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Original article

Leishmania tropica in the black rat (*Rattus rattus*): persistence and transmission from asymptomatic host to sand fly vector *Phlebotomus sergenti*

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Abstract

Black rats (*Rattus rattus*) receiving *Leishmania tropica* injected intradermally into the ear were studied for the persistence of parasites and infectivity to natural sand fly vector. The mammalian host, the parasite, and the vector all originated from the endemic focus of Urfa, Turkey. Rats did not develop lesions or any apparent signs of disease, although at the site of inoculation they harboured live parasites capable of infecting sand flies. The number of *L. tropica* amastigotes detected in the inoculated ear by quantitative real-time PCR ranged from 5×10^3 to 10^6. Parasite DNA was also present in the tail and contralateral ear, sites distant from inoculation. After feeding on the ears of asymptomatic rats, *Phlebotomus sergenti* became infected with *L. tropica*. The average infection rate was 2.9%, and rats were infective for sand flies even 24 months post infection. The infectivity of the vertebrate host for insect vector was therefore not linked to the symptomatic stage of the infection. Such lack of correlation between clinical symptoms and infectivity to sand flies was reported previously for *Leishmania infantum*, the agent of visceral leishmaniasis; for species causing cutaneous leishmaniasis, however, this is the first evidence of transmission from a host without any visible cutaneous changes. If confirmed in the field, transmission from the asymptomatic host would be of great epidemiological significance.

Keywords: Cutaneous leishmaniasis; *Rattus*; Transmission; Epidemiology; Quantitative PCR

1. Introduction

Leishmaniases are diseases caused by protozoan parasites (Kinetoplastida: Trypanosomatidae); their clinical features varying from localized cutaneous affections to generalized life-threatening visceral disease. The vectors of *Leishmania*, phlebotomine sand flies (Diptera: Psychodidae), ingest amastigotes when feeding on an infectious vertebrate host. In visceral leishmaniasis, clinically normal skin may be a source of *Leishmania chagasi* amastigotes for infection in *Lutzomyia longipalpis* [1]. Similarly, dogs naturally infected with *Leishmania infantum* were infective to *Phlebotomus perniciosus* irrespective of the extent of symptoms [2]. In cutaneous leishmaniasis, however, it is assumed that the sand fly vector acquires infection after feeding on *Leishmania*-induced skin lesions (e.g. [3]), although the parasite can persist in resistant hosts several months after lesion healing [4], and naturally infected hosts were found to harbour parasites in normal skin [5,6]. In the present study, we have investigated *Leishmania tropica* persistence in black rats (*Rattus rattus*) and their infectivity to natural sand fly vectors, *Phlebotomus sergenti*.

2. Materials and methods

2.1. Parasites and infection of rats

Two *L. tropica* isolates (MHOM/TR/1999/Vedha EP41 and MHOM/TR/1998/SU23) of identical zymodeme (MON-53) and genotype IV [7] originate from patients with cutaneous leishmaniasis in Urfa, South Anatolia, Turkey, and were typed in the laboratory of Prof. K.P. Chang, Chicago Medical School. Blood agar SNB-9 [8] from defibrinated rabbit blood, supplemented with 40 µg/ml gentamicin, was used for

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cultivation. The isolates produced lesions in golden hamsters and BALB/c mice. Black rats (*R. rattus*) were caught using Tomahawk traps (Tomahawk Live Trap Co) in Urfa, Turkey, in 1998. A breeding colony was established, and first generation progeny was used for the experiments. Rats anaesthetized with ether were inoculated intradermally into the ear or footpad with 10^7 stationary-phase promastigotes (subculture 1–2) in 10 µl of sterile saline using a microfine insulin syringe.

2.2. Monitoring Leishmania *presence in rat tissues using parasite cultivation*

Rats were killed 1, 3, 6, 9, and 12 months after *Leishmania* inoculation. Two rats, one inoculated into the ear, one into the footpad, were killed at each time point. The body surface was washed with 96% ethanol. Pieces of tissue from the inoculation site, draining lymph node, spleen and liver were cultured on blood agar [8] and checked for the presence of promastigotes weekly for the period of 1 month.

2.3. Transmission of Leishmania *from rats to sand flies*

The *P. sergenti* colony was established from about 200 gravid females caught in CDC light traps in August 1998 in Urfa. The colony was maintained at 26 °C and 14/10 LD photoperiod. Adults had permanent access to cotton wool soaked with 50% honey as a sugar source. Twice a week females were allowed to feed for 2 h on anaesthetized mice.

