*Am. J. Trop. Med. Hyg.*, 70(4), 2004, pp. 364–372
Copyright © 2004 by The American Society of Tropical Medicine and Hygiene

# MULTIFARIOUS CHARACTERIZATION OF *LEISHMANIA TROPICA* FROM A JUDEAN DESERT FOCUS, EXPOSING INTRASPECIFIC DIVERSITY AND INCRIMINATING *PHLEBOTOMUS SERGENTI* AS ITS VECTOR

LIONEL F. SCHNUR,* ABDELMAGEED NASEREDDIN,* CAROL L. EISENBERGER, CHARLES L. JAFFE, MUSTAFA EL FARI, KIFAYA AZMI, GERLIND ANDERS, MIREILLE KILLICK-KENDRICK, ROBERT KILLICK-KENDRICK, JEAN-PIERRE DEDET, FRANCINE PRATLONG, MOIEN KANAAN, TAMAR GROSSMAN, RAYMOND L. JACOBSON, GABRIELLE SCHONIAN, AND ALON WARBURG

*Department of Parasitology, The Kuvin Center for the Study of Infectious and Tropical Diseases, Hebrew University–Hadassah Medical School, Jerusalem, Israel; Institut für Mikrobiologie und Hygiene, Charité Campus Mitte, von Humboldt Universität zu Berlin, Berlin, Germany; Department of Biochemistry, Al-Quds University, Palestinian Authority; Department of Biological Sciences, Imperial College, London, United Kingdom; Laboratoire de Parasitologie and Centre National de Référence des Leishmania, Montpellier, France; Department of Biology, Bethlehem University, Palestinian Authority*

*Abstract.* The predominant sand fly species collected inside houses in Kfar Adumim, an Israeli village in the Judean Desert that is a focus of cutaneous leishmaniasis, was *Phlebotomus papatasi,* which was also caught attempting to bite humans. *Phlebotomus sergenti*, which is rarely seen inside houses, constituted the predominant sand fly species in caves near the village. *Leishmania* isolates from *Ph. sergenti* and humans typed as *Leishmania tropica*. Sand fly and human isolates produced similar small nodular cutaneous lesions in hamsters. Isolates produced excreted factor (EF) of sub-serotypes $A_9$ or $A_9B_2$, characteristic of *L. tropica* and reacted with *L. tropica*-specific monoclonal antibodies. Isoenzyme analysis consigned the strains to the *L. tropica* zymodemes MON-137 and MON-275. Molecular genetic analyses confirmed the strains were *L. tropica* and intraspecific microheterogeneity was observed. Genomic fingerprinting using a mini-satellite probe separated the *L. tropica* strains into two clusters that were not entirely congruent with geographic distribution. These results support the heterogeneous nature of *L. tropica* and incriminate *Ph. sergenti* as its vector in this Judean Desert focus.

## INTRODUCTION

*Leishmania major* and *L. tropica* both cause human cutaneous leishmaniasis (CL) in Israel and the West Bank. Cutaneous leishmaniasis caused by *L. major* is transmitted by *Phlebotomus papatasi*.[1,2] It is a zoonosis that involves desert rodents, i.e., *Psamommys obesus*, as the reservoir, the distribution of which determines that of human cases.[3,4] In comparison, cases of CL caused by *L. tropica* are less numerous, but more widely distributed throughout the region. *Leishmania tropica* infections can result in rare cases of leishmaniasis recidivans and infantile visceral leishmaniasis (VL).[5,6] Classically, *L. tropica* is considered anthroponotic, However, the relative paucity of cases and the sudden emergence of CL in foci such as Kfar Adumim and neighboring Anatot suggests that, in this region, it might also be a zoonosis. The putative reservoir host is the rock hyrax *Procavia capensis*, which is extremely abundant in the vicinity of Kfar Adumim. The DNA of *L. tropica* has been demonstrated in the skin and blood of hyraxes captured in a new focus of CL in northern Israel.[7]

*Leishmania tropica* is genetically a very heterogeneous species and strains are frequently distinguished using biochemical, antigenic, and polymerase chain reaction (PCR)–based molecular markers.[8–11] In the present study, among *L. tropica* strains isolated from sand flies and human CL cases originating in the same locality, a degree of serologic, biochemical, and DNA sequence heterogeneity is demonstrated.

*Phlebotomus* (*Paraphlebotomus*) *sergenti* is the putative vector of *L. tropica* throughout the Middle East and is a proven vector in Saudi Arabia,[12] Morocco,[13] and Afghanistan.[14] In the Galilee region of northern Israel, both, *Ph. sergenti* and *Ph*. (*Adlerius*) *arabicus* have been found infected with *L. tropica*.[7] The present study was designed to improve our understanding of the epidemiology of CL caused by *L. tropica* in desert habitats in this region. The multifarious characterization of promastigotes isolated from three female *Ph. sergenti* and their comparison with strains of *L. tropica* isolated from human CL cases verifies that this species is also the vector of *L. tropica* in the Judean Desert near Jerusalem.

## MATERIALS AND METHODS

**Study site.** Kfar Adumim is an Israeli village in the Judean Desert (Figure 1). It is located at an altitude of 350 meters above sea level approximately 5 km east of Jerusalem off the main highway that descends from Jerusalem to Jericho. Twenty CL cases were diagnosed in Kfar Adumim and its vicinity between 1989 and 1994, and at least 10 additional cases have been diagnosed since then.[15,16] Strains isolated from two of these cases, L590 from Kfar Adumim and L691 from Anatot, a neighboring village approximately 4 km to the west, were used for comparison in characterizing stocks isolated from *Ph. sergenti* (Table 1).

**Collection and identification of sand flies.** Sand flies were collected near Kfar Adumim using Center for Disease Control (Atlanta, GA) miniature light traps (John W. Hock Company, Gainesville, FL) placed inside and near the entrances of caves and crevices inhabited by rock hyraxes (Figure 1). Sand flies were also collected inside houses and off properly protected humans, using mouth aspirators. For taxonomical identification, heads and genitalia were removed and mounted on microscope slides in Berlese's fluid and identified using several keys.[17,18] Female *Ph. (Larroussius) spp*., were identified by the structures at the base of the spermathecal ducts.

**Isolation and maintenance of leishmanial stocks.** For parasite isolation, forceps and glassware were sterilized in 70% ethanol. Female flies were washed in 5% detergent solution, rinsed several times in sterile water, and placed in sterile

* These authors contributed equally to this paper.

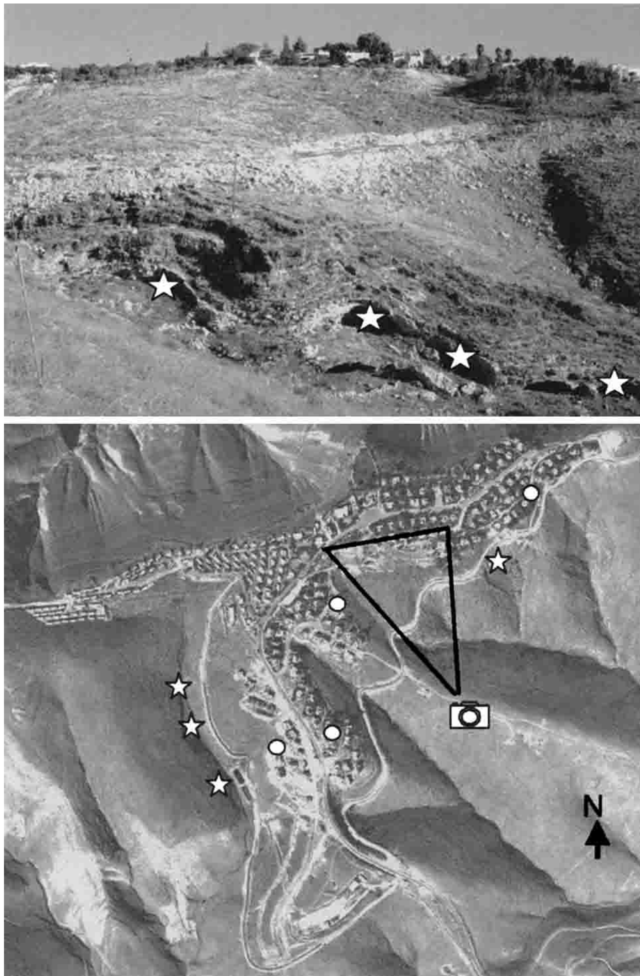


FIGURE 1. Village of Kfar Adumim (**top panel**) showing the houses and caves where sand flies were trapped (☆). Caves and crevices inhabited by rock hyraxes were between 40 and 350 meters from the nearest houses. Several houses where cases of cutaneous leishmaniasis lived are marked (○) on the aerial photograph (**bottom panel**). The **triangle** indicates from where the photograph in the top panel was taken and the angle covered.

