Serological Evaluation of Cutaneous *Leishmania tropica* Infection in Northern Israel

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Abstract. *Leishmania* spp. are medically important unicellular parasites transmitted by phlebotomine sand flies. The World Health Organization recently highlighted the importance of reliable diagnostic tools for leishmaniasis. Our study of human infection was conducted in two endemic foci of *Leishmania tropica* in the Galilee region, northern Israel. Elevated anti-*Leishmania* antibodies were present in the majority (78.6%) of *L. tropica*-PCR positive individuals. Moreover, the enzyme-linked immunosorbent assay showed high sensitivity, specificity, and negative and positive predictive values (ranging between 73% and 79%), thus fulfilling the basic requirement for future development of a serodiagnostic and screening tool. The anti-sand fly saliva antibodies used as biomarkers of exposure reflected the composition of the local sand fly fauna as well as the abundance of individual species. High levels of antibodies against vector salivary proteins may further indicate frequent exposure to sand flies and consequently a higher probability of *Leishmania* transmission.

Leishmania spp. (Trypanosomatidae) are the causative agents of leishmaniasis, a group of diseases with various manifestations attributed to the genotype of the infecting *Leishmania* species and host immune status. *Leishmania tropica* typically causes a chronic disease characterized by slow-developing, self-healing cutaneous lesions that may eventually result in persisting atrophic scars. However, human cases of recidivating or visceral manifestations due to *L. tropica* have also been reported.^{1,2} In Israel, *L. tropica* is the dominant causative agent of cutaneous leishmaniasis (CL) and is a contributor to the resurgence of CL together with *Leishmania major* in the last decade.³ Several outbreaks have been reported in northern Israel since 2003 when the incidence of CL peaked at 41 cases per 100,000 in the Kinneret subdistrict.^{3,4} Although *L. tropica* infection tends to be an urban, anthroponotic infection, in Israel it is zoonotic in nature.^{5–7} Moreover, there are two recognized vectors of *L. tropica* in Israel—*Phlebotomus sergenti* and *Phlebotomus arabicus*—making the epidemiology of this disease even more complex.^{8,9}

The World Health Organization recently indicated the serious and increasing threat of leishmaniasis and highlighted the importance of reliable diagnostic tools.¹⁰ The goal of our study was to assess whether people diagnosed with *L. tropica* infection also produce detectable serum antibody levels to this parasite. We evaluated the serologic reaction of residents from two different localities in the Kinneret subdistrict to *L. tropica* antigen as well as to sand fly saliva of three local species to demonstrate their exposure to vector sand flies by detection of anti-sand fly saliva antibodies as proven biomarkers of exposure.¹¹

Human sera were collected between May 2008 and September 2010 in an *L. tropica* endemic area in the Galilee region (northern Israel) at two locations, Korazim (north of Lake Kinneret) and Tiberias (south of Lake Kinneret). Of the 25 individual serum samples (Supplemental Table 1), 14 (group A) were obtained from *Leishmania*-PCR-positive individuals, and 11

(group B) were collected from apparently healthy individuals who were family members or neighbors of patients without history of any lesion consistent with CL. The control group (group C) consisted of 43 individuals from the Czech Republic, a sand fly-free area without autochthonous leishmaniasis. An informed consent was obtained from participants. Ethical approval was obtained from the Institutional Review Boards of the Carmel Medical Center (Haifa, Israel) (CMC-0052-07), Meir Medical Center (k-0057-08), Kupat Holim Clalit HMO (Israel), and Charles University (Czech Republic).

Anti-*L. tropica* antibody levels were measured using enzyme-linked immunosorbent assay (ELISA). Plates were coated overnight at 4°C with 75 ng/well with *L. tropica* antigen (MHOM/IL/2005/LRC-L1239). Plates were washed with PBS-0.05% Tween 20 and blocked with 2% milk for 1 hour at 25°C. Sera were diluted 1:50 in 2% milk and incubated for 1 hour at 37°C, followed by incubation with biotin-coupled protein G diluted 1:250 (Adar Biotech) for 2 hours at 37°C, followed by streptavidin–HRP (Jackson) diluted 1:250 in 2% milk. The plates were developed with 2,2-azino-bis 3-ethylbenzthiazolinesulfonic, and absorbance was read at 405 nm.

