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The recent emergence of *Leishmania tropica* in Jericho (A'riha) and its environs, a classical focus of *L. major*

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Summary Between 1997 and 2002, 49 strains of *Leishmania* were isolated from the cutaneous lesions of Palestinians living in and around Jericho. A polymerase chain reaction (PCR) amplifying the ribosomal internal transcribed spacer 1 (ITS1-PCR) was applied to their cultured promastigotes and to 207 individuals' skin scrapings spotted on filter-papers, 107 of which proved positive for leishmanial DNA. Species identification was performed by restricting the ITS1-PCR amplification products from the cultured promastigotes and the amastigotes in the scrapings with the endonuclease *Hae*III. Of the 49 cultures, 28 (57%) were *L. major* and 21 (43%) were *L. tropica*. Of the 107 dermal samples tested directly, 53 (49.5%) were infected with *L. major*, 52 (48.5%) with *L. tropica* and two remained unidentified. This is the first time *L. tropica* has been exposed in the population of the Jericho area and on such a large scale. The itinerant behaviour of some of this population precludes categorically declaring that *L. tropica* has recently become established in this classical focus of *L. major*. For this and although 88.2% of the cases of *L. tropica* claimed not to have travelled out of the vicinity of Jericho, local infected sand fly vectors of *L. tropica* must be caught, identified and, if possible, shown to harbour infections, and, if one exists, an animal reservoir host should also be exposed to endorse whether the cases caused by *L. tropica* were imported or autochthonous.

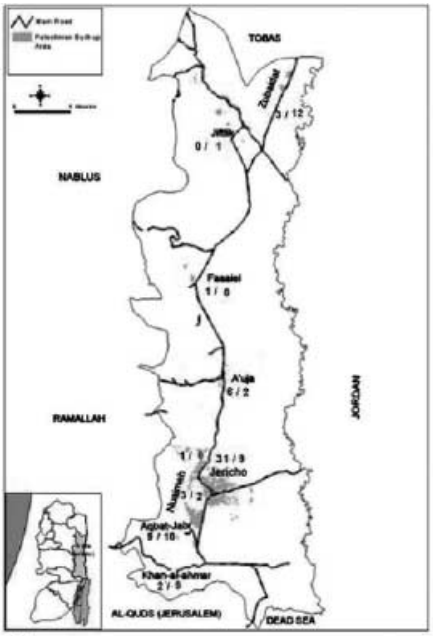
keywords *Leishmania major*, *Leishmania tropica*, ITS1-PCR, filter papers, Jericho area

Introduction

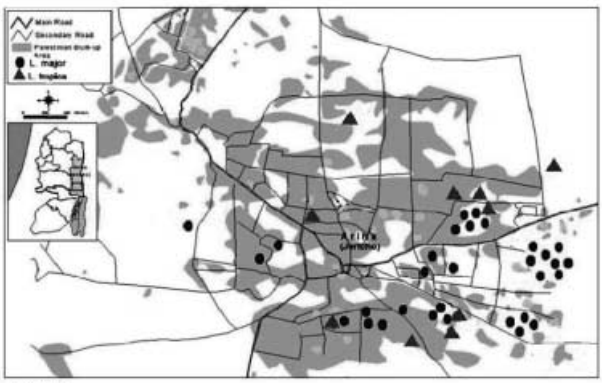
The District of Jericho (Figure 1, map 1), with the City of Jericho as its administrative centre, is the geographical area encompassed by the town of Nablus, which is just north but not part of it, the northern end of the Dead Sea in the south, the River Jordan in the east and the town of Ramallah, which is just west but also not part of it. The southern part of the Jordan Valley, which is a typical focus for zoonotic cutaneous leishmaniasis (ZCL) caused by *Leishmania major* (Schlein *et al.* 1982, 1984; Arda & Kamal 1983; Al-Jawabreh *et al.* unpublished data), runs through it. The City of Jericho itself is small and rural and not a typical urban city. It has a constant and plentiful supply of water, which gives it the appearance and ambiance of a desert oasis.

The ZCL is a reportable disease. In this part of the Jordan Valley, the animal reservoir host is the fat sand rat, *Psammomys obesus* (Schlein *et al.* 1984) and the vector the female of the sand fly species *Phlebotomus papatasi* (Schlein *et al.* 1982, 1984).

