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MAJOR ARTICLE

Sensitive Molecular Diagnostics for Cutaneous Leishmaniasis

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Background. Rapid diagnosis of cutaneous leishmaniasis (CL) and identification of *Leishmania* species is highly important for the disease management. In Israel, CL is caused mainly by *Leishmania major* and *Leishmania tropica* species.

Methods. We established an easy to handle point of care lesion-swabbing, combined with a highly sensitive multiplex real time PCR (multiplex qPCR) for accurate and rapid diagnosis of *Leishmania* species.

Results. Using three probes: one general for: Leishmania species, and two specific for *L major*, and *L tropica*, we screened 1783 clinical samples collected during two years. *Leishmania* species was found in 1086 individuals, 1008 *L major*, and 70 *L tropica*. Eight samples positive for *Leishmania* species only, were further tested using a second set of multiplex qPCR developed, and were found positive for *Leishmania braziliensis* and *Leishmania infantum/donovani* (2 and 6 samples, concomitantly).

Conclusions. Taken together, the test enabled diagnostics and better treatment of *Leishmania* infections from the Old World (1078 samples) and the New World (8 samples), and the subtyping of the dominant strains in the region, as well as in returning travelers'.

Keywords. *Leishmania major*; *Leishmania* species; qPCR; real-time PCR.

Cutaneous leishmaniasis (CL) is a zoonotic disease in which parasites of the genus *Leishmania* are transmitted from infected reservoir host to humans by phlebotomine sand flies vectors. This disease manifests as a chronic ulcer, potentially leaving unattractive scars [1]. Cutaneous leishmaniasis incidence in Israel increased dramatically in recent years. After a 15-year period of moderate illness rates, reported incidence increased from 0.4 cases per 100 000 population in 2001 to 4.4 cases per 100 000 population in 2012 [2, 3]. In Israel, CL is mainly caused by *Leishmania major* (LM) and *Leishmania tropica* (LT) species. In the Negev Desert, CL is mainly caused by LM and, until recently, was restricted to geographically defined areas [4, 5]. However, the incidence and geography of *Leishmania* infection (LM and LT) is changing, and the disease has spread to areas previously naive, including Northern Israel, the area surrounding Jerusalem, and also the Negev area in Southern Israel [2, 3]. It should be noted that CL lesions caused by LT last much longer and are more resistant to available drugs than those caused by LM. *Leishmania tropica* infections may also result in life-threatening visceral leishmaniasis, highlighting the importance of correct species characterization for the selection of proper treatment against the disease [1–3, 6].

Diagnostic methods of CL include visualization of the characteristic amastigote in smears or tissue (histopathology), parasite isolation by in vitro culture, and molecular detection of parasite's deoxyribonucleic acid (DNA). Microscopy of Giemsa-stained slides from the lesion is still the most commonly used technique to visualize the parasite. The sample should be examined by light microscopy under oil immersion for amastigotes, the tissue form of the parasite. Direct examination requires some expertise and lacks sensitivity.

In vitro culture of infected tissue is labor intensive. Samples are examined weekly for the presence of promastigotes for 4 weeks before being discarded as negative [7]. Consequently, time to result can be weeks. The "gold standard" for *Leishmania* species (LS) identification is multilocus enzyme electrophoresis (MLEE) [8]. Multilocus enzyme electrophoresis requires culture and isolation of the parasites, involving the risk of bacterial contamination. Moreover, the results of MLEE are available several weeks or even months after the diagnosis. Thus, although MLEE is essential for epidemiological studies, this technique is not rapid enough to guide first-line therapeutic decisions. Unfortunately, serology testing is not applicable for the diagnosis of CL; the sensitivity and specificity are variable, and antibody levels are generally low. Most serologic assays cannot reliably distinguish between present

