand, the *Meriones*–sandfly–*L. major* systems that have been described from Morocco (Rioux et al. 1982) to India

(Sharma et al. 1973) seem to be quite unstable, being associated with population surges of *Meriones* and outbreaks of cutaneous leishmaniasis in humans (Ashford 1986). In fact, few studies attempted to evaluate the prevalence of infection by *L. major* parasite in these species of rodents.

The aim of the present study was to compare the age structure, the morphological measurements, as well as the importance of *Leishmania* infection among natural reservoir hosts: *P. obesus* and *M. shawi* in Central Tunisia. This information is needed to evaluate the relative importance of different reservoirs in the maintenance of *L. major* transmission cycle as well as the role of *M. shawi* in the spatial expansion of the epidemic in naïve human populations outside the classic biotope of chenopods where *P. obesus* is predominant.

Materials and Methods

Study site and rodents collection

Rodents were trapped from six study sites (three sites for each species of rodents) in the endemic area for cutaneous leishmaniasis in Sidi Bouzid, Central Tunisia. *P. obesus* was captured in EL KHBINA (average Altitude 198,7296 m; N35

11490 E9 43971); EL MNARA (average Altitude 205,1304 m; N35 14698,88 E9 44818,88); and OULED MHAMED (average Altitude 310,5912 m; N35 52527,25 E9 30036,5). These sites are located on a saltflat of halophytic vegetation. Predominantly, plants of the family *Chenopodiaceae* (*Salsola*, *Suaeda*, and *Arthrocnemum* spp., with occasional *Atriplex* sp.), represented the much disturbed remnants of the edge of the sebka (Ozenda 1991).

M. shawi was captured in EL KHBINA (average Altitude 221,8944 m; N35 10775,6 E9 43050,2); EL MNARA (average Altitude 215,7984 m; N35 16163,33 E9 45354,66); and ETTOUILA (average Altitude 434.035 2 m; N34 58436,83 E9 26281,5). The vegetation of these sites is composed of *Arthrophytum* sp., *Retama* sp., *Ziziphus* mounds, and *Opuntia* hedges (WHO 1992) (Fig. 1).

Two kinds of trap were used to catch rodents: unabated pincer traps placed in the mouths of the burrows and wire-mesh cage traps baited with fresh food-plants and placed close to active burrow entrances on the residual study site. Trappings were done over a period of 1 year with 4 days/nights for each site per month. Trapped rodents were collected from the field and transported to the laboratory for examination.

FIG. 1. Study sites for the both species of rodents. (A) Location of Tunisian country in Mediterranean region. (B) Location of study area (delimited) at Sidi Bouzid Governorate (highlighted). (C) A land sat image of the study area with a zoom on bordered study sites of *Psammomys obesus* and *Meriones shawi*.

Age determination, body measurements, and clinical manifestations of *Leishmania*

The weight of the desiccated lenses of both eyes (eyes lenses weight [ELW]) was used as an indirect measure for age determination of rodents (Martinet 1966, Morris 1971, Gosling et al. 1980). Eyes were removed and preserved for at least 2 weeks in 10% formalin, and then the lens were extracted, dried for 2 h at 100°C, and weighed on a pan balance with an accuracy of 0.1 mg (Fichet-Calvet et al. 2003). Besides age determination, these specimens were analyzed as regard to their body measurements. Clinical manifestations of *Leishmania* infection were assessed by a thorough skin examination. Signs included depilation, hyper-pigmentation of the higher edge of the ear, infiltration, and dissemination of parasites with the presence of small nodules or partial destruction of organs.

