Molecular Epidemiology, Clinical Molecular Diagnosis and Genetic Diversity of Cutaneous Leishmaniasis in Jericho…

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1 author:

Aus dem Institut für Mikrobiologie und Hygiene

der Medizinischen Fakultät der Charité – Universitätsmedizin Berlin

DISSERTATION

Molecular Epidemiology, Clinical Molecular Diagnosis and Genetic Diversity of Cutaneous Leishmaniasis in Jericho, Palestine

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Von

Amer Al-Jawabreh

Aus Palästina

Dissertation Examination Committee (Gutachter):

1. Prof. Dr. med. W. Presber

2. Prof. Dr. W. Solbach

3. Prof. Dr. Ch. Jaffe

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List of Abbreviations

A	Adenine
APS	Ammonium peroxodisulfate
ARF	Annual Rainfall
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BM	Bone marrow
BSA	Bovine serum albumin
C	Cytosine
CA	Central Asia
CE	Capillary Electrophoriesis
CL	Cutaneous leishmaniasis
DAT	Direct agglutination test
DCL	Diffuse cutaneous leishmaniasis
ddH2O	Double distilled water
$D\mu^2$ ($\delta\mu^2$), Ddm	Delta mu squared
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DMSO	Dimethyl-sulfoxid
Dps	Proportion of shared alleles
EDTA	Ethylenediamine-tetra acetic acid
fg	Femtogram
FP	Filter papers
Fis	In-breeding coefficient
Fst	F-statistic
G	Guanine
HCl	Hydrochloric acid
He	Expected heterozygosity
Ho	Observed heterozygosity
IMM	Infinite Allele Model
ITS	Internal transcribed spacer
K	Number of population

KZ	Khazakistan
Kbp	Kilo base-pair
KDNA	Kinetoplast DNA
MAJ	*L. major*
ME	Middle East indicating South West Aisa
MCL	Mucocutaneous leishmaniasis
MCMC	Markov chain Monte Carlo
MLEE	Multilocus enzyme electrophoresis
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NNN	Novy-MacNeal-Nicolle medium
NW	New World
NJ	Neighbor-joining tree
NWA	North West Africa
OIF	Oil immersion field
OW	Old World
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
PKDL	Post kala-azar dermal leishmaniasis
pg	Picogram
PS	Palestine
RFLP	Ristriction fragment length polymorphism
RNA	Ribonucleic acid
RR	Relative risk
rRNA	Ribosomal RNA
RT-PCR	Reverse-transcriptase PCR
SDS	Sodium dodecyl sulphate
SMM	Stepwise Mutation Model
sp.	Species
ssU RNA	Small sub-unit RNA
T	Thymine

Taq	*Thermus aquaticus*
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	NNNN-Tetramethylene diamine
TM	Turkmenistan
U	Unit
U	Uracil
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
UZ	Usbekistan
VL	Visceral leishmaniasis
WHO	World Health Organization
$\overline{\mathbf{x}}$	Arithmatic mean

ABSTRACT

Parasitological diagnosis of cutaneous leishmaniasis is imperative before treatment. In this study we compared the sensitivity of the diagnosis of Giemsa-stained skin scrapings by standardized graded direct microscopy with that of ITS1-PCR. Out of the 60 squares scanned for amastigotes using x100 oil- immersion light microscopy, 45 (75%) gave usable results. Fifteen (25%) squares could not be microscopically scanned. Of the 23 microscopy-positive squares, 20 (87%) were positive by PCR. Of the 22 squares negative for microscopy, 18 (82%) were ITS1-PCR positive. ITS1-PCR showed a sensitivity of 87% with positive predictive value of 100% and a specificity of 100% with negative predictive value of 85%.

In-vitro cultivation using NNN medium and direct smear microscopy of Giemsa-stained slides, PCR amplifying region 1 of internal transcribed spacer (ITS1) using skin scrapings spotted on filter papers (FP) and unstained tissue smears (US) were compared on the basis of three gold standards: WHO case definition, combined and clinical gold standard. PCR using US was more sensitive than all other methods using the 3 gold standards. Of the 298 cases of CL from FP, US and *in-vitro* culture tested by PCR-RFLP using HaeIII and MnlI, 181(60.7%) contained DNA of *L. major*, 106 (35.6%) DNA of *L. tropica,* while 11 (3.7%) of the FP remained unidentified

Molecular epidemiology was used to study the distribution of *Leishmania* species in Jericho. Spatial analysis showed three statistically significant clusters of CL, one cluster for *L. major* (Auja-Fasayil villages) and two clusters for *L. tropica,* (Zubaidat village and Nabi-Musa Bedouin encampment) were recorded. In the case of space-time, four clusters were detected: Zubaidat village for four years, A'uja-Fasayil villages for one year, Nabi-Musa for three years and Nuaimeh village for one year. Clusters for *L. major* were noted in A'uja-Fasayil villages for one year and Nuaimeh village for three years. *L. tropica* clusters were in Zubaidat village for one year, Nabi-Musa for four years and Aqbat-Jaber refugee camp for one year

Microsatellites, or simple sequence repeats (SSR), are very useful genetic markers for population genetic studies. Ten pairs of PCR primers, annealing to the unique flanking regions, were designed to amplify microsatellite loci identified in the genome sequence of *L. major* on chromosomes 1, 3, 5, 21 and 35. The microsatellite repeats are (CA)n, (AT)n, (GTG)n, and (GACA)n with varying lengths. A total of 106 strains isolated in different endemic regions of Central Asia, Middle East and Africa were analysed in this study. Analysis of genetic distance revealed the existence of 7 discrete populations of *L. major* including two genetically isolated populations in the Middle East. This was confirmed by a Bayesian model-based clustering approach that assigned the individual strains to the same number of populations.

1. INTRODUCTION

Forward: Molecular epidemiology

Molecular epidemiology is a fairly new branch of science that has emerged in the last 20 years in parallel with the development of molecular biology (Tazi *et al*., 2002). To understand molecular epidemiology, it is worth knowing what epidemiology is. Last (2001) defined it as 'the study of the distribution and determinants of health-related states or events in specified populations, and the application of this study to control of health problems, in the first place the frequency, distribution and determinants of a disease'. In the case of molecular epidemiology, the definition given by Last (2001) still holds but the addition comes from the integration of molecular approaches into the conventional and classical epidemiologic studies. Molecular epidemiology becomes, then, the application of molecular biology techniques to the study of the distribution and determinants of infections and communicable and non-communicable diseases (Tazi *et al*., 2002).

In the light of the new definition, several disciplines, such as medicine, molecular biology, epidemiology, biostatistics, genetics and computer science, are conjugated with classical concepts like study designs, case control and cohort studies, and types of analyses, remaining as descriptive and analytical as they are.

1.1 Historical background

Leishmaniasis has been an antique public health problem in South-West Asia and the Arab World reported from time immemorial as the pharaohs ruled in Egypt and Assyrians in Mesopotamia. It was extensively described by Arab-Islamic scientists like Avicenna (Ibn-Sina, 980-1037 A.D.) who wrote a complete chapter in his prominent book entitled Alkanoun Fi El Tebb raising the possibility of mosquitos being involved in the transmission of the disease. Al-Rhazi (AD 850 to 923) already described cutaneous leishmaniasis (CL) as a disease endemic in Balk (Afghanistan) and Baghdad (for review see Bray, 1987; Oumeish, 1999, Morsy, 1996; Cox, 2002). Russell described CL in Aleppo-Syria in 1756 (reviewed by Klaus *et al*., 1999).

The city of Jericho is known as an old classic focus for CL, active for at least the last 130 years as confirmed by Robert Ruby's visit to Palestine who wrote in 1873: ''Then you would notice among people in Riha (*Arabic name for Jericho*) or in dealings with the Bedouins, that almost everyone in the valley had on his hands or face at least one large ugly scar''. Later on, two scientists, De Bermann, the French, (1910) and Huntemueller, the German, (1914), with

the help of Mastermann and Canaan, described cases of leishmaniasis in Jericho where the latter thought that he had made a new discovery and allowed himself to name it as *Plasmosoma jerichoense*. The people of Jericho called it, and still do, '*Habat Riha*' (Jericho button or boil).

Adler & Theodor in 1926 (1957) were the first to experimentally prove that the Phlebotomine sand fly, *P. papatasi*, is the vector for CL in Jericho. They isolated *Leishmania* parasites, called *L. tropica* at that time, from this sand fly species. This was confirmed by Naggan *et al* (1970) and Schlein *et al* (1982). Gunders *et al* (1968) showed that in Jericho the reservoir host for *Leishmania* parasite which he, then, called *L. major* was *Psammomys obesus*. Local physicians continued to see CL patients, e.g., in Jerusalem (Al-Quds) (Canaan, 1945) and Nablus (Arda, 1983).

1.2 Clinical symptoms of leishmaniases

Leishmaniases caused by obligate intracellular protozoan parasites of the genus *Leishmania*, order Kinetoplastida, are, clinically, subdivided into three distinct entities: i) cutaneous leishmaniasis (CL) caused by *L. major*, *L. tropica* and, rarely, *L. infantum* and *L. donovani*, in the Old World (OW) and *L. mexicana* in the New World (NW); ii) visceral leishmaniasis (VL) caused by representatives of the *L. donovani* complex and, rarely, by *L. tropica* in the OW and, rarely, *L. amazonensis* in the NW; and iii) mucocutaneous leishmaniasis (MCL) caused by *L. braziliensis*, *L. panamensis*, and *L. guyanensis*, in the NW, with reported cases by *L. donovani*, *L. major*, and *L. infantum* in the OW. Sometimes a special form of CL is described as a fourth independent entity: diffuse cutaneous leishmaniasis (DCL) caused by *L. aethiopica* in the OW and *L. amazonensis* in the NW (Sacks *et al*., 1995; Desjeux, 1996; Herwaldt, 1999; Saliba and Oumeish, 1999; Bulle *et al*., 2002; Ben Ami *et al.,* 2002).

CL manifests itself starting from small erythematous papules through nodules and to ulcerative lesions. Unusual clinical manifestations are sporotrichoid patterns, i.e., subcutaneous nodules developing along lymphatics, and hyperkeratosis, i.e., thick adherent scale, as well as leishmaniasis recidivans, also known as lupoid leishmaniasis. In the Middle East, it was very difficult and even impossible to discern by the clinical picture whether cases were caused by *L. major* or *L. tropica* (Klaus and Frankenburg, 1999).

Visceral leishmaniasis (VL), or Kala-azar, is associated with prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hypergammaglobulinemia. It can be impaired by serious bacterial infections and is usually fatal if left untreated (Sundar and Rai, 2002). A serious sequel to Kala-azar is post-Kala azar

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dermal leishmaniasis (PKDL) which appears within months or years of the cure of VL (Ashford, 2000).

Mucocutaneous leishmaniasis (MCL) is a severe form of CL, as it produces disfiguring lesions and mutilations of the face, nose and throat (Desjeux, 1999). They commonly appear in the mouth and nose where they erode underlying tissue and cartilage (Ashford, 2000). If the lesions spread to the roof of the mouth and the larynx, they may prevent speech. Other symptoms include fever, weight loss, and anemia. There is always a substantial danger of bacteria infecting the already open sores.

Diffuse cutaneous leishmaniasis (DCL) produces disseminated and chronic skin lesions resembling those of lepromatous leprosy. It is difficult to treat (http://www.who.int/tdr/diseases/leish/default.htm, Ashford, 2000).

1.3 Epidemiology of leishmaniasis:

1.3.1 Global view

Leishmaniases are parasitic infection caused by a range of *Leishmania* parasites supported by a wide range of vectors and reservoirs distributed on all inhabited continents (Ashford 1996 and 1999 and 2000). Recently, kangaroos in Australia have been reported to be infected by *Leishmania*-like parasites (Rose *et al*., 2004). The diseases are endemic in 88 countries, of which 66 are in the OW, 22 in the NW and 72 in the developing countries. It has been estimated that 350 million people are at risk, with 500,000 new VL cases each year. Confirmed cases of VL have been reported from 66 countries, 90% of the world's VL burden occurs on the Indian subcontinent (India, Bangladesh and Nepal), East Africa (Sudan, Ethiopia and Kenya) and Brazil (WHO 1991, 1996 and 1998)**.** There are 1.0-1.5 million cases of CL each year, with 90% of CL cases occuring in 7 countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria, showing that Middle East is a centre focus for CL (Desjeux, 1999; Desjeux, 2004; http://www.who.int/tdr/diseases/leish/default.htm).

1.3.2 Local view

In Palestine, two forms of leishmaniasis exist. One is the CL caused by *L. major* or *L. tropica* and the other is VL caused by *L. infantum*. Leishmaniasis in general is reported in all Palestinian districts except Gaza strip with an official incidence rate in West Bank of more than 10 per 100,000 in 2003 (Klaus *et al*., 1994; Baneth et al., 1998; Abdeen *et al*., 2002; Anders *et al*., 2002; Al-Jawabreh *et al.,* 2004, Jaffe *et al.,* 2004, Schoenian 2003; Ministry of Health, 2004).

1.4 Leishmaniasis: Public Health Surveillance

Leishmaniasis is a reportable infection in Palestine (Ministry of Health, 2004) and all neighbouring countries like Jordan, Syria and Saudi Arabia. Ministries of Health collect data for various reasons like therapeutic and control strategies. Despite the law mandate, underreporting is still believed to prevail partly due to passive surveillance, including only cases coming from clinics and hospitals.

Already before 1994 when the health authority was in the hands of the Israeli military rule, there were attempts to improve the reporting system in Palestine (Jaber, 1987). After 1994, the Palestinian Ministry continued the surveillance activity but suffered some disturbances between 2001 and 2005 due to the political turmoil in the area. The current situation depends on passive collection of data from health care providers in all Palestinian districts by the preventive medicine department in the Ministry of Health and presenting them either on the official website or distributing hardcopies to health stake-holders in the regions.

Several definitions for surveillance have been proposed. That by Langmuir (1963) who stated that "continued watchfulness over the distribution and trends of incidence through the systematic collection, consolidation, and evaluation of morbidity and mortality reports and other relevant data, together with dissemination to those who need to know" is the most common. It is a continuous activity and not simply a study or a survey. The aim of surveillance is to control and prevent diseases, improve epidemiological knowledge and assist policy making. The tripod of this activity is the laboratory, physicians/nurses and epidemiologist/statistician.

Data collected by active or passive surveillance should be descriptively analyzed in such a way as to provide an early warning system for an existing or coming health problem, i. e epidemic. Three methods are proposed for this purpose:

1. Shewhart's Plot: a common technique used for quality control/assurance in medical laboratories to check the performance over a period of time. It was originally designed for industrial control (Shewhart, 1930), then introduced into clinical chemistry and medical laboratories by Levy and Jennings in the 1950s (Levy and Jennings, 1950) to be applied in all clinical laboratories. Later, Westgard *et al* (1981) modified it by introducing interpretation rules called multi-rule Shewhart that became known as Westgard rules (see Material and Methods, 2.12.4).

2. Moving average: a statistical tool common in stock markets to spot the trends of the highs and lows of the security prices based on a window time period set by the analyst. This idea can be used to detect outbreaks and trends in disease distribution.

3. *SaTScan* software: detects statistically significant disease clusters based on statistical characters (see Materials and Methods, 2.12).

These and other early warning methods would be more effective if there would exist a regional network that would allow free exchange of data between countries of the region such as for instance Eurosurveillance (http://www.eurosurveillance.org), which distributes alerts and regular releases to all European countries.

1.5 Clinical diagnosis and identification

The conventional methods of clinical diagnosis of CL have ranged from clinical picture and epidemiological data, visualizing the amastigotes by microscopy of stained smears from skin touch specimens or biopsies to *in-vitro* culturing of the parasite (Reed, 1996; Herwaldt, 1999). These conventional methods are, however, limited in sensitivity, need an experienced hand and do not distinguish between *Leishmania* species which differ in virulence and, subsequently, may require different therapeutic regimes and control measures. For all this and over the last decade diagnostic tests based on molecular biology techniques i. e PCR, were introduced and proved to be more sensitive and specific (Van Eys *et al*., 1992; Wilson, 1995; Osman *et al*., 1998).

The sensitivity of different diagnostic methods was the subject of several studies. PCR protocols were given priority over conventional methods, although few of these studies gave conflicting results (Table 1.1). These discrepancies are due to factors like the different gold standards being used to define a case of CL and sampling methods.

Table 1.1 Selected studies that compared methods of diagnosis for CL

Culture%	Histopathology%	Smear%	PCR%	Reference
50-biopsy	14	19	-	Weigle *et al*., 1987
58-aspirate				
-	76	48	86	Andresen *et al*., 1996
-	-	58-wooden toothpick	100	Belli *et al*., 1998
		48- lancet		
-	-	84	-	Rajabi *et al*., 1999
-	33	42	92	Aviles *et al.,* 1999
-	-	90.4	80.8	Ramirez *et al*., 2000
-	-	95	100	Romero *et al*., 2001
-	-	85	81	Robinson *et al*., 2002
80	30	71.5	-	Sharquie *et al.,* 2002
46.5	66.2	67	95.4	Rodrigues *et al*., 2002

55.3-biopsy	-	46.7	75.7	Weigle *et al.,* 2002
46.3-aspirate				
-	-	-	92	Safaei *et al*., 2002
74	-	77	-	US military, 2002-2003

In the history of leishmaniasis, several methods were used for classification, characterization and identification of the infecting parasites, reviewed by El-Tai *et al* (2000). These included simple methods such as geographical classification, e.g. Old World versus New World, and epidemiological and /or clinical criteria. More advanced criteria began to emerge starting with EF serotyping (Schnur *et al*., 1972, 1977 and 1990), isoenzyme analysis (Miles *et al*., 1980; Evans *et al*., 1984; WHO, 1990; Andersen, 1996), and monoclonal antibodies (Noyes *et al*., 1996). Then molecular techniques were introduced such as RFLP (Restriction Fragment Length Polymorphism), kDNA and nuclear DNA/ Southern hybridization (Jackson *et al.,* 1984; Beverly *et al*., 1987; Barker, 1989; Van Eys *et al*., 1989, 1991; El-Tai *et al*., 2001), fingerprinting (Macedo *et al*., 1992), PCR fingerprinting with non-specific primers (Williams *et al*., 1990; Tibayrenc *et al.,* 1993; Pogue *et al*., 1995 a, 1995 b; Schoenian *et al*.,1996, El-Tai *et al*., 2001), molecular karyotyping (Lighthall and Gianini, 1992), and PCR-SSCP (El-Tai *et al*., 2001). However, most of these techniques lack discriminatory power or reproducibility and are not easy to compare when used in different laboratories.

1.6 Multilocus enzyme electrophoresis (MLEE)

Multilocus enzyme electrophoresis (MLEE) is considered the 'gold standard' and reference method for identification and classification of species and strains, and for studying variability within *Leishmania* (Rioux *et al.*, 1986; Russell *et al*., 1999). Promastigote mass cultures isolated from specimens are normally used for this analysis. A panel between 10 and 20 isoenzymes is utilized. The commonly used enzymes are: PGM, phosphoglucomutase, (E.C.2.7.5.1); GPI, glucose-phosphate isomerase (E.C.5.3.1.9); GOT, glutamate-oxaloacetate transaminase (E.C.2.6.1.1); ME, malic enzyme (E.C.1.1.1.40); 6PGD, 6-phosphogluconate dehydrogenase (E.C.1.1.1.49); G6PD, glucose-6-phosphate dehydrogenase (E.C.1.1.1.37); NP, nucleoside purine phosphorylase (E.C.3.2.2.1); MDH, malate dehydrogenase (E.C.1.1.1.37.), MPI, mannose phosphate isomerase (E.C.5.3.1.8); ICD, isocitrate dehydrogenase (E.C.1.1.1.42); DIA, diaphorase nicotinamide adenine dinucleotide (reduced form) (E.C.1.6.2.2); GLUD, glutamate dehydrogenase (E.C.1.4.1.3); FH, fumarate hydratase (E.C.4.2.1.2). In each run, a World Health Organization (WHO) reference strains is used. The

techniques and the zymodeme nomenclature adopted are those of Montpellier centre (Le-Blanq *et al*., 1986I &II; Nimri *et al*., 2002).

MLEE has the advantage of backing a large data set and a well-managed reference laboratory (Rioux *et al.*, 1986). MLEE has some disadvantages. It is expensive, slow and laborious, and it is not easy to compare the raw data from different laboratories. The need of mass *in-vitro* culture makes it unsuitable for high throughput analyses (Andresen *et al.*, 1996; Noyes *et al.*, 1996; Jamjoom *et al*., 2002a). A major disadvantage is that it determines phenotypes and not genotypes. In addition, any nucleotide substitution that does not change the amino acid composition remains undetected, and the same is true for changes in the amino acid composition that do not influence the electrophoretic mobility. Another disadvantage is that the house-keeping genes analysed in MLEE are most probably under selective pressure so that mutations observed are not neutral. Furthermore, MLEE relies on the assumption that the parasite's isoenzyme types (or zymodemes) represent stable multilocus genotypes. This is only true if genetic recombination is almost absent in natural populations of the parasite (Jiménez *et al*., 1997).

1.7 Microsatellites

Microsatellites or simple sequence repeats (SSRs), or Short Tandem Repeats (STRs), discovered in 1981, are tandemly repeated motifs of 1-6 nucleotides found in all prokaryotic and eukaryotic genomes. They are present in both coding and non-coding regions (Ellergan 2004). In addition to being highly variable and polymorphic, microsatellites are also easy to genotype and densely distributed throughout eukaryotic genomes, making them the preferred genetic marker for high resolution genetic mapping (Dib *et al*., 1996; Dietrich *et al*., 1996; Schuler *et al.,* 1996; Knapik *et al*. 1998; Cooper *et al*., 1999).

Dinucleotide repeats dominate, followed by mono- and tetranucleotide repeats, and trinucleotide repeats are least dominant. Repeats of five (penta-) or six (hexa-) nucleotides can also be found. Generally, among dinucleotides, (CA)n repeats are most frequent, followed by (AT)n, (GA)n and (GC)n, the last type of repeat being rare (Ellergan, 2004). Microsatellite loci are characterized by high heterozygosity and the presence of multiple alleles, which is in sharp contrast to unique DNA sequences (Ellergan, 2004). Microsatellites account for 3% of the human genome (International Human Genome Sequencing Consortium, 2001). The genome of *Leishmania* is relatively rich in microsatellites with about 600 $(CA)_n$ loci per haploid genome (Rossi *et al*., 1994). Currently a *Leishmania* genome project

(http://www.sanger.ac.uk/Projects/L_major/) is being carried out which showed that *L. major* Friedlin genome, for instance, is 32.8Mb in size, with a karyotype of 36 chromosomes.

There are more applications of microsatellite analyses other than gene mapping which are ancient and forensic DNA studies: e.g. population genetics and conservation/management of biological resources (Jarne & Lagoda 1996), assessment of population subdivision and phylogenetic relatedness (Queller *et al.*, 1993), parentage analysis, phylogenetic studies (Bowcock *et al.* 1994), studies looking at population differentiation (Paetkau *et al.*, 1995) and measuring inbreeding (Coltman *et al.,* 1998; Coulson *et al.* 1998).

1.7. 1 Mutation mechanism

Microsatellite sequence variation results from the gain and loss of single repeat units or a single nucleotide. The most plausible explanation of loss of repeat units is slippage of polymerase during DNA replication (Schloetterer & Tautz 1992).   This is transient dissociation of the replicating DNA strands followed by misaligned re-association thought to be due to DNA polymerase pausing and then dissociating from the DNA (Levinson & Gutman, 1987; Ellergan, 2004) as shown in figure 1.1. Replication slippage also occurs during PCR amplification of microsatellite sequences *in vitro*, characterised by the presence of 'stutter bands'— that is, minor products that differ in size from the main product by missing or additional repeat units (Shinde *et al.*, 2003). It is worth noting that most of these primary mutations *in vivo* are corrected by the mismatch repair (MMR) system, and only the small fraction that was not repaired ends up in the form of variable microsatellites (Strand *et al.*, 1993, You, 2002).
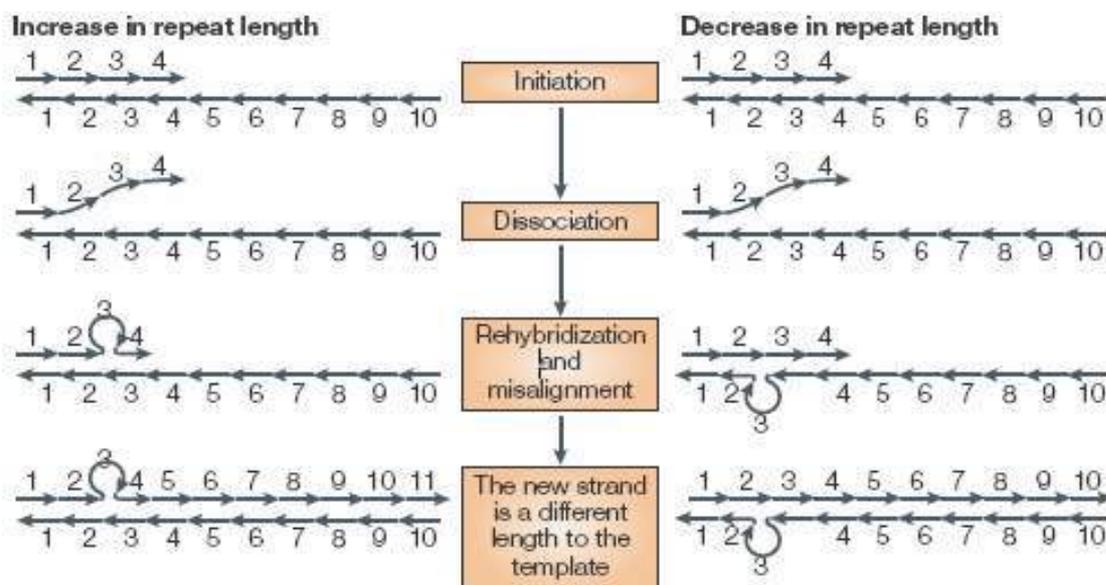
Figure 1.1 Replication slippage caused by dissociation of the two strands, re-aligning of the nascent strand out of the register (left), and then continued replication as part of the mismatch repair. This will

cause misalignment producing a loop in the nascent strand and increase the repeat length. Alternatively, the same will take place but on the template strand (right) causing decrease of repeat length (Ellergan, 2004).

The emergence of microsatellite variation is explained by the 'length and point mutation model'. It is based on the existence of two opposing mutational forces operating on microsatellite sequences: length mutations, the rate of which increases with increasing repeat count, whereas point mutations break long repeat arrays into smaller units. At equilibrium, there will be a steady-state distribution of repeat lengths governed by the rate of length mutation and the rate of point mutation (Dieringer & Schlotterer, 2003; Bell, & Jurka, 1997; Ellergan, 2004).

The rate of mutation at a given microsatellite locus is influenced by various factors: the repeated motif itself, allele size, chromosome position, GC content in flanking DNA, cell division (mitotic vs. meiotic) and the mismatch repair system (e.g. mutations at MMR genes) which is critical for the stability of the STR (You, 2002).

In addition, recombination has been presented as a potential explanation for mutation. Recombination changes the STR length/repeat number by unequal crossing over or by gene conversion (Brohele & Ellegren, 1999; Hancock, 1999; Jakupciak & Wells, 2000; Richard & P.ques, 2000; You, 2002).

1.7.2 Functions of microsatellites

Possible functions of microsatellites are illustrated in figure 1.2 adopted from You *et al.* (2002). (For more descriptions see: You, 2002 and the references therein).
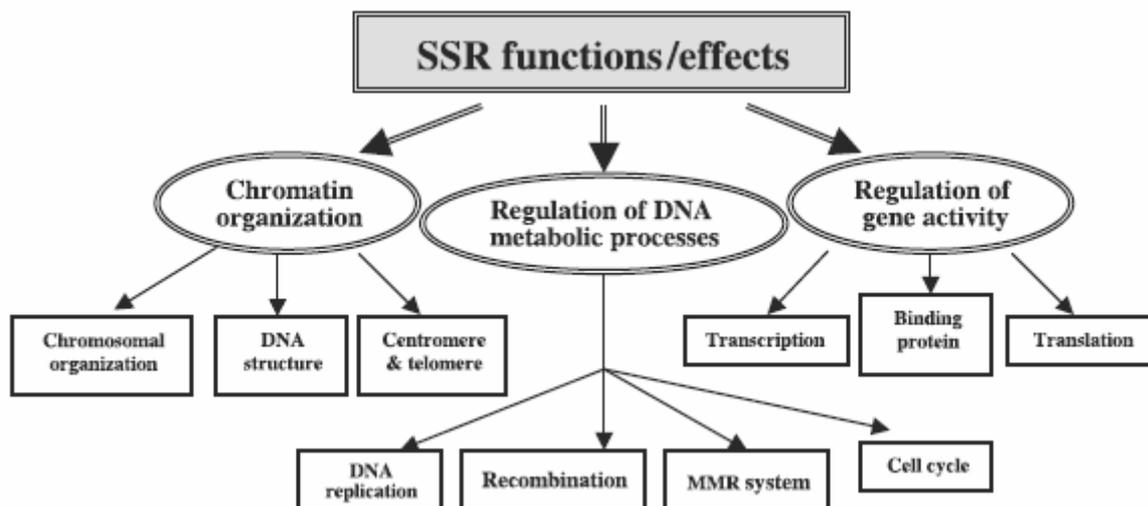
Figure 1.2 Possible functions of microsatellites (You *et al.*, 2002)

1.7.3 Application of microsatellites for *Leishmania* strain typing

To overcome the disadvantages of MLEE and of most molecular approaches used so far, methods based on microsatellite DNA loci have been developed for strain typing and population genetics studies within the genus *Leishmania* (Rossi *et al.,* 1994; Jamjoom *et al.*, 2002a & b, Schwenkenbecher *et al.*, 2004). Microsatellite analysis yields more polymorphisms (Jamjoom *et al.*, 2002a), exhibits a high level of discrimination and is suitable for characterizing closely related strains like those of *L. infantum* (Bulle *et al.*, 2002). This method is quantitative and reproducible; the output data are comparable and exchangeable between laboratories. Microsatellite analysis has the potential for high-throughput analyses. At the beginning, Russell *et al* (1999) successfully used three microsatellite markers for the discrimination of species within the NW subgenus *Viannia*. In the course of time, more and more polymorphic microsatellite markers were developed to increase the discriminatory power, e.g., 13 markers for *L. major* by Jamjoom *et al*. (2002b), 16 for *L. tropica* by Schwenkenbecher *et al*. (2004), and 20 markers for *L. donovani* and *L. infantum* by Jamjoom *et al*. (2002a). The main disadvantage of using microsatellites is that these sequences are prone to homoplasy and it is, therefore, necessary to develop a panel of 15-20 independent microsatellite markers. In *Leishmania,* microsatellite variation was shown to be species-specific (Jamjoom *et al*., 2002b, Schwenkenbecher *et al*., 2004), therefore different panels of markers should be designed for each species to be studied.

