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Original article

Visceral leishmaniasis in eastern Sudan: parasite identification in humans and dogs; host-parasite relationships

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Abstract

In 1996, an epidemic outbreak of visceral leishmaniasis (VL) started in Barbar el Fugara, a village in Gedarif State (eastern Sudan). From 1997 to 2000, regular epidemiological studies were carried out in the human population, as well as in mammals and sand flies. In symptomatic patients, 46/69 lymph node, 6/20 post kala-azar dermal leishmaniasis (PKDL) and 1/4 cutaneous cultures in NNN medium were positive. In 69 dogs, 23/79 lymph node cultures were positive. In other mammals (47 rodents, five donkeys, one mongoose and one monkey) spleen and/or blood cultures were negative. Characterization of isolated strains (by starch gel electrophoresis and isoelectrofocusing) identified three zymodemes of *Leishmania donovani*, two of *L. infantum* and two of *L. archibaldi* complexes from patient samples and three zymodemes of *L. donovani*, three of *L. infantum* and two of *L. archibaldi* complexes from dog samples. Five of them were present in both man and dog. For the first time, a strain from a PKDL case was identified as *L. infantum*, and a child had the same *L. infantum* zymodeme in VL and in subsequent PKDL. Blood samples from dogs were studied by immunofluorescent antibody test (IFAT). The seroprevalence in dogs was 72.5%, 74.3% and 42.9% in 1998, 1999 and 2000, respectively. By using CDC miniature light traps 12 745 sand flies were collected and then identified. *Phlebotomus papatasi* (7%) and *P. orientalis* (5%) were sympatric, mainly inside homes (85% and 75%, respectively). These results, the relative stability of seroprevalence in dogs and the intradomiciliar presence of *P. orientalis*, known as a vector of VL in Sudan, suggest several hypotheses: (i) man is responsible for the disease in dogs, (ii) the dog is the reservoir of VL, (iii) the dog is an intermediate host between a possible sylvatic cycle and the anthroponotic cycle. More extensive studies are needed to assess the transmission cycle of VL in this area of Sudan.

Keywords: Sudan; Visceral leishmaniasis; Dogs; Sand fly; *Leishmania donovani*; *Leishmania infantum*; *Leishmania archibaldi*; *Phlebotomus orientalis*

1. Introduction

Visceral leishmaniasis (VL) is widely distributed in the Old and the New World. The parasite causing VL in South America, the Mediterranean region, and in Asia (Iran, Pakistan and China), is *Leishmania infantum*, where the domestic dog is the reservoir host. In India and eastern Africa, the agent is *Leishmania donovani* and is considered to be anthroponotic.

In Sudan, VL is an important public health problem. The disease is endemic in several regions, with dramatic epidemic outbreaks, as in the Western Upper Nile Province, where about 100 000 deaths occurred over 5 years [1]. Another main endemic area is the eastern part of the country, particularly Gedarif State [2], where in the village of Barbar El Fugara, between 1996 and 1997, there were 657 cases of VL among a population of about 4000 inhabitants [3].

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An epidemiological survey was carried out in this focus during four field investigations between 1997 and 2000. Various aspects of the parasite cycle in nature were investigated in the human population, domestic and wild mammals, and sand fly present in the area were studied. The present paper reports the results of these investigations and discusses possible hypotheses about the reservoir host of VL in this region of Sudan.

2. Materials and methods

Two-week field trips were carried out in May 1997, May 1998, April 1999 and January 2000.

2.1. Study site

Barbar El Fugara (13°39' north and 36°8' east) is a village in the Butana area (Gedarif State, near the Atbara River and the Ethiopian border (Fig. 1) The village, at an altitude of about 500 m above sea level, has a population of about 4000 and includes several ethnic groups, comprising mostly migrants from West Africa (Haoussa and Fellata), the Western Sudan/Chad area (Aringa) and Southern Sudan (Dinka). The Haoussas and Fellatas began settling in the village in the 1940s. The Aringas and Dinkas arrived in the 1960s and 1980s, respectively. The nutritional status of this agro-pastoral population was globally correct, and HIV infection absent.