Detection of *Leishmania* by direct examination and culture

Each captured rodent was identified, sexed, and searched for cutaneous lesions in the different parts of the skin, mainly at the tail and in the ears. Both ears of each rodent caught were removed and macerated together in physiological saline. A subsample of the suspension produced from the ears of each rodent was removed in a slide, stained by May-Grunwald-Giemsa, and observed at 1000× for direct examination of *Leishmania* amastigotes. Another subsample was put on Coagulate Serum of Rabbit (CSR) for culture and the third one was inoculated subcutaneously into a hind foot-pad of a BALB/c mouse (Ben Ismail et al. 1989). The hind footpads of the inoculated mice were examined regularly until a lesion was observed or, if no lesion developed, until 5 months postinoculation. A mouse that developed a footpad lesion was declared positive for *L. major* if promastigotes are observed in cultures obtained from the swollen footpad in CSR medium.

Indirect fluorescent antibody test (IFAT)

The Indirect Fluorescent Antibody Test was standardized against *L. major* antigens. Standardization of the technique was made with progressive dilutions of positive and negative control sera (1:20 to 1:320) against progressive dilutions of the conjugate (1:50 to 1:1600) in Evans Blue. Stationary phase promastigotes were washed three times in phosphate-buffered saline (PBS), and 10 mL of a 10⁷ parasites/mL suspension was dispensed in 12-well immunofluorescence slides. The latter were air-dried for 1 h at room temperature. Samples and control sera were incubated with parasites for 30 min at 37°C. After three washes in PBS, antibody fixation was revealed with fluorescein-conjugated goat anti-rat IgG (heavy plus light chains; Invitrogen) diluted at 1:100 in 0.01% Evans blue for counterstaining. The slides were then incubated for 30 min at 37°C, washed, and examined with a fluorescence microscope (Leica, DMIL LED). Only samples that showed fluorescent promastigotes, including the flagellum, were considered positive. The antibody titers were determined for positive sera at a titer 1:20 by testing them again in serial dilutions halving the concentration (Ben Ismail et al. 1989).

Data analysis

Shapiro-Wilk test was used to check the normality of the distribution of morphological variables. Continuous variables

were compared using nonparametric tests for comparison of medians. χ^2 as well as Fisher exact tests permitted to compare association of categorical variables. STATA software version 11 was used to carry out all statistical analysis (Stata Corporation).

Results

Between November 2008 and March 2010, a total of 639 rodents: 472 *P. obesus* and 167 *M. shawi* were captured in the study area. Among the captured rodents, we found 41 pregnant *P. obesus* females and, 9 pregnant *M. shawi* females with a number of embryos ranging between 2 and 9 per female. ELW ranged from less than 20 to 80 mg for *P. obesus* and reached 100 mg for *M. shawi* (Median = 38.55 [95% confidence interval (CI) = 9–74.6] vs. 61.6 [95% CI = 20.6–113.1], respectively). ELW difference was significant between the two species (Nonparametric equality-of-medians test, $p < 0.001$).

All the morphological variables distributions for *P. obesus* and *M. shawi* lacked normality. Therefore, comparisons were based on the median for these variables and showed significant differences between the two species (Table 1). *P. obesus* had a higher weight, a longer head-body, and hind foot compared to *M. shawi*. On the other hand, ears and tails were significantly longer among the latter (Fig. 2).

TABLE 1. DESCRIPTION OF THE STUDY SAMPLE OF RODENTS: STUDY SAMPLE DISTRIBUTION BY LOCATION, COMPARISON OF DEMOGRAPHIC, AND MORPHOMETRIC PARAMETERS

