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DISSERTATION

Leishmania tropica: Molecular Epidemiology, Diagnosis and Development of an Axenic Amastigote Model

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List of abbreviations

-'3UTR	3'-untranslated region
AxA	axenic amastigotes
CL	cutaneous leishmaniasis
cpb	cysteine protease B
CVL	canine visceral leishmaniasis
DNA	deoxyribonucleic Acid
EDTA	ethylene diamine tetra acetic acid
FCS	fetal calf sera
HCl	hydrochloric acid
IFA	immune fluorescence assay
ITS	internal transcribed spacer
kDNA	kinetoplast-DNA
L.	*Leishmania*
LPG	lipophosphoglycan
NaOH	sodium hydroxide
PCR	polymerase chain reaction
Ph.	Phlebotomus, sand fly
Pkac1	protein kinase A catalytic subunit isoform 1
pmol	picomole
RFLP	restriction fragment length polymorphism
RLB	reverse line blot
RNA	Ribonucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SSC	sodium citrate
TAE	Trisacetate-EDTA
THP-1	human acute monocytic leukemia cell line
VL	visceral leishmaniasis

1. Abstract

Cutaneous Leishmaniasis (CL), or oriental sore, is mainly caused by *Leishmania tropica* and *L. major* in the Old World. CL due to *L. tropica* has become a major public health problem in different endemic foci due to recent outbreaks in several urban areas. The disease caused by *L. tropica* presents varying clinical manifestations and complications. These parasites may differ in drug susceptibility, vector and animal host specificities, and show considerable genetic heterogeneity.

In this study, a new diagnostic method was developed for the identification of *Leishmania* parasites which is based on the amplification of the ribosomal internal transcribed spacer 1 (ITS1) region followed by hybridization with species-specific probes and colorimetric detection of the hybrids (ITS1-Reverse line blot hybridization (RLB)). Using 3 species-specific probes for *L. tropica*, we were able to differentiate this parasite from the other species present in the Middle East, *L. infantum* and *L. major*, both of which co-exist with *L. tropica*. The assay was 10- to 100-fold more sensitive compared to previously used detection of PCR products on gels. The *Leishmania* RLB was used to diagnose samples from suspected CL patients in Israel and the Palestinian areas. In addition, the geographical distribution of *L. tropica* parasites was investigated in Israel, Palestine, Turkey and Morocco using the ITS1-restriction fragment length polymorphism (RFLP). This parasite was shown to cause human disease in > 15 foci in Israel and Palestine, 6/10 localities in Turkey, and in 3/6 regions investigated in Morocco.

To facilitate further studies on the intracellular amastigote form of *L. tropica*, conditions to grow axenic amastigotes were developed. Different techniques including light microscopy, macrophage infection, stage-specific antigen expression and differential display were used to characterize the *L. tropica* axenic amastigotes and compare them with the promastigote stage that resides in the sand fly vector, and with tissue amastigotes that were obtained from infected macrophages. We were able to demonstrate that pH of 5.5 and temperature $36^{o}C$ were most suitable for generating and maintaining long term cultures of axenic amastigotes (AxA). These AxA were morphologically similar to tissue amastigotes and > 15-fold more infective than stationary phase promastigotes. Western blot analysis showed that promastigote-specific monoclonal antibodies to lipophosphoglycan or flagella antigen were either absent or poorly expressed in AxA, while an amastigote-specific antibody reacted strongly with the AxA. Differential display – PCR analysis used to examine stage

specific gene expression detected amastin a gene normally expressed by amastigotes. Reverse transcriptase PCR (RT-PCR) was used to compare the expression of several genes in promastigotes, AxA and tissue amastigotes. The expression of cysteine protease B (*cpb*) and amastin genes, both highly expressed in tissue amastigotes and AxA, was down-regulated or absent, respectively, in promastigotes. Conversely, the gene for protein kinase A catalytic subunit isoform 1 (*pkac1*), a promastigote stage specific gene, was strongly expressed by the extracellular stage of the parasite and not expressed by AxA or tissue amastigotes. AxA of *L. tropica* will be useful for high-throughput screening of new drugs as well as for studies on parasite differentiation, gene regulation and metabolism.

Zusammenfassung

Die kutane Leishmaniose (CL), oder Orientbeule, wird in der Alten Welt meist durch *Leishmania tropica* und *L. major* hervorgerufen. Erkrankungen bedingt durch *L. tropica* sind in letzter Zeit durch Ausbrüche in mehreren urbanen Regioene zu einem wichtigen Gesundheitsproblem in verschiedenen Endemiegebieten geworden. Die durch *L. tropica* hervorgerufenen Erkrankungen zeichnen sich durch variierende klinische Manifestationen und Komplikationen aus. Die Parasiten können sich in ihrer Suszeptibiltät gegenüber verschiedenen Therapeutika sowie ihrer Spezifität gegenüber Vektoren und tierischen Wirten unterscheiden und sind genetisch sehr heterogen.

In dieser Arbeit wurde eine neue diagnostische Methode für die Identifizierung von *Leishmania*-Parasiten entwickelt, die auf der Amplifizierung des ribosomalen „internal transcribed spacer 1 (ITS1)"mit anschließender Hybridisierung an spezies-spezifische Sonden und kolorimetrischer Detektion der Hybride beruht (ITS1-Reverse line blot hybridization (RLB)). Durch die Verwendung von 3 spezies-spezifischen Sonden für *L. tropica*,konnten wir diesen Parasit von den anderen im Mittleren Osten vorkommenden Spezies, *L. infantum* and *L. major*, unterscheiden, die beide mit *L. tropica* koexistieren. Die Methode war 10- bis 100-fach empfindlicher als der früher angewendete Nachweis der PCR-Produkte im Gel. Der *Leishmania*-RLB-Test wurde für die Diagnostik bei israelischen und palästinensischen Patienten mit Verdacht auf CL eingesetzt. Zusätzlich wurde die geographische Ausbreitung der *L. tropica*-Parasiten in Israel, Palästina, der Türkei und Marokko mit Hilfe von ITS1-Restriktionsfragmentlängenpolymorphismen (RFLP) untersucht. Es konnte gezeigt

werden, dass dieser Parasit Erkrankungen des Menschen in > 15 israelischen und palästinensischen Foci, in 6/10 Orten in der Türkei und in 3/6 untersuchten Regionen in Marokko hervorruft.