1.8 Two models for microsatellite evolution

Two different models are used in the literature to explain the evolution of microsatellite sequences.

The **Infinite Allele Model (IAM)** was developed for the interpretation of allozyme variation. It supposes that every new mutation gives rise to a new electrophoretically distinguishable allele (or electromorph). IAM has proved very successful in explaining the observed allozyme variation (Nei 1987). Homoplasy in IAM is not accounted for as it never occurs, because mutation always creates new alleles (Rousset, 1996), which is a situation that is very different in microsatellite markers. Homoplasy is the identity between two alleles that are not identical by descent, but by state. For example, one allele has mutated by chance, but not because of a common ancestor, to the same sequence as the other, or, simply, because two different alleles cannot be distinguished by the technique used (Costantine, 2003; De Meeûs, 2004). The majority of mutations at microsatellite loci are stepwise in nature, changing allelic sizes by gaining or loosing single or very few numbers of repeats. Normally, microsatellites tend to

expand by gaining new repeats. From a certain number of repeats on natural selection does, however, counteract by decreasing the amount of repeats. Therefore, one particular number of repeats might have been due to the gain of a new repeat unit or to the loss of a repeat due to selection. That means the ancestors can be different. The homoplasy produced during microsatellite evolution leads to an underestimation of the total amount of variation and genetic distance, and to an overestimation of the similarities among populations. To take this into account, distance measures have been designed specifically to apply to microsatellites. The **Stepwise Mutation Model (SMM)** (Ohta and Kimura, 1973; Weber and Wong, 1993) has been used to simulate this situation, hence favoured when effects of mutation increase (Murray 1996).

IAM estimators are highly favoured and more accurate than SMM when populations have been only separated for a short period of time (~300 generations) in which the effect of mutation is minimal (no homoplasy) and most of the allele frequency /genetic difference should be the result of genetic drift. Also, when the alleles contain no relevant history of mutational events, as might be the case for composite microsatellites, the IAM based estimators perform better than the SMM estimators (Murray, 1996).

1.9 Genetic distance measures used in microsatellite analyses

Measurement of genetic distances can be based on proportion of shared alleles (distance of shared alleles: Dps), which is the negative logarithm of the proportion of shared alleles (Bowcock *et al.* 1994). Dps is based on the IAM model. Dps distance measure can be calculated between individuals or between populations (Chakraborty and Jin, 1993). It can be used to construct dendrograms based on microsatellite data, to correlate genetic similarity with geographic location, and to place unknown individuals into the correct subpopulations (Estoup *et al.*, 1995b).

Another measure of genetic distance is delta mu squared, Ddm, was developed by Goldstein *et al.* (1995*b*) specifically for microsatellite markers and is based on the SMM model of evolution. Loci with high mutation rates are expected to yield larger Ddm values than those with lower mutation rates. The Ddm computer simulations suggest that it is robust to fluctuations in population size (Takezaki and Nei, 1996). An advantage of using Ddm is that it is independent of the assumption of symmetry in the mutation rate (Goldstein and Pollock, 1997). However, it was shown that when using microsatellite data, distance of shared alleles (Dps) is still superior to Ddm for closely related populations (Goldstein *et al.*, 1995b).

Due to the complexity of the evolutionary process of the microsatellites themselves, there is no single estimator that is superior in all situations. Other distance measures that are useful in microsatellite analyses are:

1. Nei's standard distance $Ds = -\ln(id)$. The unbiased version for small sample sizes defined in subtracts unity from each of the estimates in the numerator and denominator (Nei, 1978).

2. *Dsw:* The stepwise weighted genetic distance measure (Shriver *et al*., 1995) is "an extension of Nei's minimum genetic distance" and is based on frequency-weighted means of the absolute value of the difference in number of repeats over pairs of alleles i and j, both within and between populations.

3. *Dkf:* The kinship coefficient *kf* is defined as the probability for a gene taken at random from I, at a given locus, to be identical by descent to a gene taken at random from J at the same locus (Cavalli-Sforza & Bodmer, 1971).

4. *Ds* or *Gst:* Nei's identity for two taxa (Slatkin, 1995).

5. *Dfs:* The fuzzy set similarity measure (Dubois and Prade, 1980) is calculated by finding the set of alleles found in population A (call it set A), the set found in population B (call it set B), and dividing the cardinality of their intersection by the cardinality of their union,

1. 10 *L. major* and *L. tropica*

1.10.1 Distribution

Cutaneous leishmaniasis (CL) caused by *L. major* or *L. tropica* is either endemic or epidemic (Ashford 1999). The endemic areas are usually identified by active or passive case reporting while the epidemic areas are usually identified by an early warning system. The distribution and epidemiology of both parasites is governed by several factors. These factors are population migration, urbanization, farming, malnutrition, climatic factors and global warming, notably bioclimatic and vegetation zones, and finally ambiguous rodent population fluctuations (Neoumine, 1996; Klaus *et al*., 1999, Ashford, 1999; Anis *et al.,* 2001).

L. major and *L. tropica* are restricted to the Old World (OW), mainly in the Mediterranean basin, East Africa, Indian subcontinent, and West and Central Asia (Table 1.2). *L. major* CL is found in low lying arid and semiarid deserts (Klaus 1999). *L. tropical* CL, by contrast, is more common in urban areas and in villages in hilly rural areas (Klaus 1999). Examples for *L. major* foci are Jericho in Palestine (Jawabreh et al., 2001; Al-Jawabreh *et al*., 2003 and 2004) and Sidi-Bozaid in Tunisia (Ben Ismail *et al.,* 1997). As reviewed by Jacobson (Jacobson,

2003), examples for urban *L. tropica* foci are Baghdad in Iraq, Aleppo in Syria, Kabul in Afghanistan and Sanliurfa in South-east Turkey. Within the past decade, the world's largest *L. tropica* focus was in Kabul (WHO, 2002). Other smaller foci for *L. tropica* can be found in Shiraz in Iran, Mosul in Iraq, Ashkhabad in Turkmenistan, and Taza in Morocco.

Table 1.2 Summary of epidemiological and clinical features of *L. major* and *L. tropica*

Species	Clinical manifestation	Geographical distribution	Incubation period	Reservoir	Vector	Resolution	Age/Gender Distribution	Habitat
L. major	30% multiple sores, 70% on cheeks, arms and legs. Moist appearance. Rare: hyperkeratosis, sporotrichoid pattern: swollen lymph glands	Western and Central and South- west Asia , Africa, India	2-8 weeks	rodents: fat sand rat (*Psammomys.obesus*), gerbils (*Meriones, Rhombomys opimus*) diverse rodents in Africa	*Phlebotomus papatasi*, North Africa and South -west Asia *Ph. duboseqi* in Africa *Ph. salehi* in India	Heals in 3–5 months with scarring	All rural populations	Semi-arid and salty areas with Chenopodiaceae, Alluvial and loess deposits. Sahel savannah
L. tropica	CL, recidivans 57% single sore, slowly enlarges, crusty appearance 50% cheeks and arms	Western and Central and South- west Asia, North and East Africa, Sub-Saharan Savanna, India	2-24 months	Humans in anthroponosis, hyraxes in Kenya and near the Lake Tiberias, dogs in Morocco .	*P. sergenti* *P. guggisbergi* in Kenya *P. arabicus* in Tiberias	Heals over 1–2 years and scars, rarely spreads	Urban and rural children and younger adults.	In cities. Rocky and semi-arid rural areas.

1.10.2 Clinical features

In most patients, the skin lesion begins as a small erythematous papule about 2 to 6 weeks after inoculation. Over the following month the papule slowly enlarges, and a crust develops in its centre (Figure 1.3). With time, the crust falls away, exposing a shallow ulcer. If no treatment is given, the nodulous ulcer remains stable for 6 to 12 months before undergoing spontaneous resolution. Usually a shallow depressed scar is left behind.

Figure 1.3 Two cases of CL from Palestinian patients in Jericho. The disfigured chin case was caused by *L. tropica*. A case of *L. major* shows 3 lesions on the leg of a Chinese worker who lived in Jericho for one year.

CL caused by *L. tropica* and *L. major* are indistinguishable on clinical bases as both erupt in the same way, the size of the lesion ranging from a few millimeters to 4 centimeter or more. The site and number of lesions(s) are an indication of the type of CL. *L. major* usually presents as multiple lesions (≥3) and *L. tropica* is more often on the nose (Klaus 1999; AL Jawabreh *et al*., 2004). It was shown that multiplicity is more frequent in *L. major* infections (30%) than in *L. tropica,* (19%). Ten percent of *L. tropica* affect the nose, compared with 4% by *L. major,* and the chin shows the same pattern. Nonetheless, these sites are not the preferred ones for either species. The preferred sites for *L. major* are cheeks, arms and legs

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which account for more than 70% of the cases, while the preferred sites for *L. tropica* are cheeks and arms forming over 50% (Al-Jawabreh *et al*., 2004).

Abnormal manifestations for *L. major* are the enlargement of regional lymph nodes which is found in about 10% of patients. At times, the infection spreads deeply into subcutaneous tissue and muscle (Al-Gindan *et al*., 1989; Vardy *et al*., 1993). Hyperesthesia or anesthesia around the lesion was reported (Satti *et al*., 1989). Another unusual clinical presentation of *L. major* CL is a sporotrichoid pattern, in which subcutaneous nodules develop along lymphatics during the course of the infections (Kubba *et al*., 1987). Other rare varieties include a hyperkeratotic type, in which a thick adherent scale develops over an otherwise unremarkable lesion, and a chronic form in which the skin lesions remain active for two years or more.

CL, generally, is adversely affected by HIV. The adverse effects are shown by diffuse widespread eruption of lesions over the body which may reach up to hundreds (Gillis *et al*, 1995). A rare form of *L. tropica* CL is known as *Leishmania* recidivans, or lupoid leishmaniasis. This is a late manifestation of an *L. tropica* infection that comes years after the infection has resolved. It presents as boggy papules in or around the scars of primary lesions. The papules transform slowly into a spreading *Leishmania* recidivans (Momeni *et al.,* 1995; Klaus *et al*., 1999). There are few reports about *L. tropica* causing visceral leishmaniasis (Kala-azar) in India (Sacks *et al.,* 1995) and canine VL in Morocco (Lemrani and Nejjar, 2002).

1.10.3 Clinical diagnosis and characterization

The clinical diagnosis of CL, according to the case definition (WHO Recommended Surveillance Standards, 1999), is confirmed by demonstrating amastigotes by stained smear microscopy and/or *in-vitro* culture. As reviewed by Klaus *et al*., 1999 and Ashford, 2000, amastigotes are intracellular parasites forms present in monocytes and macrophages, but they can also be found as extracellular parasites (Figure 3.2) Using light microscopy in a Giemsa-stained smear, the amastigotes are seen as pale-blue oval bodies, 2–5 μm in diameter, with a single violet-blue nucleus. The point-shaped kinetoplast is difficult to see under the microscope. In *in-vitro* culture, the flagellated motile promastigotes which are longer than amastigotes, 10-15μm, are usually seen under light microscopy moving rapidly in a zig-zag motion (Figure 1.4). Promastigotes are the form present in the sand-fly vector.

R. L. Jacobson, 1996

Figure 1.4 Promastigotes: flagellated motile forms found
in the vector and in culture (x1000).

The two species are two different parasites with each having a distinct transmission pattern. They can not, however, be distinguished by morphology, neither microscopically as amastigotes in smears nor as promastigotes by *in-vitro* culture. Other techniques were needed to diagnose and characterize these two along with other species of *Leishmania*. Isoenzyme analysis formed the gold standard for species and strain typing (Le Blancq and Peters 1986, Rioux *et al*., 1990). It showed that *L. tropica* is more polymorphic than *L. major*. Serological techniques like enzyme-linked immunosorbant assay (ELISA) were valueless for diagnosis (Al-Jawabreh *et al*., 2003) but others like EF serotyping were employed to serotype the two species from cultured promastigotes (Jaffe and Sarfstein 1987, Schnur *et al*. 1990).

Molecular methods were used for a more sensitive diagnosis and genotyping. PCR techniques like permissively primed intergenic polymorphic-polymerase chain reaction (PPIP-PCR) (Eisenberger and Jaffe 1999) and ITS1-PCR (El-Tai *et al*., 2001; Schoenian *et al*., 2003) were able to detect and to identify *Leishmania* parasites at species level. When these and other techniques, like single-strand conformation polymorphisms of the ribosomal internal transcribed spacer 1 (SSCP-ITS1), were used for strain typing. *L. tropica* was proven to be a more variable species compared to *L. major* (Schönian *et al.,* 2001).

1.10.4 Vector

It is commonly known that *L. major* is transmitted by females of mainly *Phlebotomus papatasi*, and *L. tropica* by mainly *P. sergenti* (Diptera, Nematocera, Psychodidae, Psychodinae) (Figure 1.5). Sand flies are smaller than 3 mm and spend the day in burrows and cracks to prevent drying out. The female flies are active during the evening, hopping around silently for their blood meals for reproduction. Sand flies have biting season during which they are active in transmission of infection. In the Middle East it extends from

April/May to September/October (Killick-Kendrick, 1999; Jacobson, 2003; Wasserberg *et al*., 2003).

WHO/TDR/stammers (www.who.int)   Khun, K. G., 1999
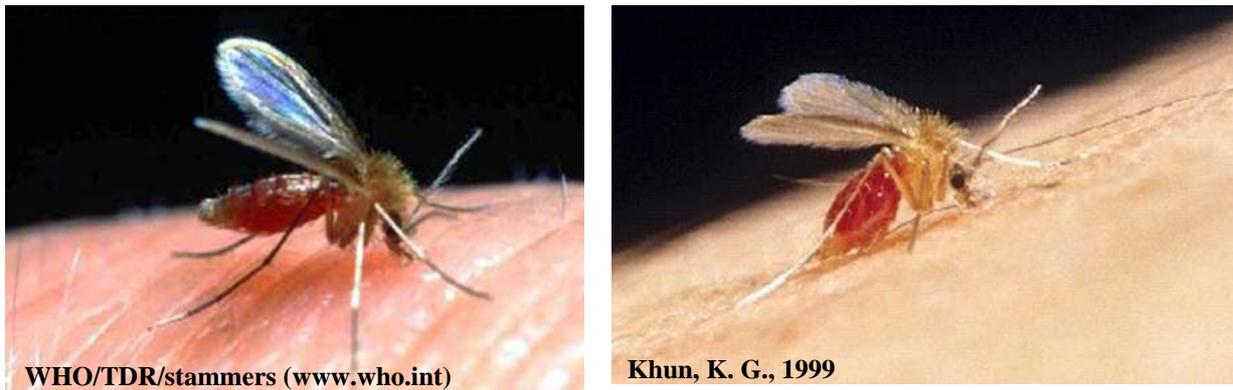
Figure 1.5 Phlebotomus species are the vectors for the Old World *Leishmaniasis*. They are small (2–3 mm), hairy, midge-like insects, with long slender legs and rather short mouth parts. They are not strong fliers and usually stay within 200 m of their breeding sites. In cities, they breed in refuse piles, in cracks in the walls, and foundations of buildings and fences. In desert areas they breed within the burrows of rodents (Killick-Kendrick, 1999; Klaus *et al*., 1999).

There are other vetors for *L. tropica* such as *P.* (*Larroussius*) *guggisbergi* in Kenya (Lawyer *et al*. 1991) with an infection rate of 4.3%, and *P.* (*L.) arabicus* in Tiberias (Jacobson *et al*., 2003) with an infection rate of 5%.

Vectors are either specific or permissive. Permissive vectors like *P. argentipes*, *P. (L.) arabicus, P. perniciosis* and *P. halepensis* have O-glycosylated receptors on their midgut epithelium supporting the adherence of different *Leishmania* species. In contrast, specific vectors like *P. sergenti* and *P. papatasi* lack these receptors and harbour only single species of *Leishmania, L. tropica* and *L. major*, respectively (Peckova *et al.,* 2005). This is in agreement with the finding of Kamhawi *et al*. (2000) that *P. sergenti* does not support *L. major* and *L. donovani*.

1.10.5 Reservoir

A reservoir host is the ecological system in which an infectious agent survives persistently (Ashford, 1996). CL is either anthroponotic, where infection is transmitted by the vector from man to man, or zoonotic, where an animal reservoir host is involved. *L. major* is completely zoonotic with various animals being confirmed as reservoirs. In North Africa and South-west Asia it is *Psammomys obesus,* a rodent living in underground burrows (Klaus 1999, Ashford, 1996) (Figure 1.6.a). In Iran and Central Asia, gerbils are the common reservoirs, either

Meriones libycus or *Rhombomys opimus*. *L. major* has also been isolated from *Meriones shawi* and *Meriones lybicus* (Rioux *et al*., 1986c; Ben-Ismail *et al*., 1987a).

(a)

(b)

Figure 1.6 (a) *Psamommys obesus*, fat sand rat, the established reservoir for *L. major*. (b) Rock hyrax from which *L. tropica* has been isolated.

CL caused by *L. tropica* is anthroponotic in urban areas and zoonotic in rural villages (Klaus *et al.,* 1999). In several rural areas in Kenya and near Lake Tiberias, *L. tropica* has been isolated from hyraxes (*Procavia capensis*) (Figure 1.6.b) (Ashford and Sang, 2001; Jacobson *et al*., 2003).

1.10.6 Control

As a role of thumb, control measures are the result of breaking one or more elements in the life cycle. There is no single method that can be used for all situations and one method may be successful in one place but not in another. In addition, cost effectiveness has to be considered before adopting a certain method. Moreover, control measures should always be revised and evaluated. Some measures target the reservoir by eliminating the rodents, by destruction of the animals' food sources, and /or ploughing burrows as in Jordan and Tunisia (Klaus *et al*., 1999; Ashford, 1996).

The sand fly vector has continuously been the target for control measures. This included the destruction of breeding sites by removing garbage and debris left near houses, and by covering cracks in buildings. In addition, spraying of residual insecticide inside houses and outside under windows were used. Plants like *Bougainvillea glabra* were shown to decrease the risk for leishmaniasis by reducing the life span for sand flies (Schlein *et al*., 2001). Impregnated bed nets with various insecticides such as Deltamethrin were applied as control measure with significant reduction in CL incidence rate (Alten *et al*., 2003).

The human host was also a means for control either by allowing the patients to be treated 2-3 weeks after the appearance of the lesion, as is the policy in Jericho, to allow immunity to develop or by leishmaniazation as in Iran (Khamesipour *et al.,* 2005). Further, there are attempts to develop *Leishmania* vaccine, but no definite results have been obtained yet (Valenzuela *et al.*, 2001)

1.10.7 Treatment

Most CL cases heal spontaneously in less than a year. However, living for one year with a lesion in the face which may be disfiguring and may complicate due to secondary bacterial infection is problematic. Treatment is the sole choice for these patients. Dowlati (1996) reviewed types of therapeutic strategies for CL. Among the methods tried with varying degrees of success are thermotherapy, cryotherapy with liquid nitrogen, and surgery (Dowlati, 1996; Al-Majali *et al.,* 1997; Reithinger *et al*., 2005). The most common treatment in most parts of the world is the intralesional application of pentavalent antimonials, e.g. sodium stibgluconate (WHO, 1990) commercially known as Pentostam (Glaxowellcom). The dose per lesion is 0.2-0.4 ml (100 mg/ml) or 15-20 mg /kg/day for 15-20 times every other day, more or less depending on the lesion and its response to treatment (Croft and Yardley, 2002). Pentostam can be given intramuscularly (IM) or intravenously (IV) depending on the progress and stage of the lesion. In the case of systemic treatment (IV), a patient should be hospitalized and liver enzymes monitored for toxicity. Meglumine antimonate or Glucantime are other forms of antimony compound that was used once.

Topical ointments have been also used. Paromomycin, in combination with methyl benzethonium chloride gave different rates of success when applied in areas where either *L. major* or *L. tropica* (Klaus *et al*., 1999) is endemic. *L. tropica* tend to be less responsive to therapy. In a few cases it took more than 6 months for large lesions on the nose to heal up (observations from patients in Jericho). To allow development of lasting immunity, patients with lesion(s) less than 3 weeks old are not treated and advised to come back after the 3-week period had elapsed.

1.11 Population genetics of *Leishmania* parasites

Population genetics dates back more than one hundred years, yet its modern form emerged only during the 1970s (Wakeley and Takahashi, 2003). Hartl and Clark (1997) defined population as a group of organisms of the same species living within a sufficiently restricted geographical area that any member can potentially mate with any other member. This

definition bounds the population by mating of same species in a restricted geography. Population genetics can be defined as 'the m athematical study of the dynamics of genetic variation within species. Its main purpose is to understand the ways in which the forces of mutation, natural selection, random genetic drift, and population structure interact to produce and maintain the complex patterns of genetic variation that are observed among individuals within a species' (Wakely, 2005).

Clonality vs sexuality debate

As for any parasite, the mode of reproduction of *Leishmania* can either be sexual or asexual (clonal). Sexual reproduction means passing half of the genes to the new progeny while asexual or clonal means passing all of the genes to the progeny (Ayala *et al*., 1998). Natural selection favours asexual reproduction, because given the same number of progeny, the asexual individual has double the fitness of the sexually reproducing one (Ayala *et al*., 1998; Victoir and Dujardin, 2002). Sexual reproduction has the advantage of creating variability for adaptation to changing environments, but has the disadvantage that advantageous gene combinations may be disrupted (Victoir and Dujardin, 2002). Since sexual recombination in *Leishmania* seems to be either absent or very rare, the "clonal theory" was proposed to explain the population structure of different *Leishmania* species (Panton *et al*., 1991; Tibayrenc *et al*., 1990; Dujardin *et al*., 1995). According to this theory, genetic variability is due to gene mutations and their selection along clonal lineages. Clonality, but without solid prove, was preferred by Lanotte *et al.* (1986) and Lainson and Shaw (1986). Studies on *Trypanosoma cruzi*, another kinetoplastid parasite, and other parasites concluded that natural populations reproduce predominantly clonally (Tibayrenc & Ayala 1988, Tibayrenc *et al*., 1990). Yet, parasites can undergo sexual recombination in the laboratory (Tibayrenc *et al*., 1990 & 1991)

The knowledge of the mode of reproduction, clonal or sexual, is important for answering many clinical, epidemiological and public health questions. Ayala *et al.,* (1998) lists three reasons: First, in a sexually reproducing organism the individual genotype is ephemeral; the entity that persists and evolves is the gene pool, and a few individuals encompass most of the genetic variability of the species. On the contrary, for a clonal organism, the entity that persists and evolves is the clonal lineage and the genetic diversity of the species can be captured only by extensive sampling of distinct lineages. Second, extensive genetic divergence among clonal lineages may reflect diverse biological characteristics, including pathogenicity, resistance to drugs and vaccines and other clinical parameters. Third, in clonal

organisms, epidemiological surveys, medical typing and drug development should be based on identification and characterization of clonal lineages, targeting those that are more pathogenic or ubiquitous.

1.12 Genetic diversity and bottleneck theory

A population bottleneck (or genetic bottleneck) is usually defined as an evolutionary event in which a significant percentage of a population or species is killed or otherwise prevented from reproducing, and the population is reduced by 50% or more. Bottlenecks reduce genetic variation and strongly disrupt the pattern of allele frequencies especially if the population has a low growth rate or high reproductively skew defined as a high variance in the reproductive success of either males or females (Hoelzel, 1999). It increases genetic drift which is inversely proportional to the population size. The overall and lasting result of a bottleneck is the reduction of genetic diversity, redistribution and reduction of allele frequencies and disappearance of rare alleles (Hoelzel 1999 and 2002). The immediate and transient observation following a bottleneck event is, however, the unexpected increase in heterozygosity level (Cornuet & Luikart, 1996) due to sudden and rapid loss of rare alleles and resulting deficit of alleles (Hoezel 2002). The bottleneck phenomenon has a detrimental effect on the population as the loss of genetic diversity and reduced polymorphism hinders the potential of a population to respond to a changing environment (Hoelzel, 1999).

This impact of bottleneck is dependent on two factors: the effective size of the population, and the duration for which the population remains small. However, the duration of the bottleneck effect can be minimized if the growth rate of the population is high (Hoelzel, 1999).

The bottleneck hypothesis was often used to explain observations of low genetic variation (O'Brien *et al.*1987; Gottelli *et al.* 1994). An important aspect about bottlenecks is that they may lead to the introduction of new species (Dodd and Powell 1985; Ringo *et al*. 1985; Meffert and Bryant 1991; Galiana *et al*. 1993), though it is not the only reason for the rise of new species (Turelli *et al*. 2001).

1.13 Objectives (Figure 1.7)

As mentioned above, use of new molecular techniques offers various possibilities for diagnostics, epidemiology and applied genetics.

Dealing with material from an endemic area, there were three main applications we were interested in: use for molecular clinical diagnosis, molecular epidemiology and to study molecular diversity. For each topic a subset of problems were to be solved:

1. *Molecular clinical diagnosis*:

   a. To apply molecular- based techniques (ITS1- PCR) for detection and identification of *Leishmania* parasites in new and old archived clinical samples collected in the vicinity of Jericho from patients to re-evaluate the epidemiology of cutaneous leishmaniasis in this area.

      So far, detection of *Leishmania* was based on microscopy and cultivation, both methods being not very sensitive. PCR detection will be used to detect and to identify the parasites at species level in all samples contained in the human clinical sample bank.

   b. Performance of the ITS1-PCR method will also be evaluated using two types of clinical samples, unstained tissue scrapings and blood and tissue scrapings on filter paper.

   c. Comparing the performance of the classical diagnostic methods (microscopy and culture) with the ITS1-PCR.

   d. Evaluating graded microscopy vs. ITS1-PCR using Giemsa-stained clinical samples.

2. *Molecular epidemiology*

   a. So far, the frequency, distribution and determinants of CL in the District of Jericho were described based on conventional epidemiology. Molecular-based methods i. e ITS1-PCR and RFLP will be used as epidemiological tools to describe the situation in what is called 'molecular epidemiology'. This includes correlating the results obtained by molecular methods to demographic and environmental factors that are known to influence the relative abundance of parasites, reservoir hosts and vectors.

   b. To check, for a purely public health consideration that includes vector control, for space-time clusters for cases of CL (spatial-temporal clusters).

Not only population growth and movements, introduction of non-immune people into area of endemic foci, enhanced urbanization, but also ecological changes, most probably the effect of global warming, are known to lead to an increase of prevalence of cutaneous leishmaniasis. Changes in the modern demographic and environmental conditions will be recorded and correlated to the molecular epidemiological data obtained in this study.

3. *Molecular Genetic diversity* :

To analyse genetic variation in strains of *L. major* isolated in Jericho area as well as from other geographical locations (Middle East, Africa, and Central Asia) by using microsatellite markers. Ten different microsatellite markers will be applied to assess genetic heterogeneity within *L. major*. Based on the information obtained, attempts will be made to analyse the genetic structure of different populations of *L. major* using appropriate software.
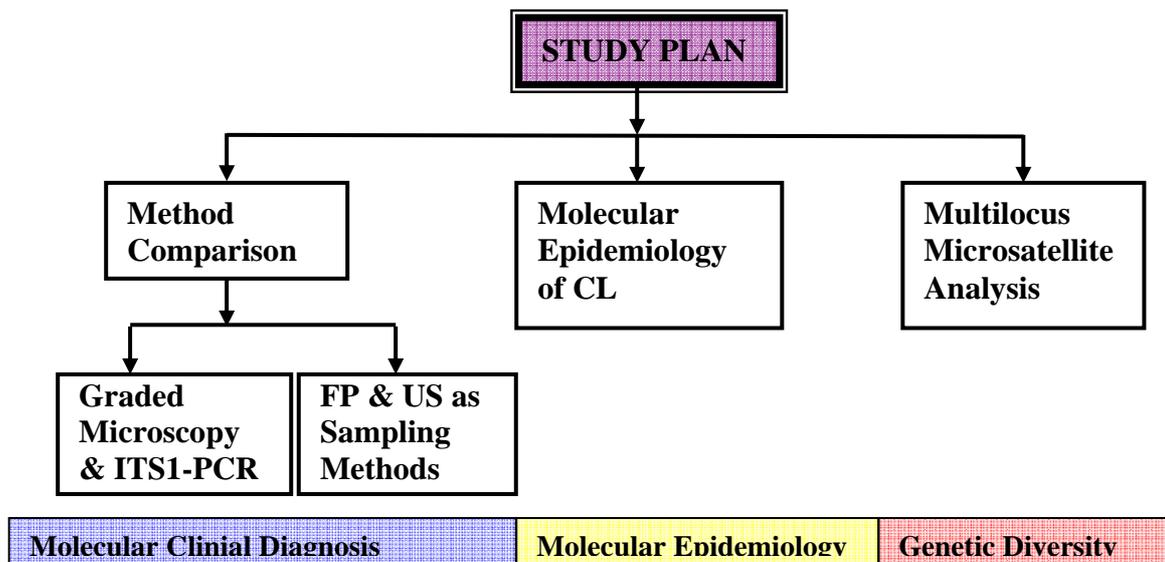
Figure 1.7 Study plan

2. MATERIALS AND METHODS

2.1 Clinical molecular diagnosis and local molecular epidemiology

2.1.1 Patients and study area:

This part of the study was conducted in the decade, 1994-2004, in the CL endemic District of Jericho (A'riha) in Palestine and its vicinity of the close hilly areas of Jerusalem (Al-Quds), Ramallah and Nablus, extending over an area of more than 593 km^2, with a population density of 73 person per km^2 and a total population of more than 40,909 (Palestinian Central Bureau of Statistics, 2005). Jericho, a strip of land extending from the Dead Sea and Jerusalem in the south to Nablus and Tubas in the north and from the River Jordan in the east to the hills of Ramallah and Nablus in the west (Figure 2.1), is a low-lying (244-398 m below sea level) arid to semi-arid area located between 29°-33° north of the equator. It is situated on the floor of the northern tip of the 6,500-kilometer-long Syrian-East African Rift, called the Jordan Valley. Climate is affected by the subtropical aridity which is characterized by long, hot and dry summers and short, cool winters. Jericho is at latitude of 31° 52´ N and longitude 35° 28´ E.

