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DISSERTATION

Development and application of molecular diagnostic assays in the epidemiology of cutaneous leishmaniasis in the Jenin District

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Table of Contents

	List of abbreviations	3
1	Abstract	4
	Zusammenfassung	
2	Introduction	6
3	Materials and Methods	7
3.1	Epidemiological data and parasite samples	7
3.2	Detection and identification of parasites	8
3.3	Development of a 7SL PCR reverse dot blot (RDB) diagnostic assay	8
3.4	Genetic, serological and biochemical characterization of Leishmania tropica from foci in northern Palestine	9
4	Results	9
4.1	Epidemiology of CL in the Jenin District	9
4.2	7SL PCR RFLP and PCR RDB diagnostic assay	11
4.3	PCR and RFLP and their use as a 'gold standard' in diagnosing Old World cutaneous leishmaniasis	12
4.4	Characterization of strains of L. tropica from foci in northern Palestine, using the kDNA-RFLP and EF serotyping, and the discovery of zymodeme MON 307	13
5	Disscussion	15
5.1	Cutaneous leishmaniasis in Jenin District	15
5.2	7SL PCR RFLP and PCR RDB diagnostic assays	17
5.3	PCR assays and their use as a 'gold standard' in diagnosing Old World cutaneous leishmaniasis	18
5.4	Characterization of strains of *L. tropica* from foci in northern Palestine and discovery of zymodeme MON 307	19
	Conclusion	22
	References	23
	Affidavit	24
	Declaration of any eventual publications	25
	Curriculum Vitae	28
	Complete list of publications	31
	Acknowledgements	33

List of abbreviations

CL	cutaneous leishmaniasis
DNA	deoxyribonucleic acid
EF	excreted factor
FCS	fetal calf sera
FH	fumarate hydratase
HCl	hydrochloric acid
ITS	internal transcribed spacer
JD	Jenin District
kDNA	Kinetoplast DNA
L.	*Leishmania*
LPG	lipophosphoglycan
MLEE	multilocus enzyme electrophoresis
MLMT	multi-locus microsatellite typing
MLST	multilocus sequence typing
NaOH	sodium hydroxide
NJ	Neighbour Joining
PCR	polymerase chain reaction
Ph.	*Phlebotomus* (sand fly vectors)
pmol	picomole
RFLP	restriction fragment length polymorphism
RDB	reverse dot blot
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SSC	sodium citrate
SL	spliced leader
TAE	Trisacetate-EDTA
VL	visceral leishmaniasis
UPGMA	Unweighted Pair Group Method with Arithmetic mean

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1. Abstract

In Palestine, cutaneous leishmaniasis (CL) is mainly caused by *Leishmania major* and *L. tropica.* From 2002 and 2009, 466 CL cases were reported in Jenin District (JD) with an average annual incidence of 23 cases per 100,000 inhabitants. Most cases presented a single lesion, generally on the face.
Diagnosis and species identification in clinical materials were done by applying PCR assays. In this study, a new approach was developed targeting the 7SL RNA gene. The causative species was identified by RFLP analysis of the 7SL product and by reverse dot blot (RDB) hybridization using five probes. One probe was a genus-specific probe and hybridized to all leishmanial species (Lc), two were specific for *L. major* (Lm1 and Lm2), one was specific for *L. tropica* (Lt) and one detected both *L. major* and *L. tropica* (Lmt). The PCR–RDB was 10 times more sensitive than 7SL PCR-RFLP and could detect <1 parasite.
The 7SL PCR was compared to widely used PCR approaches targeting kinetoplast DNA (kDNA) and ribosomal internal spacer sequences (ITS1), for its ability to detect parasites in skin tissue aspirates of 212 CL suspects. The kDNA PCR was most sensitive, detecting 156/170 of the truly-positive samples but failed in identifying the species of *Leishmania*. The 7SL PCR detected 154/170 and the ITS1 PCR only 108/170 of the truly-positive ones. Species identification was possible with either the 7SL or ITS1 PCR assays.
L. tropica was the predominant cause of CL when species identification had been performed using DNA isolated from parasite cultures (47) or skin tissue scrapings (256), followed by *L. major*. For the first time, *L. infantum* was identified as the causative agent of CL in four Palestinian patients.
Considerable intra-species genetic heterogeneity was observed when 12 strains of *L. tropica* from JD were analyzed by different analytical methods. Three of them constituted a new zymodeme, zymodeme MON-307, which seems to be unique to the northern part of the Palestinian West Bank. Other strains belonged to the known zymodeme MON-137. Excreted factor (EF) serotyping showed that MON-307 strains were sub-serotype A_2 and those of MON-137 were either A_9 or A_9B_4. The sub-serotype B_4 component appears to be unique to some strains of *L. tropica* of zymodeme MON-137. When their kDNA was digested with the endonucleases *Rsa*I and *Mbo*I, Palestinian strains were assigned to different genetic groups, in congruence to their geographical origin, and different zymodeme and EF types.

1. Zusammenfassung

In Palästina werden kutane Leishmaniosen (CL) hauptsächlich durch *Leishmania major* und *L. tropica* verursacht. Zwischen 2002 und 2009 wurden insgesamt 466 humane CL-Fälle im JD registriert, die durchschnittliche jährliche Inzidenz betrug 23 Fälle pro 100000 Einwohner. Die meisten Erkrankten wiesen nur eine Hautläsion, meistens im Gesicht, auf.

Die Diagnose und Speziesidentifizierung in klinischen Materialien wurden mit verschiedenen PCR-Verfahren durchgeführt. In dieser Arbeit wurde ein neues PCR-Verfahren entwickelt, welches das 7SL RNA-Gen amplifiziert und bei dem die unterschiedlichen Erreger entweder durch RFLP-Analysen der amplifizierten Produkte oder durch „reverse dot blot (RDB)"- Hybridisierung mit fünf spezifischen Sonden identifizert wurden. Eine Sonde war genus-spezifisch (Lc) und hybridisierte an die Sequenzen aller *Leishmania*-Arten, zwei Sonden waren spezifisch für *L. major* (Lm1 und Lm2), eine für *L. tropica* (Lt) und eine Sonde (Lmt) detektierte sowohl *L. major* als auch *L. tropica*. Diese PCR-RDB-Methode war zehnfach empfindlicher als die 7SL PCR-RFLP und konnte <1 Parasiten nachweisen.

Die 7SL-PCR wurde mit häufig angewendeten PCR-Verfahren, die die Kinetoplasten-DNA (kDNA) oder den ribosomalen internalen Spacer (ITS1) amplifizieren, hinsichtlich ihrer Fähigkeit verglichen, Parasiten in Hautproben von 212 Personen mit Verdacht auf CL nachzuweisen. Die höchste Sensitivität wurde mit der kDNA PCR erreicht, die 156 der 170 „echt" positiven Proben detektierte, aber keine Speziesidentifizierung ermöglichte. Mit der 7SL PCR und der ITS1 PCR wurden *Leishmania*-Sequenzen in 154 bzw. nur 108 der 170 „echt" positiven Proben amplifiziert, beide Methoden konnten aber für die Identifizierung der unterschiedlichen Spezies genutzt werden.

L. tropica war der häufigste Erreger von CL, wenn eine Speziesidentifizierung in DNA-Proben durchgeführt wurde, die aus *Leishmania*-Kulturen (47) oder direkt aus Hautproben (256) isoliert worden waren, gefolgt von *L. major*. Erstmalig wurde *L. infantum* als Erreger von CL bei 4 palästinensischen Patienten bestätigt.

Eine ausgeprägte intra-spezifische Heterogenität wurde für 12 *L. tropica*-Isolate aus JD mit verschiedenen analytischen Verfahren nachgewiesen. Für drei dieser Stämme wurde ein neues Zymodem, MON-307, beschrieben, das bisher nur im Norden der palästinensischen Westbank gefunden wird. Andere Stämme entsprachen dem bereits bekannten Zymodem MON-137. Die MON-307 Stämme wurden in der „excreted factor" (EF) Serotypisierung dem Sub-Serotyp A_2 zugeordnet und die des Zymodems MON-137 entweder A_9 oder A_9B_4. Die Komponente B_4 wurde bisher nur bei einigen *L. tropica*-Stämmen des Zymodems MON-137 nachgewiesen. Wenn die kDNA mit den Restriktionsendonukleasen *Rsa*I und *Mbo*I verdaut wurde, konnten die palästinensischen Stämme verschiedenen genetischen Gruppen zugeordnet werden, in Übereinstimmung mit ihrer geographischen Herkunft und ihren Zymodem- und EF-Typen.