Für zukünftige Studien an dem intrazellulären Stadium (amastigote Form) von *L. tropica* wurden Bedingungen für die Anzucht axenischer Amastigoten entwickelt. Verschiedene Techniken, wie Lichtmikroskopie, Makrophageninfektion, stadien-spezifische Antigenexpression und der "differential display", wurden benutzt, um die axenischen Amastigoten *von L. tropica* zu charakterisieren und mit den Promastigoten, die im Sandmückenvektor vorkommen, und den Gewebeamastigoten, die in infizierten Makrophagen zu finden sind, zu vergleichen. Wir konnten zeigen, dass ein pH von 5.5 und eine Temperatur von 36$^{\mathrm{o}}$C für die Herstellung und Erhaltung von Langzeitkulturen axenischer Amastigoten (AxA) am besten geeignet sind. So erhaltene AxA waren den Gewebeamastigoten morphologisch ähnlich und > 15-fach infectiöser als Stationäre-Phase-Promastigoten. Western-blot-Analysen ergaben, dass Promastigoten-spezifische monoklonale Antikörper für Lipophosphoglykan oder ein Flagella-Antigen in AxA entweder fehlten oder nur schwach exprimiert waren während ein amastigoten-spezifischer Antikörper stark mit den AxA reagierte. Die „differential display – PCR"-Analyse, die für die Untersuchung der stadienspezifischen Genexpression angewandt wurde, wies in den AxA die Expression eines normalerweise Amastigoten-spezifischen Proteins, des Amastin, nach. Mit Hilfe der „reverse transcriptase" PCR (RT-PCR) wurde die Expresssion verschiedene Gene in Promastigoten, AxA und Gewebeamastigoten verglichen. Die Expression der Gene für die Cysteinprotease B (*cpb*) und Amastin, beide stark in Gewebeamastigoten und AxA exprimiert, war in Promastigoten herab reguliert oder nicht nachweisbar. Umgekehrt wurde das Gen für die katalytische Untereinheit Isoform 1 der Proteinkinase A (*pkac1),* welches ein Promastigoten-spezifisches Gen ist, durch das extrazellulären Stadium des Parasiten stark exprimiert und nicht durch AxA oder Gewebeamastigoten. AxA von *L. tropica* können für ein "high-throughput screening" neuer Medikamente sowie auch für Untersuchungen zur Differenzierung, zur Genregulation und zum Metabolismus der Parasiten eingesetzt werden.

2. Introduction

The leishmaniases are a spectrum of different diseases caused by more than 20 species and subspecies of parasites belonging to the genus *Leishmania*. Approximately 350 million people in 88 countries are exposed to these parasites which cause an estimated 12 million infections world-wide [1]. The clinical manifestation of leishmaniasis ranges from self-healing cutaneous lesions (cutaneous leishmaniasis - CL) through metastasizing mucocutaneous to potentially lethal visceral forms [2, 3]. CL threatens ~350 million people with an annual incidence estimated at 1-1.5 million cases CL and a prevalence of 12 million people [4]. The disability-adjusted life-years (DALY) lost due to CL are close to 1.0–1.5 million cases each year (http://www.who.int/tdrold/dw/leish2004.htm). *Leishmania tropica* causes CL and occasionally viscerotropic leishmaniasis (VTL) in endemic regions of the Old World [5, 6]. This parasite has been isolated from the bone marrow and spleens of patients with VTL and visceral leishmaniasis (VL), and from dogs with VL [7]. Visceralizing strains have been isolated from patients in Israel [8], Kenya [9], India [10], Iran [11], and also from veterans of operation desert storm who served in Saudi Arabia [12, 13].

It may also cause leishmania recidivans, a recrudescent infection that fails to heal completely with new lesions emerging at the edge of the scar tissue. CL caused by *L. tropica* usually manifests as dry, small lesions, mainly located on the face, which leave permanent scars and serious disability after healing, while lesions due to *L. major* tend to present as single or multiple "wet" ulcers.

Phlebotomine sand flies (Diptera: Psychodidae) act as vector(s) actively transmitting these parasites between human (the accidental host) and the animal reservoirs, though in some cases the disease is believed to be anthroponotic rather than zoonotic. The parasite exhibits a dimorphic life cycle. The extracellular promastigote form developing in the midgut of the sand fly vector is transmitted to human or animal hosts by the bite of infected female phlebotomine sand flies during their blood meal. Promastigotes are ingested by macrophages and, once inside, transform into the intracellular amastigote form and multiply. Eventually the number of intracellular parasites increases, the host cell bursts and releases parasites which then infect other phagocytic cells.

The geographical distribution of *L. tropica* extends from India throughout central Asia, the Middle East, and southeast Europe into north and equatorial Africa. Epidemics or outbreaks due to *L. tropica* in heavily populated cities were recently described in Afghanistan, Iran, Turkey and Syria where they caused extensive morbidity [14]. *L. tropica* has also been isolated from patients in rural areas of Kenya [15], Palestine [16], Jordan [17] and Israel [6]. In the latter foci, direct transmission between people is unlikely to occur and the parasite has been isolated from rats (*Rattus rattus*) and rock hyraxes (*Procavia capensis*) suggesting that these animals may be reservoir hosts [18]. Unlike *L. major* that only causes zoonotic CL, transmission of *L. tropica* is thought to be either anthroponotic or zoonotic depending on the local ecology.

CL caused by *L. tropica*, in contrast to *L. major*, appears to be more recalcitrant to treatment with drugs and generally takes longer to heal [19], [20]. Topical treatment of lesions with paromomycin/methylbenzethonium chloride ointment was completely successful in only 37.5 - 45% of the *L. tropica* patients compared to 76 - 88% of the patients with CL caused by *L. major*. Although systemic and intralesional treatment with antimonial drugs, such as sodium stibogluconate, is generally more successful for treating CL caused by *L. tropica*, this treatment is far from ideal [19, 20]. In addition, resistance to antimonials has been reported in *L. tropica* CL patients from Iran [21].

In Israel two vectors (*Phlebotomus sergenti* and *Ph. arabicus*) were reported for *L. tropica*, representing distinct transmission cycles for two different genetic types of *L. tropica* parasites [6, 22]. In addition, *L. tropica* intraspecific lipophosphoglycan (LPG) polymorphisms were correlated with transmission by different *Phlebotomus* species [23].