In the experiments, sand flies were fed on inoculation sites of the rats at different intervals after inoculation of parasites (Table 1). Rats anaesthetized with ketamin/xylazin (150 and 15 mg/kg, respectively) were placed in the cage with sand flies for 2 h. No lesions or other dermal changes were visible on the inoculation site of any rat. Feet or ears were exposed to sand flies through an opening in a cloth bag so that the females were forced to feed on the inoculation site only. Blood-fed females were separated one day after feeding, kept at 23 °C and were provided with 50% honey. Nine days after feeding, their guts were dissected and microscopically checked for the presence of *Leishmania* promastigotes.

Table 1
Infection rates in sand flies after feeding on the ears of black rats

Months p.i.	Numbers of *P. sergenti* females		
	Dissected	Infected	Infected (%)
1	6	1	–
2	56	0	0
2	69	2	2.9
2	139	3	2.2
3	45	0	0
3	166	12	7.2
5	41	1	2.4
6	22	1	4.5
21	222	4	1.8
24	171	3	1.8
Total	937	27	2.9

Rat ears (n = 10) were inoculated with *L. tropica*, and *P. sergenti* were fed at different time intervals after inoculation.

2.4. Monitoring Leishmania *kDNA in rat tissues by quantitative real-time PCR*

At different time points following inoculation of promastigotes in the right ear, rats were killed for the monitoring of the distribution of the parasites in the different tissues, assessed by detection of minicircles of kinetoplastic DNA (kDNA). The following tissues were sampled until 24 months post infection (p.i.): both ears separately, spleen, liver, femoral bone marrow, blood, and tail. At each time point, one or two rats were killed, owing to the limited numbers of available animals. Homogenates were prepared from tissues as described [9], and stored at –20 °C until DNA extraction, performed in triplicate from each homogenate with DNeasy® Tissue Kit (Qiagen).

Detection and quantification of *Leishmania* kDNA was carried out with a LightCycler PCR assay described recently [10]. Quantitative results were expressed by interpolation with a standard curve included in each PCR run and consisting of a series of tenfold concentrations of *L. tropica* promastigote DNA, ranging from 0.1 to 10^4 parasites per PCR reaction. The reproducibility of the assay was excellent, since inter-assay variation between eight runs tested on different days was always <11%, for a given *Leishmania* DNA concentration (data not shown). We checked that the PCR assay did not amplify DNA from naive rats.

3. Results

3.1. Infectivity of L. tropica *for black rats and its long-term persistence at different inoculation sites as revealed by cultivation of parasites*

Rats inoculated with *L. tropica* did not develop lesions or any other symptoms of the disease. However, *L. tropica* was infective for black rats, as viable parasites were reisolated from the inoculated sites of all 10 rats 1, 3, 6, 9, and 12 months p.i., from both the ears and footpads. In one case, *L. tropica* was also isolated from the lymph node draining the inoculated ear 3 months p.i. Cultivation attempts from spleen and liver were negative.

3.2. Infectivity of L. tropica-*infected rats for sand flies*

In a preliminary experiment, rats inoculated with *L. tropica* in either footpad or ear were compared for their infectivity for sand flies at 6 months p.i. Only those flies fed on the ear became infected with *Leishmania*. Therefore, the experiments which followed were performed using the ear as the inoculation site.

Asymptomatic black rats were infective for *P. sergenti* which had fed at the inoculated ear at different time points, ranging from 1 to 24 months p.i. Eight out of 10 rats trans-

Table 2
Kinetics of long-term persistence of *L. tropica* in the inoculated ear of 10 rats, as estimated by real-time quantitative PCR

Months p.i.	Number of rats	Parasites/ear	S.D.
1	2	1.3×10^5	5.4×10^4
		1.2×10^6	2.7×10^5
2	2	6.7×10^5	1.6×10^5
		4.1×10^3	1.2×10^3
3	2	1.0×10^5	2.8×10^4
		6.0×10^4	1.3×10^4
10	1	7.6×10^3	3.2×10^3
16	1	9.6×10^4	2.4×10^4
18	1	6.8×10^5	6.9×10^4
24	1	5.1×10^4	1.1×10^4

Each value represents the mean C_T value (± 1 S.D.) obtained from three DNA extracts from an individual rat.

mitted the parasites, as indicated by the values of sand fly infection rates, ranging from 0% to 7% (Table 1). The average infection rate was 2.9%. All infected females except one had heavy infections with more than 1000 promastigotes per gut. Parasites were concentrated in the thoracic midgut and stomodeal valve, with the presence of forms morphologically similar to metacyclic promastigotes.