Between 1994 and 2004, the daily average temperature throughout the year was 23.3 ^{o}C. The average maximum temperature was 44.9 ^{o}C, with maximum temperature 48 ^{o}C in 2002, while the average minimum temperature is 3.6 ^{o}C with minimum temperature 0.6 ^{o}C in 1997. The annual average rainfall in the period between 1994 and 2004 is 146.29 mm and falls between October/November and March/April, with the rest of the year mostly rainless. The maximum annual rainfall was 240.3 mm in 2002/2003 season, while the minimum annual rainfall (driest season) was 40.3 mm in 1998/1999 season. (Personal communication with the Palestinian Meteorology department- Jericho, 2004; Environmental profile for the West Bank, 1995).

This area is generally characterised by the presence of springs, on which the people depend to grow banana, vegetables and palms, such as Wadi Qelt that brings water from the mountains of West Bank down to Jericho, which over-floods the city in winter and makes the area green and lush all year round. Reduced vegetation and grass is another characteristic of the area. The area is flat and composed of cracked alluvial soil with hills. Inhabitants mainly work as farmers, government employees and nomadic Bedouin shepherds who roam the area all the year round.

In the decade period between 1994 and 2004, a total of 943 patients with skin lesion(s) attended ICS-Jericho medical laboratory for clinical diagnosis and were in turn sent to the

Jericho Health Department-Ministry of Health for treatment. All patients were tested by microscopy (Giemsa-stained smears) which is the basic diagnostic method adopted by ICS-Jericho and the "gold standard". Since 1998 NNN culture and ITS1-PCR were introduced into Jericho. They were, however, used mainly for research purposes, such as collection of strains and to confirm clinical diagnosis, and not merely regular diagnostic methods. The patients were Palestinians living in the district of Jericho and the vicinity with exception of a few patients who were temporary workers on international projects.
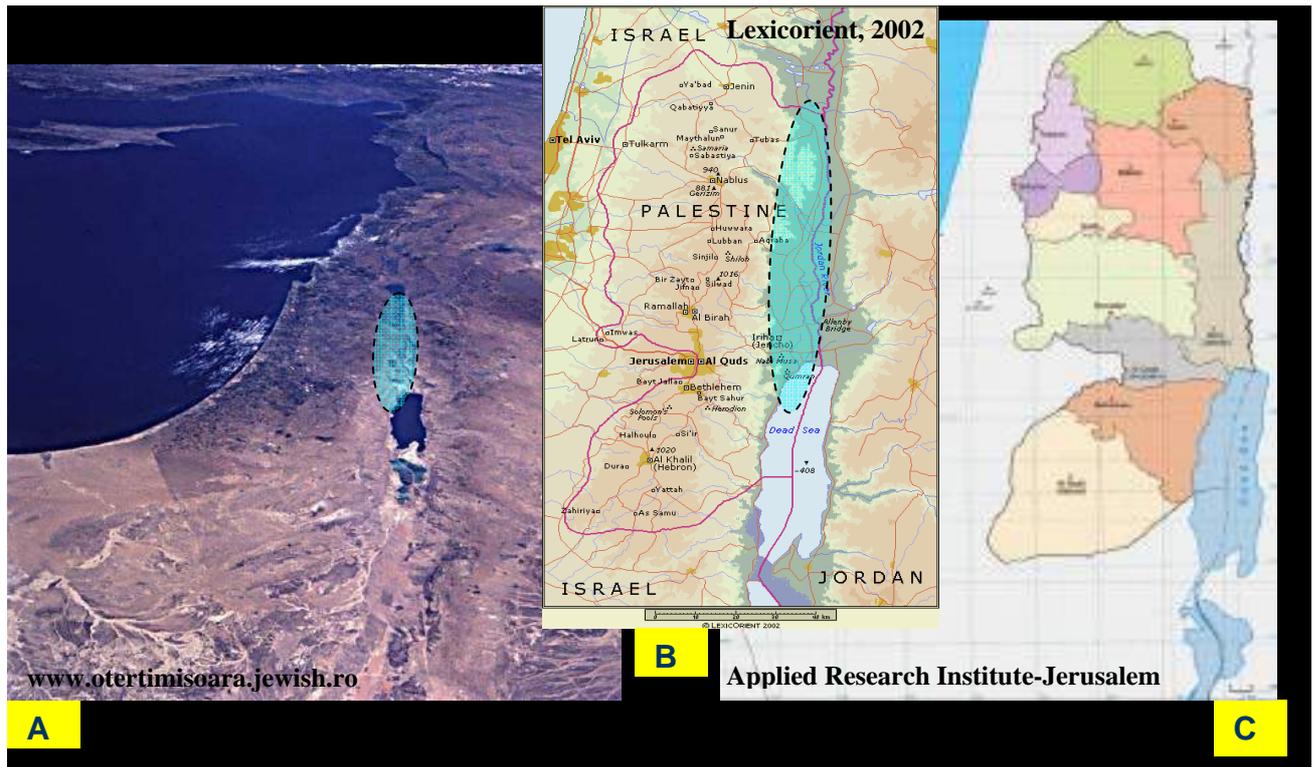
Figure 2.1 (A) Satellite image of the District of Jericho in the Eastern Mediterranean region. (B) District of Jericho. (C) Map of the Palestinian governorates according to Palestinian classification.

2.2 Sample collection

2.2.1. Patient data sheets

Data of each referred patient were collected in a questionnaire filled in by the technician, directly interviewing the patients. The questionnaire included demographic, clinical, diagnostic, and epidemiological questions. It was designed to be user friendly with a computerized input interface using Dos-based EpiInfo 6, Access-based EpiInfo 2002 and Access-based EpiInfo 2004 throughout the study period.

2.2.2. Collection and Giemsa staining of skin scrapings

For diagnostic purposes skin scrapings were collected from 943 Palestinian patients with lesions referred to Islah Medical Laboratory in Jericho in the period between 1994 and 2004. The patients were from all refugee camps, villages, Bedouin encampments and Jericho City. The tested lesion was disinfected using cotton wool immersed in 70% ethanol. Using a sterile lancet, two 3-5 mm incisions were cut on the periphery of the lesion and from the centre in case it is not infected (Rameraiz *et al*., 2000). The incisions were squeezed gently and touched with 3-5 clean glass slides. Three of the slides were air dried and fixed with methanol for a few seconds, while the remaining two are kept for further comparison study as shown below in 2.2.3.

Concentrated Giemsa stain (Finkelman, Petah Tikva, or Sigma) was applied for no more than 30 seconds and then slides were washed under running tap water and air dried. The smears were examined using light microscope with a 40 x lens and with 100 x oil immersion lens. If at least one amastigote was found the smear was declared positive. When no amastigotes were seen after 15 minutes the smear was declared negative.

2.2.3. Sampling using filter papers and unstained smears

In addition the incision cut was touched three times with an autoclaved 3 mm filter paper (Schleicher and Schüll, Germany) during the sampling process, producing three separate and thick tissue-blood spots. After air drying, the filter papers were wrapped in aluminium foil and stored at room temperature until use. Between June 1997 and July 2004, 418 filter papers were sampled. Also, between August 1998 and June 2004, 173 unstained slides were sampled in Jericho.

2.2.4. Cultivation of parasites from dermal tissue aspirates

Two hundred and seventy dermal tissue aspirates from the same incisions made for microscopy were cultured in rabbit blood–agar semisolid and NNN media. To guarantee aseptic techniques, a large Bunsen burner was lit. 0.5 ml of sterile 0.9% normal saline was injected into the incisions, mixed with the fluid there, then aspirated and discharged into two culture tubes held close to the Bunsen burner. One tube containing semisolid (SS) blood agar (Schnur and Jacobsen, 1987) and the other NNN medium, Novy-MacNeal-Nicolle medium (1.4% agar, 0.6% NaCl as described by Evan (1989)).

 Ten ml of defibrinated rabbit blood containing 100 μl gentamicin were added to 100 ml of the previous melted NNN medium. Two ml of NNN media were distributed into small sterile

tubes and kept in tilted slant position until the agar solidified. The tubes were stored at $4^{o}C$ until use. Before inoculation of samples, the tubes were adapted to room temperature. After inoculation of material, the culture tubes were incubated at 22-28°C. After one week, one drop of media was examined under the microscope. Positive cultures were followed up for mass cultures and cry-conservation by Dr. L. F. Schnur at the WHO *Leishmania* reference centre in Jerusalem, in which promastigotes from cultures were transferred into Schneider's *Drosophila* medium (Gibco, Grand Island, NY; Biological Industries Beit Haemek) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 200 μg/ml streptomycin, and 200 U/ml penicillin for further mass culturing. The parasites were cultivated in 5 ml of Schneider's medium for 2-3 days before they were transferred to a larger volume (40 ml). Negative cultures were kept for three weeks with weekly regular examination before being declared negative and discarded.

2.3. DNA extraction

2.3.1 DNA extraction of cultured parasites

For DNA preparation, parasites grown in mass culture were harvested at a density of about 2 x 10^7 parasites/ ml. The cells were centrifuged for 10 minutes at 2500 rpm and washed three times with phosphate-buffered saline, pH 7.4 (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 ml of distilled H_2O. Adjust the pH to 7.4 with HCl. Add H_2O to 1 liter and sterilize by autoclaving)

DNA extraction was performed as described by Schönian *et al.* (1996). The pellet of cultured cells was resuspended in 1 ml NET lysis buffer (50 mM NaCl; 10 mM EDTA; 50 mM Tris-HCl pH 7.4). Sodium dodecyl sulphate (SDS) (Merck, Darmstadt, Germany) was added to a final concentration of 0.5% and the mixture was shaken until the solution was viscous. RNase was added to a concentration of 100μg/ml and the mixture was incubated in a water bath for 30 min at $37^{o}C$. Proteinase K (Boehringer Mannheim, Mannheim-Germany) was added to a final concentration of 100μg/ml and the samples were incubated at $60^{o}C$ in a water bath for 3 hours or overnight. Then samples were subjected to classical phenol/chloroform extraction with subsequent ethanol precipitation. The DNA pellet dried and dissolved in100 μl TE buffer (2ml of 1 M Tris-Cl and 400μl of 0.5 M EDTA in 1 liter adjusted for pH7.6 and sterilized by autoclaving). DNA concentration was measured spectrophotometrically (OD_{260} and OD_{280}) using UV/ Visible Spectrophotometer (Pharmacia LKB. Ultrospec III). DNA samples stored at 4°C until use. Dilutions of 10ng/μl were prepared as working solutions.

2.3.2 DNA extraction of Clinical samples

Filter papers, unstained smears and stained smears

DNA was isolated as described previously by Meredith *et al.* (1993) and El Tai *et al.* (2000). Briefly, filter papers with spotted skin scrapings were punched out with a sterile paper puncher. Between each sample, sterile filter paper was punched and the puncture was cleaned with 70% ethanol to prevent carry-over of DNA contamination. Two punched out sample discs were placed into 250 µl lysis buffer (50mM NaCl, 50mM Tris- HCl; 10mM EDTA pH 7.4; 1% {vol/vol} Triton X-100; 200µg of Proteinase K per ml) and incubated in a water bath for 3 hours or overnight at 60°C. Then the mixture was subjected to phenol-chloroform extraction. An equal volume of buffered phenol was added, shaken gently for 3 minutes and centrifuged at 13000 rpm (maximum speed) for 3 minutes. The upper aqueous phase was then transferred to a new 1.5 ml Eppendorf tube. An equal volume of buffered phenol: chloroform: isoamyl alcohol: (25:24:1) was added, shaken and centrifuged as mentioned above. The upper aqueous phase was again transferred to a new 1.5 ml Eppendorf tube. An equal volume of chloroform-isoamyl alcohol (24:1) was added, shaken and centrifuged as mentioned above. The upper aqueous phase was now precipitated by mixing with 1/10 volume of 3M Na acetate and 2 volumes of absolute ethanol. The tubes were left overnight at – 20°C. Then, samples were centrifuged for 30 minutes at 13000 rpm (maximum speed). DNA pellets were washed with 70% ethanol and centrifuged for 15 min at 13000 rpm (maximum speed). The invisible DNA pellet was dried using speed vacuum dryer (Eppendorf Concentrator 5301, Nethalar-Heinz GmbH, Hamburg-Germany) for 5 minutes till the 70% ethanol was completely evaporated and re-suspended in 100 µl TE buffer (10mM Tris; 1mM EDTA pH 7.5). The pellets obtained from clinical samples were additionally purified using a commercial kit (Machary- Nagel, Düren-Germany). The 30 µl purified DNA was stored at -20°C until use.

In the case of stained and unstained smears, 50 µl of lysis buffer from the 250µl in the Eppendorf was spread on the surface of the slide and with the yellow tip the material was scratched and swept into the tube. The extraction procedure was completed as described above for filter papers.

2.4 PCR amplification: Internal transcribed spacer (ITS1)

The ITS1 region was amplified with the following primers: LITSR (5'-CTGGATCATTTTCCGATG-3')/ L5.8S (5´- TGATACCACTTATCGCACTT-3´) as described by El Tai *et al*., (2000). These primers amplify a section of the ribosomal transcribed spacer (ITS1) region, which separates the genes coding for the ssu rRNA and 5.8S

rRNA (300-350 bp) of all *Leishmania* species (Figure 2.2). Primers were synthesized commercially (TIB-MOLBIOL, Berlin, Germany).
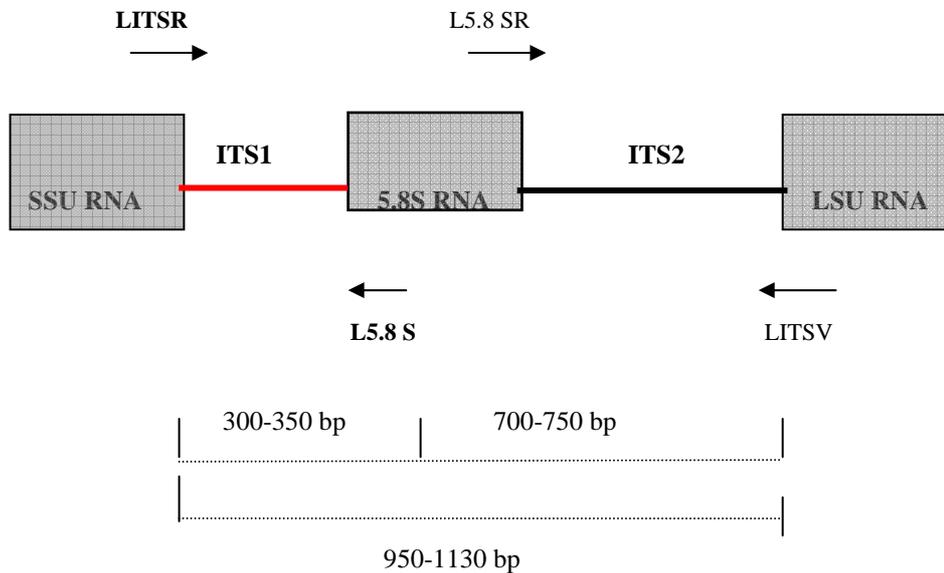
Figure 2.2 Schematic representation of the internal transcribed spacer (ITS) in the ribosomal operon with primers amplifying different parts of the spacer. Primer sequences are given in the text above. SSU = small subunit rRNA gene, LSU = large subunit rRNA gene.

Amplification reactions were performed in volumes of 50 μl (Table 2.1). On ice and under sterile conditions, 3 μl DNA from clinical samples, 2 μl DNA for re- PCR or a 10ng/μl-working solution from culture, were added to a PCR mix containing 200μM of each dNTP (Amersham Biosciences, UK limited. England); 5 μl of commercially available 10x PCR buffer (10mM Tris-HCl, pH 8.0; 50mM KCl; 1.5mM $MgCl_2$); 1-2 units of *Taq* polymerase (Applied Biosystems, Roche, USA) and 25 pmol of each primer. Samples were overlaid with sterile, light mineral oil (Sigma-Aldrich, St. Louis, USA) and amplified as follows: initial denaturation at 95°C for 2 min followed by 35 cycles consisting of denaturation at 95°C for 20 sec, annealing at 53°C for primer pair LISTR/L5.8S for 30 sec and extension at 72°C for 1 min. This was followed by a final extension cycle at 72°C for 6 min and then the thermocycler was set at 4°C for infinite.

PCR was run in Robocycler Gradient 40, Stratagene or Perkin-Elmer Thermocycler 9600. Amplification products were subjected to electrophoresis in 1% agarose NA (Amersham Biosciences, Sweden) at 100 V in 0.5x TBE buffer (0.023M Tris-borate, 0.5mM EDTA) for a

15 cm long gel tray for about 1hour, and at 140 V for a 25 cm long gel tray for about 1.5 hours and visualized under UV light after staining for 15 min in ethidium bromide (0.5μg/ml). One kilo base pair (1kbp) or 123 bp DNA ladders (Invitrogen, Life Tech, Carlsbad CA, USA), were used as molecular size markers. Amplified PCR products were documented by photography (Gene Eagle eye 11, Stratagene, Heidelberg, or GeneGenius, Syngene Europe). Re-PCR was performed to obtain sufficient PCR products.

Table 2.1 Components and quantities for the Master Mix (MM) for one sample

			ITS1-PCR		Microsatellite analysis
No.	Item	μl	Final concentration	μl	Final concentration
1	10 x PCR Buffer	5	1x	5	1x
2	dNTPs, 2.5mM each (10mM)	4	200μM	4	200μM
3	Primer, Forward, 10 pmol/μl	2.5	25 pmol	1.5	15 pmol
4	Primer, Reverse, 10 pmol/μl	2.5	25 pmol	1.5	15 pmol
5	DMSO	1.25	2.5%	-----	-----
6	Taq Polymerase, 5 units/ μl	0.2	1 units	0.2	1 unit
7	H_2O	32.55		34.8	
	Total volume of the MM	47		47	
	DNA template	3		3	
	Total	50		50	

PCR buffer is provided with 1.5 mM $MgCl_2$ in 1 x buffer

2.5 Panel of controls used in diagnostic PCR

During this study a panel of controls was used to detect contamination (false positive) or inhibition (false negative) during PCR. These controls included:

2.5.1 DNA extraction control

Human housekeeping genes were used to check for the integrity of the extracted DNA and thus for true negativity of the ITS1-PCR results, under the same conditions as described above for ITS1-PCR. The primer pair HβG-F (5`-GAA GAG CCA AGG ACAGGT AC-3`)/HβG-R (5`-CAA CTT CAT CCA CGT TCACC-3`), designed to amplify the genes corresponding to β-globin region of human genome DNA, with a product size of 268 bp, was used as a DNA extraction control (Saiki *et al*., 1988). Interchangeably with HβG, the β-actin-

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gene was used as extraction control. The primer pair: *Aco1:* (5´-ACC TCA TGA AGA TCC TCA CC-3´)/ *Aco2:* (5´-CCA TCT CTT GCT CGA AGT CC-3´) targets a 120-bp fragment within the fourth exon of the human β-actin gene (Musso *et al*, 1996). DNA extraction control becomes a necessity for ITS1-PCR negative samples.

2.5.2 Positive and negative controls

DNA from the strain of *L. turanica* (MRHO/MN/83/MNR-6), 10ng/μl, and sterile double distilled water (B. Braun Melsungen AG, Melsungen) were used as positive and negative controls, respectively.

2.5.3 Inhibition control

In order to detect possible inhibition caused by substances, which were not sufficiently eliminated by the crude extraction (such as hemoglobin), an internal control for PCR inhibition was included, where the same amount of purified *L. turanica* (MRHO/MN/83/MNR-6), 10ng/μl, DNA as in positive controls was added to each diagnostic sample. Moreover, 2.5% dimethyl-sulfoxyd (DMSO) (Carl Roth GmbH, Karlsruhe) was used as an enhancer in all diagnostic PCR runs. Other precautions such as wearing gloves, frequent cleaning and using disposables were applied to prevent contamination.

2.6 RFLP analysis of ITS1 amplicons

The amplified ITS1 region was digested using 2 different restriction enzymes (*Hae III* and *MnlI*) according to the conditions recommended by the supplier (Hybaid-UK or BioLabs Inc, New England). Briefly, for diagnostic samples 15 μl of the DNA were restricted by addition of 1μl (5 units) of each enzyme and 2 μl of the corresponding 10 x buffer and 2 μl of distilled water and incubated at 37°C for 2 hours. Culture samples were restricted in the same way but using different volumes: 5 μl of DNA are added to 1μl of enzyme and 2 μl of the buffer and 12 μl of distilled water. Restriction products (20 μl) plus 4 μl loading buffer (80% glycerine, 0.1M EDTA pH 8.0, 10 mM Tris-HCl pH 8.0) were subjected to electrophoresis in 2 % MetaPhore agarose (for fine analytical separation of small nucleic acids and PCR products, Cambrex Bio Science Rockland, Inc, Rockland ME, USA) for 2 hours at 110 Volts for medium gel tray, or 140 Volts for a large gel tray in 0.5x TBE buffer (0.023M. Tris-borate, 0.5mM EDTA). Normal agarose (Amersham Biosciences, Sweden) was also successfully used. DNA fragments were visualized under UV light after staining for 15 min in ethidium bromide (0.5μg/ml). The gel was run again for 15 min for increased resolution. Restriction

products were documented by photography (Gene Eagle Eye 11, Stratagene, Heidelberg or GeneGenius, Syngene Europe). 1 kb or 123 bp ladders were used. The restriction patterns were compared using an ITS1 restriction map.

2.7 Evaluation studies

2.7.1 Graded microscopy vs ITS1-PCR

2.7.1.1. Patients and study area

In the period between July 2002 and December 2003, a total of 86 patients living in Jericho area in Palestine presenting with skin lesions were referred to Islah medical laboratory-Jericho for the diagnosis of CL by direct smear microscopy, out of which 48 patients were randomly selected as clinically positive for graded microscopy. Stratified random sampling was used to select 20 patients from the 48 for the comparison with PCR. A total of 50% (10/20) were children (<14 years of age), 40% (8/20) were adults (>14 years of age) while 10% (2/20) were of unknown age; 55% were males.

Thirty five percent of the patients (7/20) had only a single lesion, same number had double lesion and the remaining 30% (6/20) had multiple lesions (>3). The distribution of lesions was known in 44 cases and was as follows: 57% (25/44) on the head and neck, 39% (17/44) were in the upper extremities and 4.5 % (2/44) on the lower extremities. Sixty five percent (13/20) of the lesions were ≤ 1 month old, 10% (2/20) were 1-2 months old, 15% (3/20) were 3 or more months old.

To check for the validity (sensitivity and specificity) of ITS1-PCR as a diagnostic test, fifteen EDTA blood samples were obtained from the blood bank of the Charité Hospital-Berlin as negative controls. These blood donors were all native Germans who never visited any tropical country within the last year and were negative for a set of routinely screened diseases as part of the blood bank profile. Permission was obtained from the Ethics Committee-Charite Hospital in Berlin, Germany. Each patient gave a written declaration to the blood bank service allowing the use of his/her sample for scientific research purposes.

From each blood sample, three drops/squares were thickly and separately smeared on a slide and Giemsa-stained. Outcome of staining was checked by scanning one drop (square) one slide using a 100x immersion lens.

2.7.1. 2. Sample collection and preparation:

All 48 patients showed typical lesions. These were cleaned using sterile gauze and physiologic saline and disinfected 5 times in an outward circular motion with 70% alcohol – immersed cotton. Using a sterile lancet or sterile surgical blade, 2-3 mm long superficial

incisions were cut on the margins of the lesion and pressure was maintained with finger to achieve hemostasis. For each patient, three touch-smears of dermal tissue scrapings were collected on one slide, air dried, fixed in absolute methanol and Giemsa stained.

2.7.1. 3. Standardized graded microscopy:

For all 20 Giemsa-stained slides selected for the comparative analysis, three 5 mm x 5 mm squares were marked with a fine marker on the back surface of the slide and given the designations 1, 2 and 3 (Figure 3.1). The size of the area marked corresponds to the area of about 600 - 1000 oil immersion fields (OIF), as 1000 OIF per slide have to be screened before declaring negativity (World Health Organization 1990). The squares were purposefully selected to be marked on areas of varying densities on the slide to have examples of the various outcomes of the staining procedure. Each square was completely scanned with a 100x immersion lens by the same blinded person using the same bright-field microscopy (Figure 3.1). A maximum of four slides were scanned daily to prevent exhaustion and hence subjective interpretation. The average time taken to scan a 5mm x 5mm square is 17.5 minutes. The number of amastigotes in each square was quantified and graded as compared to WHO grading used in splenic aspirate smears (World Health Organization, 1990) and the semi quantitative scaling adopted by Ramiraze *et al*. (2000) for touch smears from lesions of CL. The semi-quantitative grading to evaluate the parasitic density in each slide was performed as shown in table 2.2.

The blood of the control persons was treated exactly the same: three squares were marked on each slide.

After microscopy, DNA was extracted of the material taken from the marked area. PCR amplification for ITS1, DNA extraction control for human β-Actin housekeeping gene and the control panels were carried out as described above.

McNemar's test was used to compare the matched pairs for graded microscopy and ITS1-PCR. McNemar's test was performed using the free Quickcalcs web calculator available on www.graphpad.com.

Table 2.2 Grading of parasites in Giemsa-stained smears from skin lesions.

Grade	Average number of amastigotes	Minimum no. OIF scanned
-	0 amastigotes per square *	625 (whole square)
+	1 amastigote per square to 1 per OIF**	625 (whole square)
++	2 to 10 amastigotes per OIF	50
+++	11 to 20 amastigotes per OIF	50
++++	> 20 amastigotes per OIF	10

* Square: 5mm x 5mm corresponding to 625 OIF.   **OIF: oil-immersion field

2.7.2 Filter Paper vs Unstained smears and conventional methods vs PCR

During the study period, February 1994 to July 2004, skin scrapings spotted on filter papers and unstained tissue smears (2.2.3) were compared as potential sampling methods for ITS1-PCR. Conventional diagnostic methods including Giemsa-stained smears (2.2.3) and *in-vitro* culture (2.2.4) were compared with ITS1-PCR (2.3, 2.4 and 2.5). The comparison was based on three different 'gold standards': WHO case definition, combined (at least one positive out of the methods used) and the clinical definition which is based on the presence of a lesion and relevant epidemiological data.

2.8. Genetic microsatellite variation and global molecular epidemiology

2.8.1 Study area

For the study of genetic variation and molecular polymorphism, 106 strains of *L. major* were sampled from 9 African countries (Egypt, Sudan, Kenya, Tunisia, Algeria, Morocco, Senegal and Burkina Faso) and 10 Asian countries (Jericho area and Negev desert, Saudi Arabia, Kuwait, Iraq, Iran, Turkey, Turkmenistan, Uzbekistan and Kazakhstan) (Figure 2.3, Table 3.11).
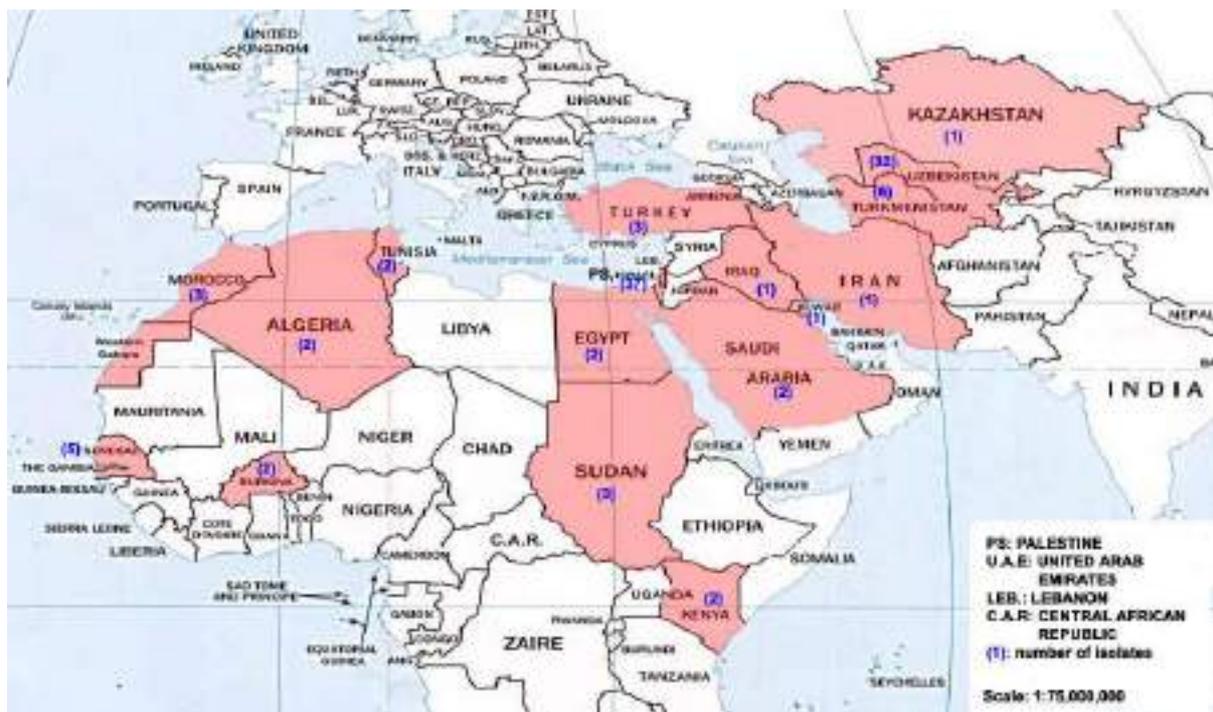
Figure 2.3 Map showing the countries of origin for microsatellite analysis. One strain is not shown on the map as the origin was an unknown country in Africa.

2.9 Microsatellite markers

Ten multilocus microsatellite markers located on 5 different chromosomes (1, 3, 5, 21 and 35) were designed previously by our group (Table 2.3). Microsatellite repeats, such as (CA)n, (AT)n, (GTG)n, and (GACA)n, that vary in length were identified within numerous sequences of *L. major* available from sequence data bases. Primers annealing to the unique flanking regions have been designed to specifically amplify microsatellite markers (Figure 2.4). To avoid the influence of insertion/deletion events in adjacent regions, primers were designed that anneal very close to the repeat (5-6 nucleotides apart from it on both sides). PCR products obtained from different *L. major* strains were separated in polyacrylamide gels and screened for length polymorphisms within the microsatellite repeats.