phosphate-buffered saline (PBS). Their guts were dissected on glass slides, covered with coverslips, and examined for the presence of promastigotes by phase-contrast microscopy with a 40× objective. The guts of infected sand flies were seeded into rabbit blood hyphenate agar NNN slants overlaid with Schneider's *Drosophila* medium (SDM) supplemented with 10% fetal calf serum (FCS) containing penicillin (200 IU/mL), streptomycin (200 μg/mL) (Biological Industries, Beit Haemek, Israel) and 5-fluorocytosine (1,500 μg/ml) (Sigma, St. Louis, MO).[19] For routine culture, parasites were grown in liquid SDM containing 10% FCS.

**Biologic characterization of leishmanial strains.** Strain L747 from a *Ph. sergenti* female and strain L590 from a CL case from Kfar Adumim were separately injected subcutaneously into the dorsal surfaces of the hind paws of three Syrian hamsters. Each paw received approximately $0.5 \times 10^6$ stationary phase promastigotes. The animals were examined periodically and the sites of infection were monitored for the development of lesions and the presence of parasites. Smears of skin and, at necropsy, spleen and liver tissue were made, stained with Giemsa, and examined microscopically for amastigotes. Tissue from these organs was also seeded into rabbit blood agar medium and the cultures were later checked for the development and growth of promastigotes.

**Serologic characterization of the leishmanial strains.** *Excreted factor (EF) serotyping.* Spent growth medium from cultures of local leishmanial isolates containing their EFs were compared with standard reference EFs as previously described.[20,21] Standard serotyping sera were used: anti-*L. tropica* L36, which reacts only with serotype A and permits the designation of A subserotypes; anti-*L. donovani donovani* LRC-L52, which reacts only with serotype B EFs without designating subserotypes; and anti-*L. donovani infantum* LRC-L47, which reacts only with serotype B and permits the designation of B subserotypes. The reference EFs were obtained from the following strains: *L. tropica* L36 for subserotype $A_2$, *L. tropica* LRC-L682 for subserotype $A_9$, *L. d. donovani* LRC-L133 for subserotype $B_2$, and *L. aethiopica* LRC-L495 for subserotype $B_1$ (Table 1).

*Species-specific monoclonal antibodies (MAbs).* Four MAbs were used to characterize *Leishmania* promastigotes using an indirect immunofluorescent test. Cultured promastigotes were washed and placed in multi-well microscope slides. Preparations were fixed briefly with cold acetone, blocked with 5% FCS in PBS for 30 minutes at room temperature, and incubated for one hour with the MAbs (undiluted culture supernatant fluid or ascites diluted $10^{-3}$) at 37°C. Fluorescein isothiocyanate–conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) was applied for 40 minutes at 37°C. Slides were washed three times in PBS, mounted in PBS/glycerol with 3% 1,4-diazabicyclo[2.2.2]octane (DABCO; (Sigma), and observed by fluorescent microscopy (Axiovet; Zeiss, Oberkochen, Germany). The MAbs used were T1 and T3, which were raised against *L. major* L137 and L251, and T11 and T15, which were raised against *L. tropica* strain L36 (Table 1). T1 reacts primarily with *L. major* while T3 cross-reacts with many *L. tropica* strains.[8] Both, T11 and T15 react specifically with surface moieties of *L. tropica*.[22] The sand fly and human strains were compared with one another and with control strains of *L. major*, *L. tropica*, and *L. infantum*.

**Isoenzyme electrophoresis.** Electrophoresis was performed in starch gels according to Rioux and others[10] and 15 enzymes were examined to construct enzyme files: malate dehydrogenase (MDH, EC 1.1.1.37); malic enzyme (ME, EC 1.1.1.40); isocitrate dehydrogenase (ICD, EC 1.1.1.42); 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44); glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49); glutamate dehydrogenase (GLUD, EC 1.4.1.3); NADH diaphorase (DIA, EC 1.6.2.2); purine nucleoside phosphorylase (NP1, EC 2.4.2.1); purine nucleoside phosphorylase (NP2, EC 2.4.2*); glutamate-oxaloacetate transaminases (GOT1 and GOT2, EC 2.6.1.1); phosphoglucomutase (PGM, EC 5.4.2.2); fumarate hydratase (FH, EC 4.2.1.2); mannose phosphate isomerase (MPI, EC 5.3.1.8); and glucose phosphate isomerase (GPI, EC 5.3.1.9).

**Molecular characterization.** *Polymerase chain reaction–based analysis of kinetoplast DNA (kDNA).* The primer pair Uni21 (5′-GGG GTT GGT GTA AAA TAG GCC-3′) and Lmj4 (5′-CTA GTT TCC CGC CTC CGA G-3′), which was based on a minicircle sequence of *L. major*,[23] were used to amplify kDNA from cultured promstigotes, whose PCR prod-

TABLE 1
Strains of *Leishmania* used in the study*

| Designation | Species | WHO International Code | Origin |
|---|---|---|---|
| LRC-L590 | *L. tropica* | MHOM/IL/1990/LRC-L590 | 11-year-old boy CL, Kfar Adumim, Judean Desert |
| LRC-L691 | | MHOM/IL/1996/LRC-L691 | 31-year-old woman CL, Anatot, Judean Desert |
| LRC-L775 | | MHOM/EG/1990/LPN65 | Human CL, Sinai, Egypt |
| LRC-L747 | | ISER/IL/1998/LRC-L747 | *Phlebotomus sergenti* female, Kfar Adumim, Judean Desert |
| LRC-L757 | | ISER/IL/1998/LRC-L757 | *Phlebotomus sergenti* female, Kfar Adumim, Judean Desert |
| LRC-L758 | | ISER/IL/1998/LRC-L758 | *Phlebotomus sergenti* female, Kfar Adumim, Judean Desert |
| LRC-L36 | | MHOM/IQ/1966/BRAY L75 | Human CL, Baghdad, Iraq |
| LRC-L43 | | MHOM/IL/1949/ABU GHOSH 123 | Human, infantile VL, Abu Ghosh, Israel |
| K27 | | MHOM/SU/1974/SAF-K27 | WHO international reference strain |
| LRC-L22 | | MHOM/IL/1959/GABAI 159 | Human CL, Negev desert, Israel |
| LRC-L725 | | MHOM/IL/1997/P963 | Human CL, Tiberias, Israel |
| LEM163 | *L. killicki* | MHOM/TN/1980/LEM163 | WHO international reference strain |
| LRC-L777 | *L. major* | MHOM/PS/2000/ISLAH 503 | Human CL, Jericho, Jordan Valley |
| LRC-L137 | | MHOM/IL/1967/Jericho II | Human CL, Jericho, Jordan Valley |
| 5ASKH | | MHOM/TM/1973/5ASKH | WHO international reference strain |
| SUDAN3 | | MHOM/SD/1990/SUDAN3 | WHO international reference strain |
| LRC-L251 | | MHOM/IL/1979/LRC-L251 | 47-year-old man with CL, Jordan Valley |
| LRC-L465 | | IPAP/IL/1984/1A2 | *Phlebotomus papatasi* female, Uvda, Negev Desert |
| LRC-L762 | | MHOM/UZ/1999/NURIYAH | Young female infected for immunization |
| IPT1 | *L. infantum* | MHOM/TN/1980/IPT1 | WHO international reference strain |
| Peking | | MHOM/CN/1954/PEKING | WHO international reference strain |
| Khartoum | *L. donovani* | MHOM/SD/????/Khartoum | WHO international reference strain |

* WHO = World Health Organization; CL = cutaneous leishmaniasis; VL = visceral leishmaniasis.

ucts were electrophoresed on agarose gels according to Anders and others.[24]

The PCR products were also column purified using a High Pure™ PCR Product Purification Kit (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer's recommendations. For restriction fragment length polymorphism (RFLP) analysis, 50-ng samples of PCR product were digested with the following endonucleases (each applied separately): *Mbo* I and *Ban* II (Promega, Madison, WI). The RFLP samples were subjected to electrophoresis in 2% Metaphor agarose gels, and the DNA was detected with Gel Star Stain (FMC BioProducts, Rockland, ME).