Leishmania species detection and identification is important for disease prognosis and prescribing appropriate treatment. Many species are associated with similar clinical pathologies, and some species cause several clinical forms of disease, thus different CL symptoms and species may overlap. In addition, CL is spreading into new regions previously free of disease where the disease may not be recognized in primary health clinics [6, 24]. Thus there is a need for new sensitive molecular methods for diagnosis rather the traditional ones such as microscopy, culture, clinical picture or travel/residence history, which all lack sensitivity and specificity [25]. Molecular techniques based on DNA amplification by PCR of various targets, either nuclear

DNA or kinetoplast DNA (kDNA), are gradually replacing standard classical methods in many laboratories [25, 26]. kDNA PCR using universal minicircle primers is considered the most sensitive diagnostic tool to date for detecting leishmaniasis [26]. Diagnostic PCR using the internal transcribed spacer 1 (ITS1) region, located between the 18S and 5.8S rRNA genes, is a sensitive and specific method for detecting *Leishmania* DNA in patients with CL or VL [25, 27]. By digesting the PCR product with restriction enzymes, it allows identification of almost all pathogenic *Leishmania* species, thus enabling direct, rapid characterization of the infecting parasite.

Studies on *L. tropica* have focused primarily on clinical description of disease pathology, treatment and diagnosis or characterization of promastigotes, the extracellular stage of the parasite. Few biological, biochemical, immunological and molecular biological analyses of *L. tropica,* especially when the intracellular amastigote stage is concerned, have been performed compared to other *Leishmania* species. Unlike many *Leishmania* species*,* convenient animal models were not available for *L. tropica* until recently. Even so, development of lesions in mice, rats and hamsters is slow or not apparent [5, 18, 28], and only few tissue amastigotes are produced. Axenic amastigotes (AxA) which are adapted to grow under *in vitro* conditions in the absence of macrophages, have been described for many *Leishmania sp*. [29]. Conditions for culturing *L. tropica* axenic amastigotes have been also described in brief [30], [31], but no characterization of these AxA was carried out. However, this would be required for using their great potential for expanding biological, biochemical and molecular studies on the intracellular stage of *L. tropica*.

Main Objectives:

1. To enhance diagnostic sensitivity and specificity of the ITS1-PCR assay for direct detection and identification of Old World *Leishmania* parasites by developing an improved detection method based on hybridization of the PCR product to species-specific probes in a reverse-line dot blot assay (RLB).

2. To apply and compare existing and new diagnostic assays for Old World leishmaniasis to samples from CL patients from different endemic regions in the Mediterranean Basin.

3. To develop and characterize *L. tropica* axenic amastigotes maintained in long-term cultures.

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3. Material and Methods

3.1 Development of ITS1-PCR RLB diagnostic assay

3.1.1. Samples and *Leishmania* reference strains

In total, samples were obtained from 180 patients referred to the Dermatology Department of the Hadassah Hospital, Jerusalem, with suspected CL between 2005-September 2009. Sixty-seven of these samples were analysed in our initial study [32]. All of the patients were infected in Israel or the West Bank region with most of the cases coming from Ma'ale Adumim or Kfar Adumim, two Israeli settlements 5 km east of Jerusalem. Of these 180 samples, 80 produced promastigotes in culture. In addition, four of the 105 female *Ph. sergenti* caught in this region produced promastigotes, three of which were used for classification: L747, L757, and L758. In addition, suspected CL cases from a new focus near Bethlehem (Tekoa, Nekodim and Herodion), 10 km south Jerusalem, were examined in 2009, of which 5 cultures were obtained.

In Morocco, tissue samples were taken from 27 patients with suspected CL that came from different parts of the country.

In Turkey, a total of 77 samples, 37 promastigote cultures and 40 tissue smears on slides, obtained from patients suspected to have CL were received from various localities in Turkey between the years 2006 and 2008 and were examined by ITS1-PCR followed by RFLP.

For DNA extraction, specimens were cut from filter paper with a disposable sterile scalpel and incubated in 250 µl cell lysis buffer for 1 hour at 56°C. 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 -20°C until use. DNA extraction using stained slides and cultures was performed as described by Schonian et al. [27].

The following strains were used as positive controls at appropriate stages: *L. donovani* MHOM/IN/1980/DD8, *L. infantum* MHOM/TN/1980/IPT1, *L. tropica* MHOM/SU/1974/SAF-K27, *L. major* MHOM/SU/1973/5ASKH.

3.1.2 Internal transcribed spacer 1 PCR - RFLP

Clinical samples were analyzed by ITS1-PCR using the primers: LITSR and L5.8S (400 nM each) [33]. The reaction was carried out using the PCR-Ready Supreme mix (Syntezza Bioscience, Jerusalem, Israel) in 25 µL total reaction. The amplicons, 300-350 bp, were analyzed on 1.5% agarose gels by electrophoresis at 100 V in 1X Tris-

acetate- EDTA buffer (0.04 M Tris acetate and 1 mM EDTA, pH 8.0) and visualized by UV light after staining with ethidium bromide (0.3 µg/ml). GeneRuler DNA ladder Mix (Fermentas, MBI) was used as DNA molecular marker. Amplification conditions were as described previously [33].

PCR products (8-15 µl) were digested with *Bsu*RI (Fermentas, MBI), a *Hae*III prototype according to the manufacturer's instructions. The restriction fragments were analyzed by gel electrophoresis at 120 V in 1X Tris- acetate- EDTA buffer (0.04 M Tris acetate and 1 mM EDTA, pH 8.0) on 4% agarose gels (FMC Bioproducts, Rockland ME) and visualized by UV light after staining with ethidium bromide (0.3 µg/ml). The size of the restriction products was determined.

3.13 ITS1-PCR followed by RLB

We took advantage of polymorphic DNA sequences in the ITS1 region to develop a reverse line blot hybridization assay (RLB) that allows the identification of multiple Old World *Leishmania* species simultaneously. Several species-specific probes were designed [32] and covalently coupled to negatively charged membranes (Biodyne C; Pall Life Sciences, MI) that were activated as described by Kong et al. [34].