2. Introduction

In Mediterranean countries, cutaneous leishmaniasis (CL) is caused by three overlapping species of *Leishmania*: *L. major*, *L. tropica* and, more rarely, *L. infantum*, which is usually associated with visceral leishmaniasis (VL). Cutaneous leishmaniasis caused by *L. major* and *L. tropica* is an important public health problem in Palestine. Palestine is a small country and people can be infected by the three species within a few hours of travel or going to their place of work.

From 1990 to 1999 the highest rate of CL occurred in the vicinity of Jericho (Palestinian Ministry of Health (PMOH)), where *L. major* is its main cause, followed by the Jenin District (JD) where *L. tropica* is its main cause. *L. tropica* has, rarely, been the cause of visceral leishmaniasis (VL) [1]. The epidemiology of CL, a zoonotic, vector-borne disease caused by *L. major*, has been studied in depth [2] whereas *L. tropica* has not. Classically, CL caused by *L. tropica* is considered to be anthroponotic, however, in Palestine, and Israel, it appears to be zoonotic, with rock hyraxes (*Procavia capensis*) serving as the reservoir host in Israel [3]. The vector for *L. tropica* has not been unequivocally identified in the JD. *P. sergenti* which is the main vector in Israel [4] is also the putative vector in Palestine. The northern region of the West Bank is considered to be a main focus of *L. tropica* and most cases were reported from the JD.

Since several species causing CL and/or VL may co-exist in the same transmission foci, it is imperative that infections are shown to actually be leishmaniases by proving the presence of leishmanial parasites and also identifying the species of *Leishmania*. The different species of *Leishmania* present the same morphology when classical diagnostic methods, such as microscopic examination of Giemsa stained smears or parasite culture are used. This led to the development of molecular approaches that combine high sensitivity for direct detection and identification in clinical specimens with species-specificity by amplifying either species-specific DNA sequences or genus-specific sequences that allow for subsequent differentiation of *Leishmania* species by RFLP, hybridization to specific probes and/or sequencing of the PCR products obtained (for review see [5]). A few studies have compared the sensitivity and specificity among different PCR-based assays. Here, methods targeting the ribosomal internal transcribed spacer 1 (ITS1) or the 7 spliced leader sequence (7SL) RNA gene have proven to be powerful methods for detecting and identifying leishmanial parasites directly in clinical samples and without requiring isolation by culture and cultivation [5, 6]. Species identification was achieved by RFLP or sequencing of the PCR products obtained.

L. tropica is a genetically heterogeneous species and strains exhibit varying antigenic, biochemical and genetic features [4], Recently microsatellite markers have been used to determine the profiles of 117 strains of *L. tropica* [7] . Of the 27 strains that came from Palestinian foci, 9 were isolated from CL cases living in the JD. These grouped in the clusters ' I: Middle East ' and ' II: Asia ' whereas strains from other Palestinian foci, e. g., the Jericho area and Samaria, were all assigned to the cluster 'I: Middle East'.

Main Objectives:

The aim of this study was to investigate the epidemiology and clinical features of CL in the JD, Palestine, including the molecular identification of the causative parasite species

and the analysis of the genetic heterogeneity of strains of *L. tropica*. For this it was attempted

1. to study epidemiological parameters and parasitological features of CL in the JD, Palestine, between 2002 and 2009, including the detection and identification of parasites causing CL cases in that focus, determination of disease prevalence in the human population and investigation of the putative vector species.
2. to develop and validate a new diagnostic PCR assay, the 7SL RNA PCR RFLP and RDB assays, for the direct detection and identification of the causative agent of CL in the JD directly in clinical samples.
3. to compare the new diagnostic assays and those existing for Old World leishmaniasis for samples from CL patients in the JD and to define a 'gold standard' for diagnosing Old World CL.
4. to study the genetic, serological and biochemical features of strains of *L. tropica* from foci in northern Palestine and compare their genetic diversity to that of *L. tropica* strains isolated in Israeli foci and to correlate the diversity/ homogeneity of these strains with their geographical distribution and the corresponding environmental and ecological conditions existing in the various locations to clarify the disease transmission and re-emergence CL in these foci.

3. Materials and Methods

3.1 Epidemiological data and parasite samples

Epidemiological data were collected on 466 CL cases seen in JD from 2002 to 2009. Clinical data were obtained for 256 patients admitted to the clinics of PMOH for treatment. A questionnaire was completed for each case presenting cutaneous lesions, recording the patient's: full name; age; sex; number of lesions; their sites and duration; the patient's address; work place and travel history and being a household member of previous CL cases. The presence of hyraxes and/or other animals near human habitations were collated for risk assessment. Statistical analysis was carried out by SPSS 17.0 software.

Tissue aspirates were taken from 212 suspect cases of CL admitted to the JD clinic and the Ibin Sina Clinic, Jericho of the Palestinian Ministry of Health. Patients were three to 80 years old. Tissue scrapings were smeared onto filter papers and tissue aspirates were collected into syringes containing 0.5 ml of saline, part of which was inoculated into normal rabbit blood-agar semisolid medium. Cultures were incubated at 26°C, examined every 2 to 4 days and only discarded as negative after one month.

Mass cultures of leishmanial parasites were grown in Schneider's Drosophila medium supplemented with 10% heat inactivated FCS with 2 mM L-glutamine, containing penicillin at 200 IU/ml and streptomycin at 200µg/ml.

The strains of *Leishmania* included WHO reference strains provided by the *Leishmania* Reference Center, Parasitology Department, Hebrew University-Hadassah Medical School Jerusalem; which were isolated in India, Turkey, Tunisia, Turkmenistan, Azerbaijan, and Ethiopia; as well as local strains from Israel and Palestine. Twenty DNA samples from dogs living in a VL focus were analysed in addition to the samples from humans.

A total of 1492 sand flies were collected in four foci of the JD in 2008 and 2009. For taxonomic identification, heads and genitalia were removed and mounted on microscope slides in Berlese's. Females belonging to *Phlebotomus* spp. were identified by the structures at the base of the spermathecal ducts. Females that had imbibed blood or swollen abdomens were dissected and examined for the presence of promastigotes.

3.2 Detection and identification of parasites

DNA extraction from cultures was performed as described by [8]. For DNA extraction from clinical samples, specimens were cut from filter paper with a disposable sterile scalpel and incubated in 200 µl cell lysis buffer for 1 hour at 56°C. Clean autoclaved filter paper was included as negative extraction control. DNA was extracted with the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's instructions. The DNA was kept at 4°C until used.

The ITS1 PCR was carried out as described by Schonian et al. [8], minicircle kDNA was amplified using primers 13A and 13B [9], and 7 SL PCR as described by Zelazny et al [10]. A PCR was considered positive when a band of the expected size, ~300 bp for ITS1, ~185 bp for 7SL and ~120 bp for kDNA, was obtained. DNA from the WHO reference strains *L. major* (MHOM/SU/1973/5ASKH), *L. tropica* (ISER/IL/1998/LRC-L747) and *L. infantum* (MHOM/TN/1980/IPT1), were amplified as positive controls.

Leishmanial species identification was by RFLP analysis of the ITS1 and 7SL RNA amplicons after digestion of ∼15 µl of the PCR product with BsuRI, without prior purification and following the instructions of the manufacturer.

DNA of *Trypanosoma equiperdum, T. evansi, T. cruzi, T. brucei, T. vivax, Crithidia luciliae, C. fasciculata and Leptomonas collosum*, kindly provided by Dr. Gad Baneth, Koret School of Veterinary Medicine, Hebrew University, were used to test the specificity of the assays.