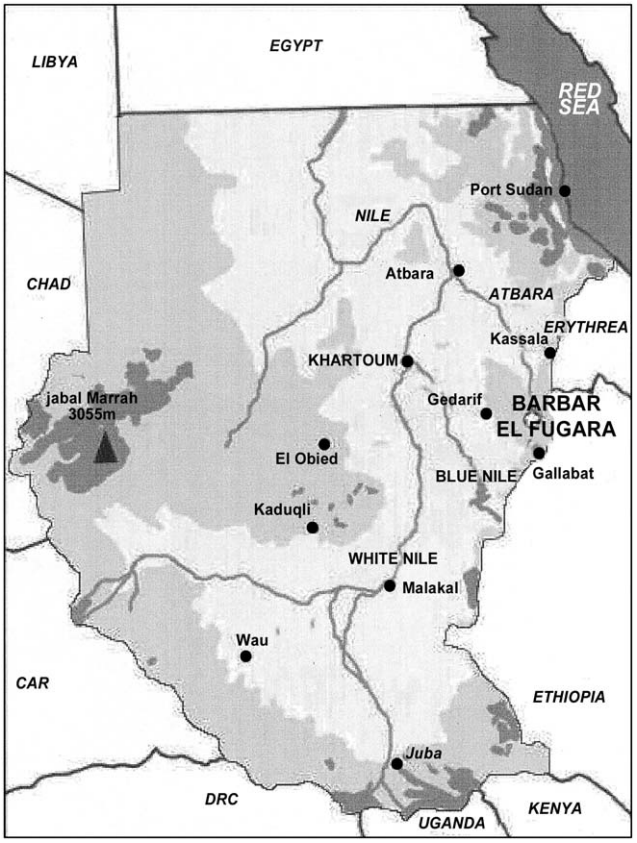

Fig. 1. Map of the Republic of Sudan, with localisation of the studied area (Barbar El Fugara village).

The characteristic vegetation of this area is wooded savanna, including *Acacia seyal* (talah), *A. senegal* (asheb) and *Balanites aegyptiaca* (lalop), associated with *Azadirachta indica* (neem) inside the village. The economy is agro-pastoral: cultures of sorghum (*Sorghum vulgare*), sesame (*Sesamum indicum*), cotton (*Gossypium* spp.) and peanut (*Arachis hypogea*), combined with farming of zebu (*Bos indicus*).

The climate is Sudanese-Sahelian (semi-arid tropical). The mean annual temperature is 28.8 °C. January is the coldest month (mean temperature: 25.8 °C) and April the warmest month (mean temperature: 32 °C). The annual rainfall is 635.9 mm, occurring mainly between June and September [4].

2.2. Ethical considerations

The study was approved by both the Federal and Gedarif State Ministries of Health and by the Faculty of Medicine, Khartoum University. Informed consent was obtained from the district authorities and from the village committee as well as from all the adults who participated in the study. For young children, consent was obtained from parents. For domestic mammals, consent was obtained from owners. After positive diagnosis, patients were treated by pentavalent antimonial (meglumine antimoniate).

2.3. Human and dog samples

A census of the village population was conducted by Bucheton et al. [5], making personal and clinical data available. The dogs were investigated with the assistance of their owners.

From 1997 to 2000, 69 inguinal lymph node samples were collected from patients with clinical symptoms of VL (mainly fever, splenomegaly and adenopathy). Also, 24 cutaneous samples were obtained by needle aspirate or by biopsy punch in 20 post kala-azar dermal leishmaniasis (PKDL) patients and four cutaneous leishmaniasis (CL) patients. Lymph node samples (79) were collected from 69 village dogs, all in poor physical condition. All these samples were cultivated in NNN medium.

Anti–*Leishmania* antibodies were investigated in a total of 121 sera collected from dogs during 3 years of study. Immunofluorescent antibody test (IFAT) was performed, using as antigen a stock of *L. donovani* MON-18 (MHOM/97/SD/LEM 3566), isolated from a VL case diagnosed in the same village.

2.4. Other mammal samples

Forty-seven rodents were trapped. Smears were made, and pathological examinations and culture in NNN medium were carried out in spleen samples from three Nile grass rats (*Arvicanthis niloticus*) in 1998 and from 34 Nile grass rats,

two spiny mice (*Acomys albigena*), five multimammate rats (*Mastomys* spp.) and three unidentified rodents (*Apodemus* spp.) in 1999. Also in 1999, five smears were made and one culture in NNN medium was carried out from blood of five donkeys (*Equus asinus*). Moreover, one smear was made and one culture from spleen of a mongoose (*Ichneumia albicauda*) and from blood of a monkey (*Cercopithecus aethiops*) were carried out.