Received 8 November 2016; editorial decision 17 February 2017; accepted 22 February 2017.
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DOI: 10.1093/ofid/ofx037

and past infection. The sensitivity of serology might be better in the setting of mucosal leishmaniasis, and antibodies may decrease after treatment [9]. Advances in molecular methods have the potential to lead to the development of improved and field-applicable diagnostic techniques. The molecular analyses are sensitive and can identify the pathogen in the absence of parasite viability. Furthermore, analysis of the polymerase chain reaction (PCR) amplicons by restriction fragment length polymorphism (RFLP) or high-resolution melting (HRM) can also type LS [10–13]. However, in addition to prolonged turnaround time and increased labor, the disadvantage of RFLP analysis in a clinical set up is the risk of contamination by amplicons, resulting in false-positive results. On the other hand, HRM reverse-transcription (RT)-PCR analysis can prevent cross-contamination in the clinical laboratory set up, but the melting curves can be affected by salt and other components of the reaction. These draw backs can be overcome by the use of quantitative real-time PCR (qPCR) useing hydrolysis probes. Moreover, various probes, each labeled with a differently colored fluorophore, can be used in a single tube enabling throughput multiplex assays (multiplex qPCR). The goal of this work was to develop a sensitive multiplex qPCR set to identify *Leishmania* and its species to improve patient management and therapy.

METHODS

Study Population

The study population comprised patients with CL and controls (non-leishmanial lesion). The study was approved by the Ethics Committee for research on human beings of the Soroka University Medical Center.

Patients with signs and symptoms suggestive of leishmaniasis were referred to the laboratory for diagnostic testing. Samples were evaluated by microscopic diagnosis for identification of (1) amastigotes in skin lesion biopsy and (2) promastigotes in lesion culture.

Giemsa-Stained Smears and Parasite Culture

Smears were taken under sterile conditions using a disposable scalpel blade (no. 11). A small incision was made in the cleaned margin of nodules and lesions with the point of the blade. The blade was scraped along the cut edge of the incision to remove and pick up skin tissue, which was smeared on a clean glass microscope slide. After smears had dried completely, they were fixed with 100% methanol, allowed to dry again, and stained with Wright's Giemsa for microscopic examination. Parasite culture was as follows: tissue material from the border of the ulcers was aspirated from the edge of the lesion with a sterile Pasteur pipette and inoculated into a culture tube containing Roswell Park Memorial Institute (RPMI) medium, supplemented with 30% fetal calf serum. The culture was incubated at 25 to 28°C for up to 3 weeks and was checked microscopically for promastigotes.

Nucleic Acid Extraction

In parallel to the microscopic examination of the stained slide, a swab applicator was taken from edge of the wound, placed into a tube containing transfer media (FLOQswabs; Copan, Murrieta, CA), and nucleic acid extraction (500 µL of sample was extracted into 50 µL of elution solution) was performed using NucliSense EasyMag (bioMerieux, Marcy l'Etoile, France), according to the manufacturer's instruction.

In-Laboratory Multiplex Quantitative Polymearse Chain Reaction for *Leishmania*

A set of forward and reverse primers combined with 5 different probes was used to detect the various LS by multiplex hydrolysis probes-based qPCR (described in Table 1). Each sample (5 µL of extracted DNA) was tested in parallel, in 1 test tube, for the following: LS, LM, and LT. Amplification (95° for 15 min, and 45 cycles of 95° for 15 seconds, 57° for 30 seconds, and 60° for 30 seconds) was carried out in a final volume of 20 µL, using the DNA qRT-PCR enzyme (Light Cycler 480 Probes Master; Roche Diagnostics, Mannheim, Germany). Primers, common to all LS, for the ITS (LS *internal transcribed spacer 1, 5.8S ribosomal RNA* gene) conserved region (see Table 1) were modified from Talmi et al [14], and the hydrolysis probes within the ITS region were designed to be a species-specific sequence. The probes in set I were designed to specifically identify 3 possibilities: (1) any LS (Yakima yellow-tagged probe), (2) LM (6-carboxyfluorescein-tagged probe [6-FAM]), and (3) LT (ROX-tagged probe). Set I also included primers and probe that detect the human gene *PHP*, an internal control (IC) that enables monitoring of the following: specimen collection, extraction, and amplification (Cy5-tagged probe; see Table 1 for details of concentrations of primers and probe sets and for *Leishmania* testing combinations). Samples that were positive for LS but no signal was detected in the FAM (LM) or ROX (LT) channels were considered positive and were tested further by set II, for *L braziliensis* (6-FAM) and *L infantum/donovani* (Cy5-tagged probe). Samples that were negative in set I to all the LS but were positive for the IC were considered as negative for *Leishmania*.