Cut-off values were determined as three standard deviations from the control (group C) mean. Specificity, sensitivity, and positive and negative predictive values of the ELISA were calculated using four categories: true positives (individuals positive for both *Leishmania*-PCR and anti-*L. tropica* antibodies), false positives (*Leishmania*-PCR-negative individuals positive for anti-*L. tropica* antibodies), false negatives (*Leishmania*-PCR-positive individuals negative for anti-*L. tropica* antibodies), and true negatives (individuals negative for both *Leishmania*-PCR and anti-*L. tropica* antibodies). Statistical significance was analyzed using the nonparametric Wilcoxon rank-sum test or Wilcoxon signed-rank test for difference in medians. Statistical analyses were performed using NCSS 6.0.21 and R softwares (<http://cran.r-project.org/>).

Overall seroprevalence of anti-*L. tropica* antibodies among the samples was 56%. Among *Leishmania*-PCR-positive individuals (group A), a total of 78.6% showed elevated anti-*L. tropica* antibodies. In general, individuals from group A revealed significantly higher ($P = 0.02$) antibody levels compared

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with *Leishmania*-PCR-negative individuals (group B) (Figure 1A, Table 1).

Current serologic tests for CL are restricted because of the poor humoral response provoked by the infection and the consequential low sensitivity.^{12,13} However, our study showed that 78.6% of tested CL patients exhibited elevated anti-*L. tropica* antibodies. Our ELISA performed with high sensitivity and high positive predictive value (both 79%), indicating a minimal number of false-positive samples. Specificity and negative predictive value were both 73% achieving minimal false-negative error rate, demonstrating its potential as a complementary diagnostic tool. In general, serologic assays usually do not distinguish between active and former infection; thus, the group assigned as false positive may actually include individuals with quiescent infection.

Higher sensitivity (91%) was found in another study for chemiluminescent ELISA detecting *Leishmania* anti- α -Gal immunoglobulin G (IgG) as a diagnostic marker of *L. tropica* infection in humans. Moreover, anti- α -Gal antibodies showed promising features as a marker distinguishing patients from cured individuals.¹⁴ Recently, Costa et al.¹⁵ introduced three antigens that performed in an ELISA with 100% sensitivity and specificity using sera of patients infected with *Leishmania braziliensis*, highlighting no cross-reactions with sera of *Trypanosoma cruzi*- or *Leishmania infantum*-infected patients. Another example of an already successfully tested serodiagnostic tool for CL is ELISA based on recombinant *L. infantum* heat shock protein 83. This test was performed without significant cross-reactivity to sera from patients with Chagas disease, toxoplasmosis, and malaria, but was unable to distinguish the infecting *Leishmania* species.¹⁶

In this study, we tested individuals also for anti-sand fly saliva antibodies, a well-proven biomarker of exposure to estimate the risk of human infection.^{11,17} Serum samples were tested for the presence of IgG antibodies against the saliva of *P. arabicus*, *P. sergenti*, and *Phlebotomus papatasi* sand flies. The anti-sand fly saliva antibodies were measured by ELISA as described in Supplemental Figure 1 using human sera diluted 1:50 and peroxidase-conjugated goat anti-human IgG (Sigma-Aldrich, Czech Republic) diluted 1:2,500. Antigens for the sand fly saliva tests were prepared from colonies of *P. arabicus* and *P. sergenti*, species serving as *L. tropica* vectors in Israel,^{8,9} originated from the same locality where human samples were tested.