*Permissively primed intergenic polymorphic (PPIP)–PCR.* The PCRs were performed as previously described[16] with minor changes, i.e., using 20 ng/μl of genomic DNA and amplified with 5 μM of the single leishmanial-specific primer 2B (5′-CAG GAG CGC GCA CAC GCA CAC ACG-3′), and two units of recombinant *Taq* DNA polymerase (MBI Fermentas, Amherst, NY).

*Amplification and digestion of the internal transcribed spacer (ITS) sequence.* This was done essentially according to Schonian and others.[25] The ITS ribosomal operon between the small subunit and large subunit ribosomal RNA genes was amplified using the primers LITSV (5′-ACA CTC AGG TCT GTA AAC-3′) and LITSR (5′-CTG GAT CAT TTT CCG ATG-3′). Aliquots of 17μl of the 900–1000 basepair products were digested for two hours with 1 μl of either *Taq* I or *Cfo* I (Hybaid GmbH, Heidelberg, Germany), according to the manufacturer's recommendations, and the fragments separated by agarose gel electrophoresis.

*Single strand conformation polymorphism (SSCP) analysis of the ITS1 sequence.* The first part of the ITS region (ITS1) was amplified with the primer pair L5.8S (5′-TGATACCACTTATCGCACTT-3′) and LITSR (5′-CTGGATCATTTTCCGATG-3′) to produce a PCR product of approximately 300 basepairs. Subsequent SSCP analyses were performed as described by El Tai and others.[26]

*DNA fingerprinting.* DNA fingerprinting was done essentially as described by Gomes and others[27] using the human multilocus probe 33.15.[28] Seven micrograms of leishmanial genomic DNA were digested completely with the restriction enzyme *Hae* III and subjected to electrophoresis. Following Southern blotting, the membrane was hybridized under low stringency with the digoxigenin (DIG)–labeled probe (probe 33.15, cloned in M13mp8, kindly provided by A. J. Jeffreys, University of Leicester, Leicester, United Kingdom). The DIG-labeled hybrids were detected with an anti-DIG–alkaline phosphatase conjugate and the chemiluminescent substrate CDP-Star (Roche, Penzberg, Germany). The relatedness of the strains was determined by analyzing the resulting banding patterns with RAPDistance Package version 1.04 using Pearson's Phi coefficient to calculate similarities (http://www.anu.edu.au/BoZo/software).

## RESULTS

**Sand flies.** Sand flies were collected in and around houses and in caves below the perimeter of the village (Figure 1). *Phlebotomus sergenti*, the most abundant species in light-trap collections from the caves, was not greatly attracted to humans, as demonstrated by the relatively small number collected off human bait in the same locations. Furthermore, aspirator collections inside houses showed that few *Ph. sergenti* entered houses. Conversely, *Ph. papatasi* was highly endophilic and anthropophagic but was not greatly attracted to light traps (Table 2). Very small numbers of three other spe-

TABLE 2
Sand flies from Kfar Adumim and its vicinity caught by different methods*

| Sand fly species | Light traps (in caves) | Human bait (close to caves) | In houses | Total |
|---|---|---|---|---|
| *Ph. sergenti* | 155 | 20 | 2 | 177 |
| *Ph. papatasi* | 10 | 106 | 123 | 239 |

* Besides *Phlebotomus sergenti* and *Ph. papatasi*, very small numbers of *Ph. tobbi*, *Ph. syriacus*, and *Ph. alexandri* were caught in light traps near caves.

cies were also collected: *Ph. (Pa.) alexandri*, *Ph. (Laroussius) tobbi*, and *Ph.* (*L.*) *syriacus.* Four of the 105 female *Ph. sergenti* caught in light traps in the caves were infected with promastigotes, from which three stocks were isolated for characterization: L747, L757, and L758 (Table 1). None of the more than 200 *Ph. papatasi* caught inside houses were infected.

**Characterization of *L. tropica* infections in hamsters.** One *Ph. sergenti* strain (L747) and strain L590 from a human case of CL behaved very similarly in Syrian hamsters. Both were infective and caused patent infections at the sites of injection of the parasites, the dorsal surfaces of the hind paws. Over a period of six months, both strains caused small, circumscribed, nodular lesions 3–4 mm in diameter without ulceration. Subsequently, some regression in the size of the lesions was observed. In both cases, heavy infections of amastigotes were observed in stained smears. Cultures of tissue aspirates from the lesions grew promastigotes within five days. In both cases, the smears and cultures of splenic and hepatic tissue were negative, indicating there was no involvement of these organs during the period tested.

**Serologic characterization by EF and species-specific MAbs.** The EF serotypes and MAb specificities of all the strains studied are shown in Table 3. The Iraqi strain L36, a reference strain of the EF subserotype $A_2$, reacted strongly with the MAb T11 and relatively weakly with MAb T15, which were raised against it and are species-specific for *L. tropica*. However, strain L36 did not react with MAbs T1 and T3 raised against *L. major*. Strain L22 isolated from a human case of CL acquired in the Negev region and included as an Israeli reference strain was also EF subserotype $A_2$, showing that this subserotype also exists in Israel. The promastigotes of strain L22 were not checked for their MAb-binding specificities with the MAbs T11, T15, T3, and T1. However, its EF did bind strongly to MAb T11 and moderately to MAb T15, but not to the MAbs T1 and T3.[21] The test strains were either EF subserotype $A_9$ or the mixed subserotype $A_9B_2$, both of which and like the EF subserotype $A_2$, are species-specific for *L. tropica.* They also all bound MAb T11 strongly. Strains L747, L757, and L758 from locally caught *Ph. sergenti* were serologically similar in not reacting with MAb T3 and separable from strains L590 and L691 from the local human cases of CL and from strain L775 from Sinai, Egypt, which bound MAb T3 strongly. Only two of the test strains, L590 and L775, bound MAb T1 relatively weakly. None of the test strains reacted with MAb T15 (Table 3).

**Biochemical characterization.** The Montpellier (MON) zymodeme numbers of the strains analyzed are shown in Table 3, and the 15-enzyme profiles associated with these zymodemes, which give the electrophoretic mobility of each electromorph, are shown in Table 4. Strains L747, L757, and L758 from *Ph. sergenti* caught at Kfar Adumim and strain L691 from a human case of CL from nearby Anatot shared the same profile, which was characteristic of strains belonging to zymodeme MON-137, the reference strain of which is L775 (Table 1). The enzyme profile of strain L590 was different from all the profiles recorded for the established zymodemes. Thus, strain L590 represents a new zymodeme, MON-275. Its profile was much closer to that of the Iraqi reference strain L36, which belongs to zymodeme MON-006 than it was to that of the strains from Kfar Adumim, which belong to zymodeme MON-137, even though it came from a human case of CL living in the village. The enzyme profiles of strains L590 and L36 shared 10 enzyme electrophoretic mobilities, whereas L590 and the strains from Kfar Adumim belonging to the zymodeme MON-137 shared only four, those of ICD, DIA, $NP_1$, and GPI (Table 4). The Israeli strain L22 also represented a new zymodeme, MON-288. Its enzyme profile was closer to that of the local strain L590 than it was to that of the Iraqi strain L36 (Table 4).