ACGGCCGGGTGGTCGTGGGT GGCGG (GTG) 12 GGGAGT CGTTCGCACGCACGCACG CA

 Figure 2.4 Schematic representation of the 45GTG marker in which the sequence consist of the repeat region in the middle, (GTG) 12, flanking regions of 5-6 bp on each side, and primer sequence of 20 bp on each side of the flanking regions.

Table 2.3 The 10 microsatellites markers used for the analysis of populations of *L. major*.

#	Marker	Primers	Ann. Temp. ºC	Chrom.	Location	Size (bp)	# Repeats in *L. major* (Friedlin)
1	4 GTG	f CGG TTT GGC GCT GAA AGC GG r CGT GAG GAC GCC AC CGAG GC	58	35	6460-530	70	7
2	27GTG	f GGA GGT GGC TGT GGT TGT TG r GCC GCT GAC GCT GCA GGC T	58	3	1440-515	75	9
3	36GTG	f AGC GAA GAA GAG TCG GGC AG r GCG CCT TCA GTG CGT CGT CC	62	1	140895-963	68	9
4	39GTG	f GTC TTG CCG CGA GGT GAC CG r CCA GCA CCA GCA CCA CCA TC	58	1	202765-851	86	9
5	45GTG	f ACG GCC GGG TGG TCG TGG GT r CGT TCG CAC GCA CGC ACG CA	58	1	59751-890	139	12
6	1GC	f CTG GCA CGC ACA CCC ACA CA r ATC TGC GCT CAT CTG GCG AG	60	3	10323-386	109	7
7	28AT	f TTG CCT ATC AAC ACA AGG CT r AGT CTC TCT CTC TCT CTA TA	42	5	27966-031	65	9
8	71AT	f TCT TGC GAA GGT GTG GTC TT r AGC CCA CGT GTA CAT GTG TG	50	21	22113-168	55	13
9	1GACA	f GAA AGG GCA GGA GGA CGG AT r CAC ACA CAC ATA CAC ACA TA	54	1	68459-534	75	7
10	1CA	f TTA GTT CCA TCA TAC ACC CG r CGT TCG ACG TGG AGA ATA AG	48	35	30151-238	87	14

Ann. =Annealing; Chrom. =Chromosome

2.9.1 Amplification of microsatellite markers by PCR

The short tandem repeats (STR) or microsatellites from the 106 cultured samples sent by the collaborating laboratories were amplified in volumes of 50 μl as described above (Table 2.1). The primers were synthesized commercially (TIB-MOLBIOL, Berlin, Germany). Three μl DNA from a 10 ng/μl- working solution were added to a PCR mix containing 200 μM of each dNTP (Amersham Biosciences UK limited. England); 5 μl of commercially available 10x PCR buffer (10mM Tris-HCl, pH 8.0; 50mM KCl; 1.5mM $MgCl_2$); 1 unit of *Taq* polymerase (Applied Biosystems, Roche USA) and 20 pmol of forward primer and 15 pmol of reverse primer. Samples were overlaid with sterile, light mineral oil (Sigma-Aldrich, St. Louis-USA) and amplified as follows: initial denaturation at 94°C for 5 min followed by 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at annealing temperature for 30 sec

and extension at 72°C for 1min. This was followed by a final extension cycle at 72°C for 10 min and then the thermocycler was set at 4°C for infinite.

PCR was run in Robocycler Gradient 40, Stratagene or Perkin-Elmer Thermocycler 9600 and subjected to electrophoresis as described in 2.4.

2.9.2 Polyacrylamide gel electrophoresis (PAGE)

As described by Sambrook (2001), long run gel glass plates (450x350 mm) were cleaned well with detergent solution and rinsed with warm water and then with deionized water and dried. The glass plates were then rinsed with 70% ethanol, dried and washed once again with 96% absolute ethanol and dried. One of the glass plates, preferably the notched one, was wiped with acrylease (Stratagene, Heidelberg) or Gelsave (Applichem GmbH, Darmstadt, Germany) to prevent sticking. Spacers (0.4 mm) were placed between the plates of both sides and the bottom side and wrapped with sticky tapes. The two glasses were fixed together using bulldog paper clips. Adhesion promoter, Silan A174 (Merck kGaA, Darmstadt, Germany) was gently added on the free side where the comb inserted by wetting a tip of a kimwip (Kimberly-Clarck Corp) and gently inserting it between the two plates to a depth equal to the teeth of the comb and then running it two times between the two ends of the glass plates.

These samples were loaded on 12% acrylamide gel 350 x 450 x 0.4 mm [45 ml 29% Acrylamide plus 1% N,N´-Methylenebisacrylamide (Rotiphorese® Gel 40 (29:1), Carl Roth GmbH &Co, Karlsruhe, Germany), 10 ml 10x TBE (10.8 %Tris, 5.5% Boric, 0.02 M EDTA pH 8), 90 ml distilled water, 750 µl 10% APS or 0.35 g of the APS powder (Ammonium peroxide sulphate, Merck, E. merck, Darmstadt-Germany)]. Then 75 µl of the gelling factor, NNNN-Tetramethylene diamine (TEMED) (Merck, Darmstadt, Germany or Serva, FeinBiochmica, Heidelberg, Germany) were added and swirled gently before pouring. The samples were subjected to electrophoresis in 0.5xTBE or 1xTBE for over-night runs (0.023M Tris-borate, 0.5mM EDTA). Electrophoresis was run successfully at 1 kV, 250mA and 7 Watts for 19 hours, depending on the length of run. Ten base-pair DNA ladder (Invitrogen, Life Tech, Carlsbad, CA USA) was used as a molecular size marker

2.9.3 Silver staining

Following electrophoresis, the gel was fixed in 1% Nitric acid for 15 min, washed in distilled water for 5 minutes and then stained in 0.2% $AgNO_3$ for 25 min. After washing for 10 min in distilled water, the gel was placed in freshly prepared developing solution (89.2 g Sodium carbonate in double distilled water (0.28 M) plus 1 ml formaldehyde) until bands clearly

appeared. The gel was washed for 10 minutes in distilled water before the reaction being stopped by 10% glacial acetic acid for 5 minutes and then washed for 5 minutes, transferred to blotting paper 460x570mm, wrapped in Saran plastic film (Dow Chemical Company) and dried in a Slab gel dryer (Savant-Hicksville, N.Y., USA) at 80°C for 2 hours. After complete drying, Saran Wrap was peeled off and the dried gel was labeled, scanned and photocopied for archiving purposes.

2.9.4 Capillary electrophoresis (CE) using CEQ^{TM} 8000 Beckman coulter

PCR products for CE were prepared as described above for PAGE, with an exception of using Dye-Labeled forward primer (Proligo). CEQ DNA Size Standard – 400 was used. CEQ Separation Gel was loaded in the CEQ^{TM} 8000 Beckman coulter and run according to manufacturer's instructions. Bands are shown in the form of peaks that show the size of the fragments.

2.10 Data analysis: clustering methods and presentation of genetic data

Genetic data were processed using two clustering methods: distance-based methods and model-based methods. Two distance-based methods were utilized: Neighbour-Joining (NJ) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA), while admixture model was used as model-based method. Three software programs were employed for drawing the NJ and UPGMA phylogenetic trees, MICROSAT, PHYLIP version 3.6 and PAUP***** version 4.0beta. *Structure 2.0* was used to classify the genetic data into discrete populations based on the admixture model.

Software used, see Table 2.4.

2.10.1 Calculating a distance matrix using *MICROSAT*

The free software *MICROSAT* (Minch *et al*, 1997) (http://hpgl.stanford.edu/projects/microsat/) was used to calculate the genetic distance between 67 multilocus microsatellite profiles obtained for the 106 strains when analysed with the 10 microsatellite markers. Changes in allelic sizes by one or more repeats were computed utilizing two measures: proportion of shared alleles (Dps$_)$ and Delta mu squared (Dμ^2).

Dps calculates multilocus pairwise distance measurements as 1 – (the total number of shared alleles at all loci/n), where n is the number of loci compared (Bowcock *et al*., 1994). The second, Dμ^2 is based on the average squared difference in allele size (Goldstein *et al*., 1995). Confidence intervals for Dps and Dμ^2 were calculated by bootstrapping (1000 replicates) over

loci. The output file containing the matrix was used for drawing the phylogenetic trees using PHYLIP and PAUP.

2.10.2 Drawing of NJ and UPGMA consensus trees using *PHYLIP/ PAUP*

The output file containing 1000 matrices for both Dps and Dμ^2 distance measures between the 67 CL genotypes were fed into *PHYLIP* version 3.6 (J. Felsenstein, 2004) available through http://evolution.gs.washington.edu/phylip.html) and *PAUP** version 4.0b8 (Swofford 2001) to construct neighbor-joining and UPGMA rooted trees.

2.10.3 Structuring populations with S*tructure 2.0*

The software S*tructure* Version 2 (Pritchard *et al*., 2000a) was implemented to classify the whole bulk of 106 CL isolates with their corresponding genetic data represented by the number of repeats for 10 microsatellite markers. The program was run using admixture model with length of burn-in period of 30,000 iterations and followed by 1,000,000 of MCMC (Markov chain Monte Carlo) repeats after burn-in. Two types of runs were conducted, one with 5 pre-defined populations (Central Asia, Middle East, North Africa, East Africa and West Africa) in which each CL isolate was assigned to one of these geographical sub-divisions. The second attempt was implemented without using the population ID (geographical origin of sample). Number of populations probabilistically assigned for the second run was determined by drawing a curve with x-axis as number of K (population) and y-axis as mean value of ln likelihood (ln Pr(x\k). The optimal number of K (population) is the first value of K at which plateau starts.

2.10.4 F-Statistic by F-STAT and GENEPOP

The degree of genetic differentiation among populations (106 isolates) was estimated by Wright's F-statistics (Wright 1978) as calculated by Weir and Cockerham (1984). Two F statistics were calculated: Fis (inbreeding coefficient) and Fst (differentiation among population) pairwise calculation. This was carried out by FSTAT software version 2.9.3.2 (Feb. 2002) (Goudet 1995) available http://www2.unil.ch/popgen/softwares/fstat.htm . Also, for double checking the results, Fst was calculated by GENEPOP (Rousset and Raymond, 1995) which is a population genetics DOS-based software package downloaded http://wbiomed.curtin.edu.au/genepop/index.html or an online calculation on http://wbiomed.curtin.edu.au/genepop/genepop_op1.htm. Number of alleles per locus was also calculated by FSTAT.

2.10.5 Descriptive statistics for markers by GDA

Descriptive statistics for the markers used to assess genetic variation were calculated using Gene Data Analysis (GDA), version 1.0 (d16c), a free software available on http://lewis.eeb.uconn.edu/lewishome/software.html (Lewis and Zaykin, 2001). The calculation was based on permutation method (Zaykin *et al*., 1995) which is useful for multiallelic microsatellite loci. The measures used to assess genetic variation were: n: average sample size; A: mean number of alleles per locus; (Ho) and expected (He) heterozygosity under Hardy–Weinberg equilibrium and Fis: the inbreeding coefficient.

2.11 Epidemiological data banking and analysis: Epi Info™

Epi Info™ is CDC free software. With this software a questionnaire was developed to customize, enter and analyze data. It is designed for public health and medical professionals. Epi Info™ was originally DOS and now it is available for Windows. http://www.cdc.gov/epo/epi/epiinfo.htm

2.12 Geographical clustering and public health surveillance

For public health surveillance, *SaTScan*™ v5.0 freeware (Kulldorff, 1998) was used to detect statistical evidence for spatial and space-time clustering of *L. major*, *L. tropica* and all CL cases (*L. major + L. tropica +* and undetermined). *SaTScan* was used to test the null hypothesis that the risk of *L. major*, *L. tropica* and CL is the same in all studied populations. Under the null hypothesis, and when there are no covariates, the expected number of cases in each area is proportional to its population size. Poisson data was analyzed with the purely spatial and the space-time scan statistics. For space-time, the unit of the time interval was one year.

The Poisson model was provided with the cases and their corresponding sampling dates as well as the population counts in 1997 (last Palestinian census) and 2004 (Palestinian Central Bureau of Statistics, 2005) for 9 geographical areas in the District Jericho (~600 km^2) with their corresponding geographical coordinates for each of those locations. Geographical coordinates were allocated as the centre of the built-areas.

The population data was specified for two years, one is the census time in 1997 and the other is its 2004 projection. For times in between, *SaTScan* does a linear interpolation based on the population at the census times immediately preceeding and immediately following. For times

before the first census time, the population size is set equal to the population size at that first census time, and for times after the last.

Using the geographical and the population data fed into the Poisson model, two statistics were used: spatial scan statistic and space-time scan statistic.

2. 12. 1 Spatial scan statistics

The purely spatial scan statistic imposes a circular window on the map. The window is in turn centred on each of several possible coordinate points (latitude/longitude) positioned throughout the study region. For each coordinate point (latitude/longitude), the radius of the window varies continuously in size from zero to upper limit of 50% of the population at risk. In this way, the circular window is flexible both in location and size. In total, the method creates an infinite number of distinct geographical circles with different sets of neighbouring data locations within them. For each circle, a likelihood ratio is computed for the alternative hypothesis that there is an increased risk of CL inside the circle against the null hypothesis that the risk of being CL (+) inside and outside the circle is the same. The candidate circle is the one with the highest likelihood ratio. The likelihood ratio-based test statistic takes multiple comparisons into account that resulted from looking for clusters in many different locations and sizes. The statistical significance ($p<0.05$) of this large circle (large likelihood ratio) is calculated by determining its distribution under null hypothesis through Monte Carlo simulation (1000 simulations). This produces a main cluster alongside other secondary clusters. Another way of looking for other cluster is to remove the cases of the main cluster from the input file and repeat the analysis. In the spatial statistic, the time is totally ignored.

2. 12. 2 Space-time scan statistics

The space-time scan statistics are defined by a cylindrical window with a circular geographic base and with height corresponding to time. The base is defined exactly as for the purely spatial scan statistics, while the height reflects the time period of potential clusters. The cylindrical window is then moved in space and time, so that for each possible geographical location and size it also visits each possible time period. In effect, we obtain an infinite number of overlapping cylinders of different size and shape, jointly covering the entire study region, where each cylinder reflects a possible cluster. The main cluster is computed as described above in the spatial statistics (Kulldorff, 2001; Kulldorff *et al.*, 2005).

2. 12.3 Adjustment for season relative risk

Based on the historical data (Al-Jawabreh *et al*., 2003) it is known that in summer months (beginning in April till the end of September) the number, in general, is reduced in all areas to approximately half.

We adjusted for this relative risk by using the adjustments file. In this file, a relative risk of 0.5 for all geographical areas for all the years included in the study (1994-2004) was applied.

2. 12. 4 Adjusting for covariates

Covariates as age group and sex were not adjusted for, since the two covariates are randomly distributed geographically, and since age group and sex distribution is uniform and constant all over the Jordan valley.

2. 13 Shewhart's Chart

Shewhart Chart or Levy-Jennings Chart was originally created for industrial applications and then applied on a large scale in medical laboratories to check the work performance. In this study we applied this technique for epidemiological monitoring and surveillance.

The mean (m, $\overline{\mathbf{X}}$) for the number of cases of CL in the period between 1994 and 2004 was calculated. This mean will form the center line representing the target value. Then, standard deviation (SD) was calculated to set up the upper and lower limits commonly know as UCL (upper control limit) and LCL (lower control limit), symmetrical about the center line. The limits are chosen so that almost all of the data points will fall within these limits as long as there is no peak or outbreaks. In a clinical laboratory the LCL and UCL are +/- 2SD. However +/-1SD can be considered for epidemiological purposes. Grubb's test was used to check for outliers.

A graph with the center mean line and UCL and LCL line is drawn and the annual CL totals are plotted on the time line graph.

Table 2.4 Software packages used in the study

program	Operating system	Purpose	Author	Download
Epi Info	Windows	Epidemiologic analysis for health data	CDC	http://www.cdc.gov/epo/epi/epiinfo.htm
MICROSAT	DOS	Calculation of distances from microsatellite data	Eric Minch	http://hpgl.stanford.edu/projects/microsat/

Phylip version 3.6	DOS	Construction of neighbor-joining and UPGMA rooted trees	J. Felsenstein	http://evolution.gs.washington.edu/phylip.html
PAUP version 4.0beta	DOS	Construction of neighbor-joining and UPGMA rooted trees	Swofford	http://paup.csit.fsu.edu/
Structure Version 2	Windows	Estimation of underlying population genetic structure	Pritchard	http://pritch.bsd.uchicago.edu/software.html
GDA version 1	Windows	Population genetic analysis package that includes estimation of LD for pairs of loci; significance tested by permutation method	P. O Lewis and D. Zaykin	http://alleyn.eeb.uconn.edu/gda/
FSTAT version 2.9.3.2	Windows	Estimation of genetic differentiation among populations	Goudet	http://www2.unil.ch/popgen/softwares/fstat.htm
GENEPOP	DOS and online	Calculation of LD, F-statistics and others	Rousset and Raymond	http://wbiomed.curtin.edu.au/genepop/index.html
SaTScan	Windows	Detection of statistical evidence for spatial and space-time clustering of leishmaniasis	Kulldorff	www.satscan.org

LD: linkage disequilibrium

3. RESULTS

3. 1. Method comparison

3.1.1 Graded microscopy and ITS1-PCR

3.1.1.1 Positivity rates and sensitivities of ITS1-PCR and graded microscopy

For comparison of graded microscopy with ITS1-PCR, random sampling method was used, in which, from the 48 scanned and graded slides, 9 microscopically positives and 11 microscopically negatives (~1:1 ratio) were randomly selected.

Seventy five percent (45/60) of the 5mm x 5mm test squares could be scanned and graded for amastigotes microscopically. The remaining 15 could not be evaluated microscopically because of staining failure: in 12 cases stain was too thick and in three cases too thin (Table 3.2). These slides were considered negative (Figure 3.1 & 3.2). Table 3.1 shows that a parasite density of (+) in the graded microscopy was observed in 21 squares, (++) in 2 squares, (-) in 22 squares. Thus, finally 38% (23/60) were positive which can be considered as the sensitivity of microscopy taking in account all samples, and 52% (23/45) skipping the 15 wrongly stained slides.

Of the 60 squares tested with ITS1-PCR, 52 (87%) were positive for leishmanial DNA (Table 3.3). The rate of positivity for ITS1-PCR among the 45 samples that could be screened by graded microscopy was shown to be 87% (39/45) too.
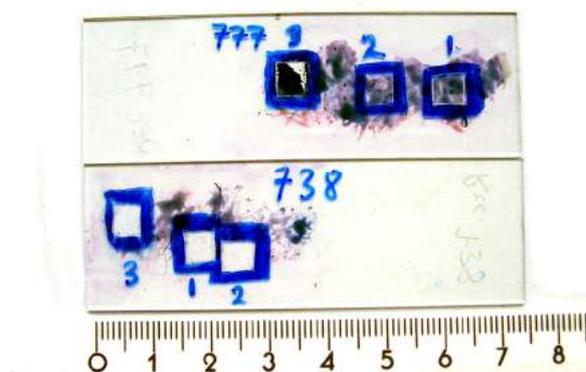
Figure 3.1 Standardized grading microscopy: Giemsa-stained smears showing the three labeled 5 mm x 5 mm squares. Slide 777 prepared for microscopy with square 3 being purposefully selected to be a darkly stained area. Slide 738 shows the slide after the material is scraped off and DNA-extracted.

3.1.1.2 Statistical comparison of sensitivities

When comparing the results obtained by ITS1-PCR with those of graded microscopy, it became obvious that the improvement in diagnostic capability is statistically very significant

by using ITS1-PCR (McNemar's test, P<0.0013). In the first group, or stratum, of the 23 microscopy positive squares 20 were ITS1-PCR positive (87%). Of the remaining three ITS1-PCR negatives, one failed in DNA-extraction, one microscopically showed only one amastigote in the whole square (more than 625 OIF), while the other was not quantified but graded as +1 (Table 3.1). In the second group (22 microscopy negative squares), 18 were ITS1-PCR positive (82%) and 4 remained negative. Out of the 60 squares scanned for amastigotes under x100 oil- immersion bright-field microscopy, 15 (25%) squares could not be evaluated due to bad staining. Of the 12 darkly-stained squares, 11 were ITS1-PCR positive and one was PCR negative. The 3 poorly–stained squares were all ITS1-PCR positive. Thus, 93% (14/15) of the slides where microscopy failed were positive by PCR. The DNA extraction control in the ITS 1-PCR negative probe was positive indicating that the Giemsa-stain did not influence the outcome of the PCR.

3.1.1.3 Diagnostic relevance of ITS1-PCR

The 45 squares that represented 15 human negative controls were all negative by microscopy and ITS1-PCR. When merging the results of negative controls with those of the patients in a 2 x 2 contingency tables, the sensitivity of microscopy becomes 37% with a positive predictive value of 100%, a specificity of 100% and a negative predictive value of 54% (Table 3.3).

In reality one would try to prepare a new slide in case of staining failure. Thus we separately calculated the data of the sufficiently stained slides (45). Microscopy then comes out a little better: sensitivity becomes 49% with a positive predictive value of 100%, a specificity of 100% and a negative predictive value of 66% as shown in Table 3.3.

Comparing the results with ITS1-PCR, the advantage is obvious. Sensitivity increases to 87% with a positive predictive value of 100%, a specificity of 100% and a negative predictive value of 85% as shown in table 3.3.

Table 3.1 Outcome of graded microscopy and ITS1-PCR using Giemsa-stained slides obtained from patients.

Microscopy grades (no. amastigotes)	No. scanned squares	Microscopy results (positive)		ITS1-PCR positive
++ (2 to 10 per OIF)	2	2	(2)	2
+ (1 per square to 1 per OIF)	21	21	(21)	18**
- (0 per square)	22	22	(0)	18
No microscopical evaluation possible*	15*	0		14
Total	60	45	(23)	52

* Failure in staining did not allow microscopy.

** One sample is counted negative as a result of extraction failure.

Table 3.2 Comparison of graded microscopy and ITS1-PCR in the 60 square-test group.

	PCR (+)	PCR (-)	Total
Microscopy (+)	20	2	
		1*	23*
Microscopy (-)	18	4	
	3 poorly stained**		
	12 darkly stained**	1	37
total	52	8	60

* One sample is counted negative as a result of extraction failure.

** No microscopical examination possible because of failed staining

Table 3.3 Sensitivity and specificity of microscopy and ITS1-PCR compared to a negative control group

	CL (+)		CL (-)	Total	
microscopy (+)	22		0	22	
microscopy (-)	38*	(23)**	45	83*	(68)**
Total Microscopy	60*	(45)**	45	105	(90)**
PCR (+)	52		0	52	
PCR (-)	8		45	53	
Total PCR	60		45	105	

* including the 15 staining failures   ** without staining failures
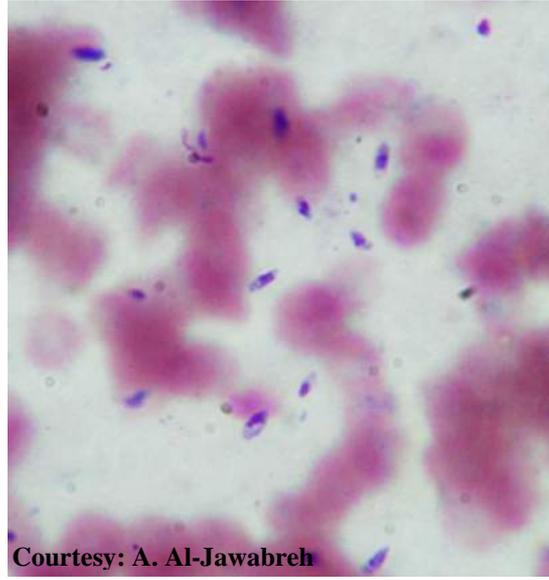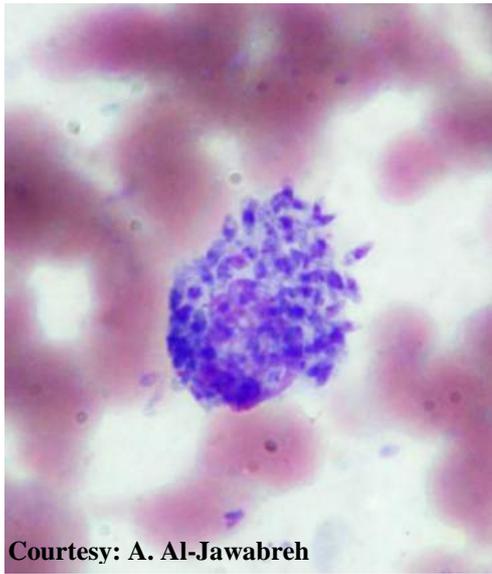
(a)   **(b)**
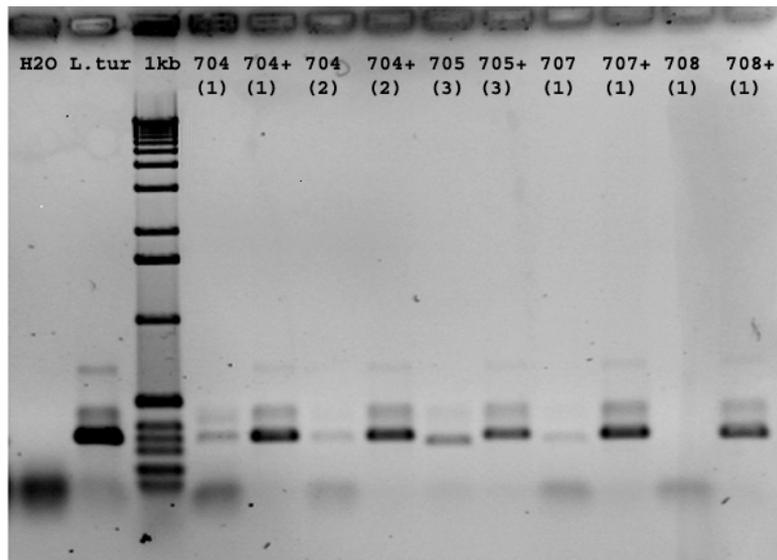
(c)

Figure 3.2 Intracellular **(a)** and extracellular **(b)** leishmanial amastigotes in a Giemsa-stained smear made from scrapings of cutaneous lesions (bright-field microscopy, x 1000). **(c)** PCR amplification of the 350 bp ITS1 region represented on 1.5% agarose gel. Distilled H_2O is the negative control; 20ng of *L. turanica* (known not to infect humans) is used as positive control. A 1 kb ladder is the molecular size marker. Numbers in brackets represent numbers of squares tested, while the (+) is the inhibition control (test sample plus *L. turanica)*.

3.1.2 Clinical diagnosis of cutaneous leishmaniasis: filter paper and unstained smears as potential sampling methods for ITS1-PCR

During the study period, February 1994 and July 2004, 418 skin scrapings spotted on filter papers, 173 unstained tissue smears, 943 Giemsa-stained smears and 270 NNN cultures were collected from patients attending the ICS-Jericho Medical Laboratory. DNA was extracted from spots on filter papers and from material on unstained smears, and diagnosed using ITS1-PCR (Figure 3.3). Giemsa-stained smears were scanned and cultures were checked for promastigotes by bright field microscopy. The species identification for DNA extracted from cultures, filter papers and smears was carried out by digesting the ITS1 PCR product with restriction enzymes *Hae*III and *Mnl*I. A CL case was, according to the WHO operational definition, a person showing clinical signs (skin or mucosal lesions) with parasitological confirmation of the diagnosis (positive smear or culture) (WHO Recommended Surveillance Standards, 1999).
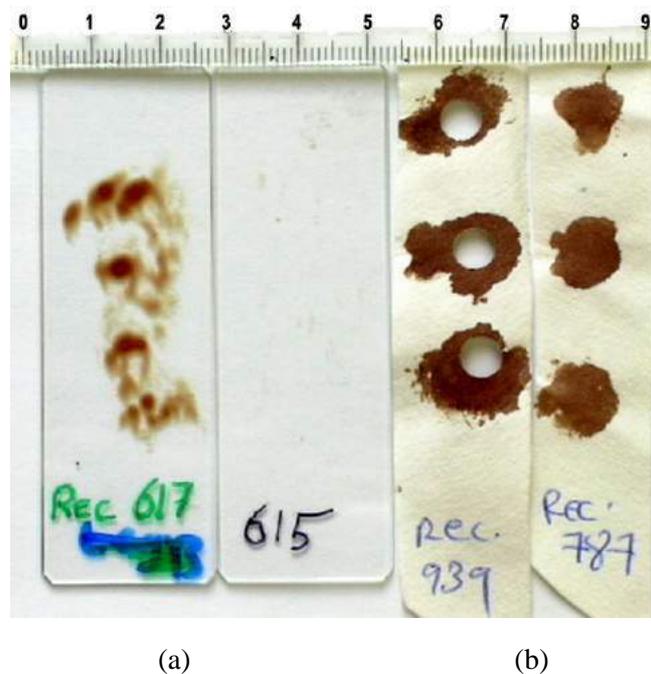
Figure 3. 3. Unstained direct tissue smears shown before and after scraping of material for DNA extraction (a) and tissue and blood spotted on filter papers shown before and after punching (b).

Table 3.4 shows the results using three different gold standards: WHO gold standard based on the operational case definition (WHO Recommended Surveillance Standards, 1999) that considers a CL cases to be the one confirmed by either microscopy or *in-vitro* culture or both. In the combined gold standard we considered a CL case to be the one confirmed by at least

one of the four methods used, direct smear microscopy, in-vitro culture, PCR using FP or PCR using US. The third gold standard is the broad gold standard which considers CL case to be the one showing lesion(s) in an endemic area with compatible clinical history.

From the 943 patients in the database, 64 had been tested using all four methods known as matched cases. Sensitivity is the number of positive results by a certain method over the number of CL cases as defined by the selected gold standard. Using the WHO gold standard, the sensitivity of the ITS1-PCR by FP is 78% while it increases when using US to 86%. Similar sensitivities for FP (77%) and US (84%) are obtained using the combined gold standard. The sensitivity of the microscopy and *in-vitro* culture using the combined gold standard was 65% and 50%, respectively.

In the broad gold standard using the 64 matched cases the sensitivity (rate of positivity) of the microscopy, in-vitro culture, PCR-FP and PCR-US were 55, 41, 66 and 72%, respectively.