3.3. Distribution and persistence of Leishmania *in inoculation site and distant tissues monitored by LightCycler PCR assay*

The parasite load of *L. tropica* in the inoculated right ear of rats was monitored by quantifying *L. tropica* kDNA (Table 2). Each point represents the mean of three extractions (with a variability <35%). In *L. tropica*-infected rats, there was a long-term persistence of parasitic kDNA at the inoculation site, until at least 24 months after infection, although the number of parasites per ear was very variable according to the individual rats, ranging from ca. 4×10^3 to 10^6 parasites per ear. Distant tissues available for sand fly feeding, i.e. the opposite ear and the tail were also analysed by PCR. Both the tissues were PCR-positive from month one until month 24 p.i. At least one of the three DNA extracts from the ear was PCR-positive at each time, indicating a dissemination and persistence at a low level. All the DNA extracts from the tail were positive throughout the 2-year followup and the density of parasites ranged from 7.5×10^3 to 6×10^4 per square centimetre of tail. In contrast, all the other tissues investigated, i.e. liver, spleen, blood and bone marrow, were PCR-negative.

4. Discussion

In the present study, we have demonstrated the long-term persistence of *L. tropica* in asymptomatic hosts, in numbers sufficient to infect its natural sand fly vector, *P. sergenti*. As a model host, we used black rats originating from the endemic focus in Urfa, Turkey, and colonized a proven vector of *L. tropica*, *P. sergenti*, originating from the same site. Rats have been reported to be susceptible to *L. tropica* infection, with different outcomes ranging from asymptomatic infection at the inoculation site (Jacobson, pers. com.) to visceral infection of a specimen caught in Iraq [11]. Black rats from Urfa could be infected with *L. tropica*. The animals never developed lesions, but viable parasites were present at the inoculation site for several months.

P. sergenti, a natural vector of *L. tropica*, acquired *Leishmania* infection while feeding on parasite inoculation sites. However, only those females that fed on ears were infected. The ear might be an optimal site for *Leishmania* transmission due to its lower skin thickness, fleshy appearance and lack of thick hairs, enabling sand flies to readily feed on it. In jirds (*Psammomys obesus*) naturally infected with *L. major*, isolation attempts from various tissues were successful only from the ears [12]. It has been shown that *L. major* inoculated into the tail of laboratory rats (*Rattus norvegicus*) is able to metastasize into the ear [13]; in mice, *L. major* DNA could be detected at sites distant from the inoculation site including the tail and the contralateral ear [9].

Black rats infected with *L. tropica* did not develop any apparent signs of disease, although they harboured live parasites capable of infecting sand flies. The infectivity of the vertebrate host for the insect vector was therefore not linked to the symptomatic stage of the infection. Such lack of correlation between clinical symptoms and infectivity to sand flies was reported previously for *L. chagasi*, the agent of visceral leishmaniasis. The infectivity of *L. infantum* to *P. perniciosus* was independent of the extent of symptoms in the host; in an asymptomatic group, three of five dogs were sources of parasites for sand flies [2]. On some occasions, the infectivity of asymptomatic dogs was even slightly higher than that of the polysymptomatic ones [14]. In cutaneous leishmaniasis, however, this is, to the best of our knowledge, the first evidence of *Leishmania* transmission from an asymptomatic host.

The relatively low percentages of sand flies infected after feeding on lesion-free skin were similar to those observed in *L. longipalpis* fed on asymptomatic dogs infected with *L. chagasi*, and *P. perniciosus* and *P. perfiliewi* fed on black rats infected with *L. infantum* [1,15]. For those rats killed immediately after sand fly feeding, no correlation could be found between the parasite number and transmissibility to sand flies. Even the rat with the lowest detected parasite number (4×10^3) in the ear was infective for sand flies. Interestingly, the repeated use of one rat for sand fly infection, i.e. 21 and 24 months p.i., resulted in exactly the same infection rate of 1.8%. Although the sand fly infection rate was low, positive sand flies were heavily infected and would probably transmit the parasites at the next feeding. *L. tropica* is usually considered anthroponotic; however, it has been isolated from several mammalian species, which might serve as reservoir hosts, e.g. dogs in Morocco, hyraxes (*Procavia johnstoni*) in Kenya [16,17]. Black rats are common in the old part of Urfa, and at present, we cannot exclude their involvement in *L. tropica* circulation. They certainly serve as

one of the blood sources for sand fly populations [18]. Although rats probably do not fit into the strict definition of the reservoir host [19], they might serve as a source of infection in new foci if appropriate vectors were present. Our study shows that *L. tropica* persists in asymptomatic rats, which are probably infectious to sand fly vectors until death. The epidemiological significance of these findings has yet to be evaluated in the field; however, the natural role of asymptomatic hosts in the epidemiology of cutaneous leishmaniasis is neglected at present.

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