Specimens were considered as true positives (T-Pos) when either at least two PCR assays were positive for leishmanial DNA or the culture produced promastigotes. Similarly, samples were considered as true negatives (T-Neg), when culture and two PCR assays were negative for leishmanial DNA. This constituted the "gold standard" applied here. The overall sensitivity and specificity for each diagnostic assay were calculated using the online calculator (http://faculty.vassar.edu/lowry/clin.html). Positive (PPV) and negative (NPV) predictive values, and the level of agreement between the different diagnostic tests, Cohen's kappa coefficient (k), were estimated (http://www.graphpad.com/quickcalcs).

3.3 Development of a 7SL PCR reverse dot blot (RDB) diagnostic assay

Five hybridization probes were designed for application in the RDB assay. For this, 32 7SL sequences of Old World *Leishmania* species, 13 of New World *Leishmania* species and 3 of other trypanosomatids were analyzed. The Old World *Leishmania* 7SL gene sequences were aligned using the multiple sequence alignment program. Two probes were specific for *L. major*, one for *L. tropica*, one detected both *L. major* and *L. tropica* and one that was a genus-specific probe. Strains of the *L. donovani* complex reacted only with the genus-specific probe. All selected probes were in-silico tested for specificity against the 48 trypanosomatid 7SL sequences retrieved from the Genbank. Sequences of leishmanial strains from different geographical areas were chosen. Probes were modified with an amine group at the 5' region covalently linked to the activated membrane which was cut into strips, each having the same probes order.

For the RDB assay, the primer TRY7SL.Rev1 was labeled with biotin at the 5' end. PCR was carried out in a total reaction volume of 25 µl using the PCR-Ready Supreme mix (Syntezza Bioscience, Jerusalem, Israel). RDB hybridization was done as described [11].

3.4 Genetic, serological and biochemical characterization of *Leishmania tropica* from foci in northern Palestine

In total, 12 strains isolated from patients in the JD suspected of being CL cases were analysed in this part of the study. Two Israeli reference strains of *L. tropica*, MHOM/IL/98/LRC-L758 and MHOM/IL/97/P963 (= LRC-L725), and one of *L. major*, MHOM/IL/67/JerichoII (=LRC-L137), were included for comparison.

Amplification of the complete leishmanial kDNA minicircle sequence was done according to Anders [12], using the primer pair Uni 21 (5'-GGG GTT GGT GTA AAA TAG GCC) and Lmj4 (5'-CTA GTT TCC CGC CTC CGA G). The PCR product was digested separately with either the endonucleases *Rsa*I or *Mbo*I. kDNA of *L. tropica* MHOM/IL/98/LRC-L758 was included as a positive reference and that of *L. major* MHOM/IL/67/JerichoII (=LRC-L137) as the outgroup. kDNA-RFLP results were analyzed using the RAPDistance Package version 1.04 (http;//www.anu.edu.au/Bozo/software/). All the fragments seen, represented by bands, were numbered and scored: 0 for the absence of a band; 1 for its presence. Identicalness and similarity were measured according to the overall profile seen after digestion with each endonuclease and the number of shared bands among each enzyme's profiles. A dendrogram was constructed based on the combined fragmentation of the restriction enzymes *Rsa*I and *Mbo*I, using the distance matrix (Neighbor joining) option.

EF serotyping was done according to Schnur and Zuckerman [13] and MLEE [14] according to where nine cultures from JD were typed by determining the mobility of 15 enzymes in starch gel electrophoresis.

4. Results

4.1 Epidemiology of CL in the Jenin District

The skin lesions observed for 466 human cases of CL were 0.5 to 5 cm in diameter, dry in appearance, and showed granulation and papules appearing at their periphery. The average annual incidence of CL was 23.0 per 100, 000 inhabitants and the prevalence 190.1 per 100,000 inhabitants.

The annual number of reported cases of CL varied between 128 in 2002 and 22 cases in 2004, with rates varying between 8.9 to over 50.5 per 100,000 inhabitants. The highest number of cases and incident per 100,000 inhabitants were, respectively, 128 and 50.5 in 2002 and 102 and 39 in 2008. Most cases had dry lesions of long duration and, of the 212 patients between the years 2006-2008, 170 (80%) developed signs and symptoms of disease between January and May, indicating a long incubation period. This situation is classically accepted as associated with CL caused by *L. tropica*.

Though the patients were coming from throughout the JD, most were from south of Jenin City, mainly Qabatya, and northwest of Jenin City from the villages of El-Yamoon and Silat El Hartheyia. In fact, the highest infection rate during the study period was observed in the area northwest of Jenin City where 63.3% of all the cases occurred with a total average annual incidence of 119.4 cases per 100,000 inhabitants; and most of these cases,

288 (61.8%), occurred in the vicinity of El-Yamoon and Silat El Hartheyia at an altitude of 140-200 m a.s.l. Lower infection rates were found in the southern part of the JD, 106 (22.7%) of cases, and in urban Jenin, 39 (8.4%). The lowest infection rates, 2.8% and 1.1%, respectively, occurred in the eastern and western parts of the JD with a total average annual incidence of 5.8 and 2.0 per 100,000 inhabitants, respectively.
Cases were of all ages. The mean age was 22 years (median 16.0, lower quartile = 9, upper quartile = 30). Of the 466 cases diagnosed as CL, 257 (55.2%) were male and 209 (44.8%) female, a ratio of 1.2:1 with no significant association to gender.
Half of the infections (49.6%), were patients under 15-years old with the incidence appearing to increase with age, 14.7% in those up to and including four years old and 26% in those above five years old, with significant differences ($P<0.05$) compared to the adult group. The lowest number (82, 17.6%) was in the group above 40 years old. Combining gender with age groups showed that adult males between 20 and 39 years old were at the highest risk ($P<0.05$).
The 466 cases presented a total of 686 lesions. The head was most commonly affected (235/466, 50.4 %), especially the cheek (173, 37%). Then the upper limb followed (168, 36.1%) and then lower limb (33, 7%). Multiple lesions involved one or more parts of the body in 30 (6.5%) cases. Single lesions occurred in 331/466 (71.0%) cases. Most of these were on the head (174/331, 52.6%), followed by the upper limb (139/331, 42.0%). Two lesions were seen in 83/466 (17.8%) cases and 52 /466 (11.2%) cases showed from three to seven lesions. Males had more lesions on the upper limb than females (117/168, 69.6%) and females had more lesions on the head (128/235, 54.4%) mainly on the cheek (101/173, 58.4%), $P<0.05$. Of the 225 children, 48.6% (161/331) had single lesions.
By using different PCR approaches on 256 clinical samples *L. tropica* was identified as the causative agent in most of them, followed by *L. major* (for details see 4.3). Surprisingly, *L. donovani sensu lato* was identified in samples obtained from four patients. Partial sequencing of ITS1 revealed identical sequences for these patients, which corresponded to that of the *L. infantum* reference strain MHOM/TN/1980/IPT1. These sequences have been deposited in Genbank under the accession numbers JN181861-JN181864. EF serotyping of used medium from one of them showed the strain was EF sub-serotype B_2, which was compatible with the DNA results.
Sixty strains were isolated from dermal tissue aspirates by culture, 13 (22%) of which were lost through concomitant bacterial and fungal contamination. Of the 47 cultures, 44 (93.6%) were *L. tropica* by ITS1 PCR-RFLP and 3 (6.4%) were *L. major*. Forty-one strains were EF serotyped, of which 38 were EF sub-serotype A_2 or A_9B_4 and therefore *L. tropica*, and three were EF sub-serotype A_1 and therefore *L. major*. Nine strains of *L. tropica* were typed by MLEE and fell into two different zymodemes: six belonged to the zymodeme MON-137 and three belonged to a new zymodeme (MON-307).
Of the 1462 sand flies trapped, 922 (63%) were species of *Phlebotomus* and 540 (37%) were species of *Sergentomyia*. The following species of *Phlebotomus* were identified: *P.* (*Paraphlebotomus*) *sergenti,* 32%; *P.* (*Phlebotomus*) *papatasi*, 25.2%; *P.* (*Larroussius*) *tobbi*, 22.3%; *P. major syriacus,* 9.4%; *P.* (*Larroussius*) *perfiliewi*, 4.6%; *P. major neglectus*, 4.2%. The remaining 2.3% belonged to the species *P.* (*Paraphlebotomus*) *alexandri*, *P.* (*Synphlebotomous*) *sp.*, *P.* (*Larroussius*) *mascitti*, *P.* (*Adlerius*) *kazeruni*, *P.* (*Adlerius*) *arabicus* and *P.* (*Adlerius*) *halepensis* . None of the female sand flies showed infections of promastigotes.