Prior to use the membranes were rotated 90 degrees and cut into 0.5 cm strips such that each strip contains parallel lanes from each probe. These strips were washed and incubated separately at 46°C for 30 min in hybridization buffer (30 mM sodium citrate-0.3 M NaCl [pH 7.0] [2× SSC] containing 0.1% sodium dodecyl sulfate [SDS]). ITS1-PCR was carried out using the 5′-biotinylated primers LITSR (5′-CTG GAT CAT TTT CCG ATG-3′) and L5.8S (5′-TGA TAC CAC TTA TCG CAC TT-3′) essentially as described by Schonian et al. [27]. The biotinylated PCR products (20 µl) were boiled for 10 min and rapidly placed on ice (2 min). Hybridization was carried out by adding one PCR product per strip in 5 ml 2× SSC-0.1% SDS buffer for 1 h at 46°C. The membrane strips were then washed with 0.75× SSC-0.1% SDS for 30 min at 46°C and incubated with streptavidin conjugated to horseradish peroxidase (1:3,500 dilution in 2× SSC-0.1% SDS [Roche, F. Hoffmann-La Roche Ltd. Basel, Switzerland]) for 20 min at room temperature. After that the strips were washed three times, for 2 min each time, at room temperature with 2× SSC-0.1% SDS and then three times with 0.1 M sodium citrate buffer (pH 5.0). The positive reactions were detected after 10 min with a solution containing 3,3′,5,5′-tetramethyl benzidine (0.1 mg/ml; Sigma Life Science) in sodium citrate buffer containing 30% H_2O_2 (1/10,000 dilution).

3.2 Production and characterization of *Leishmania tropica* axenic amastigotes (AxA)

3.2.1 Optimization of conditions for the growth of AxA

The *L. tropica* strain (MHOM/IL/1990/P283, LRC-L590) was typed by serological, biochemical and molecular techniques [35]. Promastigotes were grown at 26°C in M199 or RPMI 1640 media plus 10% heat inactivated Fetal Calf Sera (FCS), pH= 6.88 (M199+ medium, [30]). Axenic amastigotes (AxA) were cultured in RPMI 1640 supplemented with 10% FCS as described by Debrabant et al. ([30], RPMI+ medium). The final pH of the medium was adjusted to 5.0 or 5.5 at each temperature examined and the pH effect was evaluated based on growth curve at each condition. Amastigote cultures were passaged once a week by 1:10 or 1:20 dilutions in 5 ml medium according to their growth and cell density [36].

3.2.2 Characterization of AxA

For light microscopy, parasite cultures were Giemsa stained and examined using a light microscope (Olympus Optical, NY, model BX60) X 1000 magnification and captured by Olympus DP1 camera.

Ability to infect mouse BALB/c peritoneal macrophages (MΦ) for both stages was determined using eight-chamber tissue culture slides (Lab-Tek Brand Products, USA) as described [37]. Data analysis was done using Prism 4.0b. Intracellular amastigotes of *L. tropica* were prepared by infecting the human acute monocytic leukemia cell line (THP-1) essentially as described [38].

Promastigote and AxA antigen expression was examined by Western blotting [39] using monoclonal (mAb, 1:1000 dilution ascites fluid) or rabbit antibodies (rAb, 1:2500) to different antigens including lipophosphoglycan (T2), a 60 kDa flagella antigen (F3) and an amastigote specific molecule (T16) [40]. Parasites from each stage were prepared for Indirect immune fluorescence (IFA) essentially as previously described [41]. The slides were incubated for 30 min RT (1:1000 dilution) with ascites fluids from either mAb F3, M2 or T3 [41, 42]. Images were captured using a fluorescent microscope (Axioplan 2 Imaging, Zeiss, Goettingen, Germany) X 1000 magnification and using an Olympus DP1 camera.

cDNA was prepared from promastigotes, AxA and intracellular amastigotes for the differential display assay and reverse transcriptase – polymerase chain reaction (RT-PCR) as follows. RNA was isolated using the TRI reagent kit (Sigma, USA) and

treated with DNAse (Promega, USA, 1 U/ μg RNA). Double strand cDNA was synthesized at 46C^o for 1 hr using 50 U of RTase enzyme; (ABgene House, UK).

For the differential display assay the cDNA was synthesized from late logarithmic stage promastigotes and AxA as mentioned above. Specific sequences were amplified by PCR using radioactive ^{32}[P]-dTTP and different primer pairs (see table for DNA sequences). The 5'-spliced leader primer, LEISL1, was paired with different 3'- end primers, either an anchored oligo-dT primer (LEIRTG, LEIRTC or LEIRTA) or a degenerate primer found in the 3'-untranslated region (3'-UTR) associated with several amastigote specific genes [43]. Radio-labelled amplicons from promastigotes and AxA were separated on DNA sequencing gels and analyzed by radio-autography. Bands showing stage specific differential expression were excised from the sequencing gels, cloned and sequenced. DNA sequences were blasted against the *L. major* (MHOM/IL/1980/Friedlin) genome (http://www.sanger.ac.uk/Projects/L_major/) and National Center for Biotechnology Information (NCBI) database.

Stage specific gene expression by reverse transcriptase – polymerase chain reaction (RT-PCR) was done as follows. For all stages, cDNA was produced as described above. RT-PCR was done using primers (DNA sequences were as described by Nasereddin et al. [44]) for the following genes: protein kinase A catalytic subunit isoform 1 (*lpkac1)* [45], *L. tropica* amastigote specific gene identified by differential display PCR (*Ltaasp = amastin) - [L. tropica*, Accession No. EU683616, this study], cysteine protease B (*cpb)*[38], and α- tubulin Accession No. XM_001681731. The gels were analyzed with NIH Image Version 1.61 and gene expression was normalized based on the amount of cDNA included in the reactions. Negative controls for the PCR [no *L. tropica* RNA or DNA] and for the RT-PCR [*L. tropica* RNA rather than cDNA] were also included. *L. tropica* DNA was used as a positive control for the PCR.

4. Results

4.1 New molecular tool for the diagnosis of *Leishmania tropica* and other Old World *Leishmania* species

4.1.1 Development of the ITS1-PCR-RLB

ITS1-PCR followed by RFLP or RLB was carried out on identical samples in order to compare the specificity and sensitivity of the two methods of species identification. For preparing the Biodyne C membrane, the optimal concentration for probe coupling (3 pmol/µl) was determined by cross titration using 10 pg/µl *L. major* or *L. tropica* DNA in the ITS1-PCR which was then followed by the RLB.

The sensitivity of both ITS1-PCR methods was compared using decreasing dilutions of *Leishmania* DNA. PCR products prior to RFLP analysis were easily detected by gel electrophoresis down to 100 pg/µl *L. major* or *L. tropica* DNA, and faint bands were still visible at 10 pg/µl DNA. Detection after restriction enzyme digestion was less sensitive. Hybridization to species specific probes in RLB was at least 10-fold more sensitive and clearly detected at least 1 pg/µl DNA.