Reference Tests

To confirm the results of the in-laboratory (In-LAB) test, the DNA extracted was tested by qPCR for a different gene of LS: the LS *18s Ribosomal RNA gene*, using a commercially available kit (Clonit, Milan, Italy), and *Leishmania* kinetoplast DNA (kDNA), which is considered to be a highly sensitive methods to detect CL, as described [11, 15].

Statistical Analysis

Data were presented as mean ± standard deviation for continuous variables and percentage with 95% confidence interval

Table 1. Primers and Probes Used in Multiplex Real-Time qPCR Assays

Name	Primer Sequences	(nM)	Target Gene
LS-Fw	AGCTGGATCATTTTCCGATG*	125	LS-ITS
LS-Rev	TCGCACTTTACTGCGTTCTT	125	
IC-Fw	CATGGGAAGCAAGGGAACTAATG	63	Human ERV-3
IC-Rev	CCCAGCGAGCAATACAGAATTT	63	
Target	Probe Sequences		
L species	YAK-5′-CGACACGTTATGTGAGCCGTTATCCAC-BHQ-1–3′	62	ITS-LS
L major	6-FAM-5′- TCTCT/ZEN/ CCCTCCCGCCAAAAACC/3IabFQ-3′	62	ITS-*L major*
L tropica	ROX-5′-AACAAAACCGAAACGCCGTATATTTGTATA/3IAbRQSp	62	ITS-*L tropica*
PHP (IC)**	CY5-5′-TCTTCCCTCGAACCTGCACCATCAAT-BBQ	31	Human ERV-3
L species	YAK-5′-CGACACGTTATGTGAGCCGTTATCCAC-BHQ-1–3′	62	ITS-LS
L braziliensis	FAM-5′-TGTATACGCGATATGTTAACGTCGA-BBQ	62	ITS- *braziliensis*
L infantum/donovani	Cy5-5′-CGCCAAAAACCGAAACGCCGTATAT-BBQ	62	ITS-INF/DON

Abbreviations: DON, *L donovani*; ERV-3, endogenous retrovirus group 3; Fw, forward; IC, internal control; INF, *L infantum*; ITS, internal transcribed spacer; L, *Leishmania*; LS, *Leishmania* species; PHP, pseudohypoparathyroidism; Rev, reverse; RT-PCR, reverse-transcription polymerase chain reaction.

*Modified from Talmi et al [14].

**Lieberman et al [16].

(CI) for binary variables. We used χ^2 tests and *t* test to compare categorical and continuous variables, respectively. We assessed the specificity and sensitivity of the multiplex quantitative PCR compared with the commercial RT-PCR kit in subsets of the patients. Furthermore, we calculated Kappa with 95% CI for the agreement between the 2 kits.