Diagnosis of CL at the regional clinic in Jericho was performed mainly by microscopical examination of dermal tissue smears stained with Giemsa's stain and the culture of dermal tissue aspirates and biopsies in rabbit blood–agar medium. This classical method lacks maximum sensitivity and still requires identification of the infecting species of *Leishmania*. In human cases, lesions caused by *L. major* self-heal and bestow immunity to re-infection. Therapy, if applied, is usually by Pentostam® administered intra-lesionally. Treatment of CL caused by *L. tropica* is sometimes less successful. Based on the assumption that *L. major* transmitted by *Ph. papatasi* is the only cause of human CL in this region, massive spraying in the entire Jericho District for 40 days at, both, the beginning and end of the sand fly season, which are in April and September, is the adopted method of control. The severity of some cases, slow response to treatment and only partial efficacy of the control strategy have raised uncertainty concerning the cause of some of the cases of CL encountered.



Map 1



Map 2

Figure 1 Map 1 shows the distribution of *Leishmania major* (the first number) and *L. tropica* (the second number) in the District of Jericho. Map 2 shows the distribution in the City of Jericho and its immediate vicinity: the triangle = a case caused by *L. tropica* and the oval = a case caused by *L. major*.

Methods

Target group and study area

Patients with lesions suspected of being CL, who were referred to the Islah Medical Laboratory in Jericho between June 1997 and December 2002, represented all the inhabited areas of the District of Jericho. This included the city, the refugee camps, the villages and the Bedouin encampments. Because of the poor response to treatment, species other than *L. major* were suspected of being a cause of CL in this focus. Forty-nine strains were isolated by culturing dermal tissue aspirates in rabbit blood–agar semisolid and NNN media and identified by employing a polymerase chain reaction (PCR) amplifying the parasites' ribosomal internal transcribed spacer 1 (ITS1-PCR). Also, 107 cases were diagnosed by using the ITS1-PCR directly on dermal tissue aspirates spotted on filter papers.

DNA Extraction

DNA from cultured strains of *Leishmania* was extracted as described by Van Eys *et al.* (1992). Disks of filter papers with dermal tissue aspirate were aseptically punched out of clinical samples. To avoid DNA contamination, 10 disks were punched out of clean filter paper immersed in 70% ethanol before punching out the next clinical sample. Two disks were placed in 250 µl lysis buffer (50 mM NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4); 1% Triton X-100 and 200 µg proteinase K/ml) and incubated for three hours or overnight at 60 °C. Lysates were then subjected to phenol–chloroform extraction as described by Meredith *et al.* (1993). The DNA pellets were dried, using a speed vacuum dryer (savant, speedvac 100) for 5–10 min and re-dissolved in 100 µl TE buffer (10 mM Tis and mM EDTA pH 7.5). The extracted DNA was then purified, using Nucleospin® Extract (Macherey Nagel GmbH & Co. KG, Dueren, Germany). The 30 µl samples were kept at −20 °C until used.

PCR Amplification

A PCR was used to amplify the ribosomal internal transcribed spacer 1 (ITS1) region, which separates the genes coding for the ssu rRNA and L5.8S rRNA, using the primers LITSR and L5.8S for PCR-ITS1 as described by El Tai *et al.* (2000) and Schoenian *et al.* (2003). Amplification reactions were performed in volumes of 50 µl. Three microlitres of genomic DNA or 2 µl of PCR product (for re-amplification) or 3 µl genomic DNA (for the DNA extraction control) were added to a PCR Master Mix, containing 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1 unit Taq polymerase, 25 pmol of each primer and 2.5% DMSO as an enhancer. The samples were overlaid with sterile, light mineral oil and amplified as follows: initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at for 53 °C for 30 s for both primer pairs, LISTR/L5.8S and HβG-F/HβG-R, and extension at 72 °C for 1 min. This was followed by a final extension cycle at 72 °C for 6 min. The PCR was run in a Perkin Elmer Cetus 9600. PCR amplifications products were run electrophoretically in 1% agarose at 100 V in 0.5X TBE buffer (0045 M Tris-borate, 1 mM EDTA) and

visualized under UV light after staining with ethidium bromide (0.5 μg/ml) for 15 min. DNA from the international reference strain of *L. turanica* (MRHO/MN/83/MNR-6) was run alongside each sample as a positive control and sterile distilled water was the negative control. For each diagnostic sample, an internal control for PCR inhibition was included where the same amount of purified DNA as in positive controls was added to these samples. The primer pair HβG-F (5′-GAA GAG CCA AGG ACA GGT AC-3′)/HβG-R (5′-CAA CTT CAT CCA CGT TCA CC-3′), specific for human β-globin, was used as a DNA extraction control to check true negativity of the ITS1-PCR, under the same conditions described above for ITS1-PCR. All negatives were subject to DNA extraction control.

To obtain enough material for subsequent restriction analysis the ITS1-PCR products were re-amplified, using the same primer combination and PCR conditions as for the first round of amplification.

