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EPIDEMIOLOGY OF VISCERAL LEISHMANIASIS IN THE JENIN DISTRICT, WEST BANK: 1989–1998

ZIAD A. ABDEEN, SAMIR S. SAWALHA, CAROL L. EISENBERGER, HAROUN M. KHANFAR, CHARLES L. GREENBLATT, OBAIDA YOUSEF, LIONEL F. SCHNUR, KIFAYA AZMI, ALON WARBURG, KHALDOUN A. BADER, CHARLES L. JAFFE, AND GAD BANETH

Health Research Center, Faculty of Medicine, Al-Quds University, Abu-Deis, West Bank, Palestinian Authority; Kuvin Center for the Study of Infectious and Tropical Diseases, Department of Parasitology, Hebrew University-Hadassah Medical School, Jerusalem, Israel; School of Veterinary Medicine, Hebrew University of Jerusalem, Rehovot, Israel

Abstract. Fifty patients from rural areas in the Jenin district of the West Bank, Palestinian Authority, were diagnosed with visceral leishmaniasis (VL) between 1989 and 1998. Forty-nine (98%) were younger than 6 years old, the youngest being 9 months. The yearly incident rate of VL in the Jenin district was highest in 1994 (11.8/100,000) and decreased to 1.5/100,000 in 1998; a mortality rate of 4% was recorded. Seventeen (5.5%) of 308 dogs from the Jenin and Ramallah districts of the West Bank were seropositive by enzyme-linked immunosorbent assay in a survey of canine leishmaniasis. Although all the leishmanial strains cultured from humans and dogs were identified as *Leishmania infantum* by a species-specific polymerase chain reaction, further genetic analysis by restriction fragment length polymorphism of kinetoplast DNA revealed patterns of polymorphism within isolates. The findings indicate that an active focus of potentially fatal VL exits in the Jenin district of the West Bank and that the parasite, vector, and reservoir host are found in this area. The epidemiology of VL in that vicinity follows the pattern of a predominantly infantile disease traditionally found in Middle Eastern countries, without a considerable involvement of immunocompromised adults infected with HIV virus as reported in other regions.

INTRODUCTION

Zoonotic visceral leishmaniasis (VL), is a sand fly-borne disease caused by the protozoal parasite *Leishmania infantum.* Its endemicity to the Mediterranean and Middle Eastern regions has been known since 1908.[1–3] It is prevalent in a wide part of the Palearctic region, where its distribution is generally highly focal.[4] Infections occur in geographically distinct foci where vectors and canid reservoir hosts are found in the presence of suitable ecological conditions for the transmission of the parasite.

Reports on VL in the West Bank region of the Palestinian Authority are few and lack detail on the epidemiological aspects of this disease. Cases of VL from the vicinity of Bethlehem south of Jerusalem[5] and from the village of Bal'a in the north western Tulkarm district[6] have been reported during the last two decades. The apparent recent increase in the occurrence of human VL in West Bank hospitals and the emergence of this disease in neighboring Central Israel[7] warranted a current epidemiological appraisal of the existence and recent spread of this potentially fatal illness among the Palestinian population in the West Bank. Based on preliminary data from the Palestinian Ministry of Health, which indicated that VL is highly prevalent and is spreading in the Jenin district of the northern West Bank, a study on the disease in this district was initiated. The goals of the study were to detect foci of human VL in the Jenin district, to examine the prevalence of the disease in the canine population, and to genetically analyze and compare isolates of *Leishmania* from humans and dogs. It included a retrospective investigation of the disease spread in humans and a prospective component in which infection was studied in dogs from the northern West Bank.

The Jenin district covers a predominantly hilly region of 592 km^2 with elevations of 90 to 750 m above sea level. The mean temperature ranges from 17.4°C in January to 34.2°C in August and the mean annual relative humidity from 39% to 84%, with a mean annual rainfall of 528 mm.[8] The total population of the district was 201,000 in 1998. The inhabitants are predominantly villagers with a low-income rate whose livelihood depends on agriculture. Fruit orchards occupy the largest part of the agrarian lands, followed by field and vegetable crops. Most houses have a yard where sheep, goats, chickens, and other domestic animals are kept.