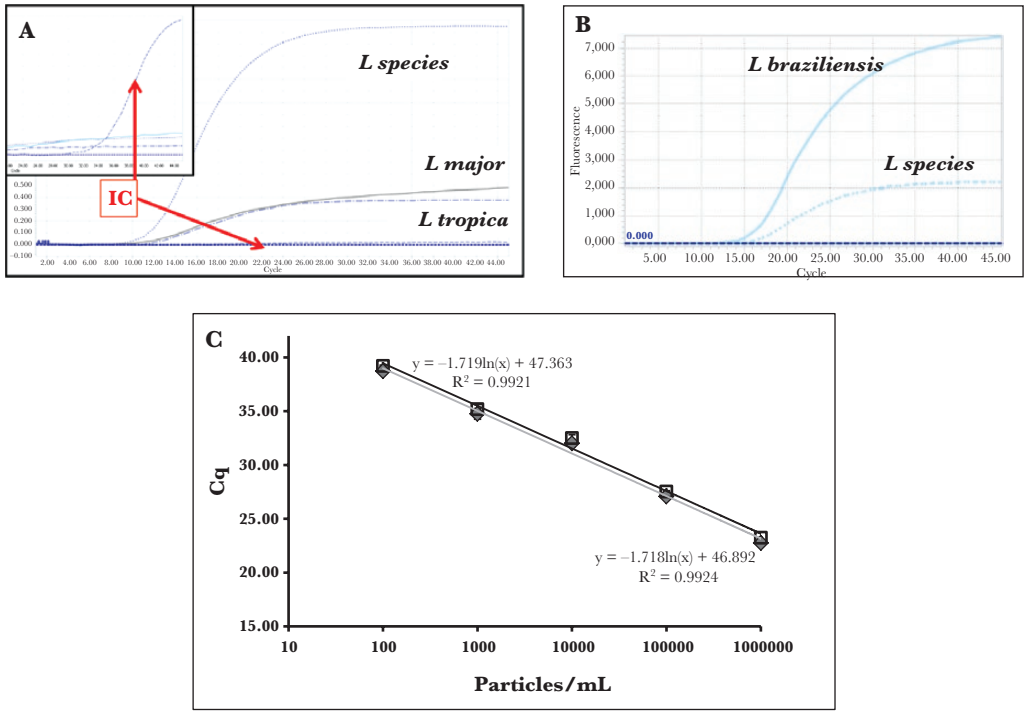

Figure 1. Multiplex quantitative polymerase chain reaction (qPCR) of set I and set II for *Leishmania* diagnosis. (A) Representative multiplex qPCR amplification of set I, positive for *Leishmania* species (Yakima yellow-tagged probe), *Leishmania major* ([*L major*] 6-carboxyfluorescein-tagged probe), and *Leishmania tropica* ([*L tropica*] ROX-tagged probe). Upper left corner demonstrates multiplex qPCR amplification of set I that was negative for *Leishmania* and positive for the human gene *PHP*, which serves as internal control (IC) for sampling, extraction, and amplification of the specimen. The concentration of the IC primers and probe was limited, to prevent competition with the sets of *Leishmania*. (B) Representative multiplex qPCR of set II for *Leishmania braziliensis* ([*L braziliensis*] 6-carboxyfluorescein-tagged probe) and *Leishmania infantum/donovani* (Cy5-tagged probe), positive for *L braziliensis*. (C) A standard curves for *L major* and *Leishmania* species (*L* species) sets. Promastigotes were grown in culture and counted and deoxyriboneucleic acid was extracted. Serial dilutions were prepared and tested in triplicates by multiplex qPCR for both: *L major* (gray diamonds, and gray trend line) and *L* species (empty black squares, and black trend line). Standard deviations are depicted for each set. Shown is 1 experiment of 3. Cq, quantification cycle.

Table 2. Analysis of CL by Multiplex Real-Time PCR vs Microscopy

		Giemsa-Stained Smears		
		Positive	Negative	Total
mqPCR	LM positive	226 (56%)	65 (16%)	291
	LM negative	0	113 (28%)	113
	Total	226	178	404

Abbreviations: CL, cutaneous leishmaniasis; LM, *Leishmania major*; mq, multiplex quantitative; PCR, polymerase chain reaction.

Table 3. Cq Values of Multiplex Real-Time PCR in Giemsa-Stained Positive and Negative Samples

	Giemsa Smears		
	Positive $N = 78$	Negative $N = 61$	*P* Value
RT-PCR Cq Values	21.21 ± 3.46	27.39 ± 3.95	<.001
Mean ± SD	20.57	27.2	
Median: Min, Max	15.52, 32.88	18.16, 35.65	

Abbreviations: Cq, quantification cycle; Max, maximum; Min, minimum; PCR, polymerase chain reaction; RT, real time; SD, standard deviation.

RESULTS

Patients

The laboratory of parasitology in Soroka University Medical Center is the only laboratory in the Negev area supplying parasitological diagnostic services to the region. In the Negev area, most of the CL is caused by LM with few annual cases of LT. However, infection with other species of leishmaniasis cannot be excluded because the laboratory also diagnoses returning travelers and patients from other regions, including endemic areas for LT.