A total of 119 (44%) strains were isolated by culturing dermal tissue aspirates in either rabbit blood–agar semisolid medium or NNN medium. Of the 418 clinical samples spotted on filter papers and checked by ITS1-PCR, 219 were positive, indicating a 52.4% positivity rate. Of the 173 unstained smears, 97 (56%) were positive by PCR (Table 3.4). The lowest rate of positivity was recorded by microscopy (42%).

Amplification of the 300–350 bp ITS1 amplicon and its subsequent digestion with the endonuclease *Hae*III did enable detection of *Leishmania* parasites and identification of the infecting species. The restriction patterns obtained for *L. major*, *L. tropica* and *L. infantum*, another species of Leishmania present in the Middle East, were clearly different (Figure 3.4). Of the 298 cases of CL from FP, US and *in-vitro* culture tested by PCR-RFLP, 181(60.7%) contained DNA of *L. major*, 106 (35.6%) DNA of *L. tropica* while 11 (3.7%) of the FP remained unidentified.

Table 3.4 Sensitivity of the four diagnostic methods using 3 types of 'gold standard' (n=64 cases)

	WHO (n=64)	Combined (n=64)	Clinical (n=64) *(n=943)*
Microscopy (Smear)	----	65%	55% (35/64) *42.1% (397/943)*
In-vitro Culture	----	50%	41% (26/64) *44% (119/270)*
PCR-ITS1-FP	78%	77%	66% (42/64) *52.4%(219/418)*
PCR-ITS1-US	86%	84%	72% (46/64) *56%(97/173)*
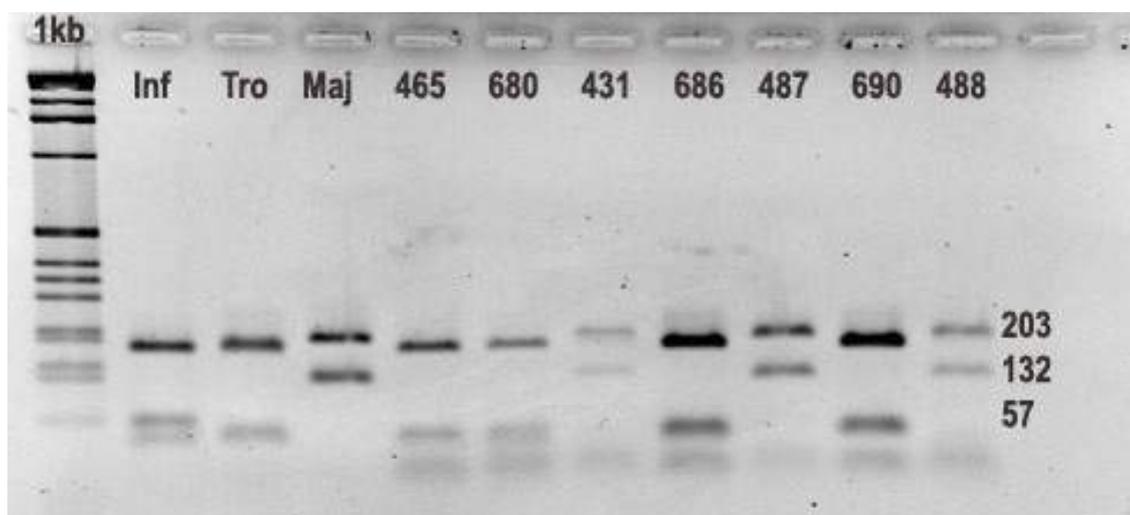
Figure. 3.4 Restriction analysis patterns of the amplified ITS1 digested with *Hae*III. Restriction pattern of reference cultured strains: Inf, Tro and Maj represent *L. infantum* MCAN/IL/97/LRC-L717 (184, 72, 55 bp), *L. tropica,* MHOM/SU/80/K28 (185, 57, 53, 24 bp) and *L. major*, MHOM/TM/82/Lev (203, 132 bp), respectively. The next 7 numbers represent DNA samples from blood and tissue blotted on filter papers isolated from different CL patients living in the District of Jericho: *L. tropica=* 465, 680, 686 and 690; *L. major=*431, 487 and 488.

The clinical diagnostic scheme for cutaneous leishmaniasis as shown in Figure 3.5 has proved to be effective for diagnosis and species identification of CL cases in Jericho and its vicinity. Yet, as clinical samples contain all possible contaminants and inhibitors, a strong control panel (Table 3.5) was utilized.

Table 3.5 The battery of controls used in the PCR to ensure validity of results

	Control	**Sample**	**Comment**
1	Negative control	dd H2O	Check for contamination
2	Positive control	*Leishmania turanica*	Efficiency of amplification
3	Inhibition control	Template DNA + *L. turanica*	To check for inhibitors i. e hemoglobin, stain, immersion oil, etc.
4	House-keeping gene	Template DNA (β-Actin primers)	Check for DNA integrity and DNA extraction failure (true negativity test).

Figure 3.5 Clinical diagnostic flowcharts for cutaneous leishmanisis
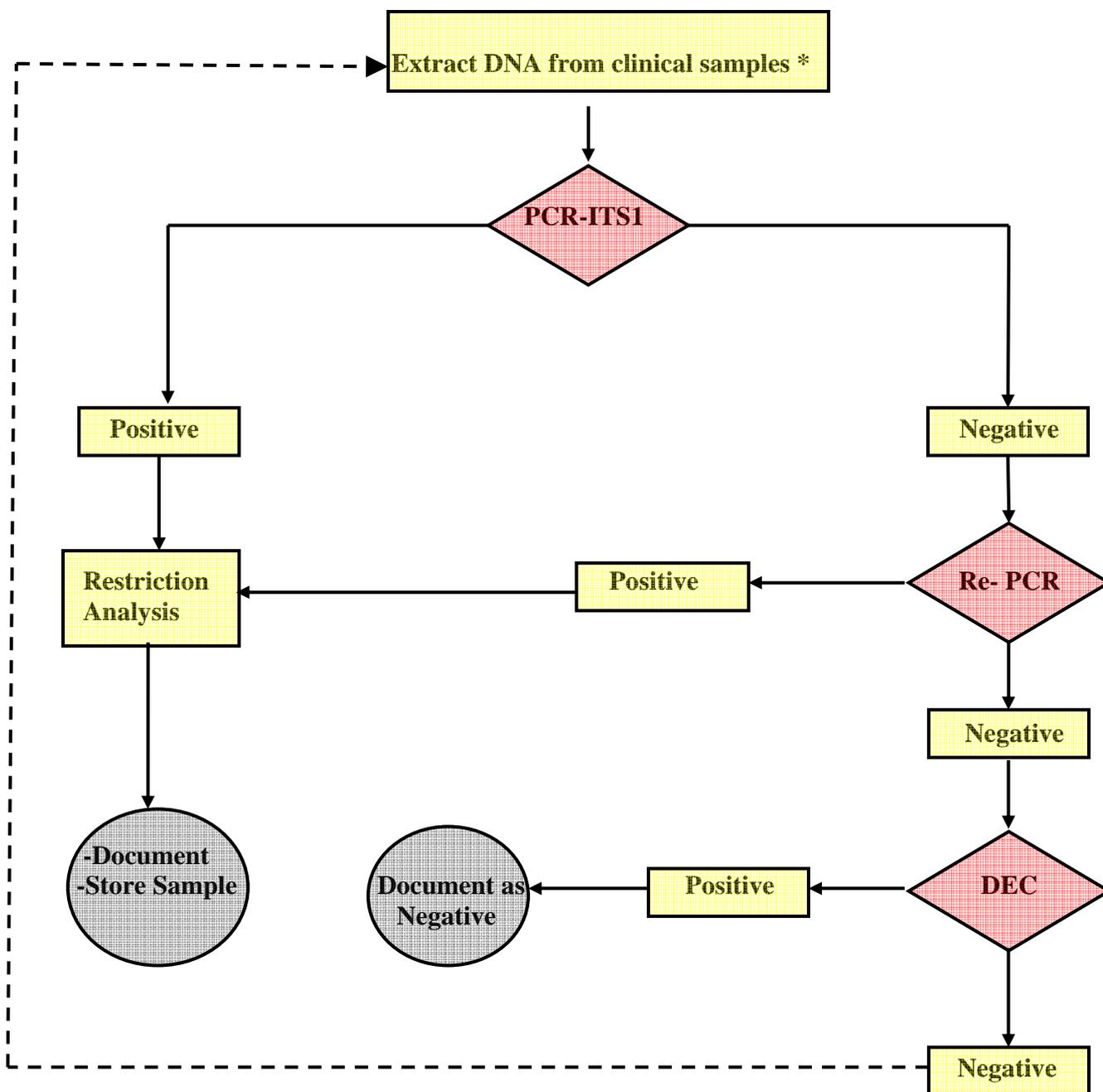
- **Clinical samples include blood spots on filter papers, unstained direct smears, stained direct smears, bone marrow aspirates, venous EDTA blood.**
- **DEC: DNA Extraction Control**

3.2 Molecular epidemiology

3.2.1 *L. major* vs. *L. tropica* in Jericho

All classical stages of the development of leishmaniomas were seen among the cases: from small erythematous papules through nodules and to ulcerative lesions; whereas unusual clinical manifestations such as the sporotrichoid pattern, i.e., subcutaneous nodules developing along lymphatics, hyperkeratosis, i.e., thick adherent scale and leishmaniasis recidivans also known as lupoid leishmaniasis were not. It was very difficult and even impossible to discern if cases were caused by *L. major* or *L. tropica* by the clinical picture. However, three severe cases led to the suspicion that they were not caused by *L. major*, as they were different from the cases of CL generally seen in the vicinity of Jericho. The three cases displayed common features. They all presented a single lesion, two of which were on the nose and one on the chin. Development was slow and all three only sought medical advice 6–12 months after the first appearance of the lesion. All three lesions resisted antimony treatment and took 6 months or more to heal leaving scars. The lesion on the chin and one of those on the nose were caused by *L. tropica.* After this, ITS1-PCR and RFLP results were continuously spotted on maps to show the distribution pattern of *L. major* and *L. tropica* in Jericho and its vicinity which showed that *L. major* is predominant in the alluvial soil/sand and chenopodiaceae-rich plains such as Jericho city and A'uja village, while the *L. tropica* is close to rural villages surrounded by rocky areas such as Zubaidat village (Figure 3.6). Jericho City contains a majority of *L. major* (76%) and the rest (24%) is *L. tropica* (Figure 3.6). A total of 94 of the 102 patients (92%), infected by *L. tropica* and answered the question whether they had been travelling out of the vicinity of Jericho during the last 3 months, claimed that they had not. The other eight cases were people who came either from the hilly regions around Jerusalem or cities like Ramallah and Jenin. Eighty-six per cent (149) of the 174 patients, infected by *L. major* and answered the question, 149(86%) also said they had stayed in the vicinity of Jericho during the last 3 months.

Most of the patients had single lesions on the cheek or upper and lower extremities (Table 3.6) and sought medical intervention within 3 months of the appearance of the lesion. As Jericho is a hyperendemic region, the inhabitants are well aware of CL, which is also partially owing to the education campaigns conducted by Islah Medical Center in Jericho over the last decade.

Table 3.6 Comparison of the clinical features of CL cases caused by *L. major* and *L. tropica* in the district of Jericho

		L. major	*L. tropica*
Duration (months)			
	≤ 3	143 *(95.3%)*	75 *(78.1%)*
	3-6	7 *(4.7%)*	12 *(12.5%)*
	> 6	0 *(0.0%)*	9 *(9.4%)*
	Total*	**150 *(100)***	**96*(100)***
Location			
	Forehead	25 (10.5%)	12(8.9%)
	Chin	11(4.6%)	15(11.2%)
	Eye	10(4.2%)	8(5.9%)
	Nose	9(3.8%)	13(9.7%)
	Ear	7(2.9%)	5(3.7%)
	Cheek	43(18.1%)	36(26.9%)
	Arm	76(32.1%)	30(22.4%)
	Leg	49(20.7%)	13(9.7%)
	Neck	7(2.9%)	2(1.5%)
	Total[*]	**237 *(100)***	**134 *(100)***
No. of lesions			
	1	74 *(41.2%)*	56 *(56.6%)*
	2	51 *(28.7%)*	24*(24.3%)*
	≥3	54 *(30.1%)*	*19(19.1%)*
	Total[*]	**179*(100)***	**99*(100)***

* Figures represent the total number of patients who answered the question related to each clinical feature.

55

Map1

Legend:
- Main Road
- Palestinian Built-up Area

TOBAS

NABLUS

ZUBAIDAT
6/28

JIFTLIK
0/3

FASAYIL
18/0

RAMALLAH

A'UJA VILLAGE
36/ 7

10/3

NUAIMEH
4/0

JERICHO
80/16

AQBAT JABER
18/16

KHAN AHMAR

AL-QUDS
(JERUSALEM)   18/16

DEAD SEA

JORDAN
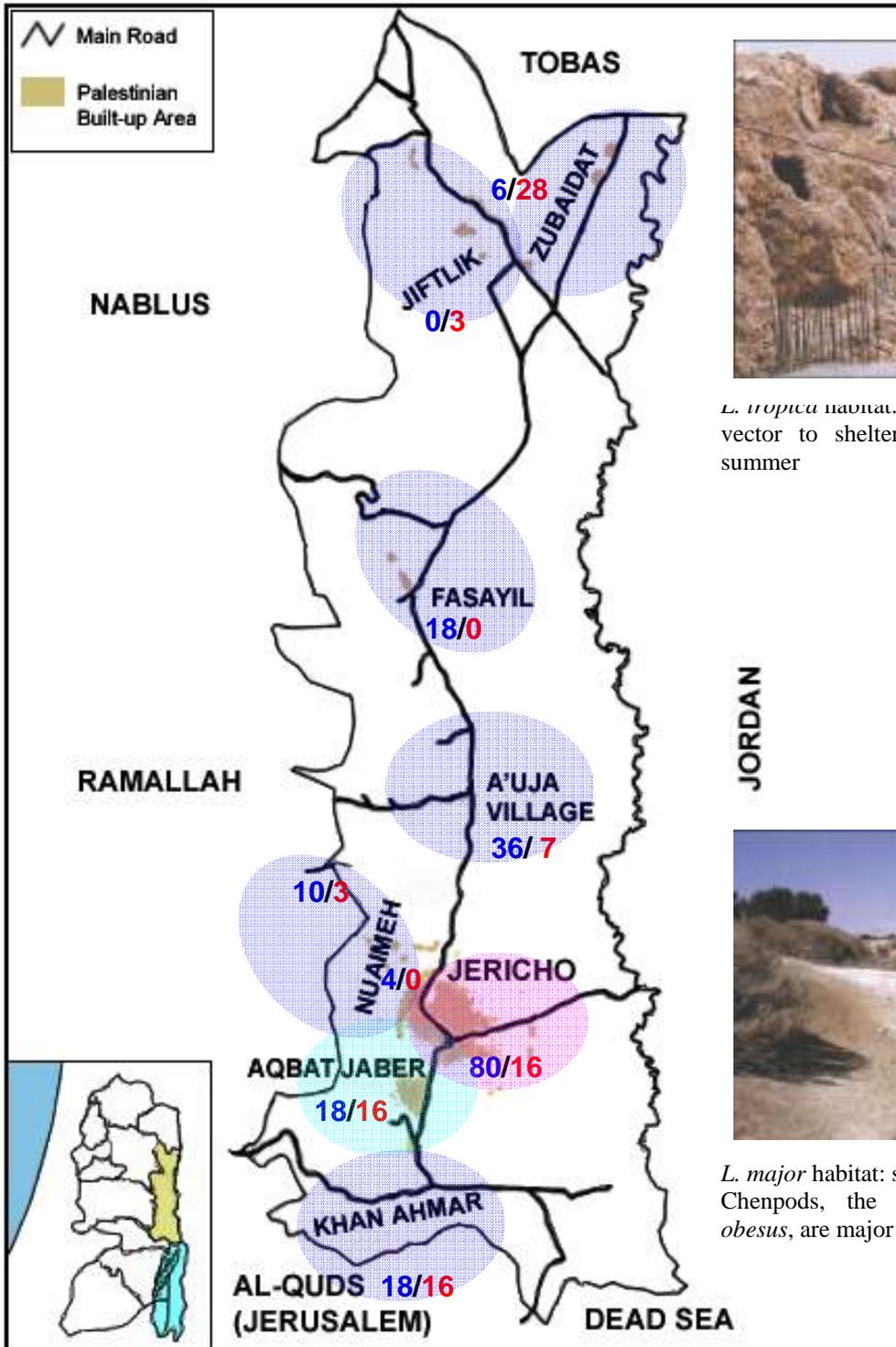
L. tropica habitat. Rocky areas with caves for vector to shelter in during the long hot summer

L. major habitat: sandy, soft alluvial plains. Chenpods, the favoured plant for *P. obesus*, are major characteristics.
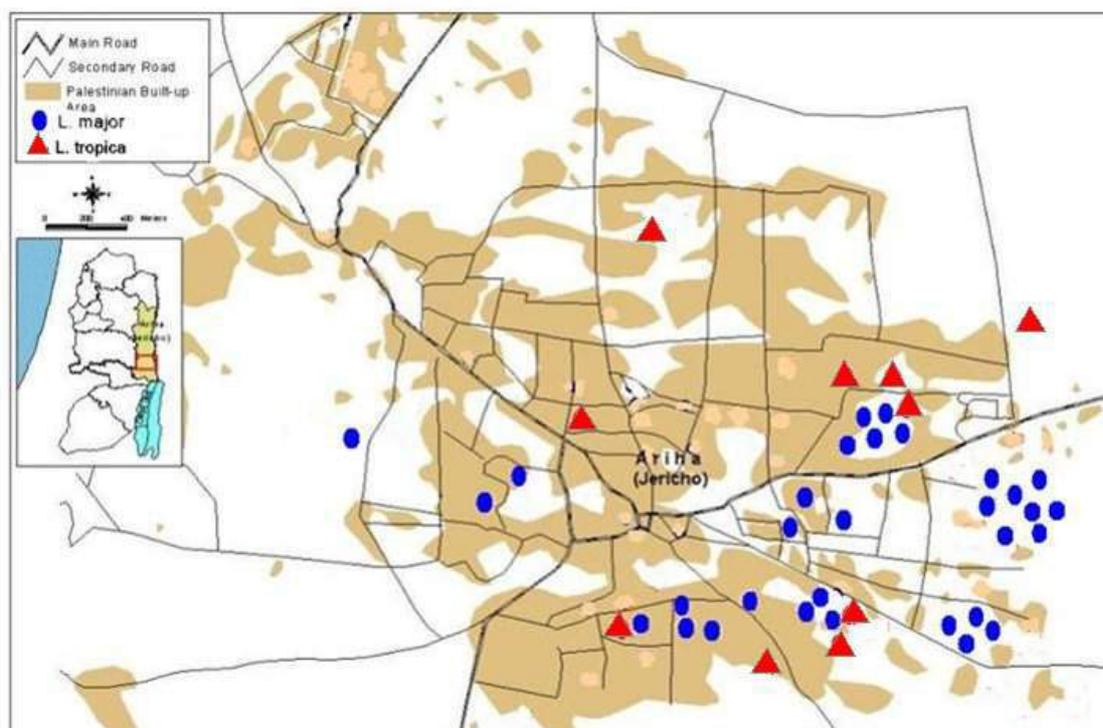
 Map 2

Figure 3.6 Map 1 shows the distribution of *L. major* (the first number in blue) and *L. tropica* (the second number in red) in the District of Jericho, 1997-2004. Map 2 shows the distribution in the City of Jericho and its immediate vicinity, 1997-2002: The red triangles represent cases caused by *L. tropica* and the blue ovals cases caused by *L. major*.

3.2.2 Sex-age group distribution

The total number of CL cases as well as those caused by either *L. major* or *L. tropica* was plotted against demographic variables, sex and age (Figures 3.7a, b and c). Children (<14) are most prone to infection with age group 0-4 being more exposed than others. The male youth (20-24) also form another risk category. In females the age group 30-34 shows an increased incidence of *L. tropica* cases. *L. tropica* has no sex tendency (M: F- 50:54) unlike the *L. major* (M: F-107:70) and total CL cases (M: F- 290:221). Children (<14) who have increased incidence rate show no defined pattern for sex distribution, yet the males in their early twenties (20-24) seem to be more prone to be infected than females.

Age group:
1: 0-4
2: 5-9
3: 10-14
4: 15-19
5: 20-24
6: 25-29
7: 30-34
8: 35-39
9: >=40
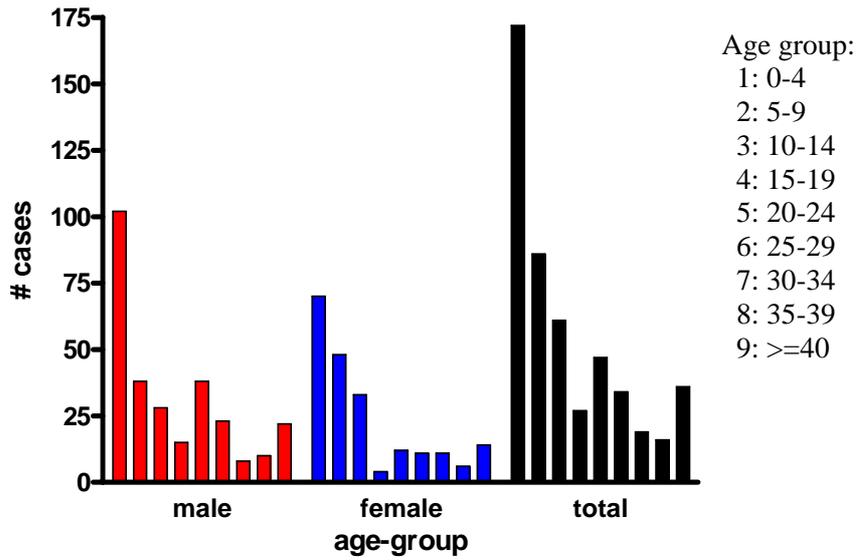
a
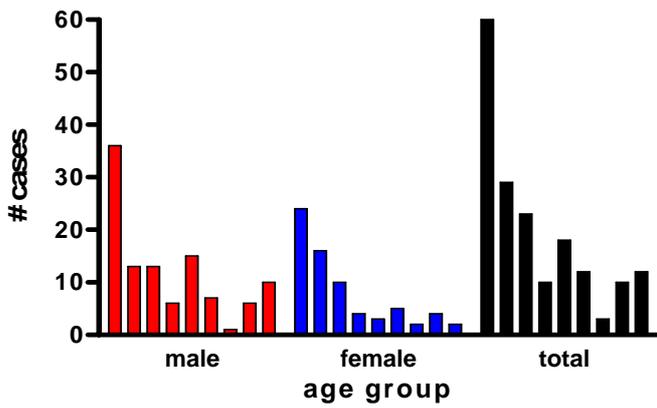
b
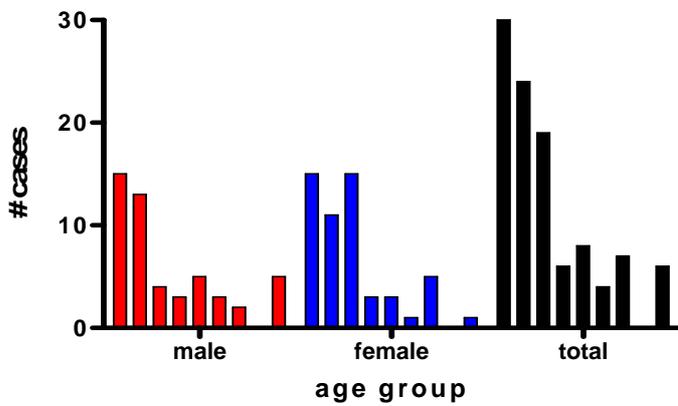
Figure 3.7 (a) Distribution of CL by age and sex in Jericho district, 1994-2004, (b) Distribution of *L. major* (c) Distribution of *L. tropica*. The bars represent the age groups arranged in the order shown in (a).

3.2.3 Annual rain fall (ARF) and CL

Number of cases of CL and annual rain fall (ARF) in mm was plotted versus year. Spearman's correlation was used to determine if there existed a significant relationship between the ARF and number of CL cases. We found a very low correlation between ARF and CL (n=11, rs= 0.300; P= 0.05) (Figure3.8-a); correlation was even lower for *L. major* (n= 7; rs= 0.270; P=0.05) (Figure 3.8-b). Also for *L. tropica* cases the correlation was insignificant (n=7; rs=-0.324; P=0.05) (Figure 3.8-c). For the total number of CL cases, which includes *L. major* and *L.tropica* as well as undetermined cases, the study period was between 1994 and 2004, while for the cases of *L. major* and of *L. tropica* the period was between 1998 and 2004 because CR diagnostics was first introduced into Jericho in 1998.
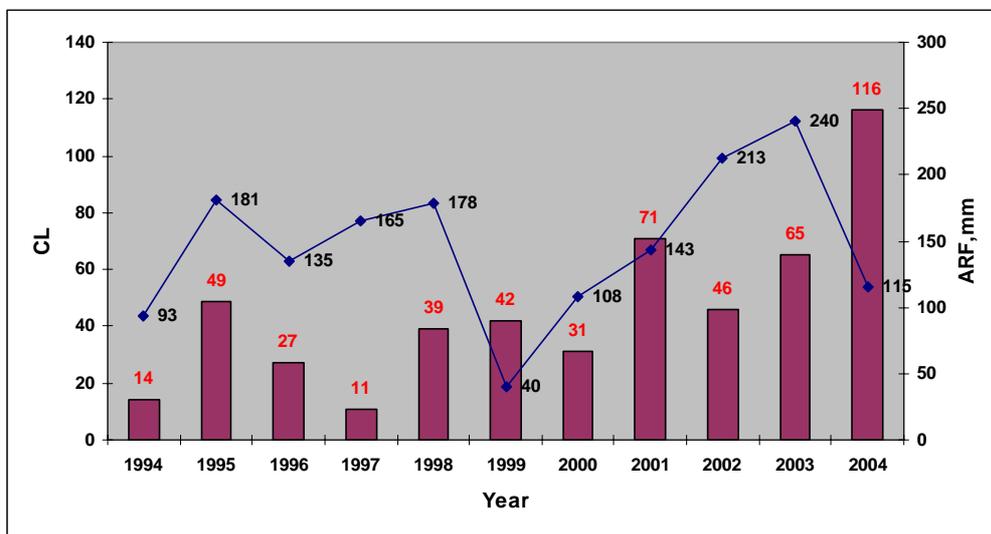
(a)
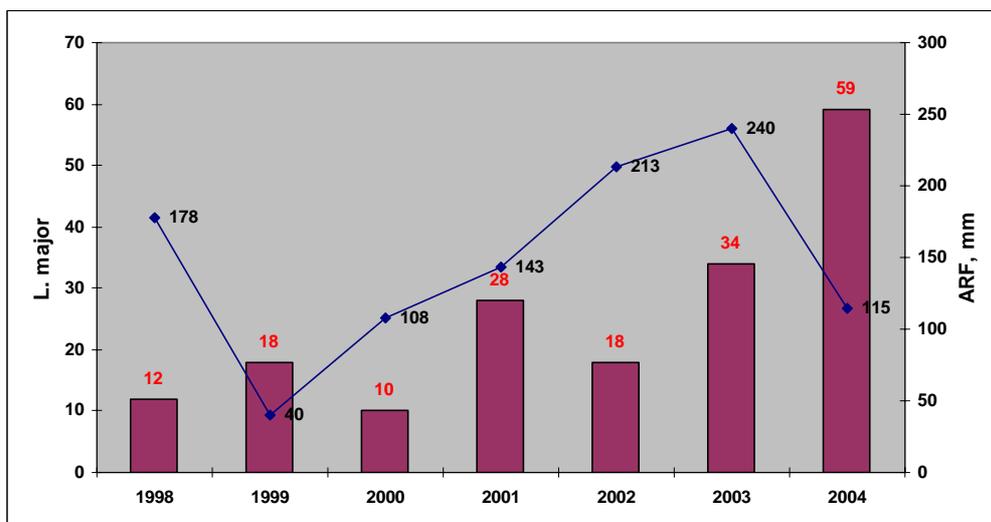
(b)
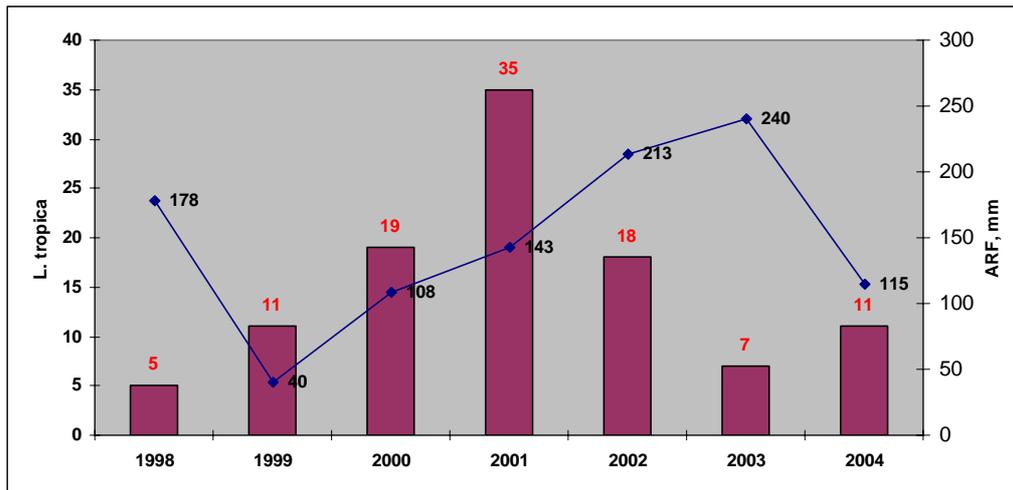
(c)

Figure 3.8 (a) shows the annual distribution of total number of CL cases versus annual rain fall (ARF) (b) *L. major* cases only (c) *L. tropica* cases only.

3.2.4 Seasonality of CL

Figure 3.9 shows that the seasonality of all CL cases as well as of those due to either *L. major* or *L. tropica* was comparable during this study. Cases always start to peak from October and declines in April.

One can notice that annual distribution of all cases of CL peaked three times: 1995, 2001 and 2004 (Figure 3.8a). However, the patterns of *L. major* and *L. tropica* cases starting from 1998 were different. *L. major* peaked in 2004 while *L. tropica* did so in 2001. This figure also demonstrates *L. tropica* to exist in Jericho area as early as 1998.