Restriction analysis

Each ITS1-PCR amplification product (15 μl) was digested at 37 °C for 2 h with 1 μl of either the two restriction enzymes *Hae*III and *Mnl*I without prior purification, using the conditions recommended by the supplier (Hybaid GmbH Heidelberg, Germany). The restriction fragments were run electrophoretically in 2% agarose gel at 100 V in 1x TBE buffer and visualized under ultraviolet light after staining with ethidium bromide (0.5 μg/ml).

Results

All classical stages of the development of leishmanomas were seen among the cases: from small erythematous papules through nodules and to ulcerative lesions; whereas unusual clinical manifestations such as the sporotrichoid pattern, i.e., subcutaneous nodules developing along lymphatics, hyperkeratosis, i.e., thick adherent scale and leishmaniasis recidivans also known as lupoid leishmaniasis were not. It was very difficult and even impossible to discern if cases were caused by *L. major* or *L. tropica* by the clinical picture. However three severe cases led to the suspicion that they were not caused by *L. major*, as they were different from the cases of CL generally seen in the vicinity of Jericho. The three cases displayed common features. They all presented single lesions, two of which were on the nose and one on the chin. Development was slow and all three only sought medical advice 6–12 months after the first appearance of the lesion. All three lesions resisted antimony treatment and took 6 months or more to heal leaving scars. The lesion on the chin and one of those on the nose were caused by *L. tropica*. Of the 68 cases caused by *L. tropica*, 60 (88.2%) said they had not travelled out of the vicinity of Jericho in the last 3 months. The other eight cases were people who came either from the hilly regions around Jerusalem or cities like Ramallah and Jenin. Of the 57 cases caused by *L. major*, 43(75.4%) also said they had not travelled out of the vicinity of Jericho in the last 3 months.

Most cases had single lesions on the cheek or arm (Table 1). Most of the patients (67%) sought medical intervention in <3 months of the appearance of the lesion as Jericho is a hyperendemic region and the inhabitants are well aware of CL, which is also partially owing to the education campaigns conducted by Islah Medical Center in Jericho over the last few years.

Forty-nine strains were isolated by culturing dermal tissue aspirates in either rabbit blood–agar semisolid medium or NNN medium. Of the 207 dermal clinical samples spotted on filter papers checked by the ITS1-PCR, 107 were positive, indicating a 52% positivity rate. Amplification of the leishmanial DNA present, in this case the 300–350 bp ITS1 amplicon, and its subsequent digestion with the endonuclease *Hae*III did enable detection of the parasites and identify the species of *Leishmania* to which they belonged. The restriction patterns obtained for *L. major*, *L. tropica* and *L. infantum* (not shown), another species of *Leishmania* present in the Middle East, were clearly different and enabled the identification of the

Table 1 Comparison of the clinical features of cases of CL caused by *Leishmania major* and *L. tropica* in the district of Jericho

	L. major (%)	*L. tropica* (%)
Duration (months)		
≤3	34 (87.2)	33 (72.2)
3–6	5 (12.8)	9 (19.2)
>6	0 (0.0)	5 (10.6)
Total*	39	47
Location		
Forehead	5 (6.7)	8 (9.4)
Chin	4 (5.3)	9 (10.5)
Eye	2 (2.6)	4 (4.7)
Nose	2 (2.6)	6 (7)
Ear	5 (6.7)	1 (1.2)
Cheek	15 (20)	22 (25.9)
Arm	23 (30.7)	24 (28.2)
Leg	17 (22.7)	10 (11.8)
Neck	2 (2.6)	1 (1.2)
Total*	75	85
No. of lesions		
1	26 (45.6)	40 (61.5)
2	15 (26.3)	11 (16.9)
≥3	16 (28.0)	17 (26.2)
Total*	57	65

* Figures represent the total number of patients who answered the question related to each clinical feature.

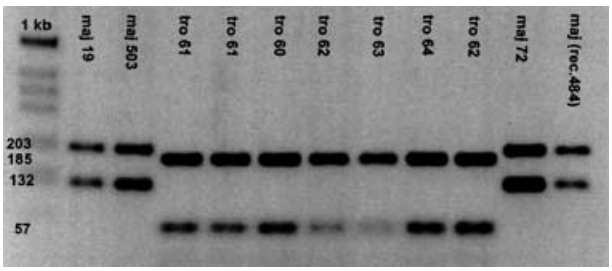


Figure 2 Restriction analysis patterns of the amplified ITS1 digested with *Hae*III. The patterns shown represent *Leishmania major* (maj) and *L. tropica* (tro) isolated from different patients living in the District of Jericho. All the DNA samples were from cultured promastigotes except rec.484, which came from a skin scraping from a lesion.

isolated strains at the species level (Figure 2). Fifty-three (49.5%) of the filter paper samples contained DNA of *L. major* and 52 (48.5%) DNA of *L. tropica*. The parasite species of the amplified DNA samples from two (2.0%) of the filter paper samples remained unidentified. Of the 49 cultured, characterized and identified strains, 21 (43%) were *L. tropica* and 28 (57%) were *L. major*. Cases caused by *L. major* and cases caused by *L. tropica* were both found in almost all the inhabited areas of the District of Jericho.