Identification of *Leishmania* Parasites by Multiplex Quantitative Polymearse Chain Reaction

For faster and most suitable diagnosis, 2 sets were designed and tested. Set I enabled the detection of any species of *Leishmania*, along with the specific identification of the common species in the region: LM or LT (for details, see Table 1, Figure 1A, and Supplementary Figures 1 and 2). In addition, set I included a set of primers and Cy5-labeled probe to identify human-ERV-3 gene, which served as IC that monitors for accurate sampling, extraction, and amplification (Figure 1A) [16]. A patient was considered to be *Leishmania* negative when all the *Leishmania* fluorescent channels were negative, whereas the IC was positive (Figure 1A). When set I was positive for LS only, further amplification with set II, which identifies *L braziliensis* and *L infantum/donovani*, was carried out (Figure 1B and Supplementart Figure 3). Polymerase chain reaction efficiency was good for all the multiplex qPCR targets, with correlation coefficients (r^2) greater than 0.993 (Supplementary Figures 1–3). To calculate the sensitivity of set I, a standard curve was performed. Promastigotes of LM where grown in RPMI (see Materials and Methods) and counted, and DNA was extracted on the EsayMag platform. The extracted DNA was serially diluted and amplified by qPCR in triplicates, using set I. Figure 1C depicts the quantification cycle (Cq) of LM and LS plotted against the concentration of premastigots/mL. When the sample was diluted to 10 particles/mL only, 1 of 3 repeats was positive (Cq = 40; data not shown). This concentration represents 0.5 particle/reaction (5 µL of extracted sample was used for the reaction). The R^2 values were 0.9924 and 0.9921 for LM and of LS, concurrently.

The sensitivity of the multiplex qPCR test and its ability to identify *Leishmania* parasites in lesions was compared with Giemsa-stained smears, to commercially available qPCR for LS and to RT-PCR of the kDNA region [10]. Of 404 samples that were tested in parallel by multiplex qPCR and Giemsa-stained smears, 226 (56%) were positive and 113 (28%) were negative, by both methods. All of the Giemsa-stained positive-smears were positive by multiplex qPCR testing. The multiplex qPCR test identified an additional 65 cases of LM (Table 2). The samples that were Giemsa negative but multiplex qPCR positive had a significantly higher Cqs values ($P < .001$), indicative of low parasites load (Table 3).

Sensitivity and Specificity of Multiplex Real-Time Polymearse Chain Reaction

To verify that the multiplex qPCR test does not give false-positive results, 69 samples were tested in parallel by a commercially available qPCR kit that tests for LS only (Clonit). Therefore, only the LS set was compared with Clonit. The assay was done using the same nucleic acid extracts that were used for the In-LAB test. As depicted in Table 4, the multiplex qPCR results were confirmed by the commercial test (100% sensitivity and specificity). Therefore, it can be concluded that the reason for the discrepancy between the Giemsa-stained smears and the multiplex qPCR might be due to a lack of sensitivity of the Giemsa-microscopy method (Table 3).

To further validate the newly developed test, 60 samples were sent to a reference center (The Israeli Ministry of Health Center for Parasitology) for confirmation by HRM analysis (Table 5). Thirty-four negative samples were all confirmed negative by the HRM testing. Of 26 multiplex qPCR-positive samples, 23 (21 LM, and 2 *L brazilazies*) were confirmed to be positive and correctly typed. Two samples of LM (Cq 34 and 32) and 1 sample of LT (Cq 25.5) that were positive by the multiplex qPCR were negative in the HRM test.

Table 4. Analysis of CL by the In-LAB mqPCR and Commercial RT-PCR[a]

	Clonit RT-PCR	
	Positive	Negative
Multiplex qPCR positive	52	0
Multiplex qPCR negative	0	17

Abbreviations: CI, confidence interval; CL, cutaneous leishmaniasis; In-LAB, in laboratory; mq, multiplex quantitative; PCR, polymerase chain reaction; q, quantitative; RT, reverse-transcription.

[a]Kappa = 1; 95% CI, 1.0–1.0.

Table 5. Analysis of CL by the In-LAB mqPCR and HRM RT-PCR[a]

	HRM RT-PCR	
	Positive	**Negative**
Multiplex qPCR positive	23	3
Multiplex qPCR negative	0	34

Abbreviations: CI, confidence interval; CL, cutaneous leishmaniasis; HRM, high-resolution melting; In-LAB, in laboratory; mq, multiplex quantitative; PCR, polymerase chain reaction; q, quantitative; RT, reverse-transcription.