MATERIALS AND METHODS

Patients. Information on the number of VL cases in the 10 districts of the West Bank was obtained from the Palestinian Health Authorities. Patient records from the two northern West Bank hospitals serving the Jenin district, Al-Watani Hospital in Nablus and the Jenin Hospital, were screened for patients diagnosed with VL between 1989 and 1998. Their personal details, date of illness onset, diagnostic tests, clinical findings, therapy, and patient's response were noted.

Visceral leishmaniasis was confirmed when amastigotes were detected microscopically in smears of bone marrow aspirate stained with Giemsa stain or when aspirate seeded into rabbit blood-agar culture grew promastigotes. All the patients with confirmed VL were traced through their last known address. When located, their parents were interviewed to determine where the patients had been living when they became ill.

Dogs. Household dogs from one village in the Jenin district and five villages in the Ramallah district were examined for skin lesions, lymphadenomegaly, and poor body condition, the last of which was identified by weight loss. Blood was taken by cephalic venipuncture to determine antileishmanial antibody levels by an enzyme-linked immunosorbent assay (ELISA). Seropositive dogs were revisited and lymph node and splenic aspirates were obtained and cultured for parasites.

ELISA. Serum antileishmanial antibodies were determined by ELISA using crude leishmanial antigen as previously described.[9] The dog sera were tested at two dilutions (1:100 and 1:1,000). The samples were incubated in antigen-coated plates for 1 hour at 37°C. The plates were then washed with 0.1% Tween 20 in 50 mmol phosphate-buffered saline (PBS), pH 7.2, and incubated with Protein A conjugated to horseradish

peroxidase (1:10,000 dilution; Zymed Laboratories, Inc., San Francisco, CA) for 1 hour at 37°C. Excess conjugate was removed by extensive washing in PBS-Tween. The plates were developed by adding the substrate 2,2′-azino-di-3-ethylbenzthiazolihne sulfonate (ABTS) (Boerhinger Manneheim, Germany). Each plate was read at wavelength 405 nm after the positive control canine serum reached a value between 0.95 and 1.0. A titration of positive and negative reference dog sera was included on each plate to monitor interassay variation. A sample was considered positive if the optical density was 2.6 times higher than the standard deviation of the control group.

Culture and genetic analysis of isolates. Biopsy samples from spleens, bone marrow, or lymph nodes were cultured on NNN rabbit blood-agar slants overlaid with Schneider's *Drosophila* medium (SDM) or on rabbit blood-agar semisolid medium.[10] Promastigotes from positive cultures were cultured in SDM supplemented with 20% fetal calf serum. Isolated strains were characterized and identified by excreted factor (EF) serotyping,[11] and the permissively primed intergenic polymorphic-polymerase chain reaction (PPIP-PCR).[12] The PPIP-PCR reactions were modified using 20 ng/μl of genomic DNA and amplified with 5 μmol of the single leishmanial-specific primer, 2B (5′-CAG GAG CGC GCA CAC GCA CAC ACG), and 2 U of recombinant *Taq* DNA polymerase (MBI Fermentas, Amherst, NY).