**Kinetoplast DNA–RFLP analysis.** The amplification of kDNA from the three sand fly strains (L747, L757, and L758) and the two human strains (L590 and L691) with the minicircle primers Uni21 and Lmj4 generated products of 800 basepairs that aligned with those of the reference strains of *L. tropica* L36 and L682. The kDNA-PCR products of the reference strains of *L. major* (L465 and L762), *L. donovani* (the Khartoum strain), *L. infantum* (IPT1), and *L. aethiopica* (L149) were of different sizes when compared with those of *L. tropica* and the test strains.

Figure 2 shows the kDNA-RFLP patterns of reference strains of *L. tropica* and several test strains following digestion with *Mbo* I (Figure 2a) and *Ban* II (Figure 2b). All strains were shown to be *L. tropica*, but strain L590 from the human case of CL from Kfar Adumim was clearly different from the sand fly strains caught in the same vicinity (Figure 2a). Moreover, among the sand fly strains, L758 and L757 were identical to one another and L747 was somewhat different (Figure 2b).

TABLE 3
Biochemical, serologic, and molecular biologic characterization of *Leishmania* strains*

| LRC designation | Zymodeme† | EF | MAb specificities | | | | Kinetoplast DNA | | | Nuclear DNA | | | | Place |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Lm/Lt* T3 | *Lt* T11 | *Lt* T15 | *Lm* T1 | Uni21/Lmj4 | *Mbo* I | *Ban* II | PPIP | SSCP | ITS + *Taq* I | ITS + *Cfo* I | |
| L590 | MON-275 | $A_9$ | 3+ | 3+ | – | 1+ | *Lt* | B | B | *Lt*A1 | A | A | A | Kfar Adumim (CL) |
| L691 | MON-137 | $A_9B_2$ | 3+ | 3+ | – | – | *Lt* | ND | C | *Lt*B1 | B | A | A | Anatot (CL) |
| L775 | MON-137 | $A_9$ | 3+ | 3+ | – | 1+ | *Lt* | ND | D | *Lt*B1 | B | A | A | Sinai (CL) |
| L747 | MON-137 | $A_9B_2$ | – | 3+ | – | – | *Lt* | C | A | *Lt*B1 | B | A | A | Kfar Adumim (CL) |
| L757 | MON-137 | $A_9B_2$ | – | 3+ | – | – | *Lt* | D | C | *Lt*B1 | B | A | A | Kfar Adumim (CL) |
| L758 | MON-137 | $A_9B_2$ | – | 3+ | – | – | *Lt* | D | C | *Lt*B1 | B | A | A | Kfar Adumim (CL) |
| L22 | MON-288 | $A_2$ | –‡ | 3+‡ | 1+‡ | –‡ | ND | ND | ND | *Lt*A1 | ND | ND | ND | Negev (CL) |
| L36 | MON-006 | $A_2$ | – | 3+ | 1+ | – | *Lt* | A | A | *Lt*A1 | A§ | A¶ | A¶ | Iraq (CL) |

* EF = excreted factor; MAb = monoclonal antibody; *Lm* = *L. major; Lt* = *L. tropica;* PPIP = permissively primed intergenic polymorphism; SSCP = single strand conformation polymorphism; ITS = internal transcribed sequence; MON = Montpellier; CL = cutaneous leishmaniasis; ND = not done.
† For the actual profiles, see Table 4.
‡ MAb reactivity with the EF of strain L22.[21] Reactivity with promastigotes was not determined.
§ Not shown in the SSCP figure, but shown in Schonian and others.[11]
¶ LRC-L36 has an extra band of approximately 300 basepairs when the ITS is digested with *Taq* I and one of approximately 250–300 basepairs when the ITS is digested with *Cfo* I.

TABLE 4
Enzyme profiles of the strains of *Leishmania* studied and specific electrophoretic differences*

| Zymoeme | Enzyme profiles | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MDH | ME | ICD | PGD | G6PD | GLUD | DIA | $NP_1$ | $NP_2$ | $GOT_1$ | $GOT_2$ | PGM | FH | MPI | GPI |
| MON-137 | 100 | 110 | 100 | 98 | 85 | 80 | 100 | 450 | 110 | 140 | 85 | 88 | 100 | 110 | 76 |
| MON-275 | 116 | 95 | 100 | 95 | 82 | 95 | 100 | 450 | 100 | 135 | 90 | 108 | 110 | 110<br>100 | 76 |
| MON-288 | 112 | 95 | 100 | 94 | 82 | 95 | 100 | 450 | 100 | 135 | 90 | 108 | 100 | 110<br>100 | 76 |
| MON-006 | 100 | 95 | 100 | 93 | 82 | 95 | 110 | 450 | 100 | 135 | 90 | 100 | 100 | 110<br>100 | 76 |
| MON-026 | 160 | 88 | 100 | 122 | 94 | 200 | 100 | 400 | 90 | 110 | 110 | 118 | 72 | 50 | 77 |

* Underlined numbers indicate shared electrophoretic mobilities. Since the enzyme profile of *L. major* L777 from the Jordan Valley has not been determined, that of *L. major* L137, also from the Jordan Valley, was used as an outgroup. It belongs to zymodeme MON-26. The data in the table correlate well with the dendrogram shown in Figure 7. MDH = malate dehydrogenase; ME = malic enzyme; ICD = isocitrate dehydrogenase; PGD = 6-phosphogluconate dehydrogenase; G6PD = glucose-6-phosphate dehydrogenase; GLUD = glutamate dehydrogenase; DIA = diaphorase; NP = purine nucleotide phosphorylase; GOT = glutamate-oxaloacetate dehydrogenases; PGM = phosphoglucomutase; FH = fumarate hydratase; MPI = mannose phosphate isomerase; GPI = glucose phosphate isomerase.

**Permissively primed intergenic polymorphic–PCR.** The PPIP-PCR patterns of all the locally isolated strains are shown in Figure 3. This analysis showed that all the strains were *L. tropica*. They were also easily distinguished from *L. major*, *L. infantum*, and *L. killicki*. Strains L747, L757, and L758 from *Ph. sergenti* caught at Kfar Adumim, strain L691 from the case of CL from Anatot, and strain L775 from Sinai, Egypt had the same PPIP-PCR type (*Lt*B1). Strain L590 from the case of CL from Kfar Adumim was different (PPIP-PCR type *Lt*A1) and identical to strains L36 and L22 from an Iraqi and an Israeli human case of CL respectively (Figure 3 and 6).

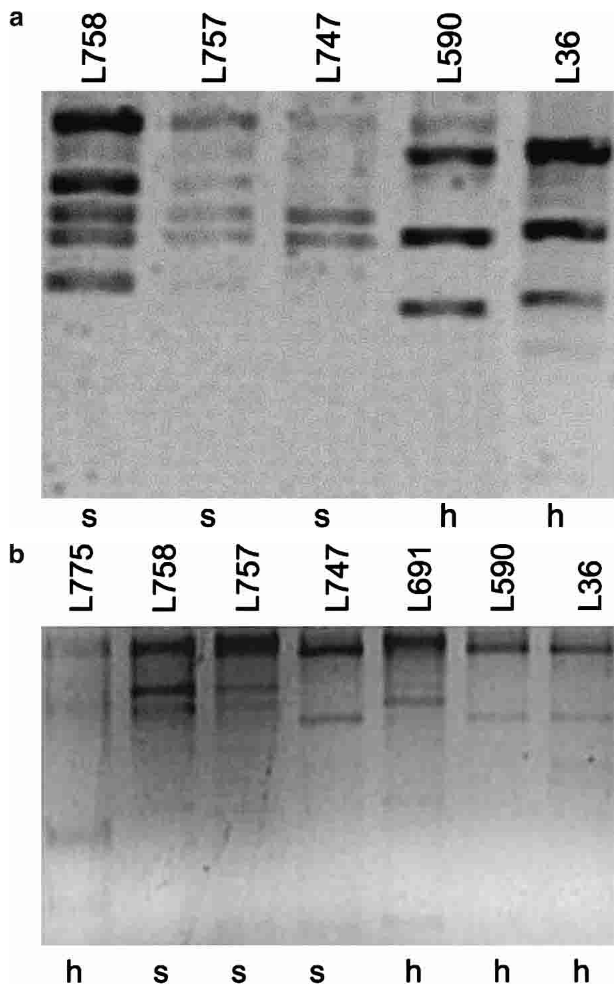


FIGURE 2. Restriction fragment length polymorphism analysis of the kinetoplast DNA polymerase chain reaction products of *Leishmania tropica* strains after digestion with *Mbo* I (**a**) and *Ban* II (**b**). s = sand fly; h = human. DNA markers (ϕX174 + *Hae* III and ϕX174 + *Hinf* II; Promega, Madison, WI) were used as size references. See Table 1 for strain designations.