Factors	P. obesus	M. shawi	p
Location of capture			
Ettouila	No observation	15 (9)	—
Khbina	201 (43)	137 (82)	<0.001
Mnara	131 (28)	15 (9)	NS
Ouled Mhamed	140 (30)	No observation	—
Total	472	167	—
Sex (%)			
Male	176 (37)	70 (42)	NS
Female	296 (63)	97 (58)	NS
Total	472	167	—
Median body measurements (IQ) ^a			
Weight (g)	117 (98–132.5)	73 (54–90)	<0.001
Ear length (mm)	16 (15–16)	17 (16–18)	<0.001
Head and body length (mm)	149.5 (139–157)	134 (119–143)	<0.001
Tail length (mm)	124 (114.5–131)	138 (128–147)	<0.001
Hind Feet length (mm)	36 (35–37)	34 (32–35)	<0.001
Age of rodents by classes of ELW (%)			
<20 mg	45 (10)	No observation	—
20–30 mg	109 (23)	11 (7)	NS
30–40 mg	96 (20)	18 (11)	NS
40–50 mg	88 (19)	23 (14)	NS
50–60 mg	85 (18)	28 (17)	NS
60–70 mg	41 (9)	26 (16)	NS
70–80 mg	8 (2)	20 (12)	NS
80–90 mg	No observation	23 (14)	—
90–100 mg	No observation	7 (4)	—
>100 mg	No observation	11 (7)	—

^aMedian is the percentile of 50% and IQ are the percentiles of 25% and 75%.

P. obesus, *Psammomys obesus*; *M. shawi*, *Meriones shawi*; NS, not significant; IQ, InterQuartile Interval; ELW, eyes lenses weight.

FIG. 2. Box plot presentation of morphological measurements among the two species of rodents. Y axis 1 in the left is in millimetres (mm), Y axis 2 in the right is in grams (g). X axis is divided in two parts: *M. shawi* measurements in the left and *P. obesus* in the right.

Infection prevalence

The importance of *L. major* infection varied among the two reservoirs according to the technique used. It was 7% versus 5% for culture (including the results of inoculated mice; p =not significant [NS]), 19% versus 16% for direct examination of smears (p =NS) and 20% versus 33% (p =NS) for IFAT among *P. obesus* and *M. shawi*, respectively.

The IFAT revealed a positive reaction at dilutions ranging from 20 to 640 for both rodents. Geometric mean (Gmean) of these titers was higher for *M. shawi* (Gmean=82.04, [95% CI=60.54 to 111.16]) than *P. obesus* (Gmean=59.07, [95% CI=49.76 to 70.12]) positive sera.

When we consider the combination of all diagnostic methods, the prevalence of infection was 34% for *P. obesus* and 41% for *M. shawi* (p =NS). Interestingly, this prevalence was significantly higher among female rodents compared to males of both populations (65% vs. 35% [p <0.001] and 66% vs. 34% [p =0.01] among *P. obesus* and *M. shawi*, respectively).

Clinical manifestations

The clinical examination of rodents captured showed various aspects of skin lesions. When we consider any clinical sign among the whole study sample (n =639), disease was detected among 222 specimens of *P. obesus* (47%) and 73 specimens of *M. shawi* (43%; p =NS). Depilation, hyper-pigmentation, ignition, and severe edema of the higher edge of the ear were the most frequent signs observed in the study sample among both rodents (79% for *P. obesus* vs. 68% for *M. shawi*; p =NS) followed by the tail (Table 2). We also noticed infiltration and more obvious thickening of the part of the pinna, dissemination of parasites with the presence of small nodules, as well as partial destruction of this part. However, the lesions were bilateral and seemed to be more destructive in *M. shawi* than in *P. obesus* (Fig. 3). Table 2 details the relative importance of clinical signs observed among both reservoirs.

Patent infection defined as the proportion of rodents with any clinical sign among those who showed a positive test by any diagnostic method was 62% (99/159) versus 59% (40/68; p =NS) among *P. obesus* and *M. shawi*, respectively. Thus, asymptomatic infection among positives by any diagnostic methods was ~40% for both rodents.

Evolution of the infection prevalence according to the season of capture and the age of rodents

The peak of the prevalence of leishmaniasis infection, among *P. obesus* population, was in November 2009 (57%), whereas the lowest was in April 2009 (17%). For *M. shawi* population the prevalence of infection was high in January 2009 (75%) and decreased subsequently. According to seasons, infection prevalence exceeded 22% and reached the maximum among both species of rodents in winter and autumn. Interestingly, during all seasons the prevalence of *Leishmania* infection seems to be higher, although not statistically significant, in *M. shawi* population compared to *P. obesus* population and exceeds 50% at the higher seasons of infection (Table 3).