These results have been published in the following paper.
K. Azmi*, G. Schonian, A. Nasereddin, LF. Schnur, S. Sawalha, O. Hamarsheh, S. Ereqat, A. Amro, SE. Qaddomi, Z. Abdeen. Epidemiological and clinical features of cutaneous leishmaniases in Jenin District, Palestine, including characterization of the causative agents in clinical samples. Transactions of the Royal Society of Tropical Medicine and Hygiene*. 2012 Sep;106(9): 554-62

4.2 7SL PCR RFLP and PCR RDB diagnostic assay

The 7SL PCR yielded a product of ~185 bp for all Old World species tested. By digestion with *BsuRI,* three fragments of 108, 59 and 17 bp were yielded for *L. tropica* and *L. aethiopica,* three fragments of 137, 31 and 17 bp for the *L. donovani* complex, and two fragments of 167 and 17 bp for *L. major*. Strains of *L. aethiopica* and *L. tropica* could be differentiated by restriction with the enzyme *Hpa*II. Three bands (158, 16 and 10 bp) were observed for *L. tropica,* and two bands (168 and 16 bp) for *L. aethiopica*. *BsuRI* did not cut any of the PCR products amplified for the different species of *Trypanosoma*, *Crithidia* or *Leptomonas*. When PCR was performed using clinical samples from humans and dogs, the restriction patterns obtained were in agreement with those of the respective reference strains.
The genus- and species-specific probes for the RBD assay were highly specific when tested *in-silico* by performing a Megablast search against 48 *trypanosomatid* 7SL RNA gene sequences available from the NCBI nucleotide database. The genus-specific probe Lc showed 100% identity with all New and Old World species of *Leishmania*, but not for the *L.* (*Viannia*) complex, and with *Crithidia luciliae*, *C. fasciculata* and *Leptomonas collosoma*. There was no match with sequences of *T. cruzi* or African trypanosomes, which was confirmed experimentally using the PCR product of *T. equiperdum*, which did not react with the Lc probe. Probes Lm1 and Lm2, were highly specific for *L. major* identifying all *L. major* DNA sequences in the Genbank. Similarly, the Lt probe identified all sequences of *L. tropica* and *L. aethiopica* in the Genbank, and matched perfectly with sequences of *L. mexicana*, *L. garnhami*, *L. amazonensis* and *L. pifanoi*. The Lmt probe designed to detect both *L. tropica* and *L. major* was 100% homologous ~~to~~ all strains of *L. major*, *L. tropica* and *L. aethiopica* deposited in the NCBI nucleotide database.
When the 7SL DNA PCR products of six strains of *L. major,* seven of *L. tropica* and 10 of the *L. donovani* complex were hybridized to the genus-specific probe, Lc, positive signals were obtained for all tested leishmanial strains. The *L. tropica* PCR product hybridized to the Lc, Lt and Lmt probes (3 positive dots). The *L. major* strains were detected by four probes, Lm1, Lm2, Lc and Lmt. Hybridization to the Lc probe was indicative for only the *L. donovani* complex. All negative controls as well as *T. equiperdum* did not hybridize to any of the probes. The RDB assay was highly specific since it identified all reference and local strains of *Leishmania* studied at the species level. The only exception was *L. aethiopica* that cross-reacted with *L. tropica*. Fortunately, *L. aethiopica* is not present in the Mediterranean countries. The sensitivity of the 7SL PCR-RDB was examined using serial dilutions of purified *L. major* DNA (5ASKH) for amplification. The detection limit of the 7SL PCR was 200 fg DNA/reaction, while the

combined PCR–RDB assay was 10 times more sensitive for Lmt and Lc and picked up 20 fg DNA/reaction**.**
Clinical samples (containing amastigotes not as cultured promastigotes) from 37 human cases of CL and 20 from canine cases of VL were analyzed by kDNA PCR, 7SL PCR and 7SL RDB. By kDNA PCR, 55 of the 57 specimens (96.4%) were positive. This included 35 of the CL and 20 of the VL samples. A total of 50 samples (87.7 %) were positive by 7SL DNA PCR. The identification of the parasites at the species level was possible in 40 of the 50 positive ones. Based on the RFLP profiles, *L. tropica* was detected in 22 samples, *L. major* in 6 and *L. infantum* in 12 samples. PCR RDB detected *Leishmania* in 53 out of the 57 specimens tested (92.9%). *L. major* was identified in 8, *L. tropica* in 27 and *L. donovani* complex in 18 specimens**.** The RDB detected parasites in nine of the ten samples for which RFLP could not be performed as well as in four samples with no visible PCR product in the gel.
These results have been published in the following paper.
K. Azmi, *A. Nasereddin, S. Ereqat, G. Schönian, Z. Abdeen. Identification of Old World Leishmania species by PCR-RFLP of the 7 spliced leader RNA gene and reverse dot blot assay. Trop Med Int Health. 2010 Aug;15(8): 872-80. Epub 2010 Jun 15.*

4.3 PCR and RFLP and their use as a 'gold standard' in diagnosing Old World cutaneous leishmaniasis.

The results of the 7 SL PCR were compared with those of the ITS1 PCR, kDNA PCR and leishmanial culture using clinical samples from 212 suspect cases of CL. Specimens were considered as true positives (T-Pos) when either at least two PCR assays were positive for leishmanial DNA or the culture produced promastigotes. Similarly, samples were considered as true negatives (T-Neg), when culture and two PCR assays were negative for leishmanial DNA. Using this definition of as "gold standard", 170 (80.2%) of the samples were true positives and 42 (19.8%) were true negatives. All 63 specimens positive by culture were confirmed by at least one of the PCR assays. By the kDNA PCR, 178 of the 212 samples were positive, of which 156 were identified as true positives and 22 as false positives. The PPV and the NPV were 87.6% and 58.6%, respectively. The kDNA PCR had the highest sensitivity of all assays (92%). The level of agreement between the kDNA PCR and the confirmed gold standard (the kappa coefficient (k) ± standard error (SE) = 0.424 ±0.172 where 0 implies no or very poor agreement and 1 perfect agreement) indicated moderate agreement.
The 7SL PCR RFLP assay corroborated 154 of the 170 true-positive samples with no false-positives. Three culture-positive samples were false negatives by the 7SL PCR. The agreement with the confirmed gold standard was good (kappa=0.792± 0.098; sensitivity and specificity 90.5 and 100%, respectively). .
The ITS1 PCR confirmed 108 of the 170 true positives with no false positives and its sensitivity was 63.5% and specificity 100%. Thirty-four of the culture positive samples were also detected by ITS1 PCR. Of the 104 negative samples, 42 were true negatives. PPV and the NPV for the ITS1 assay were 100% and 40.3%, respectively. Agreement between the confirmed results and ITS1 PCR was moderate (0.408±0.124). Both the ITS1 and 7SL assays were used to identify the species of *Leishmania* after digesting the amplified products with *Hae*III. The ITS1 PCR-RFLP assay clearly identified the species

for 78 of its 108 (72.3%) confirmed positives whereas the 7SL PCR-RFLP identified 138 of its 154 (89.6%) confirmed positives. *L. tropica* was identified in 60 of 108 (55.6%) positives by the ITS1 assay and in 115 of 154 (74.7%) by the 7SL assay. *L. major* was identified in 18 of 108 (16.7%) positives by the ITS1 assay and 23 of 154 (14.9%) by the 7SL assay. Species identification failed in 30 of 108 (27.7%) positives by the ITS1 PCR-RFLP and 16 of 154 (10.4%) by the 7SL PCR-RFLP. Identification of the leishmanial species was accomplished for 69 samples that were positive by, both, the ITS1 and the 7SL assay.

These results have been published in the following paper.