The sensitivity of the three *L. tropica* probes (RLtP, LtE1, and LtP28a) was determined and compared. Probe LtP28a had the highest sensitivity, easily detecting a PCR product at 0.06 pg/µl DNA. RLB with this probe was at least 166-fold more sensitive than detection of the ITS1-PCR product by gel electrophoresis and ethidium bromide staining.

The probes specificities were determined by checking ITS1 RLB with different Old World leishmanial DNAs (two strains each) of: *L. major*, *L. tropica*, *L. infantum*, *L. donovani*, and *L. aethiopica*. *L. guyanensis*, a New World *Leishmania* species, was also included. The *Leishmania* genus-specific probe PP3′ gave a strong signal with all *Leishmania* species tested. All species-specific probes, except for two *L. tropica* probes, were highly specific, hybridizing only with PCR products of the same species. The *L. tropica* probes hybridized to PCR products of *L. tropica,* but not to those of *L. major*, *L. donovani*, or *L. infantum*. The probes, RLtP and LtP28a, did however, also hybridize to PCR products amplified from *L. aethiopica* DNA. Comparison of the ITS1 DNA sequences of *L. tropica* and *L. aethiopica* showed that the RLtP probe sequence is present in both species; however the LtP28a sequence was found only in *L. tropica*. Thus it is not surprising that RLtP recognizes DNA amplified from *L. aethiopica*, but the reason for the unexpected hybridization of LtP28a to this PCR product is unclear. Despite the cross-reaction of LtP28a and RLtP with *L. aethiopica*

DNA, these parasites were easily distinguished from *L. tropica* by the *L. aethiopica* probes which reacted only with the latter species. Together these results demonstrate that a set of species-specific probes is available to be used in RLB for identification and distinction of Old World *Leishmania* species.

In a first study, the ITS1-PCR-RLB system was applied to 67 suspected CL patients from Israel and the West Bank. ITS1-PCR-RLB identified 61 (91%) of them as positive for leishmaniasis. The results of ITS1-PCR-RLB were compared to that of kDNA PCR which is considered to be the most sensitive molecular test for leishmaniasis but does not allow for species identification. The agreement between the two tests based on a comparison of consensus positive and negative samples, C-pos and C-neg, respectively, was excellent with Cohen's kappa coefficient being 0.925 ± 0.146 [32]. The positive predictive value (PPV) and negative predictive value (NPV) for the ITS1-PCR-RLB test were 98.3 and 100%, respectively. The main advantage of the ITS1-PCR-RLB over kDNA PCR is its ability to identify the different *Leishmania* species.

These results have been published in the following paper:

A. Nasereddin, *E. Bensoussan-Hermano, G. Schonian, G. Baneth, C.L. Jaffe, Molecular diagnosis of old world cutaneous leishmaniasis and species identification by use of a reverse line blot hybridization assay, Journal of clinical microbiology 46 (2008) 2848-2855.*

4.1.2 Molecular epidemiological studies in different CL foci in Israel, the Palestinian Authority, Morocco, and Turkey

Parasites from CL patients from different endemic regions in the Mediterranean Basin were characterized in order to determine the percentage of CL caused by *L. tropica* parasites in different areas. In a previous study in Israel and the Palestinian Authority, 28/66 (42.4%) of the samples examined were *L. tropica* [27]. More recently, 144 samples from suspected CL cases originating from 4 villages and the city of Tiberias near the Sea of Galilee [6] were tested. Of these, 13 were clinical specimens and 23 cultures from suspected human CL cases, 6 cultures from infected sand flies, and 102 clinical specimens from hyraxes. All positive cultures from humans as well as from sand flies were shown to be *L. tropica*. Eleven of the 102 hyraxes were positive by ITS1-PCR and the parasite species was identified as *L. tropica* [6, 46]. When samples from CL suspects from Ma'ale Adumim and Kfar Adumim (Israeli settlements 5 km

East of Jerusalem) [35] and P'duel settlement were studied, all ITS1-PCR products amplified from human and sand fly samples proved to be *L. tropica*. Finally, *L. tropica* was identified as the causative agent for CL in 5 human cases that occurred in a new focus near Bethlehem city (Tekoa, Nokdim and Herodion), 10 km south of Jerusalem. The species causing CL in several well-known Palestinian foci (Bethlehem, Bethany, Jericho (Jericho and Wadi Elbazan), Jenin -8 sites-, Tubas, Nablus) was identified as *L. tropica*.

A case of leishmaniasis recidivans was recorded in a Palestinian Bedouin child during this study. The patient received several different treatments (direct or intralesional sodium stibogluconate) for 14 months however, the nodules did not heal. A severe and destructive nodular pathology was observed. When the parasites were isolated the causative agent was typed as *L. tropica*. This was the first case of CL recidivans recorded in the Jericho district which was thought to be a classical focus of *L. major* and from where *L. tropica* was reported only recently.

As part of these epidemiological studies, tissue samples were obtained from 27 CL suspects living in 6 different regions of Morocco: north (Sidi Kacem), center (Beni Mellal and Boulemane), southeast (Errachidia), and southwest (Taroudant and Ouarzazate). Almost 50% (13/27) of the PCR positive samples from central and south Morocco were due to *L. tropica*. CL due to *L. infantum* was identified in one region in the center of the country, while 2 foci of *L. major* were confirmed in the south of Morocco [47].

In Turkey, *L. tropica* was identified in 37 (48%) of the 77 CL specimens from different endemic regions. In the remaining 40 patients (52%) CL was caused by *L. infantum*. CL caused by *L. tropica* was distributed all over the country (Toz and Nasereddin et al., this research study), while *L. infantum* was found only in southern Turkey in Hatay region. As yet, *L. infantum* strains have not been isolated from patient's skin lesions.

These results have been published in the following papers:

- *S. Ozensoy Toz,* **A. Nasereddin***, Y. Ozbel, H. Ertabaklar, G. Culha, N. Sevil, Z. Alkan, C. L. Jaffe, Leishmaniasis in Turkey: Molecular characterisation of Leishmania from human and canine clinical samples. Trop Med Int Health (2009) 14:1401-1406.*

- *M. Rhajaoui,* **A. Nasereddin***, H. Fellah, K. Azmi, F. Amarir, A. Al-Jawabreh, S. Ereqat, J. Planer, Z. Abdeen, New clinico-epidemiologic profile of cutaneous*

leishmaniasis, Morocco, Emerging infectious diseases 13 (2007) 1358-1360.