TABLE 2. CLINICAL MANIFESTATIONS OF *LEISHMANIA* PARASITES IN BOTH SPECIES OF RODENTS

Presence of clinical signs	<i>P. obesus</i>	<i>M. shawi</i>	<i>p</i>
All clinical signs included (%)	222 (47)	73 (43)	NS
Location of clinical signs (%)			
Ear lesions	175 (79)	50 (68)	NS
Tail lesions	77 (35)	36 (49)	NS
Belly lesions	11 (5)	4 (5)	NS
Noose lesions	1 (0)	1 (1)	—
Back lesions	1 (0)	No observation	—
Hind feet lesions	3 (1)	1 (1)	NS

FIG. 3. Examples of clinical manifestations observed among *M. shawi* (a) and *P. obesus* (b) in the study sample. (a1): Severe edema of the higher edge of the ear. (a2) Total destruction of the ear. (a3) Swelling on the proximal third of tail. (b1) Hyper-pigmentation on the ear. (b2) Depilation and thickening of the part of the Pinna. (b3) Hairless spots on the base of tail.

The trend of the prevalence of infection with age of rodents revealed a steady increase for both species from 20% for young generations to 66% and 75% for adult *P. obesus* and *M. shawi*, respectively, as shown in Figure 4.

Discussion

The present study is to our knowledge the first one in North Africa assessing the importance of *L. major* infection and its clinical manifestation among *P. obesus* as well as *M. shawi*

using a large sample size of both populations captured over 1-year period. Compared with *Psammomys* ($n=472$), the low numbers of *Meriones* ($n=167$) indicates that they are scattered in the study area, whereas the former are denser and restricted in chenopods fields.

Body measurements of the two species of rodents showed a wide range of body weight, head-body length, and tail length. All measurements of *M. shawi* were smaller than those reported by Darvish (2009). Unsurprisingly, body measurements of *P. obesus* were in agreement with those reported by Ben Hammou et al. (2006), who captured their study population in the same study area.

Age structure of the study sample of these rodents based on ELW would suggest that *M. shawi* had a longer longevity than *P. obesus* (three ELW classes surpass 80 mg for *M. shawi*). Indeed, the lowest ELW class (<20 mg) include a few rodents for *P. obesus* and zero capture for *M. shawi*. However, this parameter might be influenced by rodent's weights differences and/or the effect of method of capture used. Indeed, small rodents might escape from the unabated pincer traps technique.

Clinical expression of *Leishmania* infection ranged from small nodules to total destruction of external parts particularly the ears (average 67%) and tails (average of 27%). Surprisingly, infection seems to be more severe among *M. shawi* species. This finding, described for the second time in the literature (Ben Ismail et al. 1987a), is very striking because it would indicate either higher susceptibility or more virulence

TABLE 3. COMPARISON OF *LEISHMANIA* INFECTION PREVALENCE BETWEEN THE TWO SPECIES OF RODENTS AMONG SEASONS

Seasons	Captured <i>P. obesus</i> (% of infection)	Captured <i>M. shawi</i> (% of infection)	p
Late autumn and winter 1 ^a	157 (41)	17 (53)	NS
Spring 2009	142 (22)	22 (27)	NS
Summer 2009	79 (29)	35 (31)	NS
Autumn 2009	58 (48)	20 (50)	NS
Winter 2 and early spring ^b	36 (36)	73 (42)	NS

^aWinter 2009 and November 2008.

^bDecember 2009, February 2010, and March 2010.

FIG. 4. Infection prevalence among different classes of eyes lenses weight (ELW: method used to estimate the rodent age) in each species of rodents ($\pm$ standard error).

of *L. major* strains among this population of rodents. These hypotheses might be addressed in future studies.