***K. Azmi**, A. Nasereddin, S. Ereqat, LF. Schnur, G. Schonian, Z. Abdeen. Methods incorporating a polymerase chain reaction and restriction fragment length polymorphism and their use as a 'gold standard' in diagnosing Old World cutaneous leishmaniasis. Diagn Microbiol Infect Dis. 2011 Oct;71(2): 151-5. Epub 2011 Aug 15.*

4.4 Characterization of strains of *L. tropica* from foci in northern Palestine, using the kDNA-RFLP and EF serotyping, and the discovery of zymodeme MON 307

Amplification of the kDNA minicircle sequences of 12 Palestinian and the two Israeli reference strains of *L. tropica*, (MHOM/IL/98/LRC-L758 and MHOM/IL/97/P963 =LRC-L725), gave the same-sized (872 bp) PCR product, which differed from the smaller-sized (650 bp) PCR product of *L. major* (MHOM/IL/67/JerichoII =LRC-L137). RFLP analysis of the *L. tropica* PCR products after their digestion with RsaI produced different kDNA RFLP profiles that consisted of seven to nine bands ranging in size from 800 bp to 150 bp, albeit of two basic kinds: *Ltro*-kD1 that had a ~200 bp component but no ~ 417 bp component; and *Ltro*-kD2, that had a 417 bp component but no 200 bp component. These two types of profiles were reproducible in different gels and separated the strains into two clusters. Digestion of the *L. tropica* PCR products with MboI also produced different kDNA RFLP profiles but consisting of three to nine bands. The RAPDistance Package version 1.04 (http;//www.anu.edu.au/Bozo/software/) used to analyze the kDNA RFLP fragments seen after digestion separately with RsaI and MboI exposed nine different genetic variants, three of which, i. e., *Ltro*-kD1/1-3, formed one branch of a dendogram, Clade A, and six of which, i. e., *Ltro*-kD2/1-6, formed a second branch, Clade B. The analysis of the kDNA RFLP profiles also exposed a further level of micro-heterogeneity.

EF serotyping revealed that five of the 12 Palestinian strains reacted only with the anti-serotype A serum and were serotype A strains, three being sub-serotype A_2 and two sub-serotype A_9. The other seven reacted with, both, the anti-serotype A and the anti-serotype B sera and produced EFs with an A and a B component, and were of the mixed sub-serotype A_9B_4.

Seven of the ten Palestinian strains (LRC-L882, -L883, -L885, -L886, -L887, -L889, -L893) examined by MLEE had enzyme profiles identical to those of the two Israeli reference strains (MHOM/IL/98/LRC-L758 and MHOM/IL/97/P963 =LRC-L725), showing that they belonged to the zymodeme MON-137 [4]. The other three strains (LRC-L884, -L890, -L891) were identical in their enzyme profiles, and constituted a new zymodeme, MON-307. Ten of the electrophoretic mobilities of the 15 enzymes in their enzyme profile were different from those of the strains of the zymodeme MON-137.

A dendrogram showing the taxonomical relationship of the zymodemes of *L. tropica* revealed the existence four sub-groups: (a); (b); (c); (d) Pratlong [14]. The new zymodeme of *L. tropica*, zymodeme MON-307, fell into the sub-group (b); and, within the sub-group (b) itself, the strains in zymodeme MON-307 were closest to those in the zymodeme MON-76, which were isolated in Syria.
These results have been published in the following paper.
K. Azmi*, L. Schnur, G. Schonian, A. Nasereddin, F. Pratlong, F. Baidouri, C. Ravel, JP. Dedet, S. Ereqat, Z. Abdeen. Genetic, serological and biochemical characterization of Leishmania tropica from foci in northern Palestine and discovery of zymodeme MON-307. Parasites and Vectors*.

5. Disscussion

5.1 Cutaneous leishmaniasis in Jenin District

The JD has been an active focus of leishmaniasis since the 1970s. Cases of CL and VL occur, however, published information on both is sparse. The incidence rate of CL in the JD has increased since then. At a local level, the overall annual incidence rate differed between urban localities, ranging from 2.0 per 100,000 in the west of Jenin City to 119.4 per 100,000 in the northwest of JD, possibly, owing to different distributions of reservoir animals and sand flies. These are affected by annual rainfall [2], which ranged between 600-800 mm/year in the western parts and 350-550 mm/year in the eastern parts. In the 1960s, cases of CL were low in the eastern part. From 1990 to 2000, [15] noted an abundance of potential vectors of CL in the eastern and central parts of the JD when, according to the PMOH, there was an increase in CL cases. The latter could be explained by lifestyle changes, e. g., ceasing burning animal manure for cooking, which used to produce heavy smoke that kept sand flies away.
Infection rates appear unrelated to altitude but are related to specific topographical and climatic characteristics of locations. For example, from 1990 to 2000, 135 cases and from 2002 to 2009, 288 cases, respectively, were recorded from the vicinity of El Yamoon and Silat El Hartheyia, two neighbouring towns at an altitude of 140-160 m in the northwestern part of the JD. At the same time, *P. sergenti*, the putative vector of *L. tropica*, was the most abundant sand fly species. Most cases were from the Mountain of Abu Zreek, an undeveloped area between El Yamoon and Silat El Hartheyia. Only three cases were recorded from Deir Abu Da'if in the eastern part of the JD at an altitude of 200 m, from where 32 cases had been recorded between 1990 and 2000 [15]. During the intervening period, rainfall dropped considerably causing drought conditions, which were more severe in the eastern part.
Till 1983, no cases had been reported from Qabatya but later 62 cases that were diagnosed clinically. Here, 66 cases from Qabatya were diagnosed parasitologically and/or by molecular biological methods. The occurrence of cases from new foci in Meselya, Jalbun, Hashemeya, Araqa and Arabeh could be related to either the introduction of the vector and/or the arrival of reservoir animals from existing foci. Caves and cervices in the rocks, piles of wood and stones, and stone walls around houses on the periphery of towns and villages, supposedly, offer good conditions for sand flies to rest

and, possibly, breed. Rock hyraxes, *Procavia capensis*, are considered the natural animal reservoir of CL caused by *L. tropica* [3]. The piles of boulders cleared from arable land provide a perfect habitat though fewer in the central parts of the residential areas where hyraxes have also been seen. Towns expanded massively during the 1990s. New houses were constructed at town peripheries where sand flies are more abundant. Peripheral areas receive poorer services, e. g., less refuse collection, exposed wastewater and solid waste dumps that attract stray dogs and wild animals, including wild canines possibly serving as reservoir hosts.

Cases of CL caused by *L. tropica* in the JD occur sporadically at the periphery of human habitation and are unlikely to be anthroponotic. This rather suggests zoonotic transmission as already described in Israel [3].

Here, the increase of the annual incidence between the peaks of infection in the years 2002 and 2008 might indicate a 6- to 7-year cycle. Prior to this and taken from older public health records, there was a peak in the number of cases in 1995, where the incidence reached almost 16 per 100,000, declining to 1.9 per 100,000 by 2000 and 2001, possibly, modulated by more efficient spraying against sand flies after a year of more cases. All age groups were involved with more cases among children that increased with age. More males aged 20 to 39 were affected. Most lesions were on the head and upper limbs, owing to human behavior during summer months, when people spend the early evening outdoors. This is also the sand fly season. People do not use bed nets. Females were more often infected on their faces than males, who are more often infected on their hands and upper limbs. This gender difference is statistically significant and is, probably, associated with dress codes, type of employment, and behavior between age groups and between males and females.

Clinics of the PMOH did not differentiate between CL caused by *L. major* and *L. tropica*. *L. major* was accepted as the cause of all cases of CL which is called Jericho boil in Palestine and the area around Jericho is a classical focus of CL caused by *L. major*. This study, taking account of the need to differentiate leishmanial species, attempted to combine detection of leishmanial parasites in biopsy aspirates by a kDNA PCR with species identification by an ITS1 PCR-RFLP.

The discrepancy between the high percentage (97.3%) of positive samples exposed by the kDNA PCR compared with the low percentage (53.9%) exposed by the ITS1 PCR is probably due to the higher copy number of this target, ca. 10^4 minicircles per parasite [9] and perhaps also to the shorter length of the amplification product [6].