**Single strand conformation polymorphism analysis of ITS1.** The results of the ITS1-SSCP analysis are shown in Figure 4. Again, based on the patterns seen, all locally isolated strains were *L. tropica* and easily distinguished from *L. major*, *L. infantum*, and *L. killicki*. All of these strains of *L. tropica* displayed identical patterns except for L590 from the case of CL from Kfar Adumim, which was identical to that of strain L43 isolated from a human case of VL from the village Abu Ghosh approximately 23 km west of Kfar Adumim. Thus, the results of the SSCP analysis corresponded well with those of the PPIP-PCR and isoenzyme electrophoresis.

**Restriction fragment length polymorphism analysis of the ITS sequence.** The restriction patterns of the amplified ITS region of different strains of *Leishmania* following digestion with the restriction enzyme *Taq* I are shown in Figure 5. This generated identical patterns for strains L590 from the case of

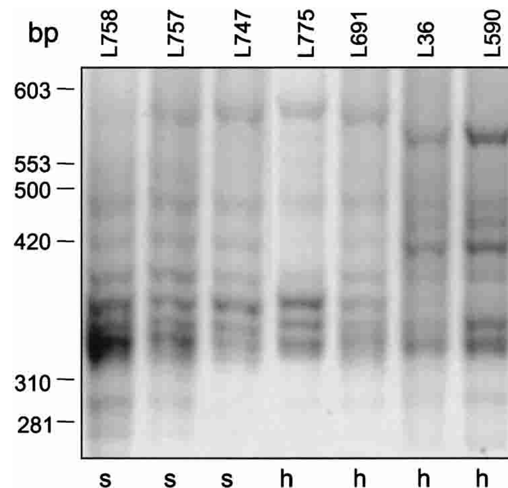


FIGURE 3. Genomic DNA analysis using the permissively primed intergenic polymorphic–polymerase chain reaction. bp = basepairs; s = sand fly; h = human. See Table 1 for strain designations.

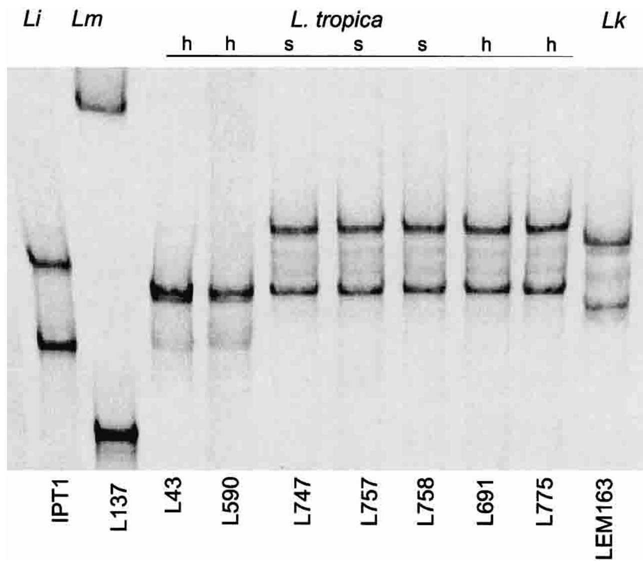


FIGURE 4. Single strand conformation polymorphism analysis of the internal transcribed spacer 1 sequence of various species and strains of *Leishmania*. *Li* = *L. infantum*; *Lm* = *L. major*; h = human; s = sand fly; *Lk* = L. killicki. See Table 1 for strain designations.

CL and L747 from a *Ph. sergenti* from Kfar Adumim and the reference strain of *L. tropica* K27. After digestion with the restriction enzyme *Cfo* I, the restriction patterns of strains L590 and L747 were also identical to one another. Both types of pattern distinguished these strains of *L. tropica* from the reference strains of *L. infantum* and *L. major*. The ITS-RFLP analysis of strains L691 and L775 from human cases of CL from Anatot and Sinai, Egypt, respectively, and strains L757 and L758 from *Ph. sergenti* produced the same patterns, also confirming their identity as *L. tropica* of the same type. However, *L. tropica* L36 from a human case of CL from Iraq had an extra band of approximately 300 basepairs after digestion with *Taq* I and another band of approximately 250–300 basepairs after digestion with *Cfo* I.

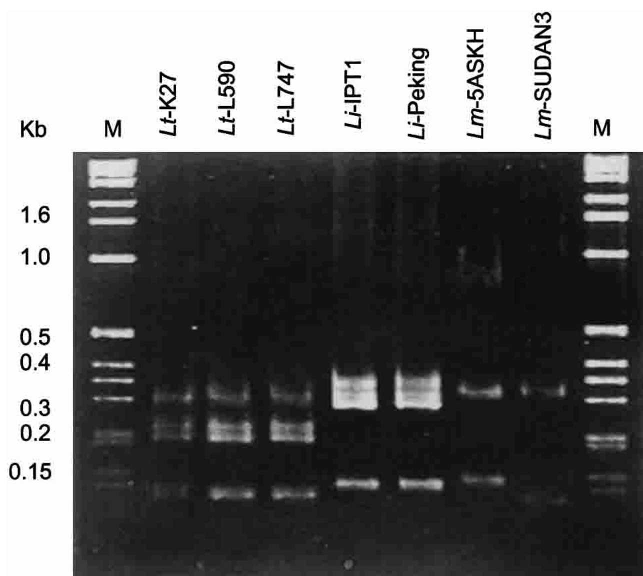


FIGURE 5. Restriction analysis of the ribosomal internal transcribed spacer sequences of different *Leishmania* strains using *Taq* I. Kb = kilobases; lanes M = molecular markers; *Lt* = *L. tropica*; *Li* = *L. infantum; Lm* = *L. major*. See Table 1 for strain designations.

**DNA fingerprinting.** The dendrogram in Figure 6 shows the inter-relationship of various strains of *L. tropica* according to their genomic DNA RFLP patterns after digestion with *Hae* III and hybridization with the minisatellite probe. All strains of *L. tropica*, including L36 from Iraq, grouped together and formed a single closely-knit cluster relative to the strain of *L. major* L777 used as an outgroup. This clustering of the strains of *L. tropica* was similar to the clustering of the strains based on 1) specific binding of MAb T11, 2) PCR-based kDNA analysis using the primer pair Uni21 and Lmj4, and 3) amplification and restriction of the ITS sequence with either *Taq* I or *Cfo* I (Table 3). This main cluster divided into two subclusters (I and II), which was supported by 1) EF serotypes, 2) enzyme profiles, 3) PPIP-PCR types, and 4) ITS1-SSCP types (Table 3). The strains within each subcluster displayed further intraspecific microheterogeneity based on 1) the specific binding of the MAbs T15, T3, and T1, and 2) restriction of amplified kDNA with either *Mbo* I or *Ban* II (Table 3). It is noteworthy that the strains of *L. tropica* in subcluster II belonged to zymodeme MON-137 and EF serotype $A_9B_2$, and had the PPIP-PCR pattern *Lt*B1and the ITS1-SSCP pattern B (Figure 6 and Table 3).