Previous studies carried out on the rodents reservoirs of *Leishmania* parasite in the old world showed a broad range of the infection prevalence ranging from 3% to 100% for microscopy and from 1% to 40% for culture (Nadim and Faghieh 1968, Edrissian et al. 1975, Elbihari et al. 1984, Githure 1986). This high variability might be partly explained by the cross-sectional study designs used in most studies that could not account for heterogeneity of transmission through time, as well as the small sample size that lead to high sampling fluctuations. Indeed, for *M. shawi*, the only study where the sample size exceeded 100 rodents, the prevalence of infection was of 2% by culture parasite method (Rioux et al. 1986). The prevalence of cutaneous leishmaniasis in the present study was 19% versus 16% by microscopy and 7% versus 5% by parasite culture for *P. obesus* and *M. shawi*, respectively. These estimates are in the same range when compared to those reported in Tunisia and elsewhere for these species (Rioux et al. 1982, Schlein et al. 1984, Ben Ismail et al. 1987a, 1989, Fichet-Calvet et al. 2003).

Few studies used serological methods to evaluate the prevalence of leishmaniasis infection among rodents. To our knowledge, only five studies were carried out on different species of rodent reservoir hosts of leishmaniasis, including one study related to *P. obesus* and *M. shawi* (El Nahal et al. 1982, Morsy et al. 1982, 1990, Zovein et al. 1984, Ben Ismail et al. 1989). The values reported in our study for serological diagnosis by IFAT were 20% and 33% among *P. obesus* and *M. shawi*, respectively. These values are in agreement with those reported by Ben Ismail et al. (1989) for the same method but they were very low compared with the serological study of *Rhombomys opimus* where a very high rate of infection was found (Zovein et al. 1984). Different estimations of infection prevalence according to the diagnostic method might also reflect variations in sensitivity and specificity of each method.

The overall prevalence of 34% for *P. obesus* and 41% for *M. shawi* showed in this study was high compared to those observed in other studies of both reservoir hosts in Tunisia (Ben Ismail et al. 1987a, 1989, Fichet-Calvet et al. 2003). Approximately 60% in both species of rodents had detectable lesions by clinical examination. Discordance between clinical manifestations and presence of leishmaniasis infection (average of 50%

for both species of rodents) might be explained either by non-specific clinical signs for leishmaniasis or lack of sensitivity of diagnostic tools used in the present study. This study shows the importance of asymptomatic infection ($\sim$ 40%) in both rodents' species. This finding, proven for the first time in *M. shawi*, was already reported for *P. obesus* (Fichet-Calvet et al. 2003). It might be explained either by young age of captured rodents showing asymptomatic infection or a variability in the *Leishmania* parasites infecting rodents. In agreement with previous studies, female rodents revealed higher infection prevalence for both species ($\sim$ 65%) (Rechav 1970, Ben Ismail et al. 1987a).

The combined results of diagnostic methods showed a high variation in the prevalence of leishmaniasis infection among both rodents through time with a lowest level in April and a highest level in November for *P. obesus*, which corroborates the findings of Fichet-Calvet et al. (2003). According to our study, the infection was absent in September and November and reached 70% in January for *M. shawi*. This result could imply an alternation of the parasite between these two rodent reservoir hosts to continuously maintain the transmission of the parasite within a sylvatic cycle.

In the present study, *Leishmania* infection increased proportionally with age and became high among adult rodents of both species (60% and 70% for *P. obesus* and *M. shawi*, respectively), suggesting a low death rate among infected rodents. This result is reported for the first time for *M. shawi* species.

The present study confirmed that *M. shawi* plays an important role in the transmission of *L. major* in Tunisia. The relatively high prevalence shows that this reservoir, because of its movements, its longevity as well as its close relationship to human settlements, could contribute significantly in the dispersal of *Leishmania* parasites leading to the emergence of epidemics in new foci among naïve populations.

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Disclosure Statement

No competing financial interests exist.

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