Moving average is an indicator that shows the average value number of cases of CL over a period of time. This useful analytical tool aims at spotting trends in distribution. It is common in the stock markets. Moving average with a window period (time span) of four months (season) has been used to spot trends in the distribution of CL cases in Jericho area. Moving average (Figure 3.10) confirms the possibility of having more than one peak a year, a major (primary) and minor (secondary) peaks such as 1994 and 2002**.** It also shows that three major peaks took place during the study period (1994-2004): 1995, 2001 and 2004. The 2004 peak is unprecedented. However, a peak is witnessed every year except in 1997-1998 season where the number of cases was low, hence, a peak was absent. As a rule of thumb, the sensitivity for detecting trends and patterns of distribution of CL can be increased by shortening the time span. The longer the time span, the less sensitive or the more smoothed the moving average will be. Nevertheless, this largely depends on the incidence rate of the infection. With

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increasing incidences the time span can be reduced down to one month or even weeks, which is impossible in the case of rare infections.
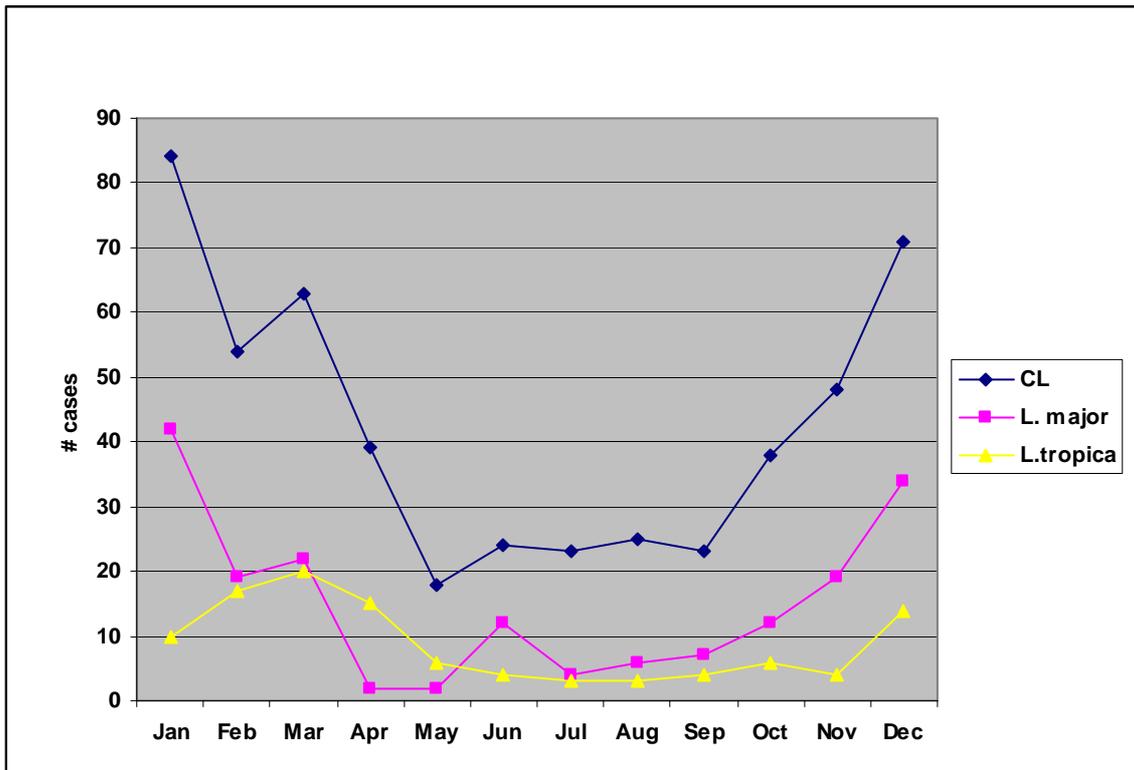
Figure 3.9 Line graph comparing seasonality for CL, *L. major* and *L. tropica*.
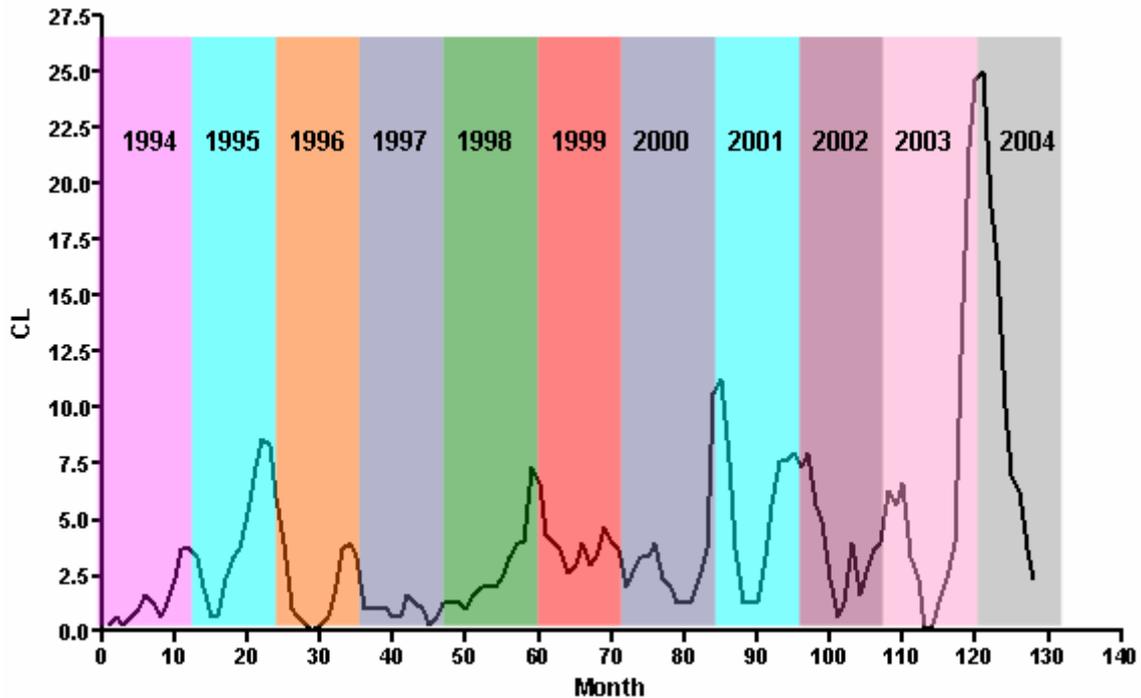
Figure 3.10 Moving average for CL cases in Jericho with a window period of four months, 1994-2004

3.3 Public health surveillance and cluster analysis

3.3.1 Descriptive data of CL cases

This part of the study is an observational epidemiological investigation of CL incidence in Jericho-Palestine. The total population at risk was 31,089 in 1997 to 40,909 in 2004 (Palestinian Central Bureau of Statistics, 2005) (Table 3.7).

During the period under investigation (3.2.1994 and 12.8.2004) PCR-ITS1 and RFLP (restriction fragment length polymorphism) were used to genotype CL cases in Jericho area. The number of geographically mapped cases was 181 *L. major*, 100 *L. tropica* and 492 CL (*L. major*, *L. tropica* and undetermined cases). Undetermined cases are CL cases which were not typed to be *L. major* or *L. tropica* either due to failure of genotyping by RFLP or lack of samples for further testing.

Relative risk was adjusted for seasonality of CL infection, in which during the summer the number of cases is reduced to half. Although large proportion (318/479) of the total cases of CL was children below 14, age-adjustment was not conducted because this pattern was uniform in the whole study area during the study period.

Table 3.7 Geographical and population data of the 9 study areas in the District of Jericho-Palestine

	location	Latitude	Longitude	population	
				1997	2004
1.	Nabi-Musa [(1)]	31.79604	35.43941	150	200
2	Aqbat-Jabr-RC	31.83960	35.44545	4521	5949
3	Jericho	31.85909	35.46404	14551	19147
4	Nuaimeh [(2)]	31.89170	35.43568	830	1192
5	A'uja	31.94818	35.46125	2858	3761
6	Fasayil	32.02624	35.44339	641	844
7	Jiftlik	32.14683	35.48001	3136	4127
8	Zubaidat	32.17590	35.53135	955	1257
9	Ein -assultan RC	31.87717	35.44692	1451	1909

(1) Includs Khal-al-ahmar, (2) includes Duke Village.

3.3.2 Purely spatial analysis, adjusted for season

Cluster analysis by *SaTScan* examines geographic variations in a ten-year period (1994-2004) using both purely spatial and space-time models to determine whether observed fluctuations in incidence rates are random or whether fluctuations represent statistically significant deviations from randomness.

The purely spatial scan statistic which is time independent was performed for all CL cases including cases of *L. major*, *L. tropica* and non-genotyped. Table 3.8 shows numbers of observed and expected cases, relative risks (RR) and p-values for the purely spatial analysis of cases adjusted for season. Expected case counts can be calculated by dividing the observed count by the RR. Using Poisson probability model, time precision of one year, number of Monte Carlo replications of 1000, maximum spatial cluster of ≤ 50 % of population and p-value=0.05, the purely spatial statistic revealed 4 statistically significant (p=0.001) clusters of high CL case numbers. Zubaidat village is shown to be the most likely significant cluster. Other secondary clusters of statistical significance were the villages of A'uja, Fasayil and the Bedouin encampments in Nabi-Musa and Khan-al-ahmar on the Jerusalem-Jericho highway. Nuaimeh village is another, but not statistically significant, cluster as the risk is increased there by 69.4%. Figure 3.11 summarizes the purely spatial season-adjusted analysis of CL mapped in terms of significance. Jericho being the most populous (20,000) area in this study did not form a cluster and the RR was 0.692 (data not shown).

Table 3.8 Purely spatial analyses: season-adjusted statistics of CL, *L. major* and *L. tropica* cases, District of Jericho, 1994-2004, RR: relative risk.

Area	Observed	Expected	RR	p-value
CL				
1. Zubaidat	61	16.48	4.084	0.0010
2. Nabi-Musa	29	3.58	8.106	0.0010
3. Fasayil	95	10.83	2.581	0.0010
3. A'uja	95	48.36	1.471	0.0010
4. Nuaimeh	23	13.85	1.694	0.1310
L. major				
1. Fasayil	17	4.00	4.607	0.0010
1. A'uja	38	17.87	2.438	0.0010
2. Nuaimeh	11	4.32	2.234	0.1110
L. tropica				
1. Zubaidat	34	3.35	14.866	0.0010
2. Nabi-Musa	17	0.73	27.962	0.0010

Fasayil and A'uja villages were shown to be the most likely statistically significant cluster for *L.major* with 4.607 and 2.438 times more cases than expected, respectively. Nuaimeh, as in CL, was a border line cluster with RR of 2.234 but a p=0.1110 (Table 3.8, Figure 3.11). Two *L. tropica* clusters were found, the major cluster in Zubaidat village, in the far north, and a secondary cluster in Nabi-Musa/Khan-al-ahmar Bedouin encampment in the south.

(a)　　　　　　　　　　(b)　　　　　　　　　　(c)
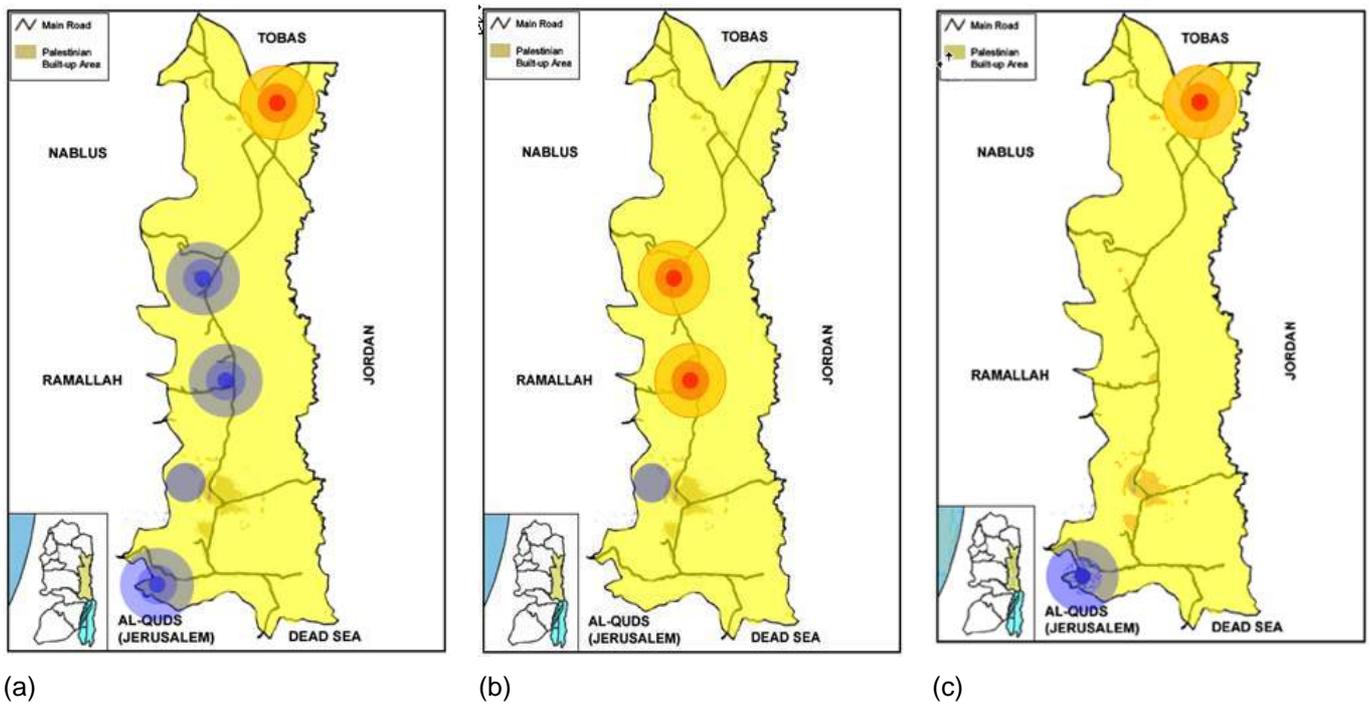
Figure 3.11 Spatial distributions of the CL (a), *L. major* (b) and *L. tropica* (c) in the district of Jericho in Palestine between 1994 and 2004. The most likely clusters are shown in red circles while the secondary clusters are shown in blue with p-value= 0.0010. The two small blue circles in (a) and (b) have border line significance.

3.3.3 Space-time analysis, adjusted for season

Table 3.9 and Figure 3.12 show space-time, season-adjusted results of *SaTScan* for CL, *L. major* and *L. tropica* cases with clusters shown as circular windows. These clusters are flexible both in location and size and their exact borders remain uncertain (Kulldorff *et al.,* 1997). Also, each of them has maximum 50% of the number of cases in that area. This maximum size circle is ideal as it detects both small and large clusters (Kulldorff *et al.,* 1998 b). Four areas are found to be significant clusters for CL. Season-adjusted space-time analysis showed more clusters than the purely spatial did. Nuaimeh became for example a significant cluster with risk increasing by 100%. Jericho city in 1995 had less observed cases (200) than expected (245) with RR=0.686 indicating about 31% fewer cases than expected and thus did

not form a significant cluster. It was only between 2000 and 2004 that Zubaidat, Fasayil, A'uja, Nabi-Musa and Nuaimeh formed apparent clusters.

Table 3.9 Space-time analyses: season-adjusted statistic of CL, *L. major* and *L. tropica* cases, District of Jericho, 1994-2004. RR: relative risk.

Area	Cluster Time	Observed	Expected	RR	p-value
		CL			
1. Zubaidat	2001–2004	61	16.55	4.066	0.0010
2. Fasayil	2003-2004	27	10.87	2.569	0.0010
2. A'uja	2003-2004	68	48.57	1.464	0.0010
3. Nabi-Musa	2000-2003	29	3.60	8.492	0.0010
4. Nuaimeh	2003-2004	23	11.74	2.006	0.0020
5. Jericho	1995	200	245.81	0.686	0.7620
		L. major			
1. Fasayil	2003-2004	17	4.00	4.587	0.0010
1. A'uja	2003-2004	38	17.87	2.426	0.0010
2. Nuaimeh	2001-2004	11	4.32	2.646	0.0030
3. Jericho	2001-2004	84	90.43	0.867	0.0140
		L. tropica			
1. Zubaidat	2001-2002	34	3.36	14.799	0.0010
2. Nabi-Musa	2000-2004	17	0.73	27.769	0.0010
3. Aqbat-Jabr-RC	2001-2002	16	15.63	1.029	0.015

Of the *L. major* clusters, Fasayil and A'uja remained, as in purely spatial results, the most likely significant cluster. As stated above, Nuaimeh became a significant cluster with 2.646 fold increase in relative risk indicated by more cases than expected between 2001 and 2004. Within the same period, Jericho city had, for the first and only time, almost as much observed cases as expected: 84 observed cases and an RR of 0.867, indicating approximately 13% fewer cases than expected. For *L. tropica,* the two clusters identified in the spatial analysis remained as they are. In addition, the Aqbat-Jabr refugee camp appeared as cluster in 2001-2002 with very slight excess of observed cases (16 cases) compared to expected cases (15.63).

(a)   (b)   (c)
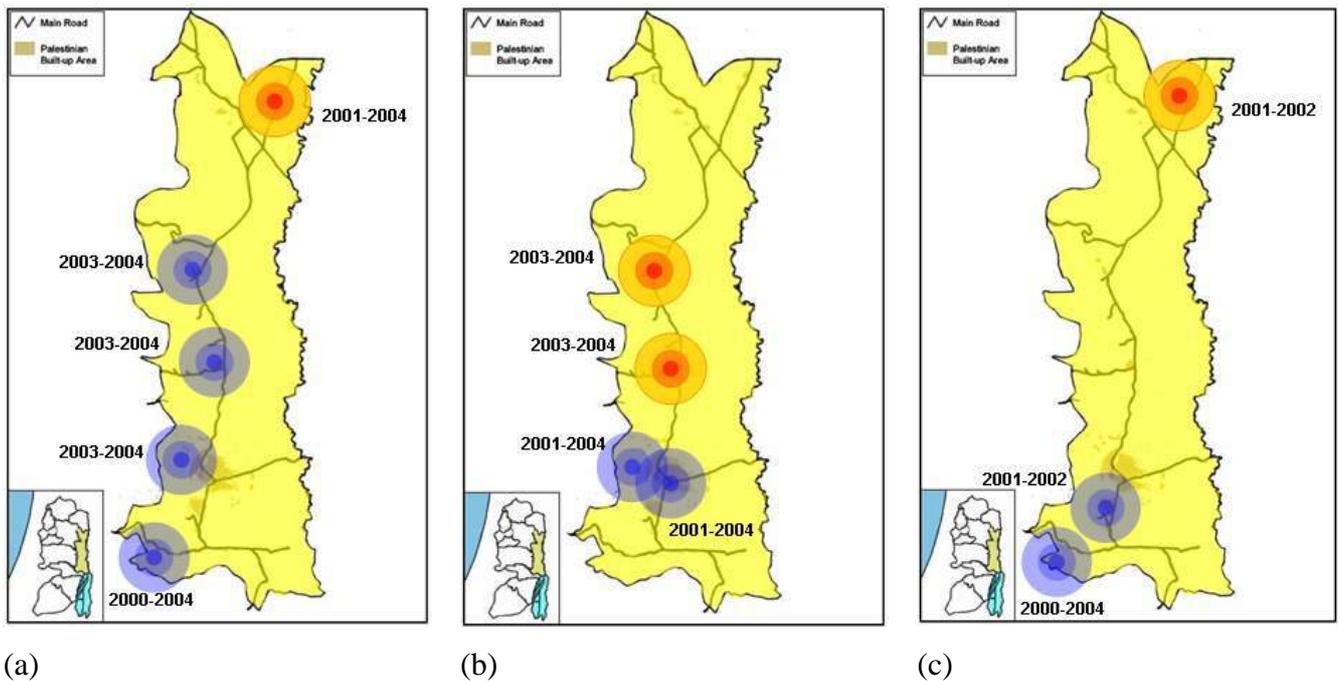
Figure 3.12. Space-time distribution of the CL (a), *L. major* (b) and *L. tropica* (c) cases in the district of Jericho in Palestine between 1994 and 2004. The most likely clusters are shown in red circles while the secondary clusters are shown in blue with p-value= 0.0010. The time period shown close to each circle represents the time frame during which this geographical site was considered a statistically significant cluster.

3. 4 Shewhart's Plot: Early warning system

Shewhart Plot (Levy and Jennings, 1950; Westgard *et al*., 1981) is another graphical display that depicts trends and peaks of leishmaniasis, in addition to the moving average in (3. 2. 4) and *SaTScan* (3. 3) techniques mentioned above. Mean ($\overline{X}$) and standard deviation were calculated for the annual total number of cases (14, 49, 27, 11, 39, 42, 31, 71, 46, 65 and 116). Grubb's test was conducted to check for outliers at the significant level of p=0.05 and none were detected. The mean ($\overline{X}$) was 46.25 and standard deviation was 29.65. With lower and upper limits being 1SD below or above the mean, cases in 1994 (14) and 1997 (11) were below mean whereas cases in 2004 were exceptionally above mean (116) indicating an outbreak or peak (Figure 13.3). The year 2001 was close to becoming a peak.
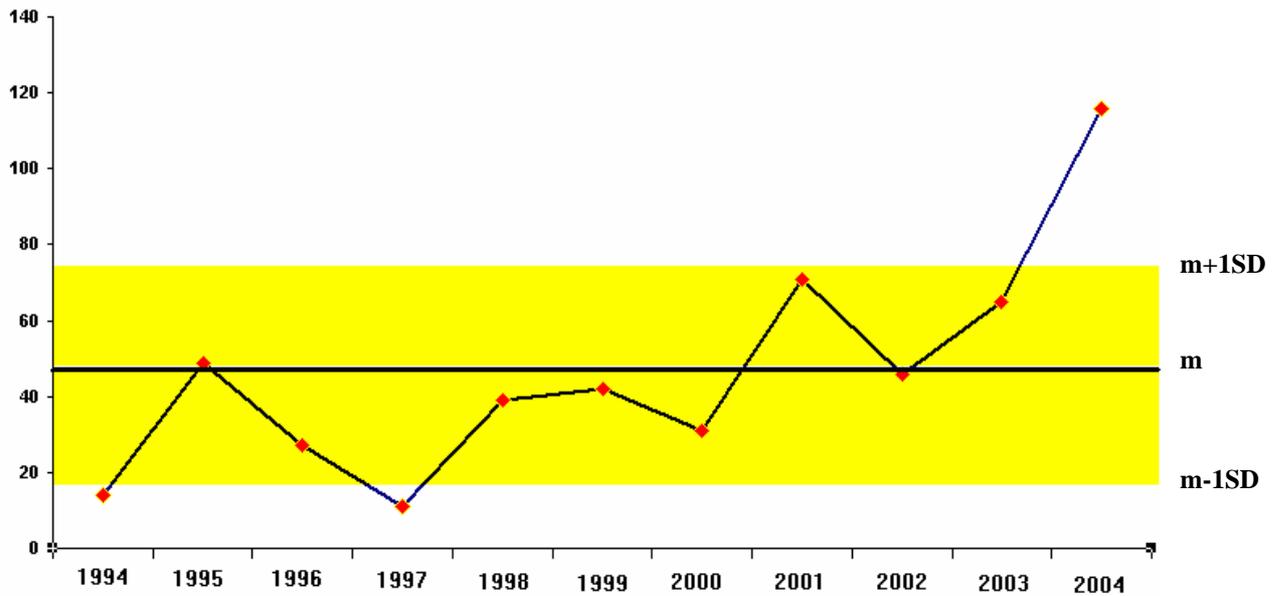
Figure 3.13 Shewhart's Chart for cases of CL in Jericho District, 1994-2004. Mean (m) is 46.25 and SD=29.65.

3.5 Genetic variability within *L. major* as revealed by Multilocus Microsatellite Analysis (MLMT)

3.5.1 Description of the microsatellite markers used in this study

Ten microsatellite markers were isolated from a genomic library of *L. major* using the Basic Local Alignment Search Tool (BLAST) (http://www.sanger.ac.uk/Projects/L_major/) based on loci located on five chromosomes (Table 2.3). Even if found on the same chromosome, loci were located at long distances from each other and thus considered to be independent. Five of the markers represented trinucleotide repeats (GTG), 4 different dinucleotide stretches and one was a tetranucleotide repeat. PCR primers of 20 bp size were designed at a distance of five nucleotides from both ends of the microsatellite repeats to make insertions/deletions in the flanking regions unlikely. The ten markers were tested using 106 strains of *L. major* (Table 3.11) from 19 countries in Asia and Africa (Figure 2.3). To search for polymorphism, the PCR products were screened on PAGE and/or 3.5% MetaPhor agarose gel electrophoresis, and sized by capillary electrophoresis (CE) using an automated sequencer (Figure 3.14). The degree of polymorphism differed in the markers from a minimum of 3 in markers 4GTG and 1GC to maximal 10 alleles in markers 45GTG, 71AT and 1CA within the strains analyzed (Table 3.10).

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(a)
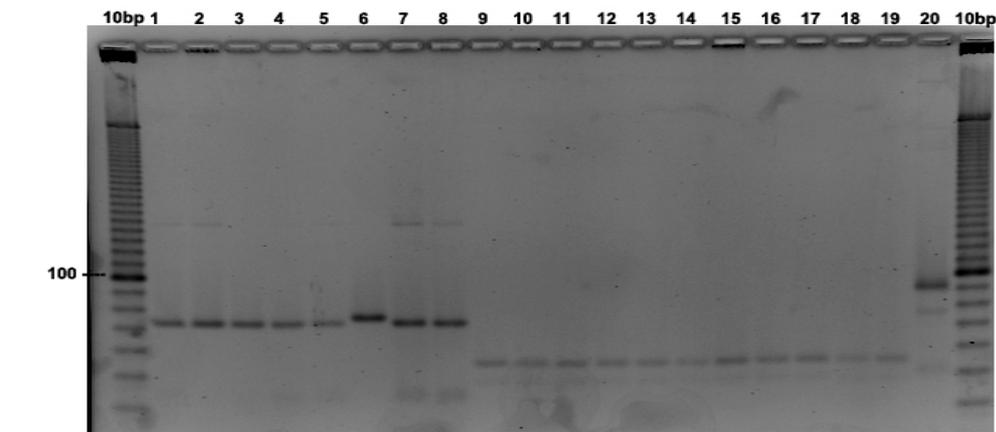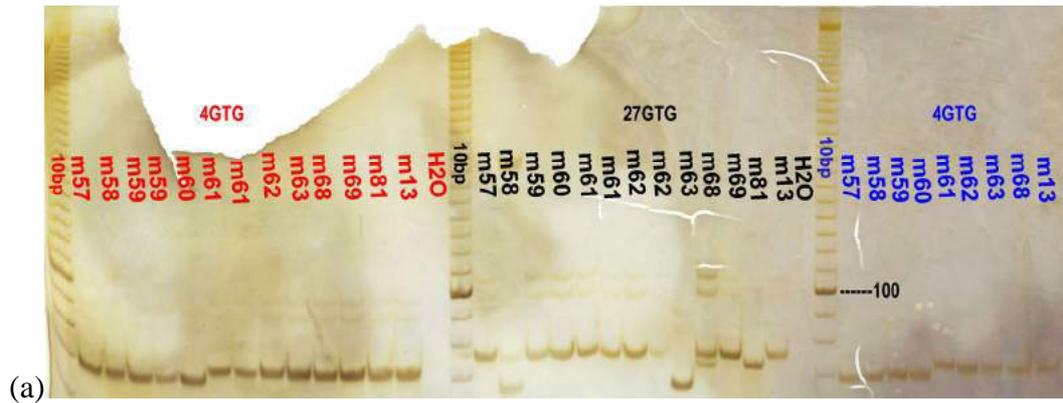
(b)
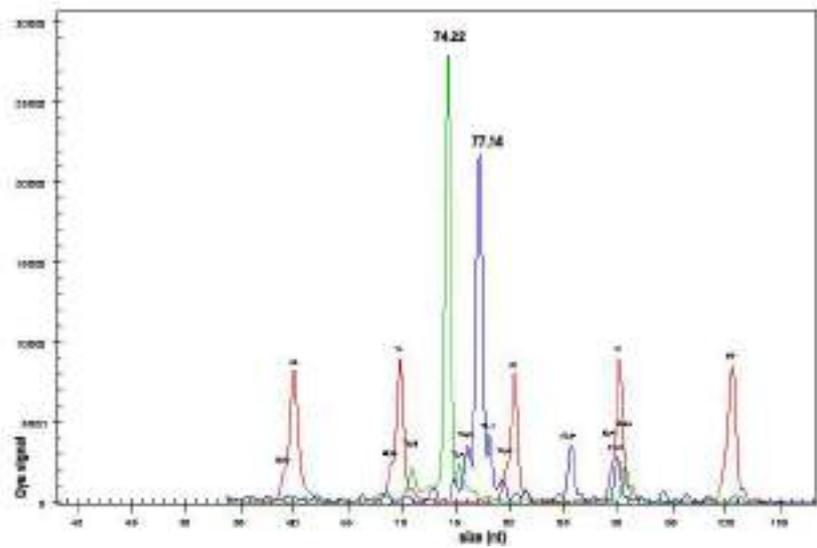
(c)

Figure 3.14 Different techniques were used to allocate microsatellite variation. (a) A PAGE run showing two markers 4GTG, 27GTG with 10 bp ladder as molecular size standard. (b) 3.5% MetaPhor agarose gel: Lanes 1-8 represent 27GTG marker with the *L. major* Friedlin reference strain in lane 6. Lanes 9-20 represent 39GTG marker with the Friedlin reference strain in lane 20. (c) CE run for *L. major* 74 from Jericho -Palestine using Beckman coulter CEQ8000. The blue peak

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shows the fragment size for the 1GACA marker and the green peak for the 39GTG marker. The red peaks represent the size marker

Table 3.10 shows that the number of alleles varies between loci, ranging from 3 as in 4GTG and 1GC to 10 as in 45GTG, 71AT and 1CA. Increased degree of inbreeding within loci (FIS, Fis or f) ranging from 0.874-1.00 (P<0.05) with a mean of 0.976 is witnessed.