## DISCUSSION

*Phlebotomus papatasi* was found to be the predominant species in houses of Kfar Adumim. However, only *Ph. sergenti* females were found infected with promastigotes, and they were collected in or near caves at the periphery of the village. Only a few *Ph. sergenti* were caught in houses or on human bait (Table 2). The reluctance of *Ph. sergenti* to enter houses and bite humans probably explains the paucity of CL cases despite the high infection rate among females of this species (4%). Although *Ph. papatasi* was notably endophilic and constituted an important pest, there is no evidence for its having a role in the transmission of CL in this focus. However, *Ph. papatasi* is the vector of *L. major* in the Jordan Valley, which is not far from Kfar Adumim but at a lower altitude, where *P. obesus* is the animal reservoir host.[1,2] The absence of *P. obesus* from rocky terrain is a reasonable explanation for the lack of locally acquired infections of *L. major* in residents of Kfar Adumim and Anatot.

Female *Ph. sergenti* from Kfar Adumim were distinguishable from females of the same species from other countries. The most prominent differentiating feature was the more swollen appearance of the distal segment of their spermathecae, which was consistent and easily seen after dissection. This feature is not readily apparent in mounted specimens and appears to have been previously overlooked. The taxonomic relevance of this differentiating morphologic feature remains to be determined.

Before the introduction of more modern serologic, biochemical, and molecular biologic methods for identifying leishmanial strains, their infectivity, virulence, dissemination, and pathogenicity in laboratory animals were used as diagnostic characteristics. The Syrian hamster, despite not being a proven natural host of any species of *Leishmania*, can harbor

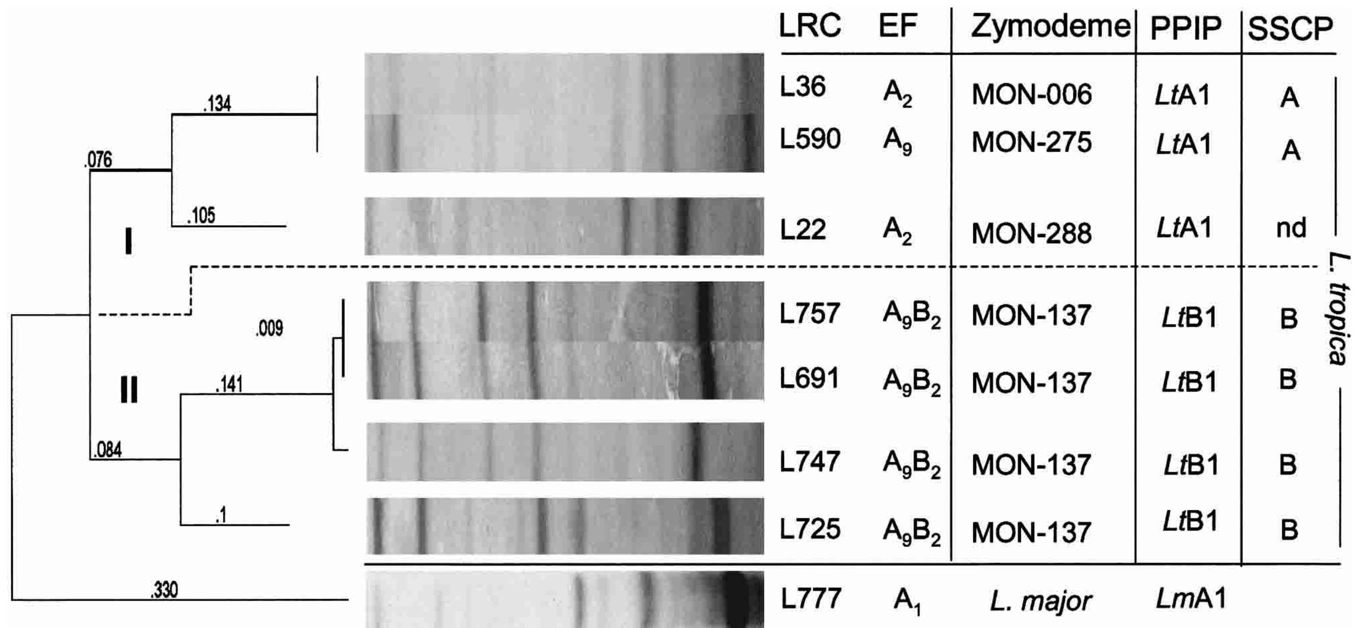


FIGURE 6. Genomic fingerprinting of different strains of *Leishmania tropica* using a mini-satellite probe. **Left panel**, dendrogram generated from a Southern blot demonstrating clustering of strains of *L. tropica*. The length of the branches indicate the degree of similarity in fingerprints among the strains of *L. tropica* (*Lt*) relative to the strain of *L. major*, which served as an outgroup in this analysis. **Right panel**, definition of the same strains by excreted factor serotyping, isoenzymes, permissively primed intergenic polymorphic–polymerase chain reaction, and single strand conformation polymorphism electrophoresis. nd = not done. See Table 1 for strain designations and origins and Table 4 for the full enzyme profiles.

infections of most species, including some strains of *L. tropica*.[29] In our experiments, infection and pathogenesis caused by the two chosen strains, L590 from human CL and L747 from *Ph. sergenti*, was essentially the same. The infections were dermatotropic without involvement of the spleen or the liver. Pathogenesis differed substantially from that caused by *L. major*.[30]

The three sand fly and two human strains of *Leishmania* from the Kfar Adumim focus proved to be *L. tropica* by all the approaches used. Two serologic tests were used: EF serotyping and analysis with species-specific MAbs. An EF comprises all immunogenic molecules secreted by promastigotes growing in culture. The main components of EF are the naturally released lipophosphoglycans.[31] The presence of the antigenic components $A_2$ or $A_9$ in an EF and reactivity with MAb T11 are typical of strains of *L. tropica.* It was noted that the first three strains listed in Table 3, all from human cases of CL, also reacted strongly with MAb T3, which although raised against *L. major*, binds to the promastigotes of both *L. major* and *L. tropica*, whereas the three strains from the sand flies did not. No explanation is currently available for this finding. Isoenzyme electrophoresis confirmed that most of the locally isolated strains belonged to the previously known zymodeme MON-137 of *L. tropica*. This included strains from a human case of CL and those from *Ph. sergenti*. Strain L590 was the single exception. Its enzyme profile was different from the rest and it represented a new zymodeme, MON-275, but was sufficiently similar to those of the reference strains of *L. tropica* L36 and L22 (Table 4) to confirm its identity as *L. tropica*. Direct amplification of the kDNA from all the local strains with the primer pair Uni21 and Lmj4 generated a product of approximately 800 basepairs, confirming they were all *L. tropica*.[24] After digestion with *Mbo* I (Figure 2a), differences were seen between the RFLP pattern of the locally isolated strains, which is discussed later in the context of microheterogeneity of the strains. Furthermore, genomic DNA analyses using PPIP-PCR (Figure 3), ITS1-SSCP (Figure 4), and ITS-RFLP (Figure 5) all generated profiles readily identifying the test strains as *L. tropica* and separating them from other species of *Leishmania*.