Most biopsies were positive for CL by the kDNA PCR but species identification was achieved in only 53.9% of the PCR positive samples. Most of the CL cases from the JD were caused by *L. tropica*, as confirmed by PCR-RFLP and EF serotyping for all strains, and MLEE of nine strains. Eleven of the 24 cases of CL diagnosed in local clinics as having been caused by *L. major* said that they had visited known foci of *L. major* in the Jordan Valley and the Negev area. One was unsure of the geographical origin and 12 maintained that they had not travelled to areas where *L. major* circulates. Whether *L. major* parasites are present at low level in the JD or whether these 12 patients got infected by *L. major* through the bites of sand flies that had fed on other human cases caused by *L. major*, who had contracted their infections in well-known foci of *L. major*, remains to be established.

Four cases of CL were caused by parasites of the '*L. donovani* complex', supposedly, *L. infantum*. These patients said they had not travelled out of the JD. CL caused by *L. infantum* is well-known in other Mediterranean countries. However, until this survey, *L. infantum* had caused only human infantile and canine VL in the JD. This was the first record of CL caused by *L. infantum* in a Palestinian focus.

The species composition of sand flies collected in the JD during 2008-9 showed that *P. sergenti*, the proven vector of *L. tropica* in several countries, including neighbouring Israel [4], and the putative vector in Palestine, was the most abundant species. This correlated well with the occurrence of human cases of CL. *P. tobbi* and *P. perfiliewi* the proven vectors of *L. infantum* in other countries were also caught in the JD. *P. papatasi*, the proven vector of *L. major* throughout North Africa and the Middle East was also caught. *Psammomys obesus*, the known animal reservoir of *L. major* does, however not exist in the JD. In Israel, about 12 km from the JD, a new focus of CL caused by *L. major* has emerged where *Ps. obesus* is not present but the parasites were detected in female *P. papatasi* and rodents, i. e., voles (*Microtus guentheri*) and jirds (*Meriones tristrami*) (Fainman et al., personal communication).

Regarding human CL in the JD, the following limitations are brought to readers' attention. This study was based on people with cutaneous lesions visiting physicians and clinics and being defined clinically as cases of CL. However, CL is, usually chronic, self-limiting and self-curing and many cases do not bother to visit physicians and attend clinics so the numbers of cases referred to here are probably lower than actually occurred during 2002-9. An active home by home survey and referral of suspected cases to clinics for diagnosis and parasite identification would rectify this. Publication of information like that presented here should help to create greater awareness and, thus, improve future studies and assessments. Some patients with CL caused by *L. major* denied visiting any areas where *L. major* circulates. Possibly, because, as stated above, they do not understand that even a brief visit, i. e., driving through and just stopping at a viewing point or roadside café at the right/wrong time is sufficient to get bitten by an infected sand fly. Physicians must be sure to point this out to their patients. The epidemiological study was based wholly on human cases. No leishmanial strains have been isolated from sand flies or animals that might have proven to be vectors and reservoir hosts. Nor was DNA extracted from sand flies that might have implicated species of sand flies as vectors of any of the species of *Leishmania* causing human CL. The molecular biological diagnostic tests applied here to human clinical samples might have proved as useful in this as it did in the diagnosis of human cases. The sand fly collections referred to here could be used for this in a further study.

5.2 7SL PCR RFLP and PCR RDB diagnostic assays

Classical methods, such as microscopic examination of Giemsa-stained slides and in-vitro culture, have many limitations for leishmaniasis diagnosis as they lack sensitivity and do not differentiate the *Leishmania* species. Some PCR RFLP approaches depend on the detection of smaller fragments, as differentiation between *L. tropica* and *L. infantum* in ITS1 PCR RFLP [8], which may become difficult in case of weak amplification products.

A new PCR assay has been established for identification of the infecting agent of CL at

species level by amplification of partial sequences of the 7SL RNA gene followed by either RFLP or reverse dot blot hybridization (RDB). It allows differentiation between the 3 *Leishmania* species, *L. major*, *L. tropica* and *L. infantum* that are most prevalent in the Mediterranean region and overlap geographically in Eastern and Southern Mediterranean countries, including Palestine and Israel. Digestion of the 7SL amplicon with the restriction enzyme *BSuRI* differentiates between them. It was possible to rely just on the sizes of the upper fragments to safely distinguish them. .
The RDB could reliably distinguish the species *L. infantum*, *L. major* and *L. tropica* using three different probes, but also provided a ten-fold increase in the sensitivity of detection. The genus-specific probe gave positive hybridization signals with all species of *Leishmania* tested. Probe Lmt was specific for *L. major*, *L. tropica* and *L. aethiopica*. *L. major* was detected by two specific probes; Lm1 or Lm2. Differences were seen in the intensity of colorimetric signals for the two *L. major* specific probes. This may be related to different structures of the single stranded DNA that may hinder the probe annealing. The high sensitivity of the PCR-RDB makes it a very promising diagnostic test, although kDNA-PCR was still slightly superior for the detection of leishmanial parasites in clinical samples. This is not surprising because kinetoplast minicircle DNA is a multicopy target whereas 7SL is a single copy gene. The 7SL PCR-RDB assay has the advantage over the kDNA-PCR that it can directly determine the species and offers the possibility of diagnosing large numbers of samples. Blots are reusable; and simplicity and rapidity can be maximized. Dot blot preparation, hybridization and detection can be completed within a day. The RDB is more convenient for species identification than RFLP since it is more sensitive, can be used in case of weak amplification product and does not require electrophoretic equipment and toxic chemical agents.
L. tropica or *L. major* were the causative agents in all the CL cases diagnosed by the 7SL PCR, which was expected because in Palestinian and Israeli foci CL was so far thought to be caused exclusively by these two species. In our study, *L. infantum* was shown to be another causative agent of CL in the JD. It was also possible to identify species of *Leishmania* in samples from dogs that could not be diagnosed by PCR-RFLP. All dogs had symptoms of VL and were infected by *L. infantum* except for one that was infected by *L. tropic*a as shown by the 7SL PCR-RFLP and RDB assays. This was the first identification of *L. tropica* in dogs in the region encompassing Israel and Palestine where both CL due to *L. tropica* and VL due to *L. infantum* are prevalent.

5.3 PCR assays and their use as a 'gold standard' in diagnosing Old World cutaneous leishmaniasis.

When three different PCR assays, namely 7SL PCR RFLP , ITS1 PCR RFLP and kDNA PCR were compared using DNA samples extracted from the same series of tissue aspirates, kDNA PCR was the most sensitive assay, followed by the 7SL PCR assay and then the ITS1 PCR . Sensitivity of the PCR seems to depend mainly on the target used for amplification. From a statistical and epidemiological point of view, calculations of sensitivity, specificity and predictive values should ideally include results obtained for a true negative cohort from the same endemic area or, since this is difficult in the case of leishmaniases, from a non-endemic area. Unfortunately we had no access to samples obtained from healthy non-endemic volunteers.

The ITS1 PCR and 7SL PCR assays both use genus-specific PCR primers and species identification is done by RFLP or hybridization to specific probes as in the RDB. The 7SL PCR assay proved more sensitive than the ITS1 PCR assay for, both, the diagnosis of CL and the species identification. Species identification by the ITS1 PCR RFLP depends on differences in small restriction fragments while the 7SL PCR-RFLP depends on bigger fragments that are more informative in species differentiation. The 7SL PCR-RFLP assay identified the leishmanial species in 85.9% of the samples; the ITS1 assay in only 73.7%. *L. tropica* was always the most dominant species detected.

The evaluation of new diagnostic methods has the problem of not having a good gold standard. The WHO considers a CL cases to be confirmed by either positive microscopy or *in-vitro* culture or both (WHO Recommended Surveillance Standards, 1999). This definition of a gold standard is, however problematic since both methods have medium to low sensitivity, with culture being least sensitive. PCR is most efficient in detecting symptomatic and asymptomatic infections and, therefore, considered as possible gold standards for the diagnosis of CL and VL. PCR approaches using different genomic and kDNA targets are gradually replacing the traditional methods for diagnosing leishmaniases in laboratories and clinics [5].

The mode of preserving samples and the time of storage until use were shown to affect the results of the ITS1 [6]. Possibly, the number of parasites in samples was too few for detecting their DNA by the ITS1 PCR, although that number was sufficient for detecting their DNA by the kDNA and 7SL PCR assays. The sensitivity of ITS1-PCR can be improved by using a nested PCR approach [8]. Nested PCRs are, however very risky for routine clinical diagnosis owing to the high risk of contamination.