- A. Al-Jawabreh, **A. Nasereddin**, *Leishmaniasis recidivans in a Palestinian Bedouin child, Skinmed 6 (2007) 250-252.*

4.2 Cultivation and characterization of *Leishmania tropica* axenic amastigotes

AxA for several *Leishmania* species have been generated and cultured *in vitro* by mimicking those environmental signals, temperature and pH, that parasites encounter in the macrophage (MΦ) phagolysosomal vacuole. Promastigotes were transferred to RPMI+ medium at 36°C and pH 5.5. The parasites continued dividing and >90% of the cells differentiated within 2-3 passages into forms that were morphologically similar to amastigotes. Parasite morphology in the culture was examined daily by inverted light microscopy and subsequent Giemsa staining. By light microscopy, the AxA appeared oval shaped with the flagellum absent or truncated, similar to what was observed for *L. amazonensis, L. mexicana* and *L. pifanoi* axenic and tissue amastigotes [48, 49]. These AxA converted back into flagellated promastigotes by diluting (1:10) in M199+ medium, pH 6.88, and incubating at 26°C.

The percentage of infected MΦ using AxA was consistently higher than that when using promastigotes regardless of the infection ratio employed. In a typical experiment (5:1 parasite:MΦ ratio) AxA infected >7 fold more macrophages than promastigotes, 52 ± 3.6% versus 7 ± 0.6% infected macrophages, respectively. Likewise, the number of parasites per infected MΦ was higher when AxA rather than promastigotes were used (2.3 ± 0.09 versus 1.1 ± 0.08 per MΦ, respectively). Total MΦ parasite load was 15.5-fold higher if AxA rather than promastigotes were used to infect the macrophages under these conditions (unpaired t test, $p < 0.01$). When instead a 10:1 parasite:MΦ ratio was used, total MΦ parasite load was 5.2-fold higher with AxA, also significantly higher than that found using promastigotes (unpaired t test, $p < 0.01$).

IFA and Western blotting were used to examine the expression of stage specific antigens on the parasites. The monoclonal antibody (mAb) T3 was used as a positive control, since it was previously shown to react with *L. major* promastigotes as well as amastigotes isolated from BALB/c mice, and to cross-react with *L. tropica* [42]. As expected, the flagella specific mAb F3 [50] only reacted with promastigotes and no staining of AxA was observed. Staining of the promastigotes was evenly distributed

over the full length of the flagellum. No staining of *L. tropica* promastigotes or amastigotes was observed using the *L. amazonensis* specific mAb M2.

The reaction of several Abs with both promastigotes and AxA was examined by SDS-PAGE and Western blotting, these included: T2, a lipophosphoglycan (LPG) promastigote specific mAb [42]; F3, an anti-flagella mAb; T16, an amastigote specific mAb [40]; rabbit anti-HSP83 and α-tubulin present in both parasite stages [51]. An equal number of cells was used for each stage (5×10^5 cells). The anti-LPG and -flagella antibodies showed strong specific reactions with promastigotes, approximately 10.9- and 3.35-fold higher than with amastigotes, respectively. For T2, a broad band at approximately 70 kDa and a second weaker band at 60 kDa were seen. F3 also recognized a doublet in promastigotes with a prominent band at about 60 kDa and a second weak band at 50 kDa. The amastigote-specific mAb T16 reacted, as expected, with a distinct 60 kDa band only seen in the AxA stage. The positive control, Ab against HSP83, showed essentially the same reaction with either stage of *L. tropica* parasite, 1.03-fold amastigote:promastigote, as reported for *L. donovani* AxA and promastigotes [52].

For differential display, cDNA was synthesized using 4 primer sets containing the same 5'- spliced leader primer, but a different 3'- primer. Anchored oligo(dT) 3'-primers were used in three of the primer sets, while the fourth 3'-primer was based on a 3'- UTR region found adjacent to several amastigote stage specific genes [43]. Overall the pattern obtained using AxA or promastigote cDNA for each oligo(dT) primer set, for instance LEISL1 and LEIRTC, was similar. However, differences could be noted between promastigotes and AxA in the intensity of some amplified bands. Differences in the size and intensity of the bands amplified using each oligo(dT) primer set were also apparent. While only a few bands were amplified using the spliced leader (LEISL1) – 3-UTR primer pair, several distinct differences between promastigote and AxA cDNA were apparent. One AxA specific DNA band (*Ltaasp*) was eluted from the gel, re-amplified, and partially sequenced. The 230 bp DNA sequence (EU683616.1) obtained showed 84% identity (170 / 202 bp) with the 3'-UTR region of the amastin-like surface protein genes from *L. infantum* (AF195531) on chromosome 34.

Several genes have been shown to code for promastigote and amastigote stage-specific proteins. These include protein kinase A catalytic subunit isoform 1 (*pkac1*), the cysteine proteinases (*cpa/b*) [53] and *amastin* [54]. Expression of the *pkac1* and

cpb genes, as well as the *L. tropica* amastin ortholog (*Ltaasp*), was compared between the different *L. tropica* stages. Expression of *α-tubulin* was included as a positive control. Expression was normalized based on the amount of cDNA included in each reaction. The expression of the *pkac1* gene in promastigotes was 2.80 ± 0.17 higher than that observed in AxA. On the other hand, *cpb* gene expression was either extremely weak or not observed when cDNA from promastigotes was used, and at least a 3.10 ± 0.16 - or 6.44 ± 1.12 - fold increase in expression was measured when cDNA from AxA or infected THP-1 cells (iTHP-1), respectively, were used. RT-PCR was also carried out using primers to the *L. tropica* amastin gene (*Ltaasp*). The 200 bp amplicon was only seen when axenic amastigotes or iTHP-1 cells containing intracellular amastigotes were used, and no PCR product was observed when either promastigote or THP-1 cell cDNA was used as template. RT-PCR using cDNA from THP-1 cells as control was negative when primers for the other leishmanial genes, *α-tubulin* or *cpb*, were used. Likewise, RT-PCR using promastigote, AxA, iTHP-1 or THP-1 RNA was negative in all reactions.