Restriction fragment length polymorphism (RFLP) was performed on kinetoplast DNA (kDNA). Two primers[13] were modified[14] to Uni21 (5′-GGG GTT GGT GTA AAA TAG GCC) and Lmj4 (5′-CTA GTT TCC CGC CTC CGA G) and used for amplifying full leishmanial minicircle kDNA. Reactions were performed in 50 μl containing 75 mmol trishydrochloride (pH 8.8), 20 mmol $(NH_4)_2SO_4$, 0.01% Tween 20; 1 μmol each primer; 20 ng leishmanial DNA template; 1.5 mmol magnesium chloride; 200 mmol each deoxyadenosine triphosphate, deoxycytidine triphosphate, 2-deoxyguanosine-5′-triphosphate, deoxythymidine triphosphate; and 2 U recombinant *Taq* DNA polymerase (MBI Fermentas, Amherst, NY). Amplifications were performed in a Minicycler (M. J. Research, Watertwon, MA) with an initial 5-minute denaturing step at 95°C followed by 35 cycles; denaturing for 1 minute at 94°C, annealing for 1 minute at 60°C, elongation for 1.5 minutes at 72°C, and final primer extension for 5 minutes at 72°C. PCR products were column purified using High Pure™ PCR Product Purification Kit according to the manufacturer's recommendations (Boehringer Mannheim Corp. Indianapolis, IN). Fifty-nanogram samples of DNA were then subjected to restriction with RsaI or HpaII enzymes (Promega, Madison, WI). The individually restricted DNA samples were then electrophoresed using 2% Metaphor agarose (FMC BioProducts, Rockland, MN) and stained with Gel Star (FMC BioProducts, Rockland, MN). DNA markers, ϕx 174/HaeIII and ϕx 174/HinfII (Promega, Madison, WI), were used as size references. Uncut and restricted fragment patterns were visualized under ultraviolet light as previously described.[12]

RESULTS

From 1990 through 1999, 127 cases of VL were recorded among the Palestinian population in the West Bank with a mean of 12.7 cases/year (annual range = 3–32). The distribution of reported cases by district is shown in Table 1. The Jenin district, which had the fifth largest population among the West Bank districts during the 1990s, had the highest number of VL cases (50/127, 39%) followed, in decreasing order, by the Hebron, Tulkarm, and Ramallah districts.[15] One district (Jericho) did not have any VL cases, and five others had one to three cases.

TABLE 1

Distribution of human cases of visceral leishmanaisis cases in the West Bank reported by the Palestinian Ministry of Health over 9 years (March 1990–February 1999)

District	No. cases	Total cases (%)
Jenin	50	39.3
Hebron	32	25.2
Tulkarm	17	13.3
Ramallah	15	11.8
Nablus	3	2.4
Salfeet	3	2.4
Tubas	3	2.4
Qalqilia	3	2.4
Bethlehem	1	0.8
Jericho	0	0
Total	127	100

Eleven of the 50 patients from the Jenin district were diagnosed by culture and 39 by microscopical examination of bone marrow. Thirty-eight of the patients were hospitalized at the Al-Watani Hospital in Nablus, and the remaining 12 were hospitalized in the Jenin Hospital. Two of the patients died in hospital, and the others recovered after therapy. All patients presented the following clinical manifestations commonly associated with VL infection: fever, hepatosplenomegaly, anemia, and leukopenia.

The distribution of human cases of VL in the Jenin district by year of diagnosis, locality of origin, and yearly incidence rate per 100,000 population is shown in Table 2. The average number of patients diagnosed annually from 1989 to 1998 was 5 and the average annual rate per 100,000 inhabitants in the district was 2.79. The highest number of cases, 21, were reported in 1994, and the incident rate was 11.8 cases per 100,000 inhabitants. Analysis of the month of onset of clinical disease for the 50 patients revealed that 28 (56%) became clinically ill between January and June. Figure 1 gives the age distribution of the 50 cases at illness onset, which ranged from 9 months to 46 years (median age = 24 months). Forty nine cases (98%) were between 9 months to 6 years of age, and the female-male ratio was 1.4:1. In comparison, 26% of the district population during the study period (1989–1998) were younger than 6 years, and the female-male ratio was 0.99.[16]

The families of all the patients confirmed for VL were located and interviewed. Although the patients were distributed throughout the Jenin district at the time of hospitalization (Figure 2), the majority were either from villages in the southern part of the district (Jaba'a, Maythalun, Qabatiya, and Jadeida) or the village of El-Yamun in its northwestern part. The families of the patients were farmers of low economic status. Forty eight (96%) lived in single story houses with walls made of stones.