[a]Kappa = 0.90; 95% CI, 0.78–1.00.l

Table 6. Analysis of CL by the In-LAB mqPCR and kDNA PCR Analysis[a]

	kDNA RT-PCR	
	Positive	**Negative**
Multiplex qPCR positive	28	1
Multiplex qPCR negative	1	16
Total	29	17

Abbreviations: CI, confidence interval; CL, cutaneous leishmaniasis; In-LAB, in laboratory; kDNA, kinetoplast deoxyriboneucleic acid; mq, multiplex quantitative; PCR, polymerase chain reaction; q, quantitative; RT, reverse-transcription.

[a]Kappa = 0.91; 95% CI, 0.78–1.00.

The sensitivity of the multiplex qPCR In-Lab test that is based on the ITS region was as good as the PCR for the kDNA region. Of 46 samples that were tested in parallel by both tests, 44 were in agreement (28 positives and 16 negatives). Discrepancy was found in 2 samples: 1 was positive by the kDNA but negative by the multiplex qPCR and 1 was negative by the kDNA but positive by the multiplex qPCR.

Applying the In-Laboratory Multiplex Quantitative Polymearse Chain Reaction Test for the Diagnosis of Cutaneous Leishmaniasis

During 2 years (July 2014 to August 2016), the laboratory for Clinical Parasitology tested 1783 samples, and 1086 (61%) were found to be positive for *Leishmania* using the generic probe (Figure 2). *Leishmania major* was the dominant species comprising 57% of the samples, whereas LT was positive in 4% (70 samples). Two samples were positive for *L braziliensis* and 6 for *L infantum/donovani*.

Higher positive rates were associated with seasonality, peaking in the winter. During September to February 2014–2015, 464 cases were LM positive of 615 that were sent to the laboratory (75%), and during the same period in 2015–16 529 samples were recorded and 319 were positive (60%) (Figure 3).

DISCUSSION

We report here on a highly sensitive, newly developed multiplex qPCR to detect and type *Leishmania*. The disease CL can cause a wide spectrum of cutaneous manifestations, from a self-healing localized skin lesions caused by LM to severe mutilating mucocutaneous leishmaniasis by *L braziliensis*. Furthermore, *L infantum*, which is also found in Mediterranean countries, can lead to both CL and visceral *Leishmania* in humans and to canine leishmaniasis in dogs. Visceral *Leishmania* is a lethal condition if untreated, with parasites affecting the liver, spleen, and bone marrow [1, 17]. In Israel, CL is mainly caused by LM and LT species [2, 4]. However, sporadic cases of *L infantum* were reported [6]. Fourteen percent of the population of Israel habitats the southern part of Israel. The major cause of CL in this region is LM, whereas LT is distributed in the area of Jerusalem and in Northern Israel, and its outcome is more severe. In recent

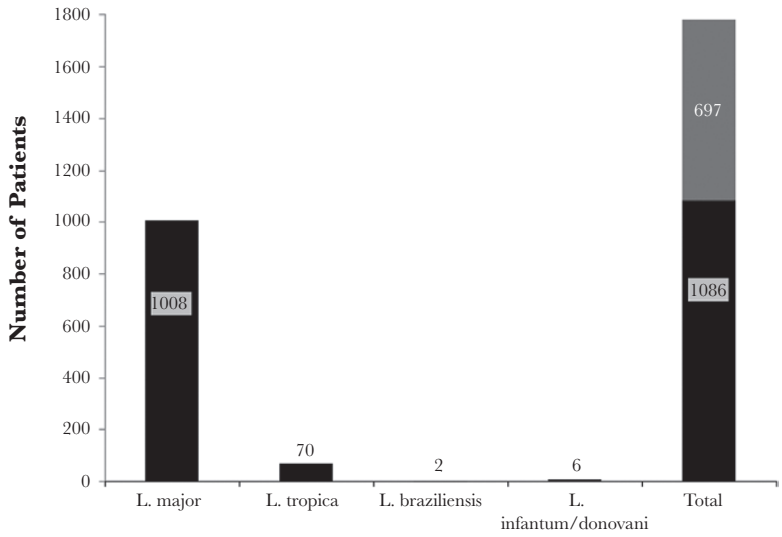

Figure 2. Diagnosis of cutaneous leishmaniasis by in-laboratory multiplex quantitative polymerase chain reaction test. The bars represent the number of samples tested during July 2014 to August 2016. Depicted are (1) total number of patients tested and (2) the *Leishmania* species samples that were found to be positive (black bars) and negative (gray bar).