Discussion

Restriction analysis of the ITS1-PCR positive samples revealed the co-appearance of *L. major* and *L. tropica* in Jericho and its immediate vicinity (Figure 1). The existence of *L. major* in the lower Jordan Valley, including the margins of Jericho, and the human CL it causes as zoonoses has been well-documented (Schlein *et al.* 1982, 1984; Al-Jawabreh *et al.* 2004). The presence of *L. tropica* in the human population of the Jericho area is a novel finding, although it has been recorded as causing human cases of CL in a focus at a higher altitude halfway between Jerusalem (Al-Quds) and Jericho (A'riha) (Klaus *et al.* 1994; Jawabreh *et al.* 2001). Further, there is clear overlapping of the two species in almost all the populated areas included in this study, particularly in Jericho and the closely adjacent areas, i.e., the refugee camps.

This change in the distribution of leishmanial species in the area studied probably started more than a decade ago after the withdrawal of the Israeli Army when there was more extensive movement of the Palestinian population to and from the Jericho area. This includes security forces, internal tourism, workers, farmers and Bedouin shepherds from all the other Palestinian districts, including the Gaza Strip. Because of the significant difference in climatic conditions between the Jordan Valley and the mountains to the west of the Jordan Valley in both summer and winter, many of these people live in the Jericho area during the winter and spend the summer in the hills surrounding Ramallah, Nablus, Jenin and Hebron (Al-khalil). Therefore, they arrive in the Jericho area after the sand fly season has ended and transmission of CL has ceased; and they are in the hills when there are sand flies and transmission is occurring in both the mountains and the Jordan Valley. As *L. major* does not circulate in the hills, but in the Jordan Valley where sand rats, the animal reservoir, abound, the cases occurring in this migrant population are incurred in the hills and are caused by *L. tropica*, which they then bring with them to the Jericho area in winter. However, with the absence of sand flies at that time of the year, they are unlikely to be a source of CL caused by *L. tropica* in the Jericho area. For the present, and although 88.2% of the individuals with *L. tropica* and 75.4% with *L. major* claimed that they did not leave Jericho during the 3 months prior to the appearance of their lesion, one can assume that the cases of CL caused by *L. tropica* are imported cases. The only way to determine otherwise would be to show, categorically, that some of these cases were autochthonous. This could be done by showing that they had not travelled out of the area for at least one sand fly season, as the incubation period of CL caused by *L. tropica* is said to be much longer than that of CL caused by *L. major*. Alternatively, one could show that sand fly vectors such as *Ph. sergenti*, the vector of *L. tropica* at a higher altitude just east of Jerusalem (Schnur *et al.* 2004), infected with *L. tropica* were present in the area during the sand fly season and, if there is one, also in the infected animal reservoir host.

What is significant is that even before and since the Israeli Army took over the 'West Bank region' people travelled between the hills and the Jordan Valley, yet cases of CL caused by *L. tropica* were extremely rare throughout the whole District of Jericho, including the mountainous regions. Now, more and more cases caused by *L. tropica* are being seen in many foci of this part of the Eastern Mediterranean region (Klaus *et al.* 1994; Anis *et al.* 2001; Nimri *et al.* 2002; Jacobson *et al.* 2003; Schnur *et al.* 2004) so there has definitely been a change. However, this, it appears, has taken place in the hills rather than in the Jordan Valley. So far, there has been no evidence of *Ph. sergenti* existing in and being a potential vector of *L. tropica*, in the City of Jericho and its environs, although Schlein *et al.* (1984) found it 10–15 km south of Jericho at Ein Gedi on the western shore of the Dead Sea and in the Arava, which is even further south.

Prior to 1998, the diagnosis of Palestinian cases of CL was restricted to microscopical examination of smears stained with Giemsa's strain, which did not lend itself to the identification of different species of *Leishmania*. Later culture and PCR-based diagnosis and leishmanial species identification directly from dermal scrapings on filter

papers were introduced. This is the first study in the District of Jericho to use new molecular biological diagnostic technology and to such an extent. Species identification is a vital step in the diagnostic procedure, especially in areas where more than one species of *Leishmania* occurs. This impinges on control strategy, therapy and determining the epidemiology and dynamics of the disease. The presence and increasing numbers of human cases caused by *L. tropica* are a definite change compared with the past when all cases from this area, from which parasites were isolated and identified, were shown to harbour *L. major* and none were shown to harbour *L. tropica*.

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