A majority of the canine population in the village of El-Yamun in the Jenin district and in five villages in the Ramallah Region of the West Bank was screened for clinical signs of VL and for antileishmanial antibodies. Three of 57 (5.3%)

TABLE 2
Distribution of human cases of visceral leishmaniasis in the Jenin district by locality, year of diagnosis, and rate per 100,000 inhabitants, 1989–1998

Locality	Year of diagnosis										
	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	Total
Al Shuhada			1								1
Arrana						1					1
El Mughayir						1					1
El Yamun	1	2	1		1	4	1	2	1		13
Jaba'a						2				2	4
Jadeida			1		1	4	1	2	1		10
Jenin					1	1			1		3
Kafr Dan						1					1
Kafr Ra'i						1					1
Kufeirat										1	1
Maythalun						3					3
Qabatiya			1	1			1				3
Sanur									1		1
Siris						2			3		5
Um El Tut							1				1
Ya'bad						1					1
Total	1	2	4	1	3	21	4	4	7	3	50
District population ($\times 10^3$)	153	158	163	168	173	178	184	18	195	201	
Rate per 100,000 inhabitants	0.65	1.26	2.46	0.6	1.74	11.8	2.18	2.11	3.58	1.49	

and 14 of 251 (5.6%) dogs from the Jenin and Ramallah districts, respectively, were seropositive. All of the sereopositive dogs were reexamined for clinical signs of disease and subject to biopsy to check for parasites. Promastigotes were cultured from 11 (65%) of the 17 seropositive dogs that underwent biopsy. Two isolates of *Leishmania* from the bone marrow of human infants were also cultured. All isolates were EF subserotype B_2, which is typical of *L. infantum.* In addition, PPIP-PCR analysis of the DNA from human and canine isolates showed that they were identical to the international reference strain of *L. infantum* and differed from the other Old World *Leishmania* species: *L. tropica, L. donovani, L. major,* and *L. aethiopica* (Figure 3). RFLP analysis revealed polymorphism among the isolated strains. Restriction of the kDNA PCR products of the reference strain of *L. infantum* (MHOM/TN/80/IPT1), the human strain LRC-L742, and most of the dog strains, by RsaI or HpaII, resulted in two major bands, whereas the human isolate LRC-L773 displayed an additional large and small band (Figure 4).

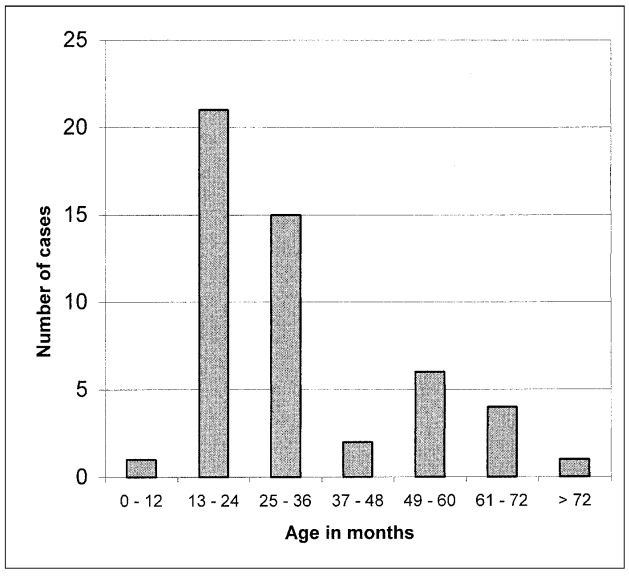

FIGURE 1. The age distribution of people with visceral leishmaniasis in the Jenin district of the West Bank, 1989–1998.