*Leishmania tropica* is recognized as a very heterogeneous species of *Leishmania* and intraspecific microheterogeneity has been readily demonstrated by many investigators.[4,9,10,11,16,21] In terms of isoenzyme profile variation, Kreutzer and others used 21 enzyme systems on viscerotropic strains of *L. tropica* isolated from soldiers returning from Operation Desert Storm.[32] In the present study, despite the small number of isolates and the restricted geographic region studied, strains of *L. tropica* were shown to comprise two different zymodemes. Most of the strains belonged to zymodeme MON-137, the reference strain of which is strain L775 from Sinai (Table 1). Other strains of this zymodeme have been isolated from CL cases in Jordan.[33] The only other local strain isolated and characterized was strain L590. Its enzyme profile was very different and it constituted a new zymodeme, MON-275. Of the 15 enzymes tested, the electrophoretic mobilities of four of them corresponded with those in the profile associated with the zymodeme MON-137, while 11 differed. However, L590, the reference strain of the new zymodeme MON-275, was much more similar to the reference strain of *L. tropica* (L36 from Iraq, zymodeme MON-6), with which it shared 10 and differed in five enzyme electrophoretic mobilities. It was even more similar in its profile to reference strain L22 from Israel that represented the new zymodeme MON-288, where it differed in only three enzyme electrophoretic mobilities, those of MDH, PGD, and FH. Strains L36 and L22

differed between themselves in four enzyme electrophoretic mobilities, those of MDH, PGD, DIA, and PGM. All these similarities and differences are seen and compared in Table 4.

The RFLP analysis of kDNA after digestion with *Mbo I* and *Ban II* (Figure 2 and Table 3) showed that the strain L590 from the case of CL from Kfar Adumim was notably different from the strains isolated from *Ph, sergenti* from the caves below Kfar Adumim, and more similar to strain L36 isolated many years ago from a human of case CL from Baghdad. The RFLP pattern of strain LRC-L747 and that shared by strains L757 and L758 were different, even though these three strains came from sand flies caught in the same focus within one month in 1998. However, the sand fly from which strain L747 was isolated was caught on a separate occasion than the sand flies from which strains L757 and L758 were isolated. The genetic microheterogeneity of kDNA among the various strains of *L. tropica* studied here is not surprising because microheterogeneity of kDNA has been reported even among clones from the same strain of *L. major*.[34]

Microheterogeneity was also disclosed by the genomic DNA analyses. The PPIP-PCR (Figure 3) and ITS-SSCP (Figure 4) patterns of strain L590 from the human case of CL from Kfar Adumim also differentiated it from strain L691 from the human case of CL from Anatot and strains L747, 757, and 758 from the sand flies caught near Kfar Adumim, which were all identical. In addition, strain L590 was very similar to the Iraqi reference strain L36 and, in the case of its PPIP-PCR pattern, the Israeli strain L22. However, ITS-RFLP analyses did not detect differences among all these strains (Figure 5).

DNA fingerprinting using a minisatellite probe placed L590 in one subcluster together with the Iraqi strain L36 and the Israeli strain L22, and separating it from the other local strains from Kfar Adumim and Anatot (Figure 6). The two subclusters of strains of *L. tropica* in the dendrogram were congruent with results obtained by PPIP-PCR, ITS1 SSCP, isoenzyme electrophoresis, and serologic profiles (Figure 6 and Table 3). In summary, microheterogeneity was evident among the locally isolated strains examined, and strain L590 appeared sufficiently different from the other local strains to suggest that the person involved, although he lived in the village of Kfar Adumim, might have contracted his CL elsewhere.

Table 4, which includes the enzyme profiles encountered, shows that the enzyme profile of zymodeme MON 026 associated with the strain of *L. major*, which served as an outgroup in the dendrogram (Figure 6), was very different from the profiles associated with the zymodemes of *L. tropica.* These profiles are consistent with the dendrogram based on DNA fingerprinting and with the data generated by the other methods used.

The various methods of characterization used here disclosed different degrees of similarities and differences among the strains studied. However, a direct connection between the DNA profiles obtained by examining kinetoplast and nuclear DNA and the phenotypic characteristics discerned by serotyping and enzyme analysis cannot be made. Nevertheless, it is interesting to note that the genetic and phenotypic characteristics identified here are consistent with one another (Figure 6 and Table 3). The methods employed seem to separate into two types irrespective of exposing either genotypic or phenotypic criteria: those that identify leishmanial parasities at the species level and those that reveal intraspecies varation. All of these methods are valid at whichever level they operate. Some of the methods are more useful as tools in the diagnosis of disease, while others would be more useful in analyzing the genetic composition of leishmanial parasite populations. Table 3 shows a full comparison of the methods and the criteria they reveal and assess, the clustering of the strains of *L. tropica* by DNA fingerprinting, and how the various methods used support this clustering (see results of DNA fingerprinting).

During the preparation of this report, a new focus of human CL caused by *L. tropica* was investigated in the Galilee region of northern Israel. The sand fly vector there was identified as *Ph.* (*Adlerius*) *arabicus* and the causative agent as *L. tropica*, which was different from all the other variants of *L. tropica* described throughout the entire geographic range of the species.[7] Similar to Kfar Adumim, this new focus was also associated with relatively recent development of the area and the establishment of new villages that caused localized ecologic and environmental disruption. It appears that human encroachment on natural zoonotic foci is leading to the emergence of human CL in the entire region.

The infected female sand flies caught in the vicinity of Kfar Adumim were identified as *Ph. sergenti* and the strains of *Leishmania* isolated from them and the local human cases of CL were identified as *L. tropica*. *Phlebotomus sergenti* has long since been the putative vector of *L. tropica* in the region encompassed by Israel and the West Bank. The overall result of this comparative study establishes it as an actual vector in this region.

Received July 2, 2003. Accepted for publication October 9, 2003.

Financial support: This research was supported by grant SO 220/5-1 from the Deutsche Forschungsgemeinschaft (DFG): "The German-Israeli-Palestinian Cooperative Project on Leishmaniosis in Israel and The West Bank" by grant number 235/99-16.2 from "The Israel Science Foundation" of the Israeli Academy of Sciences and Humanities, and a grant from the Israeli Ministry for the Environment. The Montpellier Centre National de Reference des *Leishmania* is supported by the French Ministry of Health.

Authors' addresses: Lionel F. Schnur, Abdelmageed Nasereddin, Carol L. Eisenberger, Charles L. Jaffe, Gerlind Anders, Tamar Grossman, Raymond L. Jacobson, and Alon Warburg. Department of Parasitology, The Kuvin Center for the Study of Infectious and Tropical Diseases, Hebrew University–Hadassah Medical School, PO Box 12272, Jerusalem 91120, Israel, Telephone: 972-2-675-8081. Fax: 972-2-675-7425, E-mails: schnurl@cc.huji.ac.il and warburg@cc.huji.ac.il. Mustafa El Fari and Gabrielle Schonian, Institut fur Mikrobiologie und Hygiene, Charite Campus Mitte, von Humboldt Universitaet zu Berlin, Dorotheenstrasse, Berlin, Germany, Telephone: 49-30-2093-4741, Fax: 49-30-2093-4703. Kifayia Azmi, Department of Biochemistry, Al-Quds University, Palestinian Authority. Mireille Killick-Kendrick and Robert Killick-Kendrick, Department of Biological Sciences, Imperial College at Silwood Park, Acsot SL5 7PY, United Kingdom. Jean-Paul Dedet and Francine Pratlong, Laboratoire de Parasitologie and Centre National de Référence des Leishmania, 163 Rue Auguste Broussonet, 34090 Montpellier, France. Moien Kanaan, Department of Biology, Bethlehem University, Palestinian Authority.

## REFERENCES

1. Schlein Y, Warburg A, Schnur LF, Gunders A, 1982. Leishmaniasis in the Jordan Valley. II. Sandflies and transmission in the central endemic area. *Trans R Soc Trop Med Hyg 76:* 582–586.