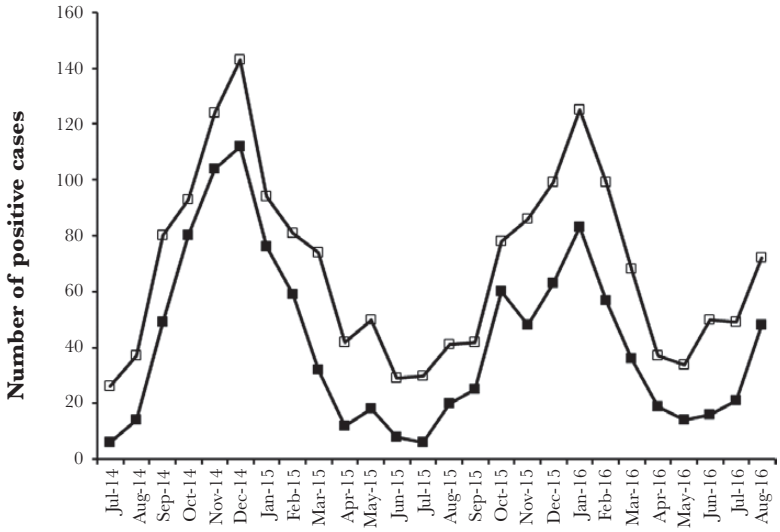

Figure 3. Seasonality of *Leishmania major* infections in Israel Southern District. The graphs depicts the number of samples that were sent for diagnosis (open squares) and the number of positive samples for *L major* (black squares), in each month, during July 2014 to August 2016.

years, the disease has spread to new areas previously considered to be free of infection. In addition, more cases resistant to available treatment have been demonstrated, probably due to LT in this region [3, 18]. In the past, no parasite characterization was performed, because it was assumed that leishmaniasis cases in the Negev in Southern Israel are limited to LM infection. Therefore, primary diagnosis of CL was mainly done by clinical or microscopic examinations of lesions. However, the changes in species pattern in recent years, the spreading of LT to the South, and sporadic infections of travelers from other geographical areas emphasized the need for accurate diagnosis of *Leishmania* and the individual species typing.

The goal of the study was to develop a molecular test applicable to clinical set up that ensures a sensitive and specific diagnostic testing of *Leishmania* genus, which differentiates LM from LT infection. This technique has the potential to significantly accelerate species-adapted therapeutic decisions regarding treatment of leishmaniasis. To that end, a sensitive multiplex-based real-time PCR (multiplex qPCR) first screen (set I) was developed. Using the advantage of hydrolysis probes together with real-time PCR, we identified in a single test LS, along with the prevalent species in the South Region: LM and LT. In most cases (99.7%), the first screen was sufficient. The succeeding test (set II) was only done in rare cases (8 samples), when the sample was positive for *L* species but negative for LM or LT, mostly in cases of returning travelers, suspected of visceral or mucocutaneous leishmaniasis.

Limitations of the study were as follows: we had only a few cases of *L braziliensis* [2] and *L infantum/donovani*. Although the assay was tailored made for our region, the use of the "species-probe" in set I, once validated on additional LS, will be applicable to other regions as well [12].

CONCLUSIONS

As expected, the multiplex qPCR was more sensitive than the Giemsa-stained slides [19] and less labor demanding. It could detect even one particle of promastigote. The turnaround time for the diagnosis was dramatically improved compared with culture techniques, allowing results within 1 day. The sensitivity and accuracy of the newly developed test was confirmed in 3 ways: retesting by a commercial test (Clonit), testing 60 samples by HRM-PCR in a reference laboratory (Israeli Ministry of Health, Jerusalem) [14], and by kDNA PCR analysis [10]. It was previously shown that swab sampling combined with DNA extraction was the most efficient recovery method for *Leishmania* DNA [20]. We used point-of-care lesion-swabbing and automated nucleic acid extraction combined with sensitive qPCR to improve the service to the medical personal and to the patient.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

Financial support. This work was supported by the Ministry of the Environment grant "Combatting Leishmaniasis" (Research No. 131-4-1).

Potential conflicts of interest. All authors: No reported conflicts. No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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