In the field, all cultures were maintained in an ice box wrapped in a wet towel in order to maintain a temperature of around 25 °C (ambient temperature was 45 °C). In the lab, the isolated strains were mass cultivated and tested biochemically by starch gel electrophoresis using 15 enzyme systems [6] and by isoelectric focusing [7]. The isolates were compared with four reference strains: MHOM/FR/78/LEM 75 (*L. infantum* s.s. MON-1); MHOM/ET/72/GEBRE1 (*L. archibaldi* MON-82); MHOM/ET/67/HU3 (*L. donovani* s.s. MON-18); and MHOM/SD/82/GILANI (*L. infantum* s.s. MON-30).

2.5. Sand fly collections

Sand flies were collected in May 1997 and 1998, April 1999, and January 2000 in 153 CDC miniature light traps (1997: 39; 1998: 24; 1999: 36; and 2000: 54). The light traps were placed inside houses and granaries (storehouses for cereal-sacks) and outdoors, under shelters ("roukouba") or close to trees. The collected sand flies were preserved in 70 °C alcohol. After clearing in potassium chlorate, staining with fuchsine and mounting in Canada balsam, identification was based on the morphology of the male genitalia and female spermatheca [8].

3. Results

3.1. Human samples

Forty-six out of 69 lymph node cultures were positive, and 45 were identified—a single culture being contaminated by fungi. Six out of 20 and one out of four cultures were positive, respectively, from PKDL and CL cases, and identified (Table 1).

3.2. Dogs' samples

Out of 79 lymph node cultures, 23 were positive and 20 were identified (three cultures were contaminated). The mean age of dogs with positive lymph node cultures was 5.2 years in 1998, 4.1 years in 1999, and 5.2 years in 2000. Some dogs had positive cultures each year, for two or even three successive years (Table 1).

3.3. Identification of isolated strains

Ten zymodemes belonging to three "phylogenic" complexes of Rioux et al. [6] (*L. donovani* s. str., *L. infantum*, and *L. archibaldi*) were identified. Five zymodemes were common to humans and dogs: *L. donovani* MON-18 and MON-274; *L. infantum* MON-30 and MON-267 and *L. archibaldi* MON-257. Two zymodemes were only found in humans: *L. donovani* MON-276 and *L. archibaldi* MON-258. *L. donovani* MON-277, *L. infantum* MON-278, and *L. archibaldi* MON-82 were only found in dogs (Table 2). For the first time, *L. infantum* MON-267 was isolated from a PKDL case.

In humans, *L. donovani* was predominant (33 out of 52 isolates), compared to 12/52 for *L. infantum* and 7/52 for *L. archibaldi*. In dogs, *L. infantum* was predominant (10 out of 20 isolates), compared to 4/20 for *L. donovani* and 6/20 for *L. archibaldi*.

This year-to-year study lead to zymodeme follow-up in both humans and dogs. The same zymodeme, *L. infantum* MON-30, was found twice in a young boy, who had been treated for VL in 1997 and for PKDL in 1999. Similarly, zymodeme MON-267 (complex *L. infantum*), was found in a single dog two successive years, in 1999 and 2000. Two dogs showed two different zymodemes of the same complex: *L. donovani* MON-18 (in 1999) and MON-277 (in 2000); *L. archibaldi* MON-82 (in 1999) and MON-257 (in 2000). Two dogs had two different zymodemes of two different complexes: *L. archibaldi* MON-257 (in 1999) and *L. infantum* MON-278 (in 2000); *L. infantum* MON-267 (in 1999) and *L. archibaldi* MON-257 (in 2000). And one dog had a positive culture in three consecutive years: *L. infantum* MON-267 in 1998; an unidentified positive culture in 1999 (fungi contaminated), and *L. infantum* MON-278, in 2000.

3.4. Seroprevalence of the disease in dogs

The seroprevalence (IFAT antibody titer ≥1/40) for leishmaniasis in dogs was 72.5% in 1998, 74.3% in 1999, and 42.9% in 2000 (Table 3). The mean age of dogs ≥1/40 was 4.7 years in 1998; 4.4 years in 1999 and 5.6 years in 2000. The age of positive dogs decreased over the years: 54.3% of

Table 1
Human and dog samples from Barbar El Fugara (Sudan)

Year	Man			Dog	
	Lymph node culture, positive/number	PKDL culture, positive/number	Skin culture, positive/number	Lymph node culture, positive/number	Mean age (years) positive dogs
1997	28/37	3/7	1/3		
1998	16/16	2/6	0/1	4/9	5.2
1999	2/8	1/3		8/35	4.1
2000	0/8	0/4		11/35	5.2
Total	46/69	6/20	1/4	23/79	

Results of NNN cultures.