These results have been published in the following paper:

A. Nasereddin, *C. Schweynoch, G. Schonian, C.L. Jaffe, Characterization of Leishmania tropica axenic amastigotes. Acta Trop (2010) 113:72-79.*

5. Discussion

5.1 **New molecular tool for the diagnosis of *Leishmania tropica* and other Old World *Leishmania* species**

ITS1-PCR followed by RLB takes advantage of the polymorphisms in the ITS1 region to identify Old World *Leishmania* species. Detection of a positive amplification reaction via hybridization of the biotinylated PCR product to species-specific probes and subsequent signal amplification increases the detection limit 10- to >100-fold over that of RFLP. The sensitivity of the PCR RLB (98.3%) was comparable to that obtained using the kDNA PCR (100%) and significantly better than that of the ITS1-PCR-RFLP. ITS1-PCR-RLB detected several positive patients missed by the PCR-RFLP assay. In addition, the RLB successfully characterized the *Leishmania* species in ~90% of the positive cases, also confirming reactions observed with the genus probe, compared to 84% of positive cases that were identified by RFLP. All of the CL patients examined during the validation of the PCR-RLB assay were infected with either *L. tropica* or *L. major* [32]. Studies in foci where other Old

World species, such as *L. infantum*, *L. donovani*, or *L. aethiopica*, are endemic would be useful for further validation of the RLB assay.

Colorimetric or chemiluminescent substrates can be used interchangeably, allowing direct detection of the reaction product. The *Leishmania* RLB is simple to carry out and, except for a PCR thermocycler, does not require any additional equipment for gel electrophoresis, UV detection, image capturing or for disposal of ethidium bromide, a carcinogen. Analysis of the PCR reaction product and determination of the parasite species in positive samples required 1/3 less time (RLB takes <2 h) than PCR followed by RFLP. In addition, there exists the potential for further simplification of the RLB technology by adapting it to PCR-oligochromatography and developing dipsticks, similar to those used to detect animal and human trypanosomes or toxoplasmosis, where detection can be obtained within 5 min of completion of the PCR. This would further simplify the analysis of CL samples. This diagnostic test will be useful for epidemiological studies where large number of samples need to be screened.

5.2 *Leishmania tropica* molecular epidemiology

The epidemiology of CL caused by *L. tropica* has not been fully explored. We applied molecular biological techniques for leishmanial species identification in collaboration with research groups in Israel, Palestine, Turkey and Morocco. Our results show that > 50% of human CL cases in these countries are caused by *L. tropica* which is significantly higher than previously recognized.

In Israel and the Palestinian Authority, CL caused by *L. tropica* is spreading into new areas and encroaching on urban populations in cities such as Tiberias, Ma'ale Adumim, Bethlehem (Tekoa, Herodion and Nokdim), Bethany, Jericho, (Jericho and Wadi Elbazan), Jenin (8 sites), Tubas, Nablus. *L. tropica* foci are expanding, primarily in the center and north of both Palestine and Israel. This may be due to changes in the local ecology due urbanization and agricultural development. Housing construction, irrigation and other changes in the environmental and ecological conditions have produced favorable habitats near human dwellings for rock hyraxes, a protected animal, which was shown in previous studies by PCR to be a putative reservoir host for *L. tropica* [46]. This has lead to an increase in the hyrax population and created favorable conditions for parasite transmission by bringing the infected hyraxes, sand flies and humans into close contact producing a peridomestic cycle of

transmission. We conclude that CL due to *L. tropica* is a dynamic disease, while *L. major* appears to be primarily restricted to old foci such as Jericho.

Our study in Morocco indicated that CL caused by *L. tropica* is found in 50% (3/6 regions) of the foci examined that are located in the centre of the country stretching from the Atlantic Ocean along the length of the Atlas Mountains almost to the Mediterranean Sea. CL caused by *L. major* is present in the desert region south of the Atlas Mountains in a strip bordering the Sahara Desert. In addition, and for the first time, we reported that CL due to *L. infantum* exists in specific regions of the country. In Turkey CL due to *L. tropica* is widespread throughout the country and was the most common cause of CL. No CL focus due to *L. major* was identified. However, just as in the case of Morocco, we showed for the first time that in Hatay province in southeast Turkey, human CL was due to *L. infantum*, not *L. tropica* as previously thought. This, together with a previous report about a focus of *L. infantum* CL in Cukurova [55], indicates that this parasite is responsible for CL over a wider area in Turkey than originally believed and that species identification is important in diagnosing this disease. In summary, it appears that two species (*L. tropica*, *L. infantum*) are responsible for the majority of endogenous CL and VL cases in Turkey.

In this study, we show that ITS1-PCR followed by RFLP or RLB is a reliable method for diagnosis and identification of the *Leishmania* species causing CL in different countries in the Mediterranean Basin i.e., Turkey, Morocco, Israel and the Palestinian Authority. *L. infantum* and *L. tropica* occur together in several endemic foci and the identification of the parasite species is important not only for the appropriate therapy, but also for understanding the epidemiology of the disease and parasite behavior in different areas of endemicity.

5.3 *Leishmania tropica* axenic amastigote (AxA) transformation, culture and characterization

L. tropica is a neglected leishmanial species, despite the fact that it causes CL throughout North Africa, the Middle East, Africa and Asia where it is responsible for epidemic outbreaks of urban disease. This species is considered to be a potential threat to Europe since it has been identified in Greece [56] and its vector (*Ph. sergenti*) was found in several places in southern Europe, including Spain [57], Portugal and Cyprus [58]. It is responsible for both anthroponotic and zoonotic leishmaniasis, and is more resistant to treatment than *L. major* species that causing CL

in the Old World [6]. The availability of AxA for this species, for which efficient animal models are lacking, would facilitate studies on drug screening, parasite biology and differentiation.

Optimal conditions for the growth and differentiation of *L. tropica* AxA were identified, pH 5.5 and 36°C, and found to be similar to those reported for other species, pH 4.5 - 7.2 and 31 - 37°C [29]. *L. tropica* promastigotes required temperatures higher than *L. amazonensis* in order to convert into AxA, but unlike *L. donovani* and *L. infantum* were sensitive to core body temperatures.