Observed (Ho) heterozygosity among loci was in all populations extremely low compared with the heterozygosity (He) expected under assumption of Hardy–Weinberg equilibrium. The greatest difference between He and Ho is in the locus 45GTG (0.784). The greater this difference, the higher was the corresponding inbreeding coefficient (Fis) as Fis = 1-Ho/He (Table 3.10)

The mean number of alleles per locus (A) within each population ranged from 3 to 10 with a mean of 6.7. The highest (A) is noticed in the 45GTG, 71AT and 1CA indicating increased heterogeneity in these loci.

Table 3.10 Characterisation of the 10 microsatellite markers used for population studies in *L. major*.

locus	No. Alleles[§]	Descriptive statistics**				
		n	A	He	Ho	Fis
4GTG	3	105	3	0.362	0.0095	0.974
27GTG	6	105	6	0.631	0.000	1.000
36GTG	7	101	7	0.711	0.000	1.000
39GTG	7	93	7	0.737	0.011	0.986
45GTG	10	104	10	0.784	0.000	1.000
1GC	3	103	3	0.330	0.000	1.000
28AT	8	104	8	0.533	0.029	0.946
71AT	10	105	10	0.604	0.076	0.874
1GACA	3	103	3	0.548	0.009	0.982
1CA	10	102	10	0.509	0.000	1.000
All	67	102.5	6.7	0.575	0.014	0.976

§ Number of alleles per locus as shown by FSTAT software.

** (n) Average sample size, (A) average number of alleles per locus, (He) expected proportion of heterozygotes, (Ho) observed proportion of heterozygotes (under hardy Weinberg equilibrium), inbreeding coefficient (Fis), were obtained using GDA software.

3.5.2 Assignment of multilocus microsatellite profiles to the strains of *L. major* under study

The sizes of the 10 microsatellite markers were estimated for the 106 strains of *L. major* studies herein by either using the fragment analysis tool of the Beckman/Coulter sequencer by comparing with reference strains. The strain MHOM/IL/1980/Friedlin which was sequenced in the *Leishmania* genome project was included in all experiments and served as reference for calculating of the repeat numbers. A multilocus microsatellite profile, also called genotype later on, which summarizes the repeat numbers obtained for the 10 markers, was assigned to each of the 106 strains analysed as some of them were uniquely represented by single strains (Table 3.11). A total of 67 (including the *L. tropica* out group) different genotypes were obtained for these strains. Eleven isolates from Termiz-UZ were represented by 1 genotype, 2 strains from Mubarek-UZ were represented by 1 genotype and another 7 from the same area were represented by 1 genotype, 2 strains, one from UZ and the other from TM were, also, represented by 1 genotype, 2 strains from Jericho were represented by 1 genotype, 9 isolates from Jericho, Arava, and Qetziot (Negev) were represented by 1 genotype, another 4 strains from Jericho were represented by 1 genotype, 2 strains from Beersheba were represented by 1 genotype, 7 strains from UZ, TM and KZ were represented by 1 genotype and, finally,1 genotype represented 2 strains from Negev and 1 from Sinai-Egypt. Only individual profiles were found for the African strains.

Table 3.11 The multilocus microsatellite profiles of the strains of *L. major* analysed in this study

#	WHO code	Lab. code	Site/Country	Source	4 GTG	27 GTG	36 GTG	39 GTG	45 GTG	1 GC	28 AT	71 AT	1 GACA	1 CA
1	MHOM/SU/1973/5ASKH	MAJ-01	Ashgabad- Turkmenistan	LRC	7	8	10	2	4	7	9	10	6	14
2	IPAP/TM/1991/M-97	MAJ-26	Tezeel- Turkmenistan	MARTS	7	8	10	2	4	7	9	10	6	14
3	MHOM/TM/1987/Rod	MAJ-39	Bakharden- Turkmenistan	MARTS	7	8	10	2	4	7	9	10	6	14
4	MRHO/TM/1995/T-9537	MAJ-35	Serax- Turkmenistan	MARTS	7	8	--	2	4	7	9	10	6	14
5	MHOM/TM/1982/Lev	MAJ-36	Geok-Tepe- Turkmenistan	MARTS	7	8	10	2	4	7	8/10	10	6	14
6	MHOM/TM/1986/ER	MAJ-37	Tejen- Turkmenistan	MARTS	7	8	10	2	4	7	9	10/11	6	14
7	MHOM/UZ/1999/Nuriya	MAJ-25	Mubarek-Uzbekistan	LRC	7	8	10	2	4	7	9	10	6	14
8	MHOM/UZ/1987/BUR	MAJ-27	Karaulbasar-Uzbekistan	MARTS	7	8	10	2	4	7	9	10	6	14
9	MRHO/UZ/1987/KK29	MAJ-29	Takhtakupyr-Uzbekistan	MARTS	7	8	10	2	4	7	9	10	6	14
10	MHOM/UZ/87/Kurb	MAJ-33	Karshi Uzbekistan	MARTS	7	8	--	2	4	7	9	10	6	14
11	MHOM/UZ/2002/Isv M-22h	MAJ-79	Mubarek Uzbekistan	ISAEV	6	8	10	2	4	7	10	13	6	14
12	MHOM/UZ/2002/Isv M-17h	MAJ-80	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	10	13	6	14
13	MHOM/UZ/2002/Isv M-12h	MAJ-81	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	10	13	6	14
14	MHOM/UZ/2002/Isv M-10h	MAJ-82	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	9	13/16	6	14
15	MHOM/UZ/2002/Isv M-30h	MAJ-85	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	10	13	6	14
16	MHOM/UZ/2002/Isv M-29h	MAJ-86	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	9	13	6	14
18	MHOM/UZ/2002/Isv M-28h	MAJ-87	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	10	13	6	14
19	MHOM/UZ/2002/Isv M-27h	MAJ-88	Mubarek Uzbekistan	ISAEV	7	8	9	2	4	7	9/10	13	6	14
20	MHOM/UZ/2002/Isv M-26h	MAJ-89	Mubarek Uzbekistan	ISAEV	7	8	9	2	4	7	9/10	13	6/7	14
21	MHOM/UZ/2002/Isv M-25h	MAJ-90	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	10	13	6	14
22	MHOM/UZ/2000/Isv T-03h	MAJ-102	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13/16	6	14

23	MHOM/UZ/1998/Isv M-09h	MAJ-103	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	10	13	6	14
24	MHOM/UZ/1998/Isv M-08h	MAJ-104	Mubarek Uzbekistan	ISAEV	7	8	9	2	4	7	9	--	6	14
25	MHOM/UZ/1998/Isv M-04h	MAJ-105	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	9	13	6	14
26	MHOM/UZ/1998/Isv M-01h	MAJ-107	Mubarek Uzbekistan	ISAEV	7	8	10	2	4	7	10	13	6	14
27	MHOM/UZ/1998/Isv M-02h	MAJ-115	Mubarek Uzbekistan	ISAEV	7	8	9	2	4	7	--	16	6	14
28	MHOM/UZ/03/Th21	MAJ-150	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
29	MHOM/UZ/03/Th24	MAJ-151	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
30	MHOM/UZ/03/Th29	MAJ-152	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
31	MHOM/UZ/03/Th32	MAJ-153	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
32	MHOM/UZ/03/Th35	MAJ-154	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
33	MRHO/UZ/03/Th6g	MAJ-155	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
34	MRHO/UZ/03/Th20g	MAJ-156	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
35	MRHO/UZ/03/Th23g	MAJ-157	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
36	MRHO/UZ/03/Th38g	MAJ-158	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
37	MRHO/UZ/03/Th44g	MAJ-159	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
38	MRHO/UZ/03/Th37g	MAJ-160	Termez Uzbekistan	ISAEV	7	8	9	2	4	7	9	13	6	14
39	MRHO/SU/59/NealP	MAJ-123	??? Central Asia	MARTS	7	8	9	--	5	7	9	11	6	14
40	MRHO/KZ/1988/Tur-27R	MAJ-38	Turkestan	MARTS	7	8	10	2	4	7	9	10	6	14
41	MHOM/WA/87/NEL2	MAJ-02	???-East and West Africa	KIT	6	9	7	3	10	8	10	9	7	23
42	MTAT/KE/????/NLB089A	MAJ-06	Marigat-Kenya	LRC	6	9	7	3	10	7	13	13	7	17
43	MTAT/KE/195?/T4	MAJ-22	Baringo-Kenya	LRC	6	9	7	3	10	7	13	7	7	17
44	MHOM/SN/????/DK99	MAJ-44	Keur Moussa-Senegal	LRC	6	10	7	10	18	8	10	11	7	17
45	MHOM/SN/????/106	MAJ-45	Podor des Fleuve- Senegal	LRC	6	10	7	10	18	8	10	13	7	17
46	MHOM/SN/2000/DK74	MAJ-58	Senegal	LSHTM	6	9	6	2	10	7	12	13	7	18
47	MHOM/SN/1996/LEM3181	MAJ-48	Thies- Senegal	LEMPP	6	10	7	10	18	7	5	14	8	8

#	Strain	MAJ	Location	Source	6	10	7	10	14	7	10	13	8	4
48	MHOM/SN/1996/DPPE23	MAJ-49	Thies- Senegal	LEMPP	6	10	7	10	14	7	10	13	8	4
49	MHOM/MA/1992/LEM2463	MAJ-46	Ain Beni Mathar-Morocco	LEMPP	6	10	--	--	18	--	8	9	6	--
50	MHOM/MA/1995/LEM2983	MAJ-47	El Rachidia- Morocco	LEMPP	6	9	9	2	10	7	10	13	7	28
51	MHOM/MA/1981/LEM265	MAJ-57	Marocco	LSHTM	6	9	9	2	10	7	10	13	8	28
52	MHOM/BF/1996/LIPA538	MAJ-50	Ouagadougou-Burkina Faso	LEMPP	6	10	8	10	18	7	9	15	8	8
53	MHOM/BF/1998/LPN166	MAJ-55	Ouagadougou- Burkina Faso	LEMPP	6	10	7	10	19	7	11	15	8	8
54	MHOM/TN/1997/LPN162	MAJ-51	Sfax-Tunisia	LEMPP	6	10	9	--	18	7	11	12	6	14
55	MHOM/TN/1994/GLC7	MAJ-54	Gafsa-Tunisia	LEMPP	6	10	9	--	18	7	8	12	6	14
56	MHOM/DZ/1998/CRE95	MAJ-52	M'sila-Algeria	LEMPP	6	10	9	--	18	7	8	10	6	14
57	MHOM/DZ/1998/LPS13	MAJ-53	Biskra- Algeria	LEMPP	6	10	9	--	18	7	5/8	9/12	6	14
58	MHOM/TR/1993/HA	MAJ-41	Manisa-Turkey	K.P. Chang	6	10	7	10	18	8	10	15	7	22
59	MHOM/TR/1993/SY	MAJ-42	Aydin-Turkey	K.P. Chang	6	10	7	10	18	8	10	15	7	22
60	MHOM/TR/1994/HK	MAJ-43	Kars-Turkey	K.P. Chang	7, 5	9	9	9	12	7	9	13	7	14
61	MHOM/IL/1967/Jericho	MAJ-09	Jericho area	LRC	7	9	9	9	12	7	9	13	7	14
62	MHOM/IL/1986/Blum	MAJ-10	Jericho area	LRC	7	9	9	9	12	7	9	13	7	14
63	MHOM/IL/1990/LRC-L585	MAJ-12	Jericho area	LRC	7	9	9	9	12	7	9	13	7	14
64	MHOM/PS/1998/PAL1	MAJ-19	Jericho area	ISLAH	7	9	9	9	12	7	9	13	7	14
65	MHOM/IL/2000/L779	MAJ-23	Jericho area	LRC	7	9	9	9	12	7	9	13	7	14
66	MHOM/PS/2000/ISLAH503	MAJ-24	Jericho area	ISLAH	7	9	9	9	12	7	9	13	7	14
67	MHOM/PS/1998/PAL2	MAJ-20	Jericho area	ISLAH	7	9	10	9	12	7	9	13	7	14
68	MHOM/PS/1998/PAL3	MAJ-21	Jericho area	ISLAH	7	9	10	9	12	7	9	13	7	14
69	MHOM/IL/1980/Friedlin	MAJ-13	Jericho area	LRC	7	9	9	9	12	7	9	13	7	14
70	MHOM/PS/2000/ISLAH 506	MAJ-66	Jericho area	ISLAH	7	9	10	9	12	7	9	13	7	14
71	MHOM/PS/2001/ISLAH 657	MAJ-70	Jericho area	ISLAH	7	7	9	9	12	7	9	13/16	7	14
72	MHOM/PS/2001/ISLAH 658	MAJ-71	Jericho area	ISLAH	7	9	10	9	12	7	9	13	6	14

No.	Strain	MAJ code	Location	Lab										
73	MHOM/PS/2001/ISLAH 659	MAJ-72	Jericho area	ISLAH	7	9	10	9	12	7	9	13	7	14
74	MHOM/PS/2002/ISLAH 662	MAJ-73	Jericho area	ISLAH	7	9	9	12	12	7	9	13	7	14
75	MHOM/PS/2002/ISLAH 697	MAJ-76	Jericho area	ISLAH	7	9	9	9	12	7	9	13/16	7	14
76	MHOM/PS/2002/ISLAH690	MAJ-77	Jericho area	ISLAH	7	9	9	9	12	7	9	13/16	7	14
77	MHOM/PS/2002/ISLAH 691	MAJ-78	Jericho area	ISLAH	7	9	9	9	12	7	10	16	7	14
78	MHOM/PS/2003/ISLAH 718	MAJ-99	Jericho area	ISLAH	7	9	10	9	9	7	9	11	7	14
79	MHOM/PS/2001/ISL600	MAJ-116	Jericho area	ISLAH	7	9	8	9	12	7	`8/9	13	7	14
80	MHOM/PS/2003/ISL720	MAJ-120	Jericho area	ISLAH	7	9	8	12	7/9	7	9	13	7	14
81	MHOM/IL/03/LRC-L962	MAJ-111	Negev???	LRC	7	9	8	9	12	7	9	13	7	14
82	MHOM/IL/2003/LRC-L949	MAJ-98	Jericho area	LRC	7	9	9	9	9	7	9	13	7	14
83	MHOM/IL/03/LRC-L964	MAJ-113	Beer Sheva	LRC	7	9	8	9	12	7	9	13	7	14
84	MHOM/IL/2003/LRC-L965	MAJ-114	Negev desert-Qeziot	LRC	7	9	9	9	12	7	9	13	7	14
85	MPSA/IL/1983/PSAM398	MAJ-16	Arava	LRC	7	9	9	9	12	7	9	13	7	14
86	IPAP/IL/1984/Uvda	MAJ-11	Negev-Uvda	LRC	7	9	9	12	9	8	9	13	7	19
87	IPAP/IL/1998/LRC-L746	MAJ-14	Negev-Uvda	LRC	7	9	9	12	9	8	9	13	7	19
88	IPAP/IL/1998/LRC-L464	MAJ-15	Negev-Uvda	LRC	7	9	9	12	9	7	9	13	7	19
89	MHOM/IL/2001/LRC-L846	MAJ-74	Jericho area	LRC	7	9	9	12/14	12	8	9	13	7	19
90	MHOM/IL/2003/LRC-L952	MAJ-94	Negev-Queziot	LRC	7	9	9	12	10	8	9	13/16	7	19
91	MHOM/IL/02/LRC-L946	MAJ-97	Negev-Yerucham	LRC	7	9	8	12	8	8	10	8	7	19
92	MHOM/IL/2002/LRC-L940	MAJ-101	Negev-Yerucham	LRC	7	8	9	12	12	8	9	16	7	19
93	MHOM/IL/2003/LRC-L958	MAJ-108	Arava-En Yahaf	LRC	7	9	5	12	9	8	9	13	7	19
94	MHOM/IL/2002/LRC-L862	MAJ-69	Negev???	LRC	7	9	5	12	9	8	9	16	7	19
95	MHOM/IL/03/LRC-L960	MAJ-110	Negev-Revivim	LRC	7	9	8	12	9	8	9	13	7	19
96	MHOM/IL/03/LRC-L963	MAJ-112	Negev-Shifta	LRC	7	9	8	12	9	8	9	14	6	19
97	MHOM/IL/2003/LRC-L1000	MAJ-122	Qaliya, Dead Sea	LRC	7	9	8	12	9	8	9	11	7	19

98	MHOM/EG/1984/LRC-L505	MAJ-18	Sinai-Egypt	LRC	7	9	--	12	9	8	9	13	7	19
99	IPAP/EG/1989/RTC-13	MAJ-17	Sinai-Egypt	LRC	7	9	9	12	9	8	9	13	7	19
100	MRHO/IR/1976/vaccine-strain	MAJ-59	Iran	LSHTM	6	9	6	12	9	7	16	13	6	24
101	MHOM/IQ/1986/BH012	MAJ-60	Iraq	LSHTM	7	9	6	12	10	7	16	16	6	23
102	MHOM/KW/1976/P47	MAJ-61	Kuwait	LSHTM	7	9	9	9	12	6	9	13	7	14
103	MPSM/SA/1989/SABIR-1	MAJ-62	Saudi Arabia	LSHTM	7	6	5	12	9	7	9	13	6	14
104	MHOM/SA/1984/KFUH68757	MAJ-63	Saudi Arabia	LSHTM	7	6	7	12	9	7	9	13	6	14
105	MHOM/SD/2004/MW1	MAJ-166	Sudan-Gadaref	M. Mukhtar	7	9	6	8	10	8	11	11	7	22
106	MHOM/SD/2004/MW38	MAJ-167	Sudan-Gadaref	M. Mukhtar	7	9	6	8	10	8	10	13	7	22
107	MHOM/SD/2004/MW94	MAJ-168	Sudan-Gadaref	M. Mukhtar	6	9	6	8	10	8	10	12	7	23

MARTS = Martsinovsky Institute of Medical Parasitology and Tropical Medicine, Sechenov Medical Academy Moscow, Russia (M.V. Strelkova); LRC = Leishmania reference centre, Hadassah Medical School, Hebrew University Jerusalem (L. F. Schnur); ISAEV = Isaev Insitute of Medical Parasitology, Samarkand, Uzbekistan (S.A. Razakov); LSHTM = London School of Hygiene and Tropical Medicine, UK (I. Mauricio); LEMPP = Centre National de reference des Leishmaniosis, Montpellier, France (F. Pratlong); KIT = Royal Tropical Institute Amsterdam, The Netherlands (H. Schallig). ISLAH: Islah medical laboratory in Jericho-Palestine.

IPAP: from *Phlebotomus papatasi*, MOHM: Human orign, MPSA: from *Psammomys obesus*, MRHO: from *Rhombomy opimus*

3.5.3 Population structure of *L. major* using *Structure*

3.5.3.1 Estimation of population structure using non-predefined populations

To estimate the optimal number of populations in our data set, a batch run was performed using values of K from 1 to 12. The runs were based on burn-in length period of 30,000 iterations and 1000000 MCMC (Markov Chain Monte Carlo) repeats. The mean values of ln likelihood, ln Pr (X\K) for K=1, 2… 12 were estimated and plotted against K (Figure 3.15). At K= 7, the curve started to form a plateau and the optimal K is, therefore, expected to be 7.

To understand the dynamics of population structure we have tested the outcome of clustering for K values from 2 to 7.
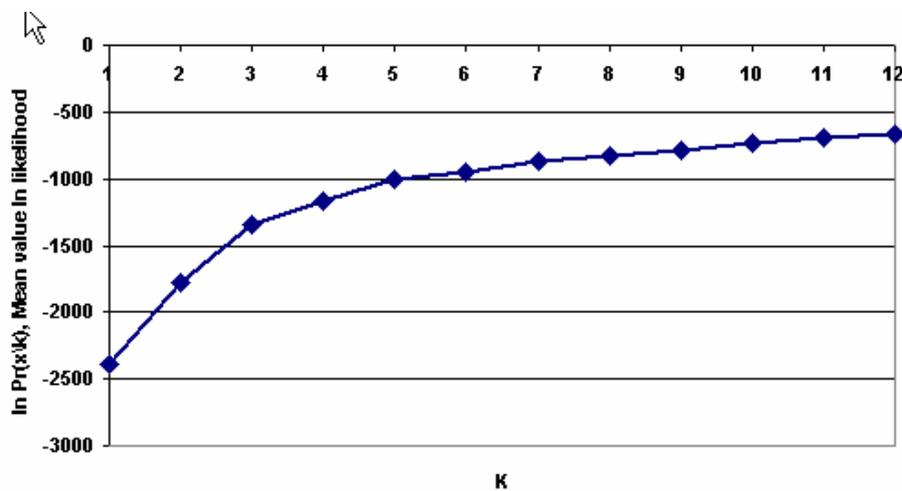
Figure3.15. Curve of the mean value Ln likelihood, ln Pr (x\K) showing a plateau starting at K=7.

Figure 3.16 displays membership coefficient which depicts each subgroup in a different colour with each individual as a fixed-length vertical line sometimes partitioned into K coloured segments which reflect membership coefficient of the individual isolates in the various subgroups (Rosenberg *et al.*, 2002). At K=2, the method correctly inferred that the underlying population structure consists of two subpopulations, the Central Asian (CA) and 'the rest'. Each increase in K splits one of the clusters obtained with the previous value. At K =3, the clusters were anchored by Central Asia, Middle East - South West Asia- (ME) and Africa regions, but 4 Middle Eastern (ME) isolates, 2 Turkish (TR), 1 Iraqi (IQ) and 1 Iranian (IR), grouped with the African cluster. With further splitting at K=4, African strains were divided into two clusters, AF1 (13 genotypes: 1IL (Jericho), 1IR, 1IQ, 5EA (East Africa), 2NA (North Africa), 1WA (West Africa), 2TR) and AF2 (11 genotypes: 3NA, 1WA, 5EA, 1TR, 1IQ). One *L. major* from Negev desert (IL) deserted

its Middle Eastern cluster in K=3 to group with AF1. However, this isolate had partial membership in both clusters with membership coefficients of 0.4 in the Middle Eastern cluster and 0.6 in the AF1 cluster; reflecting continuous variation in allele frequencies across regions or admixture of neighbouring groups. Two of the three Turkish isolates were with Africa 1 (AF1) while the third with Africa 2 (AF2) cluster.

At K=5, the Middle Eastern cluster broke down into to distinct clusters, interestingly, with strains from the Jordan valley as ME1 (27 genotypes: 20PS (Palestine) +1TR+5 IL-Negev+1KW (Kuwait)) and those from the Negev and Sinai deserts as ME2 (16 genotypes: 12 IL -Negev+2 PS+2SA). The two SA (Saudi Arabia) genotypes from ME2 at K=6, 1 IQ 1IR in addition to 3 samples from CA formed a new cluster called ME3. The Central Asian cluster, CA from UZ, TM, and KZ, remained intact and so did the two African clusters AF1 (13 genotypes: 5NA+6WA+ 2TR) and AF2 (11 genotypes: 2NA+2WA+ 5EA+ 1IR+1IQ) apart from minimal change in AF1. So far, relative correspondence between regional affiliation and genetic ancestry has been noticed. At K=6 clusters remained as they are except for Central Asian which split into two clusters, CA1 (23 genotypes: 12 Mubarak, 1Termez, 6TM (Turkmenistan), 1KZ (Kazakhstan), 3UZ (Uzbekistan) and CA2 (16 genotypes: 10Termez+5 Mubarak+1SU (USSR)) which were re-joined again at K=7. And in K=7, African strains grouped into three (Figure 3.16) different clusters. These were AF1 (6 genotypes: 2WA, 5EA, 2NA), AF2 (5 genotypes: 1WA, 4NA) and AF3 (8 genotypes: 6 WA, 2 TR). Around 75% (20/27) of the ME1 cluster come from the Jordan Valley focus while 16% (4/24) come from the Negev focus. One TR and 1 KW joined this cluster. ME2 cluster is mostly (10/14=71%) composed of genotypes from the triangle-shaped Negev desert extending from the Jordanian borders in the east to the Egyptian borders in the west. The rest come from the Jordan Valley focus.
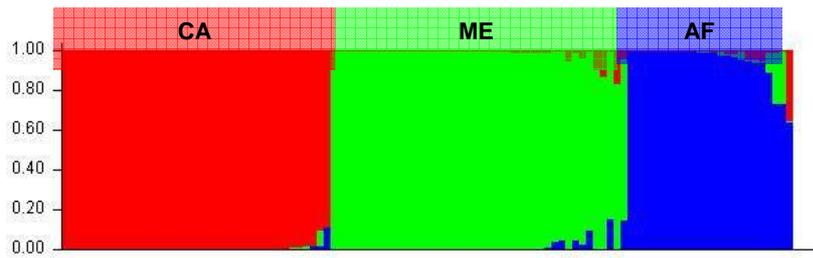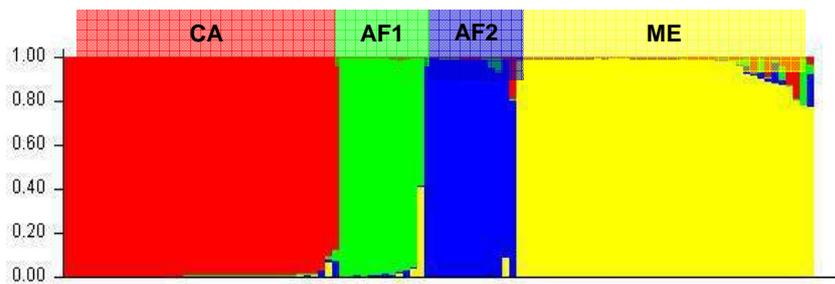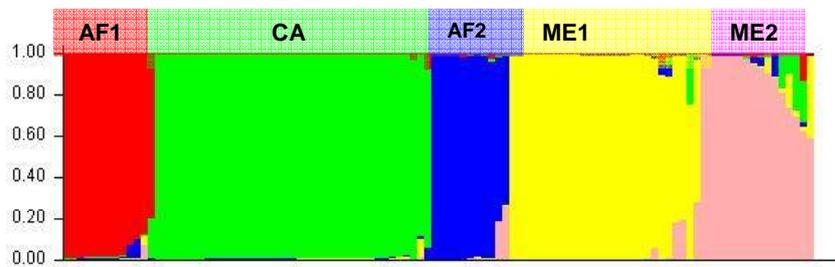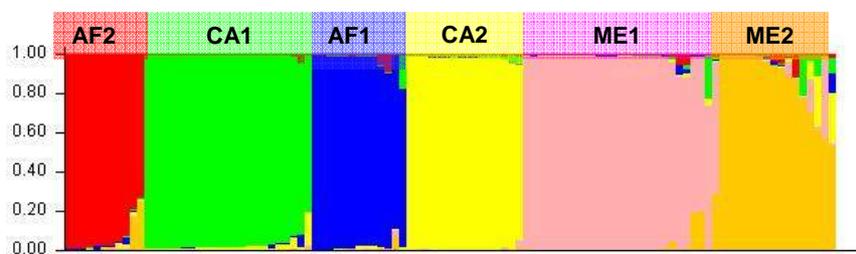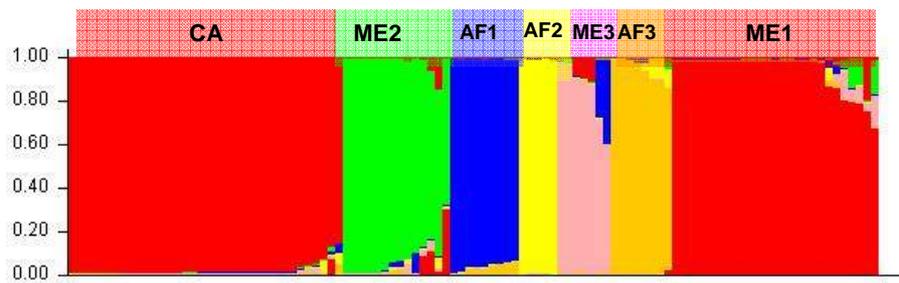
K=7

Figure 3.16. Estimated population structure shown as plots of Q (estimated membership coefficient for each sample) at K 2 to 7 which is represented by a single vertical line. Colored segments represent the sample's estimated membership in each of the K inferred clusters. Individual isolates can have membership in multiple clusters, with membership coefficients summing to 1 across clusters. CA= Central Asia; ME=Middle East; AF=Africa.

The analysis of multilocus genotypes without relying on information about their geographical origin allowed inference of their genetic relationships. The application of the model-based clustering algorithm, *Structure* computer software, allowed to allocate seven clusters subgroups in our sample set with distinctive allele frequencies. Using Wrights F-statistic in pairs, seven clusters (K=7) were proved to be the optimally differentiated populations (Table 3. 17).

3.5.3.2 Estimation of population structure using predefined population
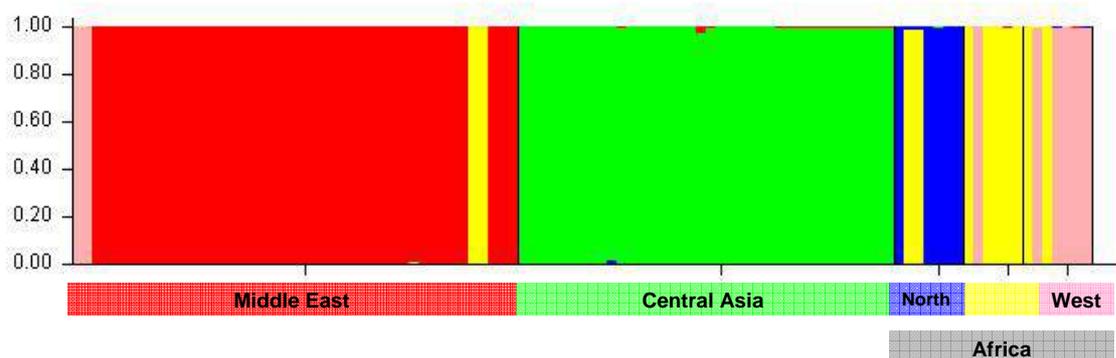
Figure 3.17 Population structure as shown by plots of Q (the estimated membership coefficient for each sample) using five populations predefined according to their geographical origin. Regions may have more than one colour. For instance, strains from ME represented by yellow colour geographically belong to ME but genetically to East Africa.