5.4 Characterization of strains of *L. tropica* from foci in northern Palestine and discovery of zymodeme MON 307

Leishmania tropica is a very heterogeneous species and its intraspecific micro-heterogeneity has been demonstrated by several investigators in different ways and examining various parasite constituents. Three methods were used in this study for the characterization of strains of *L. tropica*: EF serotyping [13]; kDNA analysis [12]; and MLEE [14] which all confirmed the main sub-division previously detected by multi-locus microsatellite typing (MLMT) [7]. Two of these methods, EF serotyping and kDNA RFLP are however, much easier and quicker to perform. Furthermore, EF serotyping and MLEE revealed that the strains assigned to different genetic groups present different phenotypes which, possibly, reflect differences in leishmanial parasite – sand fly vector interrelationships.

kDNA-RFLP analysis enabled the separation of *L. tropica* from the JD into the kDNA Clades *Ltro*-kD1 and *Ltro*-kD2, respectively. The analysis of the kDNA RFLP profiles also exposed further micro-heterogeneity and differentiation whereby three different sub-types were detected in Clade *Ltro*-kD1 and six in Clade *Ltro*-kD2. Furthermore, this micro-heterogeneity among the mini-circle kDNAs showed partial congruity with the local geographical distribution of the cases from which these strains of *L. tropica* were isolated.

The results of EF serotyping correlated with those of the molecular biological analyses just mentioned, showing that the 12 strains from JD analyzed herein were strains of *L. tropica* of two antigenic types and exposed two serological sub-groups that coincided

exactly with the two sub-groups delineated by kDNA- RFLP analysis. This serological sub-division was based on the A component of strains' sub-serotypes: one sub-group possessing the EF sub-serotype A_2; the other the EF sub-serotype A_9 or A_9B_4 In fact, also the presence of the B_4 component in the EF sub-serotype of some of the strains in the latter sub-group but not in other strains in this sub-group indicates a further antigenic sub-division. It is interesting to note that antiserum raised in rabbits against living promastigotes of the strain *L. donovani*, LRC-L52 has never before differentiated serotype B components of EFs into B sub-serotypes except in the case of strains of *L. tropica* of the mixed sub-serotype A_9B_4 as shown in this study. Where enzyme profiles have also been determined, the B_4 component seems to be associated solely with strains of *L. tropica* belonging to the zymodeme MON-137 and the B_4 component, it seems, is always present together with the A_9 component.

EFs are glyco-conjugates, bearing antigenic carbohydrate moieties that confer serological specificity on the different antigenic types of leishmanial parasite, which, approximately, coincides with leishmanial species designations. For example, a strain of *L. tropica* from a focus just north of the Sea of Galilee, characterized as belonging to zymodeme MON-265 and EF sub-serotype A_4, survived and grew in female sand flies of the sub-species *Phlebotomus* (*Adlerius*) *arabicus* [16], a proven vector in that focus, but did not survive in female sand flies of the sub-species *Ph.* (*Paraphlebotomus*) *sergenti*, the proven vector in other Israeli foci. Conversely, a strain of *L. tropica* from a more southerly focus near Tiberias, producing EF of the sub-serotype A_9B_4 and, therefore, probably belonging to the zymodeme MON-137 survived and grew well in females of both sand fly species. In this case, two very different vectors were involved in the transmission of the two different sub-types of *L. tropica*, supposedly, associated with ecological differences in the two habitats from which the two different sand fly species and two sub-types of *L. tropica* came. Since the strains of *L. tropica* of both EF sub-serotypes A_4 and A_9B_4 survived in the sand fly species *Ph. (A.) arabicus*; but only the strain producing EF of the sub-serotype A_9B_4 survived in *Ph. (P.) sergenti*, one might assume that the lack of survival of the strain of the EF sub-serotype A_4 in *Ph. (P.) sergenti* is a parasite-based effect rather than a sand fly vector-based one. EF sero-typing of 12 Palestinian strains of *L. tropica* revealed that they were of two serological/antigenic types: EF sub-serotype A_2, congruent with zymodeme MON-307 and the minicircle DNA sequence *Ltro*-kD1, and serotype A_9B_4, congruent with zymodeme MON-137 and the minicircle sequence DNA *Ltro*-kD2. Each of the two EF types is, supposedly, bearing a surface lipophosphoglycan with the same antigenic determinants that enables successful attachment in their specific natural vector. This indicates the possibility that two sand fly species, or just sub-species, might be involved in transmitting the two Palestinian types of *L. tropica* in the foci of CL in the JD. It would be interesting to identify the sand fly vector(s) transmitting the two sub-types of the Palestinian strains of *L. tropica* described in this study to know if they are transmitted by the same vector or each sub-type of *L. tropica* has its own species or even just sub-species of the same vector.

Despite the introduction of many new methods for characterizing and identifying strains of *Leishmania*, MLEE is still the widely accepted standard means for differentiating leishmanial species and exposing biochemical micro-heterogeneity and interrelationships among strains, within the species. The report given here is the first on the enzyme analysis of Palestinian strains of *L. tropica* and the zymodemes to which they belong. Ten

of the 12 Palestinian strains were subjected to MLEE that also showed the strains were strains of *L. tropica*, according to the electrophoretic mobilities of certain enzymes in their enzyme profiles, notably NP1 and GOT1. The electrophoretic mobilities of other enzymes in their enzyme profiles separated the ten strains into two sub-groups: seven belonged to the zymodeme MON-137; three to the new zymodeme MON-307, which are the reference strains of this zymodeme. These sub-groups were fully congruent with those identified by kDNA analysis, EF serotyping and the MLMT done by Schwenkenbecher [7]**.** Interestingly, the strains of *L. tropica* belonging to zymodemes MON-137 and MON-307 overlapped geographically within the limited region from where the strains came.

Of the 15 enzymes used in formulating the enzyme profiles, one, fumarate hydratase had an electrophoretic mobility (FH^{105}) different from the electrophoretic mobilities of all the known variants of FH found so far among strains of *L. tropica* examined by MLEE. However, FH of this mobility (FH^{105}), while being unique to the strains of *L. tropica* belonging to the zymodeme MON-307, has been found in all the strains of *L. aethiopica* analyzed by this method of MLEE [14]. The seven Palestinian strains belonging to the zymodeme MON-137 were definitely *L. tropica* and strains of *L. tropica* belonging to this zymodeme also occur in Israel [4]; the Sinai Peninsula, Egypt, and Jordan [14]. The UPGMA dendrogram indicates that strains belonging to zymodeme MON-137 have a more marginal enzyme profile than most other strains of *L. tropica* and are closer to strains of *L. tropica* (syn. *L. killicki*), from Tunisia in the zymodeme MON-8 and strains of *L. tropica* from Algeria, Yemen and Kenya in the zymodemes MON-301, MON-71 and MON-119, respectively, that are all found in cluster (a) of the dendrogram. Pratlong [14] showed that strains of *L. tropica* belonging to the zymodemes MON-137 and MON-265, also found in Israel and Jordan , clustered more closely with one another than they did with any of the other strains falling into their '*L. tropica* complex' despite significant differences in geographical distribution, sand fly vector specificity, EF serotypes, enzyme profiles and some of their molecular biological criteria [4, 7, 16]. Correspondingly, strains of the zymodemes MON-137 and MON-265 subjected to MLMT clustered together in the sub-population I: Middle East, but those of zymodeme MON-137 belonged to the microsatellite sub-sub-groups A1a and A1b and those of zymodeme MON-265 belonged to the sub-sub-group A2b, a grouping into which no Palestinian strains fell. Combining results from MLEE and MLMT showed that the zymodeme MON-137 encompassed strains in the microsatellite sub-sub-groups A1a and A1b and zymodeme MON-307 encompassed strains in the sub-sub-groups B1a and B1b, demonstrating the greater discrimination of MLMT. A similar degree of discrimination was previously shown for strains of the zymodemes of *L. infantum*, which were studied using MLMT and *L. donovani*, which were studied using multilocus sequence typing (MLST) [5]. Strains with the same enzyme profile are not necessarily a genetically monolithic group. Enzymes of identical electrophoretic mobility can have different amino acid sequences, and that the resulting changes in molecular mass and charge can in some cases balance one another, conferring the same electrophoretic mobility, and indistinguishable zymodemal phenotypes can result from distinct genotypes.