2. Schlein Y, Warburg A, Schnur LF, Le Blancq SM, Gunders A, 1984. Leishmaniasis in Israel: reservoir hosts, sandfly vectors and leishmanial strains in the Negev, Central Arava and along the Dead Sea and transmission in the central endemic area. *Trans R Soc Trop Med Hyg 78:* 480–484.
3. Wasserberg G., Abramsky Z, Kotler BP, El Fari M, Schoenian G, Kabalo I, Schnur L, Anders GA, Warburg A, 2002. The Eco-epidemiology of cutaneous leishmaniasis in the Western Negev of Israel: heterogeneity in host prevalence and its underlying ecological mechanisms. *Int J Parasitol 32:* 133–143.
4. Fryauff DJ, Modi GB, Mansour NS, Kreutzer RD, Soliman S, Youssef FG, 1993. Epidemiology of cutaneous leishmaniasis at a focus monitored by the Multinational Force and observers in the northeastern Sinai Desert of Egypt. *Am J Trop Med Hyg 49:* 598–607.
5. Schnur LF, Le Blancq SM, 1986. The serological and enzymological characterization of aetiological agents of leishmaniasis in Israel. Rioux J-A, ed. The *Leishmania. Taxonomie et Phylogenese. Application Eco-Epidemiologiques.* Montpellier: Colloque International CNRS/INSERM/OMS, 1984, IMEEE, 347–355.
6. Schnur LF, Chance ML, Ebert F, Thomas SC, Peters W, 1981. The biochemical and serological taxonomy of visceralizing *Leishmania. Ann Trop Med Parasitol 75:* 131–144.
7. Jacobson RL, Eisenberger CL, Svobodova M, Baneth G, Sztern J, Carvalho J, Nasereddin A, El Fari M, Shalom U, Volf P, Votypka J, Dedet JP, Pratlong F, Schonian G, Schnur LF, Jaffe CL, Warburg A, 2003. Outbreak of cutaneous leishmaniasis in northern Israel*. J Infect Dis 188:* 1065–1073.
8. Jaffe CL, McMahon D, 1983. Monoclonal antibodies specific for *Leishmania tropica*. I. Characterization of antigens associated with stage- and species-specific determinants. *J Immunol 131:* 1987–1993.
9. Le Blancq SM, Peters W, 1986. *Leishmania* in the Old World: Heterogeneity among *L. tropica* zymodemes. *Trans R Soc Trop Med Hyg 80:* 113–119.
10. Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J, 1990. Taxonomy of *Leishmania*. Use of enzymes. Suggestions for a new classification. *Ann Parasitol Hum Comp 65:* 111–125.
11 Schonian G, Schnur LF, El Fari M, Oskam L, Kolesnikov AA, Sokolowska-Kohler W, Presber W, 2001. Genetic heterogeneity in the species *Leishmania tropica* revealed by PCR-based methods. *Trans R Soc Trop Med Hyg 95:* 217–224.
12. Al-Zahrani MA, Peters W, Evans DA, Chin C, Smith V, Lane RP, 1988. *Phlebotomus sergenti*, a vector of *Leishmania tropica* in Saudi Arabia. *Trans R Soc Trop Med Hyg 82:* 416.
13. Guilvard E, Rioux J-A, Gallego M, Pratlong F, Mahjour J, Martinez-Ortega E, Dereure J, Saddiki A, Martini A, 1991. *Leishmania tropica* au Maroc. III. Role vecteur de *Phlebotomus sergenti*. A propos de 89 isolates. *Ann Parasitol Hum Comp 66:* 96–99.
14. Killick-Kendrick R, Killick-Kendrick M, Tang Y, 1995. Anthroponotic cutaneous leishmaniasis in Kabul, Afghanistan: the high susceptibility of *Phlebotomus sergenti* to *Leishmania tropica. Trans R Soc Trop Med Hyg 89:* 477.
15. Klaus S, Axelrod O, Jonas F, Frankenburg S, 1994. Changing patterns of cutaneous leishmaniasis in Israel and neighbouring territories. *Trans R Soc Trop Med Hyg 88:* 649–650.
16. Eisenberger CL, Jaffe CL, 1999. *Leishmania*: identification of Old World species using a permissively primed intergenic polymorphic-polymerase chain reaction. *Exp Parasitol 91:* 70–77.
17. Theodor O, 1958. Psychodidae-Phlebotominae. Lindner E, ed. *Die Fliegen der Palaearktischen Region*. Volume 9. Psychodidae. Stuttgart, Germany: Nagele/Obermiller, 1–55.
18. Lewis DJ, 1982. A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). *Bull Br Mus Nat Hist 45:* 121–209.
19. Schnur LF, Jacobson RL, 1987. Appendix III. Parasitological Techniques. Peters W, Killick-Kendrick R, eds. *The Leishmaniases in Biology and Medicine*. Volume I. London: Academic Press, 449–541.
20. Schnur LF, Zuckerman A, 1977. Leishmanial excreted factor (EF) serotypes in Sudan, Kenya and Ethiopia. *Ann Trop Med Parasitol 71:* 273–294.
21. Schnur LF, Sarfstein R, Jaffe CL, 1990. Monoclonal antibodies against leishmanial membranes react with specific excreted factors (EF). *Ann Trop Med Parasitol 84:* 447–456.
22. Sarfstein R, Jaffe CL, 1989. Identification of *Leishmania tropica* by species specific monoclonal antibodies. *NATO Adv Sci Inst Ser A 163:* 925–929.
23. Smith DF, Searle S, Ready PD, Gramiccia M, Ben Ismael R, 1989. A kinetoplast DNA probe diagnostic for *Leishmania major* sequence homologies between regions of *Leishmania* minicircles. *Mol Biochem Parasitol 37:* 213–224.
24. Anders G, Eisenberger, CL, Jonas F, Greenblatt CL, 2002. Distinguishing *Leishmania tropica* and *Leishmania major* in the Middle East using the polymerase chain reaction with kinetoplast DNA specific primers. *Trans R Soc Trop Med Hyg 96 (suppl 1):* 87–92.
25. Schonian G, Akuffo H, Lewin S, Maasho K, Nylen S, Pratlong F, Eisenberger CL, Schnur LF, Presber W, 2000. Genetic variability within the species *Leishmania aethiopica* does not correlate with clinical variations of cutaneous leishmaniasis. *Mol Biochem Parasitol 106:* 239–248.
26. El Tai NO, Osman OF, El Fari M, Presber W, Schonian G, 2000. Genetic heterogeneity of ribosomal internal transcribed spacer (ITS) in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-stranded conformation polymorphisms and sequencing. *Trans R Soc Trop Med Hyg 94:* 575–579.
27. Gomes RF, Macedo AM, Pena SD, Melo MN, 1995. *Leishmania* (*Viannia*) *braziliensis*: genetic relationships between strains isolated from different areas of Brazil as revealed by DNA fingerprinting and RAPD. *Exp Parasitol 80:* 681–687.
28. Jeffreys AJ, Wilson V, Thein SL, 1985. Hypervariable "minisatellite" regions in human DNA. *Nature 314:* 67–73.
29. Bastian P, Killick-Kendrick R, 1992. *Leishmania tropica* infection in hamsters and a review of the animal pathogenicity of this species. *Exp Parasitol 75:* 433–441.
30. Schnur F, Zuckerman A, Montilio B, 1973. Dissemination of Leishmanias to the organs of Syrian hamsters following intrasplenic inoculation of promastigotes. *Exp Parasitol 34:* 432–447.
31. McConville MJ, Schnur LF, Jaffe CL, Schneider P, 1994. Structure of *Leishmania* lipophosphoglycan: inter- and intra-specific polymorphism in Old World species. *Biochem J 310:* 807–818.
32. Kreutzer RD, Grogl M, Neva FA, Fryauff DJ, Magill AJ, Aleman-Munoz MM, 1993. Identification and genetic comparison of leishmanial parasites causing viscerotropic and cutaneous disease in soldiers returning from Operation Desert Storm. *Am J Trop Med Hyg 49:* 357–363.
33. Saliba EK, Saleh N, Oumeish OY, Khoury S, Bisharat Z, Al-Ouran R, 1997. The endemicity of *Leishmania tropica* (zymodeme MON-137) in the Eira-Yarqa area of Salt District, Jordan. *Ann Trop Med Parasitol 91:* 453–459.
34. Greenblatt CL, Schnur LF, Juster R, Sulitzeanu A, 2002. Clonal heterogeneity in populations of *Leishmania major. Isr J Med Sci 26:* 129–135.