DISCUSSION

This study confirms that an important and active focus of VL has been in existence in the Jenin district of the West Bank for at least 10 years. The leishmanial species that infect people in the West Bank and Israel are *L. major, L. tropica,* and *L. infantum.*[17] *L. major* causes cutaneous leishmaniasis (CL) and, to our best knowledge, has not been reported to visceralize. *L. tropica,* which usually causes CL, may occasionally cause VL.[18–20] *L. infantum* is the predominant visceralizing *Leishmania* species in the Mediterranean region.[4] The identification of *L. infantum* as the probable cause of VL in the Jenin region is supported by molecular characterization using PCR of leishmanial isolates from the bone marrow of

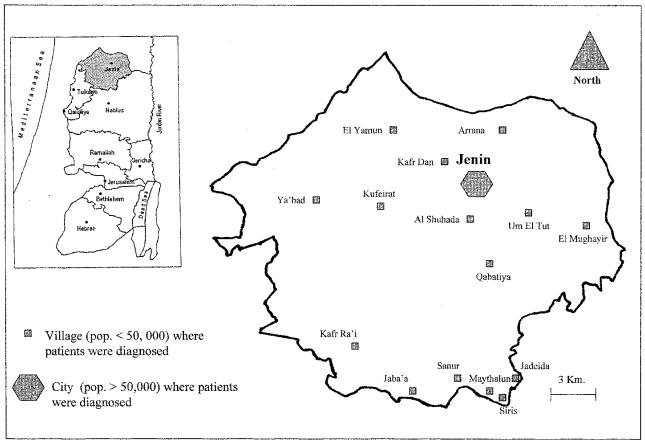

FIGURE 2. The geographic distribution of 50 cases of visceral leishmaniasis in the Jenin district, 1989–1998.

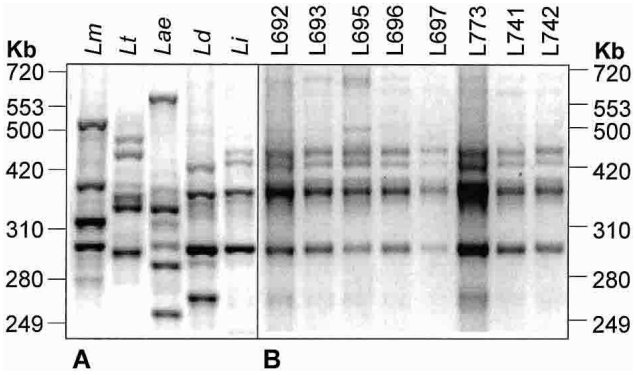

FIGURE 3. Identification of leishmanial strains isolated from humans and dogs from the West Bank by the permissively primed intergenic polymorphic-polymerase chain reaction. DNA purified from six different dog isolates (L692, L693, L695, L696, L697, L741) and two human isolates (L773 and L742) were examined and compared with DNA from reference strains: *L. major* MHOM/IL/67/JerichoII (*Lm*), *L. tropica* MHOM/IQ/66/L75 (*Lt*), *L. aethiopica* MHOM/ET/72/L102 (*Lae*), *L. donovani* MHOM/ET/67/HU3 (*Ld*), and *L. infantum* MHOM/TU/80/IPT1 (*Li*).

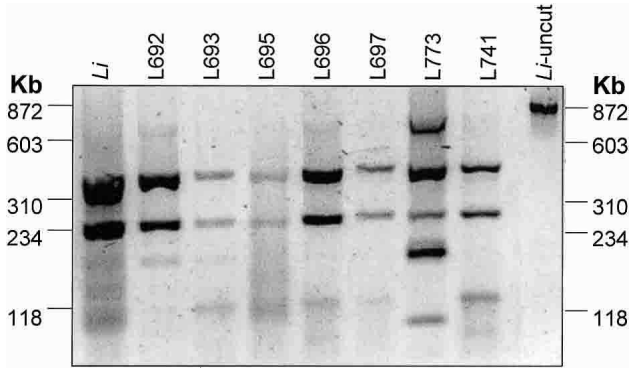

FIGURE 4. Restriction fragment length polymorphism (RFLP) of the full minicircle kinetoplast DNA of the seven leishmanial isolates shown in Figure 3 restricted with RsaI. The left column shows restriction of the reference strain of *Leishmania infantum* MHOM/TN/80/IPT1 (*Li*). The right column shows uncut DNA from *L. infantum.*