Strains of zymodeme MON-137 seem to be restricted to the western part of the Middle East and those of zymodeme MON-307 appear unique to the northern part of the Palestinian West Bank but, on comparing enzyme profiles and microsatellite profiles of

strains of *L. tropica*, those of the new zymodeme MON-307 are taxonomically closer to strains of *L. tropica* that are geographically much more distant from the foci in the JD. Only three enzymes had different mobilities (MDH^{112}, $GLUD^{95}$ and FH^{110}) in the 15 enzyme profile of Syrian strains of zymodeme MON-76, compared with these enzymes (MDH^{100}, $GLUD^{80}$ and FH^{105}) in the 15 enzyme profile of strains of zymodeme MON-307; and only three enzymes had different mobilities ($GLUD^{95}$, PGM^{100} and FH^{100}) in the 15 enzyme profile of some Lebanese and Iraqi strains, compared with these enzymes ($GLUD^{80}$, PGM^{108} and FH^{105}) in the 15 enzyme profile of strains belonging to the zymodeme MON-307**.** This might suggest importation of this type of *L. tropica* but it is sufficiently different from its geographically distant relatives to think otherwise.
The enzyme and microsatellite profiles of the strains LRC-L888 and -L1324 in kDNA Clade B are not known. However, since the nine strains belonging to the zymodeme MON-137 fell into the kDNA Clade B, had microsatellite profiles that fell into the sub-group A_1, and either the EF serotype, A_9 or A_9B_4, one can surmise that the strains LRC-L888 and -L1324 would belong to the zymodeme MON-137.
kDNA analysis, EF serotyping and MLEE, were able to split the 12 Palestinian strains into the same two sub-groups as could MLMT. The presence of two separate sub-types of *L. tropica*, possibly, indicates two separate transmission cycles involving two separate types of phlebotomine sand fly vector. Further research should be directed at collecting potential vectors, checking for infections and isolating and characterizing leishmanial isolates from them to clarify this and to see if these leishmaniases are anthroponoses or zoonoses by searching for infected domestic, e. g., dogs, and wild, e. g., hyraxes, animals.

Conclusions

Cases of CL have been increasing in the northern part of the West Bank region and some areas have a higher density of infections than others. Most cases have been caused by *L. tropica*. Cases caused by *L. major* occurred occasionally and were, probably, acquired in foci outside the JD. Four cases of CL, without concomitant signs of VL, were found to be caused by *L. infantum.* The existence of three species of *Leishmania*, *L. tropica*, *L. major* and *L. infantum*, in close proximity, makes species identification essential in the diagnosis of CL. The introduction of new and fast methods of diagnosis and consecutive identification of their causative agents enables a different, more precise approach to prognosis, treatment as and response to the latter. It will also play an important role in deciding control strategies. PCR RFLP and PCR RDB assays are valuable tools for identification of the parasites from culture and in naturally infected clinical samples with the PCR-RBD test being more sensitive and easier to perform. Compared with the ITS1 PCR RFLP, the 7SL PCR RFLP assay was more sensitive and reliable for diagnosing leishmaniases and detecting and identifying leishmanial parasites in clinical samples. The kDNA PCR assay is recommended for confirming truly negative samples.
A high degree of congruity was seen among the results of the three methods used here to characterize and differentiate Palestinian strains of *L. tropica*: EF serotyping; MLEE; and analysis of kDNA mini-circle sequences, and also with those from MLMT that was applied in a previous study, even though the types of criteria examined were very different from one another, having been derived from different cellular components with different intra-cellular origins within the parasites. The results from the kDNA analysis

corresponded well with those of MLMT and could, possibly, replace the more complicated and labor intensive application of MLMT to leishmanial parasites from this area. The Palestinian strains that were assigned to different genetic groups also differed in their MLEE profiles and their EF types. A novel outcome of this study was the discovery of a new zymodeme, MON-307 that, at this time, seems to be unique to the northern part of the Palestinian West Bank. As two sub-types of *L. tropica* are circulating in the JD, future studies could investigate whether they are transmitted by the same sand fly species or indicate the presence of two different transmission cycles, involving separate sand fly vector species; and whether the two different phenotypes reflect differences in the biological functions of the two parasitic sub-types, such as virulence, in different human hosts and attachment and survival in sand fly vectors.

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Nr.	Publikation	Impact factor
1	***K. Azmi***, *G. Schonian, A. Nasereddin, LF. Schnur, S. Sawalha, O. Hamarsheh, S. Ereqat, A. Amro, SE. Qaddomi, Z. Abdeen.* Epidemiological and clinical features of cutaneous leishmaniases in Jenin District, Palestine, including characterization of the causative agents in clinical samples. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2012 Sep; 106(9): 554-62.	**2.443**
2	***K. Azmi,*** *A. Nasereddin, S. Ereqat, G. Schönian, Z. Abdeen.* Identification of Old World *Leishmania* species by PCR-RFLP of the 7 spliced leader RNA gene and reverse dot blot assay. Trop Med Int Health. 2010 Aug; 15(8): 872-80.	**2.795**
3	***K. Azmi***, *A. Nasereddin, S. Ereqat, LF. Schnur, G. Schonian, Z. Abdeen.* Methods incorporating a polymerase chain reaction and restriction fragment length polymorphism and their use as a 'gold standard' in diagnosing Old World cutaneous leishmaniasis. Diagn Microbiol Infect Dis. 2011 Oct;71(2): 151-5.	**2.528**
4	***K. Azmi***, *L. Schnur, G. Schonian, A. Nasereddin, F. Pratlong, F. Baidouri, C. Ravel, JP. Dedet, S. Ereqat, Z. Abdeen.* Genetic, serological and biochemical characterization of Leishmania tropica from foci in northern Palestine and discovery of zymodeme MON-307. Parasites and Vectors. 2012. 18;5: 121.	**2.94**

Prof. Dr. Wolfgang Presber **Kifaya Azmi**

Affidavit

I, Kifaya Azmi Suleiman certify under penalty of perjury by my own signature that I have submitted the thesis on the topic „Development and application of molecular diagnostic assays in the epidemiology of cutaneous leishmaniasis in the Jenin District“. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date: 26/01/2013 ______________________________ Signature

Declaration of any eventual publications

Kifaya Azmi Suleiman had the following share in the following publications:

Publication 1:
K. Azmi, G. Schonian, A. Nasereddin, LF. Schnur, S. Sawalha, O. Hamarsheh, S. Ereqat, A. Amro, SE. Qaddomi, Z. Abdeen. Epidemiological and clinical features of cutaneous leishmaniases in Jenin District, Palestine, including characterization of the causative agents in clinical samples. Trans R Soc Trop Med Hyg. 2012; 106(9): 554-62.
Contribution in detail: K. Azmi is the ***corresponding author*** of this paper. She designed the study, was involved in the collection of samples, prepared media for isolating and culturing of parasites, extracted DNA from cultures and filter papers, carried out all the techniques including typing the strains by ITS1-RFLP, collected and analyzed the demographic data and interpreted the data, and drafted the manuscript.

Publication 2:
K. Azmi, A. Nasereddin, S. Ereqat, G. Schönian, Z. Abdeen. Identification of Old World *Leishmania* species by PCR-RFLP of the 7 spliced leader RNA gene and reverse dot blot assay. Trop Med Int Health. 2010;15(8): 872-80

Contribution in detail: K. Azmi is the ***corresponding author*** of this paper. She designed the study, developed and performed the7SL PCR RFLP technique with cultured strains and clinical samples, designed the probes, prepared the membranes, did the hybridization, analysed, and compiled the results, and drafted and revised the manuscript.

Publication 3:
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Contribution in detail: K. Azmi is the ***corresponding author*** of this paper. She designed the study, was involved in the collection of samples and, extraction of DNA, performed the kDNA PCR-RFLP, analysed and interpreted the data, and drafted and revised the paper.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Publications:

K. Azmi, *G. Schonian, A. Nasereddin, LF. Schnur, S. Sawalha, O. Hamarsheh, S. Ereqat, A. Amro, SE. Qaddomi, Z. Abdeen.*
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Complete list of publications

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