Table 2
Identification and comparison of strains isolated from man and dog in Barbar El Fugara

Leishmania species	Zymodeme	Host			
		Man			Dog
		VL	PKDL	CL	
L. donovani	MON-18	26	4	1	1
	MON-274	1			1
	MON-276	1			
	MON-277				2
L. infantum	MON-30	10	1		2
	MON-267		1		6
	MON-278				2
L. archibaldi	MON-82				2
	MON-257	6			4
	MON-258	1			
Total		45	6	1	20

Table 3
Results of serology (IFAT) from dogs of Barbar El Fugara

Year	IFAT					Total	Frequency IFAT ≥ 1/20 (%)	Frequency IFAT ≥ 1/40 (%)
	<1/20	1/20	1/40	1/80	>1/80			
1998	5	9	15	15	7	51	90.2	72.5
1999	3	6	21	2	3	35	91.4	74.3
2000	10	10	7	7	1	35	71.4	42.9

positive dogs were <5 years in 1998, vs. 50% in 1999 and 20% in 2000.

3.5. Samples from other mammals

All the collected samples from rodents, donkeys, mongoose and monkey were negative on smears, and/or pathological examination, and/or cultures.

3.6. Sand fly collections

Out of 12 745 sand flies collected, five species of the genus *Phlebotomus* (1625 samples) and 19 species of *Sergentomyia* (11 120 samples) were identified (Table 4) [8]. The two most frequent species of the genus *Phlebotomus* were *P. papatasi*: 901/12 745 (7%) and *P. orientalis*: 629/12 745 (5%) and were mainly sympatric inside homes: 763/12 745 (5.9%) and 473/12 745: (3.7%), respectively.

4. Discussion

Although East African VL, particularly in Sudan, is thought to be anthroponotic, there is good circumstantial evidence for a residual zoonotic reservoir. There is a high frequency of PKDL, as a distinctive clinical feature, and there are periodic epidemic outbreaks.

The present study sheds new light on the epidemiological aspects of the disease in this area, concerning parasite diversity, since seven zymodemes were identified from human samples. Apart from *L. donovani* s.s., *L. infantum* and *L. archibaldi* were demonstrated, for the first time, to be respon-

Table 4
Captures of sand flies from Barbar El Fugara

Species	1997		1998		1999		2000		Total
	M	F	M	F	M	F	M	F	
P. papatasi	438	228	70	15	86	53	6	5	901 (7%)
P. orientalis	123	110	63	112	103	96	10	12	629 (5%)
P. rodhaini	14	20	13	14	13	11	2	3	90
P. duboscqi			1		1		2		4
P. bergeroti					1				1
Sergentomyia (19 species)	1004	2006	880	974	2157	2042	490	1567	11 120
Total	1579	2364	1027	1115	2361	2202	510	1587	12 745
	3943		2142		4563		2097		

sible for VL in an East African focus [9]. Moreover, a strain from a PKDL case was identified for the first time as *L. infantum* and a child had the same *L. infantum* zymodeme in VL and in subsequent PKDL. During the same study, *L. donovani* was also identified as responsible for a CL case. Only a few cases of CL have previously been reported as due to *L. donovani*: in Yemen [10] and Sri Lanka [11]. This extensive study of parasite diversity during an outbreak of VL in a single village did not provide evidence for any clear correlation between the zymodeme and its pathogenicity (VL or PKDL). Note that for CL, only one *Leishmania* stock was isolated. These results indicate that isoenzymatic identification of *Leishmania* is not sufficient to identify specific strains of the parasite implicated in the determination of the clinical outcome. Molecular techniques could possibly be more appropriate to achieve this goal. However, these results also favour another hypothesis that host-related factors play an important role in the evolution of the host–parasite interactions. Indeed ethnic and familial factors were shown to have played an important role in the distribution of VL cases in this village population [5].