The study included human sera collected from inhabitants of Tiberias and Korazim that differ in composition of sand fly fauna and sand fly vector species. Tiberias has been identified as a new emerging focus of *L. tropica* in early 2000.^{4,8} *Phlebotomus sergenti*, the vector species, is the most abundant sand fly, comprising more than 90% of caught specimens.⁹ Conversely, *P. papatasi* is a minor species (2%) and *P. arabicus* is absent in Tiberias.⁹ The presence of antisaliva antibodies in the serum of Tiberias residents (Table 1) corresponds well with the sand fly species composition; none of the samples reacted with the salivary antigen of *P. arabicus*.⁹

Korazim, another emerged *L. tropica* focus, is located in the northern Galilee region, and although only 10–15 km northeast from Tiberias, it has some different characteristics. The sand fly fauna consists of seven sand fly species, including *P. sergenti* (up to 30% of collected sand flies), *P. arabicus* (22%), and *P. papatasi* (2.5%).^{8,9,18} Two species have been found infected in this area—*P. sergenti* and *P. arabicus*—the

FIGURE 1. Human antibody response against *Leishmania tropica* and sand fly saliva. Human serum samples from Korazim and Tiberias, two *Leishmania* foci in northern Israel, and samples from the sand fly-free area, the Czech Republic (CZ), were screened for the presence of antibodies against (A) *Leishmania tropica* and against saliva of three sand fly species: (B) *Phlebotomus arabicus*, (C) *Phlebotomus sergenti*, and (D) *Phlebotomus papatasi*. Asterisks above y axes indicate significant differences ($P < 0.05$) between *Leishmania*-PCR-positive (open triangle) and *Leishmania*-PCR-negative (open circle) individuals from the same locality. Asterisks below y axes indicate significant differences ($P < 0.05$) between localities (regardless the *Leishmania* status). Samples were evaluated in triplicates and are presented as mean values.

TABLE 1
Serology data

Location	n	anti-leish	anti-ARA	anti-SER	anti-PAP
Korazim	12	7	1	2	2
Group A	5	5	1	2	2
Group B	7	2	0	0	0
Tiberias	13	7	0	2	1
Group A	9	6	0	1	1
Group B	4	1	0	1	0
Total	25	14	1	4	3

Data are presented as the number of individuals seropositive for anti-*Leishmania tropica* immunoglobulin G (IgG) (anti-leish), anti-*Phlebotomus arabis* IgG (anti-ARA), anti-*Phlebotomus sergenti* IgG (anti-SER), and anti-*Phlebotomus papatasi* IgG (anti-PAP). Total number of residents in a given locality or in an assigned group is indicated (n); group A = *Leishmania*-PCR-positive individuals; group B = *Leishmania*-PCR-negative individuals. Cut-off values were determined as three standard deviations from the mean optical densities (OD) of the control sera (group C). The cut-off values were as follows: anti-*L. tropica*, OD = 0.092; anti-*P. arabis* IgG, OD = 0.261; anti-*P. sergenti* IgG, OD = 0.297; and anti-*P. papatasi* IgG, OD = 0.303.

latter identified as a new vector of *L. tropica*.^{8,9} Similar to Tiberias, the antibodies against sand fly saliva in human sera reflected the occurrence of sand fly species present in Korazim (Table 1). Moreover, patients from Korazim presented higher levels of anti-*P. arabis* IgG than healthy individuals from the same place ($P < 0.05$, Figure 1B), supporting the employment of antivektor antibodies as a risk marker of *L. tropica* infection.

To exclude the possible cross-reactivity of antisaliva antibodies with heterologous antigens, we used a mouse model. In accordance with our previous results,^{17,19,20} mouse antibodies elicited by bites of a single sand fly species were highly species specific (Supplemental Figure 1). The results from Tiberias indicate similar species specificity also for human sera; none of the sera showed reactivity with salivary antigen of *P. arabis*, the species that is absent in this focus.⁹

In conclusion, our study demonstrated elevated anti-*Leishmania* antibodies present in the majority of *L. tropica*-infected patients, thus fulfilling the basic requirement for future serodiagnostic tool. We have also shown that anti-sand fly saliva antibodies reflected the composition of the local sand fly fauna and thus could be used as a biomarker of exposure to the vector sand fly in epidemiological studies. Further studies are required to reinforce the diagnostic potential of anti-sand fly saliva antibodies as a risk marker of *L. tropica* infection.

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