L. tropica AxA showed many molecular and biological markers typical of tissue amastigotes. Macrophage infectivity with AxA was 15.5 fold higher than with promastigotes and similar to that described for amastigotes [59]. Amastigote-specific biological and antigenic markers, and genes specifically expressed in the amastigote stage were appropriately up-regulated in AxA similar to what was reported for amastigotes from other species. Conversely, promastigote-specific markers and genes were down-regulated in AxA accordingly. Western blotting using an anti-LPG mAb (T2) showed a major decrease in LPG expression by *L. tropica* AxA compared to promastigotes. This was accompanied by atrophy and loss of the flagellum, observed both microscopically and by western blotting with a mAb (F3) against proteins located in the paraxial rod of the flagellum [50]. Concurrent expression of an amastigote stage specific antigen (T16) by *L. tropica* AxA was observed, as in intracellular amastigotes of *L. major*, *L. mexicana* and *L. donovani* [40]. Differential display PCR, using several sets of primers, was used to compare and identify specific gene expression in *L. tropica* promastigotes and AxA. Interestingly, the pair containing the 3'-UTR 450 nt amastigote regulatory element amplified significantly fewer products than the oligo dT anchored primer sets. This might be due to either the limited number of this element in the genome (85 in *L. major*) or to the only 60 to 70% identity shared by different 450 nt conserved sequences. However, differential display PCR and RT-PCR identified at least one gene, *amastin*, containing this sequence that is differentially expressed in the *L. tropica* amastigote stage. The amastin gene was previously shown to belong to a large gene family, the majority of which are differentially expressed by intracellular amastigotes of *Leishmania* [54]. It can be used as a marker for *L. tropica* AxA, similar to the A2 gene for *L. donovani* AxA [60]. In order to further demonstrate the similarity between the *L. tropica* AxA and intracellular amastigotes, we used RT-PCR to characterize the expression of

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several known stage-specific genes including *cpb*, *pkac1* and *amastin*. *Leishmania cpb* is a multi-copy gene family with variable copy numbers depending on the parasite species [53]. Its expression is strongly up-regulated in amastigotes of *L. mexicana* and *L. pifanoi*, and also in amastigotes (6.44 ± 1.12 fold) and AxA (3.10 ± 0.16 fold) of *L. tropica* as shown in this study). On the other hand, *pkac1* is more strongly expressed by *L. tropica* promastigotes compared to AxA, similar to what has been demonstrated in *L. major* and *L. donovani* where this gene is preferentially expressed by promastigotes [45]. Lastly, we confirmed that the *amastin* gene is indeed specifically expressed by AxA and amastigotes as shown by differential display PCR.

In conclusion, optimal environment was established for culturing *L. tropica* AxA. Different molecular parameters were used to characterize these parasites and to identify an amastigote stage-specific gene, *amastin*. *L. tropica* AxA will be used in future studies on drug screening and on stage-specific metabolic processes.

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Agreement

This doctoral thesis (publication thesis) is based on the publications published in the journals listed below.

1. **A. Nasereddin,** C. Schweynoch, G. Schonian, C.L. Jaffe, Characterization of Leishmania tropica axenic amastigotes. Acta Trop (2010) 113:72-79.

2. S. Ozensoy Toz, **A. Nasereddin**, Y. Ozbel, H. Ertabaklar, G. Culha, N. Sevil, Z. Alkan, C. L. Jaffe, Leishmaniasis in Turkey: Molecular characterisation of Leishmania from human and canine clinical samples. Trop Med Int Health (2009) 14:1401-1406.

3. M. Rhajaoui, **A. Nasereddin**, H. Fellah, K. Azmi, F. Amarir, A. Al-Jawabreh, S. Ereqat, J. Planer, Z. Abdeen, New clinico-epidemiologic profile of cutaneous leishmaniasis, Morocco, Emerging infectious diseases 13 (2007) 1358-1360.

4. **A. Nasereddin**, E. Bensoussan-Hermano, G. Schonian, G. Baneth, C.L. Jaffe, Molecular diagnosis of old world cutaneous leishmaniasis and species identification by use of a reverse line blot hybridization assay, Journal of clinical microbiology 46 (2008) 2848-2855.

5. A. Al-Jawabreh, **A. Nasereddin**, Leishmaniasis recidivans in a Palestinian Bedouin child, Skinmed 6 (2007) 250-252.

Erklärung über den Eigenanteil an den Publikationen

Nr.	Publikation/ Erläuterung des Anteils von Herrn A. Naser Eddin	In %
1.	*A. Nasereddin, C. Schweynoch, G. Schonian, C.L. Jaffe, Characterization of Leishmania tropica axenic amastigotes. Acta Trop (2010) 113:72-79.* A. Naser Eddin designed the study protocol, carried out all the techniques including culturing of parasite AxA and promastigotes, microscopy, macrophage infection, stage-specific antigen expression, differential display, analyzed the data and drafted the manuscript.	**70%**
2.	*S. Ozensoy Toz, A. Nasereddin, Y. Ozbel, H. Ertabaklar, G. Culha, N. Sevil, Z. Alkan, C. L. Jaffe, Leishmaniasis in Turkey: Molecular characterisation of Leishmania from human and canine clinical samples. Trop Med Int Health (2009) 14:1401-1406.* A. Naser Eddin **contributed equally to the first author**. He typed the strains by ITS1-RFLP, compiled and analyzed the results, and helped in drafting the manuscript.	**45%**
3.	*M. Rhajaoui, A. Nasereddin, H. Fellah, K. Azmi, F. Amarir, A. Al-Jawabreh, S. Ereqat, J. Planer, Z. Abdeen, New clinico-epidemiologic profile of cutaneous leishmaniasis, Morocco, Emerging infectious diseases 13 (2007) 1358-1360.* A. Naser eddin is the **corresponding author** of the paper and **contributed equally to the first author**. He extracted DNA from the Moroccan Giemsa stained slides, amplified and cut the ITS1 region, compiled the results and made the analysis, drafted the manuscript.	**50%**
4.	*A. Nasereddin, E. Bensoussan-Hermano, G. Schonian, G. Baneth, C.L. Jaffe, Molecular diagnosis of old world cutaneous leishmaniasis and species identification by use of a reverse line blot hybridization assay, Journal of clinical microbiology 46 (2008) 2848-2855.* A. Naser Eddin amplified the strains with ITS1 labeled primers, designed the probes, prepared the membranes, did the hybridization, collected the results and run the analysis, compiled the results and took part in drafting and revising the manuscript.	**70%**
5.	*A. Al-Jawabreh, A. Nasereddin, Leishmaniasis recidivans in a Palestinian Bedouin child, Skinmed 6 (2007) 250-252.* A. Naser Eddin prepared the semisolid media for cultures, collected the DNA from slides and cultures and performed the RFLP analysis, compiled the results and took part in revising the manuscript.	**50%**

Prof. Dr. Wolfgang Presber   **Abedelmajeed Naser Eddin**

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version

meiner Arbeit nicht veröffentlicht

Erklärung

„Ich, Abedelmajeed Naser Eddin, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „*Leishmania tropica*: Molecular Epidemiology, Diagnosis and Development of an Axenic Amastigote Model" selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

Berlin, den 01.11.2010   Unterschrift
                                                        Abelmajeed Naser Eddin

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