humans and dogs in the region. This concurred with the typical generalized symptoms of VL, which were seen primarily in children, as in other regions where *L. infantum* infections occur and included hepatosplenomegaly, leukopenia, anemia, and even death in some cases. The presence of dogs infected with VL in the vicinity of this focus of infection in the West Bank complies with the zoonotic epidemiological pattern of VL caused by *L. infantum* in the Mediterranean region, where canines are considered the main peridomestic reservoir of this parasite.[4]

In addition to the presence of a suitable animal reservoir, a survey of the sand fly fauna in the Jenin region (Abdeen and others, unpublished data) indicated that several sand fly species proven or suspected to be vectors of *L. infantum* in other parts of the Middle East and the Mediterranean are present in the district. These include *Phlebotomus tobbi, P. syriacus,* and *P. perfiliewi.*[21]

The polymorphism demonstrated among the leishmanial isolates by RFLP of kDNA, whereas genomic DNA analyzed by PIP-PCR from the same strains did not show genetic heterogeneity, is an interesting finding. Parasite polymorphism may be attributable to variations among parasites from different geographical locations or clusters.

The overall annual incidence rate of 2.79 per 100,000 population in the Jenin district during 1989 to 1998 is relatively high, and the rate of 11.8 per 100,000 inhabitants during 1994 increased alarmingly. In the endemic Mediterranean VL focus of Malta, where a similar study of hospitalized and reported cases was performed, the overall incidence was 0.9 per 100,000 people between 1994 and 1998.[22] In the Wilaya of Tizi-Ouzou in the Grand Kabylie region of Algeria, the annual incidence rate of VL between 1985 and 1990 was 5 human cases per 100,000 during a resurgence of the disease.[23] The high incidence rate of human VL in the Jenin focus differs from the reported situation of VL in the neighboring Israel, where the disease is mainly found in canines and only three autochthonous human VL cases were reported between 1994 and 1998.[7]

In contrast to the current epidemiological pattern of VL in the southern European countries bordering the Mediterranean Sea, where it is now a disease primarily of immunocompromised adults,[24–27] VL in the Jenin district is predominantly a disease of infants; only 10% of the patients are older than 5 years. HIV infections are relatively rare in the Palestinian Authority, and the incidence of this infection among the West Bank population was only 1.5/100,000 in 1998.[15] Because of the primarily pediatric nature of VL in the Jenin district, the high recovery rate from the disease (96%), and the low prevalence of HIV in the region, it appears that VL in this area follows the classical epidemiological pattern of infantile kala-azar as found in the Mediterranean region before the emergence of HIV or in regions where the latter disease is rare.

Several factors, both environmental and ecological, may have contributed to the increase in VL incidence in the Jenin region during the 1990s. These include the expansion of towns and villages, the establishment of new settlements, and increased agricultural and poultry production. These conditions have led to an increase in refuse and solid waste providing good habitats for sand fly vectors and also attracting stray dogs and wild canid reservoir hosts. A study of wild jackals and foxes in Israel suggested that they are probably associated with the epidemiology of VL in Israel.[7] The presence of an active focus of VL in the Jenin district of the West Bank warrants the implementation of control measures in the region and bringing this disease to the awareness of health care providers and public health officials.

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Gad Baneth, School of Veterinary Medicine, Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel.

Reprint requests: Ziad A. Abdeen, Department of Community Health, Health Research Center, Faculty of Medicine, Al-Quds University 8, Nur Al-Din Street, P.O. Box 51000, Jerusalem, Israel, Tel: 972-2-6261490, FAX: 972-2-6277166, E-mail: zabdeen;caplanet.edu.

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