Many investigations have been carried out in East Africa to find wild and/or domestic reservoir hosts of human VL. In Sudan, Bousfield found a dog with amastigotes of *Leishmania* in Gallabat [12], 50 km south-east of Barbar El Fugara. In 1924, Archibald and Susu (in [13]) described one positive dog in Kassala. In 1956, R. Kirk found a single positive dog out of fifteen in Tapotha, Kapoeta in the south of Sudan and one positive out of four sand foxes (*Vulpes pallida*) with a cutaneous lesion [13]. Also, in Kapoeta, Zaki related an unconfirmed report of two infected dogs (in [14]). But in 1937, Archibald and Mansour [15] found no positive dog out of 200 examined. As for other mammals, Marshall in 1911 [16] and Kirk in 1956 [13] found *Leishmania* in monkeys. In 1969, Hoogstral and Heyneman found, by inoculation into hamster, several wild mammals infected: 4/117 Nile grass rats (*A. niloticus luctuosus*), 1/144 spiny mice (*A. albigena*), one serval (*Felis serval philipsi*) and one genet (*Genetta genetta senegalensis*) [14]. All the dogs studied in this area by these authors were negative. The same authors also described one infected *A. albigena* (1962) and one *Rattus rattus* (1961) in Malakal city [14]. More recently, in 1987, Sixl et al. [17] found one infected jackal (*Canis* sp.). But in all these cases, there was no parasite identification. Elnaim et al. [18] described 2/14 Egyptian mongooses (*Herpestes ichneumon*), 1/168 *A. niloticus* and 1/8 *Mastomys natalensis* positive by smear detection and PCR. The first mention of specifically identified *L. donovani* in dogs was made by Mutinga et al. [19], who isolated two stocks from Kenyan dogs, one of them (MCAN/KE/00/D2) related to *L. donovani* MON-36 [20]. In Iraq, another canine strain (MCAN/IQ/81/SUKKAR-2) isolated by Sukkar et al. [21] was identified as *L. donovani* MON-3 [20].

Within the epidemiological survey carried out in Barbar El Fugara, the first identification of *L. infantum* and *L. archibaldi* strains was reported in dogs in Sudan [22]. In the present study, we demonstrate the presence of sympatric *L. donovani*, *L. infantum* and *L. archibaldi* in man and dog not far from Gallabat and Kassala, where Bousfield [12] and Archibald and Susu (in [13]), respectively, also found infected dogs.

The fact that the zymodemes isolated in this area are in ancestral positions on the phylogenetic tree [9] supports the idea previously expressed by Ashford et al. [23], that Sudan should be considered as the possible original focus of VL, from which the disease has spread north and east for *L. donovani* and north and west for *L. infantum*. But the recognition of these systematic complexes on the basis of GOT enzyme variation was contested by some authors, who suggest grouping the zymodemes belonging to these taxa in a single group, *L. donovani sensu lato* [23].

Furthermore, the presence of different zymodemes of the same or different complexes, in the same dogs for several consecutive years is an original observation which can be explained by different hypotheses: (i) reinfections, (ii) switch of parasite clones, according to the clonal theory of infectious agents [24], subsequent to a point mutation or the cytogamy process [25].

Other canine diseases are present in this focus: erlichiosis, *Bartonella vinsonii* infection, Carré disease, dirofilariasis, babesiosis, Q fever (B. Davoust, personal communication). The presence of one or other of these infections could have altered the susceptibility of dogs to leishmaniasis.

The present work opens new dimensions about the parasite's dynamics and circulation in its hosts, and the generation of epidemic outbreaks, in this particular area. For many authors, the presence of *Leishmania* in wild mammals and in humans is in favour of a sylvatic cycle of the disease contracted outside of the villages, in the *Acacia–Balanites* forests [26]. But, the following facts: (i) *Leishmania* of the same complex and, sometimes, of the same zymodeme, were present in man and dog simultaneously, (ii) some infected dogs were found still alive in two or even three successive years and had positive cultures, (iii) the seroprevalence of leishmaniasis in dogs was regular from year to year, and (iv) *P. orientalis* was collected principally inside the houses of the village where *Acacia* and *Balanites* trees are present, could be strong arguments for intradomiciliary transmission of the parasite by *P. orientalis* from a dog-based reservoir to man in this area.

P. papatasi was found inside a village where CL has not been previously reported. The joint occurrence of this sand fly, which is a known vector species of CL, and of the reservoir host, *A. niloticus*, also trapped in this focus, should draw attention to the possible occurrence of outbreaks of *L. major* CL in this area.

Once the step of intradomiciliary transmission has been achieved, the generation of epidemic outbreaks could possibly be related to individual human genetic factor(s) that would determine the fraction of VL-susceptible subjects in the population, as suggested by Bucheton et al. [5,27].

But it is also possible that sylvatic, domestic and anthroponotic cycles are present together in this area of Sudan. More extensive studies are needed to know whether (i) man is responsible for dog infection, (ii) dog is the reservoir host of VL, or (iii) dog is an intermediate host between a possible sylvatic and anthroponotic cycle.

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