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The sample collection areas in Asia and Africa were divided into 5 major regions: Central Asia, Middle East (Southwest Asia), North Africa, West Africa and East Africa. Boundaries between these regions mostly corresponded to major physical barriers (seas and Sahara). Assigning each *L. major* isolate to one of these areas, corresponding to its collections site, without any idea about the underlying genetic relationships was tested by S*tructure 2.1* using admixture model. The Central Asian cluster was shown to be ideal, genetically and geographically, with all central Asian isolates having a very high coefficient of membership of 0.999 (Figure 3.17). Two of the three Turkish and single Iraqi and Iranian strains which are geographically closer to the Middle East (Southwest Asia) belong genetically to West Africa and East Africa, respectively, while the third geographically belonged to East Africa but genetically to West Africa. Using Chi-square (Table3.12) it was proven that genetic clusters corresponded significantly to the geographically predefined regions (X^2 =288.4, P<0.0001, df 16). A sample of unknown origin (MHOM/WA/87/NEL2) in which the patient caught CL while roaming between west and east Africa was shown to originate from East Africa.

Table 3.12 The number of *L. major* isolates in the five predefined clusters inferred by *Structure v. 2*.

	Cluster 1	Cluster2	Cluster3	Cluster4	Cluster5
Middle East	43	0	0	0	0
Central Asia	0	39	0	0	0
North Africa	0	0	5	0	0
East Africa	2	0	2	6	1
West Africa	2	0	0	1	5

(X^2 =288.4, P<0.0001, 16 df)

3.5.4 Analysis of *L. major* population structure using distance-based methods

For the 67 genotypes of *L. major,* the repeat numbers were organised in an input file in a diploid form to calculate the genetic distances. Two different measures for genetic distance both implemented in the *MICROSAT* software have been used: proportion of shared alleles, Dps and Delta mu squared alleles, Ddm (or Dμ^2); the latter has been developed especially for the analysis of microsatellite variation (Zhivotovsky *et al*., 2000). NJ and UPMGA trees were constructed based on the distance matrices obtained. The statistical significance of each node on the

dendrogram was assessed by bootstrap analysis generating 1000 randomly re-sampled subsets of the data. A strain of *L. tropica* was used as an out-group.

Both the NJ and UPMGA cladogram based on Dps (Figure 3. 18) displayed exactly the same pattern, albeit with different bootstrap support, showing six major clusters and sub-clusters. *Structure* at K=7 revealed seven clusters as shown in figure 3.15: CA, ME1, ME2, ME3, AF1, AF2, and AF3**.** The Central Asian cluster includes all the 39 *L. major* isolates coming from (UZ) Uzbekistan, (TM) Turkmenistan and (KZ) Kazakhstan**.** The strains from Middle East were assigned to two clearly separated groups with genotypes from the Jordan Valley focus forming the clade Middle East 1 (ME1) and those from the Negev, with 1 genotype from the Sinai desert, belonging to Middle East 2 (ME2). A third ME cluster, ME3 composed of 4 from ME (SA, IQ and IR) and 3 CA (UZ). The African strains formed three clusters. First, AF1 representing genotypes from North, East and West (NEW) Africa as SD, KE, MA and SN, the second is AF2 which consists of genotypes from West Africa as SN and BF and North Africa. The third is a cluster, AF3, consisting of strains from West African countries in addition to 2 TR strains. The Dps-based cladograms showed largely congruent topology compared to the results of S*tructure* analysis at K=7. However, CA2 (Figure 3.16) was not well supported in the trees (Figure 3.18). Strains from SA, IQ, IR and central Asia that were assigned ME3 by S*tructure* were scattered all over the trees. The two SA strains sub-clustered close to CA and the IR and IQ strains sub-clustered close to AF1 Africa, while the three strains from UZ clustered in the CA clade**.** Two of the three Turkish isolates are grouping with the AF3 cluster, while the third Turkish strain is found in the cluster ME 1. It was observed that bootstrap support for the African clades was better compared to the other clades in the Dps trees.

The UPGMA cladogram based on delta mu squared distance, Ddm ($D\mu^2$) (Fig 3.18c); showed very poor congruence with the results of *structure* analysis. Despite this, the UPGMA-Ddm tree still highlights a well-distinct Central Asian and two Middle Eastern (Southwest Asia) clades.

(a) NJ-DPS-boot1000 genotypes
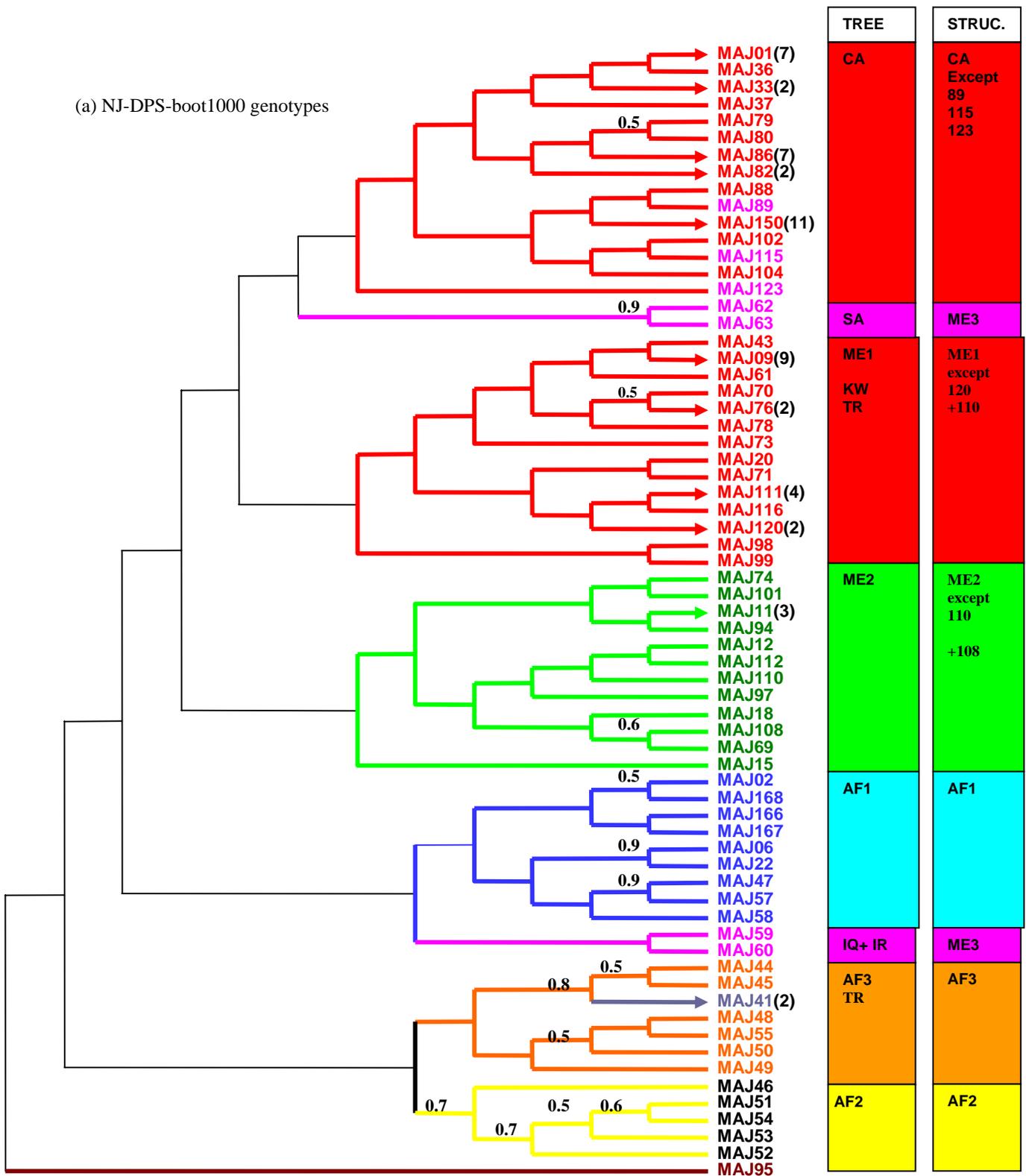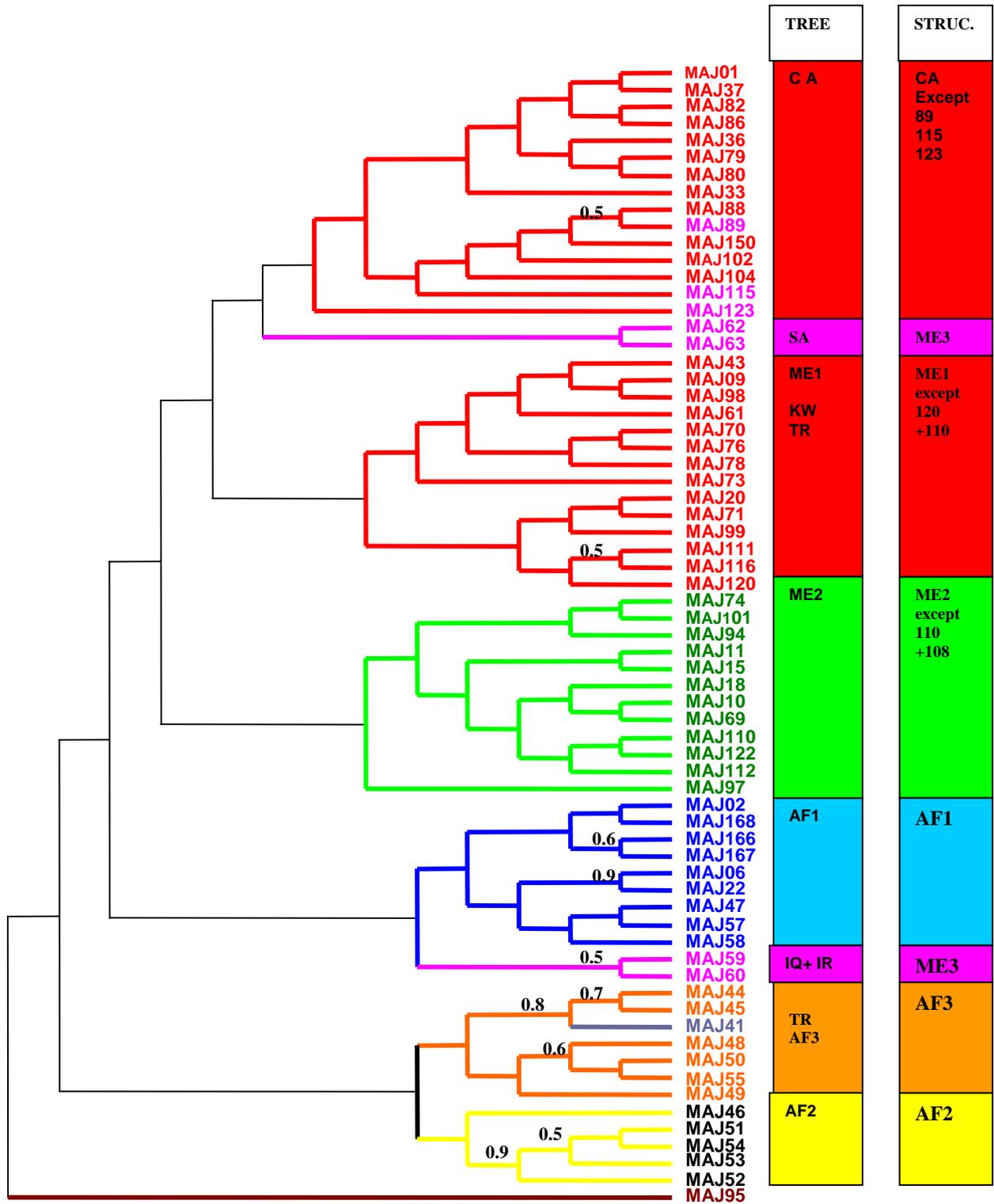
(b) UPGMA-DPS-boot1000 genotypes

(c) UPGMA-Ddm-boot1000 genotypes
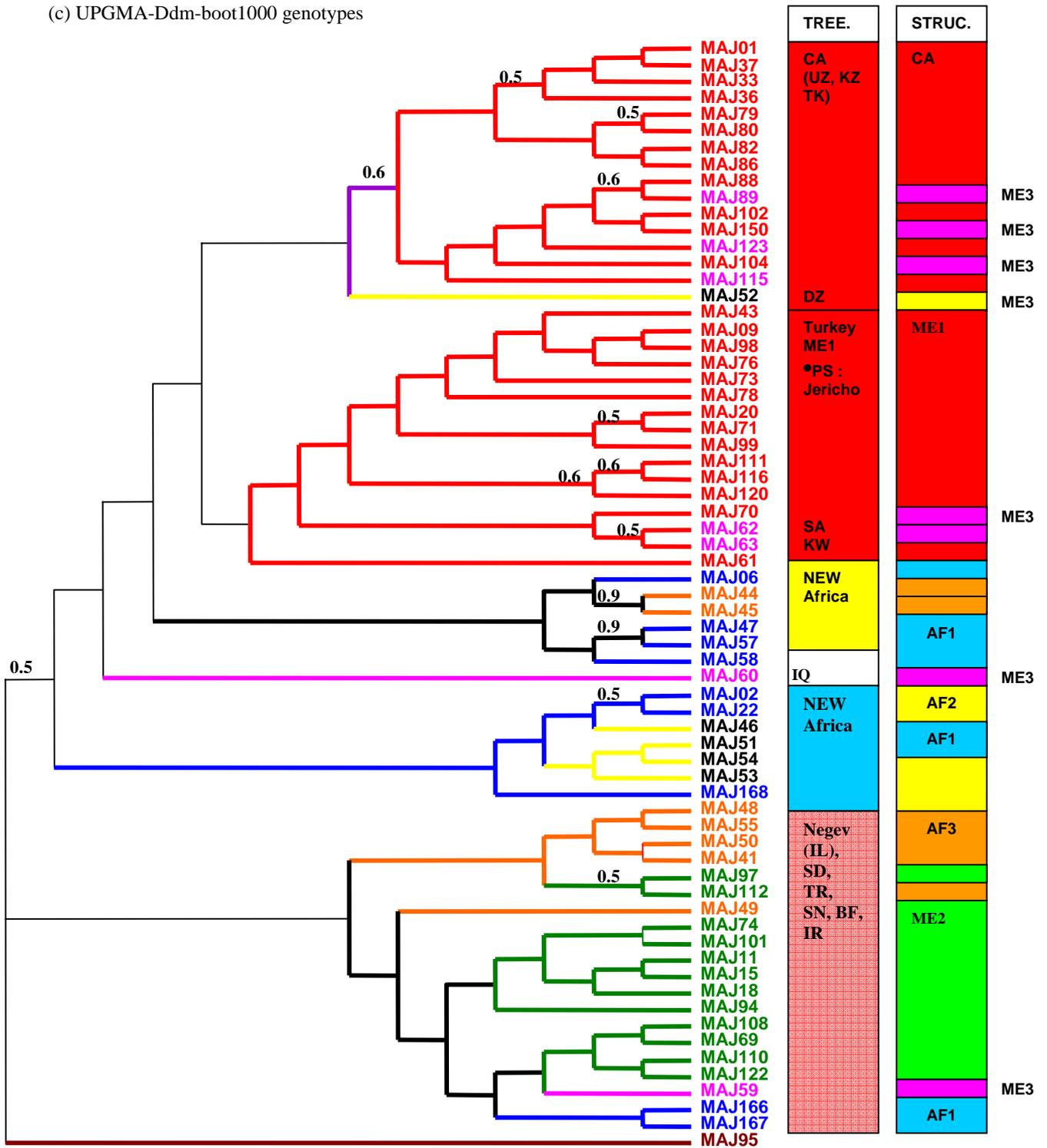
Figure 3.18 Dendrograms for the 67 *L. major* genotypes with *L. tropica* MHOM/IL/2002/LRC-L943 as out-group, calculated with 1000 bootstraps. (a) Consensus neighbour-joining tree calculated using distance of proportion of shared alleles (Dps). (b) Consensus UPGMA tree using Dps (c) consensus UPGMA using Delta mu squared (Ddm) distance. Bootstrap values above 0.5 are shown with 1 representing 100%. CA= Central Asia, ME=Middle East, AF=Africa, IQ=Iraq, IR=Iran, KW=Kuwait. End branches with arrows are genotypes representing 2 or more strains as indicated by the number in brackets.

3.5.5. Genetic isolation of the *L. major* populations identified in this study

Fst, as a measure for genetic differentiation between populations, was calculated in a pairwise manner using FSTAT, GENEPOP and GDA software. The calculations were based on the optimal number of populations K=7 as defined by *Structure* analysis. As shown in Table 3.13, all are separated by a very great genetic isolation with significant departure from zero (Fstat > 0.25). Using predefined geographic clustering into the five major regions, the differentiation was moderate between West and North Africa (0.1459) from one side and between West and East Africa (0.1258) from the other.

Table 3.13 Estimates for Fst, measures of genetic differentiation (above diagonal), for all loci between populations of *L. major* as measured by FSTAT. Below diagonal is the corresponding calculated migration rate, Nm. (a) optimal population at K=7. (b) Predefined population according to the five major geographic regions.

(a)

	CA	ME2	AF1	AF2	ME3	AF3	ME1
CA		0.7417	0.7075	0.7394	0.5500	0.7685	0.7249
ME2	0.0871		0.4585	0.7097	0.4399	0.6088	0.6026
AF1	0.1034	0.2953		0.5784	0.3931	0.3817	0.6124
AF2	0.0881	0.1023	0.1822		0.4507	0.4859	0.7800
ME3	0.2045	0.3183	0.3860	0.3047		0.5154	0.5889
AF3	0.0753	0.1608	0.4050	0.2645	0.2351		0.7419
ME1	0.0949	0.1649	0.1608	0.0705	0.1745	0.0870	

CA: 36: UZ+TM+KZ; **ME2:** 10IL-Negev & Sinai +4 Jericho; **AF1:** 6 genotypes: 2WA, 5EA, 2NA; **AF2:** 5 genotypes: 1WA, 4NA; **AF3:** 8: 6WA, 2TR; **ME3**: 3CA, IR, IQ, 2 SA; **ME3:** 3CA, 1IR, 1IQ, 2SA; AF3: 6 WA, 2 TR; **ME1** (27): 20PS+1TR+5 IL- Negev+1KW.

(b)

	M. East	C. Asia	N. Africa	E. Africa	W. Africa
M. East		0.5112	0.4224	0.3616	0.3867
C. Asia	0.2390		0.6114	0.7228	0.6841
N. Africa	0.3419	0.1589		0.3544	0.1459
E. Africa	0.4414	0.0959	0.4554		0.1268
W. Africa	0.3966	0.1154	1.4962	1.7216	

Fst can be used to estimate genetic flow or migration rate, Nm, as Nm = 1-FST/4 FST (Souto and Premoli, 2003). Genetic flow migration (or gene flow) refers to the movement of individuals among subpopulations and can set a limit as to how much genetic divergence can occur. At K=7 genetic flow or migration is low ranging from 0.0705 to 0.4050. At the predefined population identification, there was a clear genetic flow (migration rate) between the African regions (1.4962 and 1.7216).

3.5.6. Estimation of allele numbers in geographical groups of strains

The mean number of alleles was plotted against the three main groups of strains that were derived from Central Asia, Middle East (Southwest Asia) and Africa (Figure 3.19). It is shown that the African group has the highest number of alleles (41) followed by Middle East (25) and Central Asia (15). This comparison was based on allele numbers counted for 17 African strains, 50 from Middle East and 39 from Central Asia. To overcome the problem with different numbers of strains from the three areas, a shuffling procedure was developed. An increment window was selected based on the region having the lowest number of strains which was Africa with 17 strains. The 17-increment window was serially moved over the strains of the two other regions. Each time the window was moved, the number of alleles was counted. Based on this, it moved 22 times over the 39- strain -Central Asian region and 34 times over the 50- strain- Middle East region. Mean and standard deviation for Central Asia and Middle East were estimated, which were 15 and 25, respectively, for mean and 0.72 and 6.2, respectively, for standard deviation. The gradual decrease in allele numbers on the geographic line from Africa via Middle East to Central Asia reflects a decreased genetic variation. The mean number of African alleles was highest, despite that the number isolates available was the lowest.
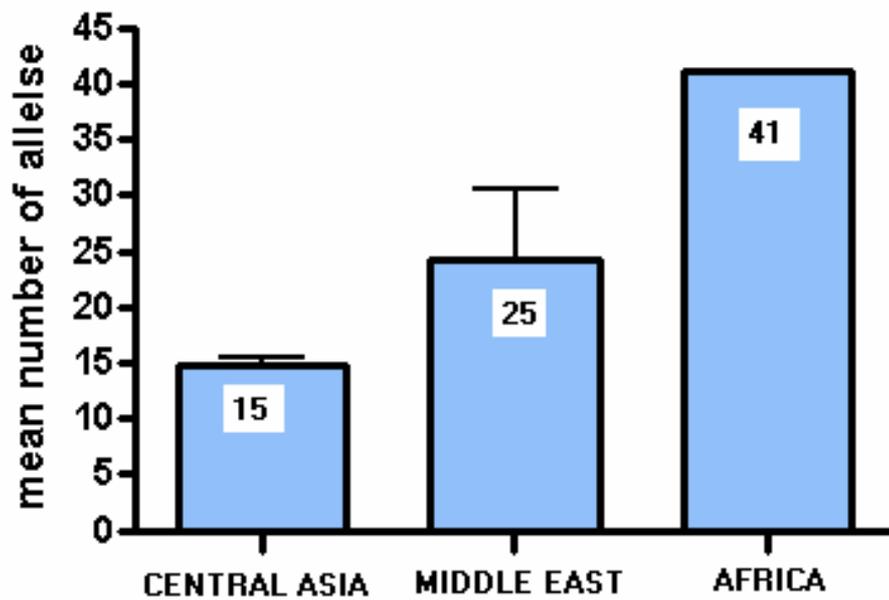
Figure 3.19 The mean number of alleles in the three major geographical regions. Standard deviation is shown to be minimal in the Central Asia, 0.7162, while as high as 6.36 in the Middle East.

4. DISCUSSION

This is the first molecular epidemiological study of leishmaniasis in the Jericho area. Molecular epidemiology is the application of molecular biology techniques to the study of the frequency, distribution and determinants of infectious and non-communicable diseases. For the first time, molecular diagnostic tools have been applied that lend themselves to the identification of different species of *Leishmania* as well as to the differentiation of strains of *L. major* of different origin.

It is in this study that *L. tropica* was first recognised as causative agent in Jericho, a classical *L. major* focus, as early as in 1998. The other striking finding came from the microsatellite analysis which showed that two genetically distinct populations of *L. major* are present in the Middle East. The Jordan Valley and the Arava/Negev desert populations genotypes are found in geographically close areas, less than 100 km apart from each other.

Molecular epidemiological studies will impact the look on leishmaniasis in Jericho district, as it showed differences in epidemiology patterns for both *L. major* and *L. tropica* species most likely reflecting different life cycles. This finding will, if taken into consideration by the authorities in Palestine, lead to a revision of control measures and even treatment regimens.

4. 1. Method comparison

4.1.1 Graded microscopy and ITS1-PCR

It has always been a dilemma to compare a newly proposed diagnostic method that is expected to be superior especially with regard to sensitivity, as in the case of ITS1-PCR, with other methods in the absence of a valid (highly sensitive and specific) or even acceptable gold standard. All conventional methods employed for the diagnosis of cutaneous leishmaniasis have modest to low rates of positivity, such as microscopy 42% by Aviles *et al.* (1999); 46.7% by Weigle *et al.* (2002); 48% by Andresen *et al*. (1996); 66.7% by Rodrigues *et al.* (2002); and 72% by Rodriguez *et al*. (2002), histopathology 33% by Aviles *et al*. (1999); 76% by Andresen *et al*. (1996) and 66.2% by Rodrigues *et al*. (2002) and culture 46.5% by Rodrigues *et al*. (2002) . Part of this broad range may be explained by the differing parasite densities in various types of materials. Thus, Andresen *et al*. (1996) had the best results with histological sections (76%) but only 48% in smears from ulcerations.

In the case of ulcerations, more invasive specimen sampling (biopsies versus scrapings) will yield better results. To overcome this problem, a broad case definition for CL was adopted, which depended on the presence of skin lesion(s), epidemiology of the area and compatible clinical history of the patient. This way we obtained the case group of Palestinians, living in an endemic area with high probability to become infected, all presenting typical lesions.

Skin-scrapings in comparison to other materials showed heterogeneity with regard to the number of parasites in a smear, as the parasites are not equally distributed in the tissue and the number of cells fixed on the slide may differ too.

In addition, in all cases of leishmaniasis, the host defense influences the density of parasites. In leishmaniasis diagnostics, the real problem is the cases with only few parasites in the material. To diagnose these cases correctly, one needs a very sensitive method. The method we have used was ITS1-PCR which has been proven as sensitive and specific method for direct detection and identification of *Leishmania* parasites in different clinical specimens (Schönian *et al*. 2003). The fact, that there were no positive PCR reactions in the negative group and that all negative controls were negative, confirmed that we obtained real positive results.

Whereas diseases that mimic CL such as leprosy, Blastomycosis, Lupus vulgaris, Yaws, tropical ulcers or skin cancers (Singh and Sivakomar, 2003; MOH, 2004) have not been reported in Jericho, skin disease like *Streptococcus pyogenes* (Streptococcus group A) infection, benign tumors, and infected wounds stings by insects and flies have been reported (Ministry of Health-Palestine, 2004).

ITS1-PCR proved to be more sensitive (87 %; 52/60) in comparison to graded microscopy regardless whether all samples (37%; 22/60), or only those that were stained correctly (49%; 22/45) were used.

In general, the sensitivity of ITS1-PCR was consistent with results of other studies that evaluated microscopy and PCR-based methods and revealed sensitivity ranging from 75.7% to 100% depending on the targeted DNA sequence amplified, i. e kinetoplast DNA, ribosomal RNA genes, minicircle DNA etc. the type of primers, i.e. genus-specific, sub-genus specific or species-specific and the nature of clinical sample (Piarroux *et al.,* 1994; Andersen *et al.,* 1996; Belli *et al.,* 1998; Aviles *et al*., 1999; Weigle *et al*., 2002; Rodrigues *et al*., 2002; Motazedian *et al*., 2002).

However, the peculiarity of this study in comparison to the others cited above lies in the standardized procedure by testing the same area and quantity of Giemsa-stained skin scrapings by

both graded microscopy and ITS1-PCR and subsequently statistically analyzing the results in matched pairs, which was meant to strengthen and validate the comparison process.

ITS1-PCR as a diagnostic test on skin scraping is not only advantageous over graded microscopy in its increased sensitivity, but also to the following aspects: (a) darkly- stained areas which are not favorable and in most cases difficult to screen by microscopy do not form any barrier against testing with ITS1-PCR. (b) Most of the clinically positive cases that were graded as negative by microscopy were positive by ITS1-PCR, which makes grading, particularly (-) and (+1) that formed most of the results, not a valid diagnostic tool. ITS1-PCR was not only able to diagnose the minimum number of amastigotes, down to one amastigote in 3 of the cases, but also it was sensitive enough to detect traces or sheds of leishmanial DNA which are impossible to detect by grade microscopy. (c) Procedural mishaps such as poor staining hindered or prevented diagnosis using graded microscopy in 3 squares, while it did not prevent diagnosis by ITS1-PCR which saves re-sampling and consequently time, effort and materials. There were two squares which were microscopy positive, grade (+1), and ITS1-PCR negative. One was proved to be PCR-false-negative as a result of DNA extraction failure, which may happen in some cases. In the other case microscopy showed only one amastigote in the whole square (>625 OIF). Larger series have to prove whether there may be a risk of false positive results in microscopy too.

There remain more critical points concerning our evaluation study. First, the predictive values depend on the incidence of a given event (disease). As we selected 20 cases with 3 test areas each and 15 controls, we set an incidence, which is far from real. Thus, it is only allowed to compare the negative predictive values within this study which speaks even more for the PCR. The second point is more serious: our clinical and the control group came from an endemic and a non-endemic area, respectively. That means that they belonged to two different populations but were treated here as samples of one population. We think this is justified because the control group thus comes from an area where the clinical diagnosis was valid. This could be not the case for many endemic regions where there will be always people showing no signs of the disease but bearing the parasite which might be detectable with a very sensitive method such as the PCR. That means if one needs a truly negative group, it can only be found in a non-endemic area.

If we arbitrarily consider the demonstration of amastigotes in a stained smear to be the 'golden standard' and compare it with the PCR, then the sensitivity of ITS1-PCR is 87% (52/60) (Table 3.1), compared with 37% in case of microscopy counting the badly stained smears as negative. Of the 45 correctly stained squares tested by graded microscopy, 22 were smear-positive, giving

a rate of positivity of 49%. This rate of positivity can be considered as the sensitivity for graded microscopy when assuming the broad CL case-definition based on clinical picture. It is probably time to substitute microscopy as "Gold Standard" of leishmaniasis diagnostics by PCR.

For epidemiological, therapeutic and control reasons PCR is recommended. However, this does not abolish microscopy and *in-vitro* culture.

The genus-specific PCR primer pair used in this study which was described by El Tai *et al.* (2000) (see Material and methods) made it possible not only to diagnose leishmaniasis, but to differentiate all relevant old world *Leishmania* spp. including *L. major* and *L. tropica*, the predominating species in the district of Jericho-Palestine (Al-Jawabreh *et al.,* 2004). However, a battery of controls including distilled water and *L. turanica* as negative and positive controls, respectively, inhibition and DNA extraction controls should be included to ensure reliability and validity of the run.

The results showed that ITS1-PCR-based techniques offer a very sensitive diagnostic method for Giemsa-stained smears collected from patients as compared to graded microscopy similar to that adopted by the WHO for VL (WHO, 1990), also indicating that Giemsa-stained smears used for microscopy can be re-used for ITS1-PCR and that oil, stain, debris and possible inhibitors do not form any obstacle for PCR diagnosis. Yet, making ITS1-PCR and PCR-based methods, in general, more economically and technically attractive in regions of high endemicity and bringing them from research institutions into daily clinical use remains a challenge.

4.1.2 Clinical diagnosis of cutaneous leishmaniasis

The problem of gold standard is faced by almost all studies that compare different approaches for the diagnosis of *Leishmania* (Table 1.1). Of the 64 patients tested by all four methods, the sensitivity of the methods using WHO gold standard (positive by either microscopy or *in-vitro* culture or both) and the combined gold standard (at least one method out of four was positive) was comparable (Table 3.4). However, the gold standard based on clinical and epidemiological features gave low sensitivity for all methods used, either by the 64 patients as the denominator or the entire lot tested by that particular method such as 943 for microscopy or 270 for *in-vitro* culture. This shows that using the entire database as the denominator for the calculation of sensitivity leads to lowered values due to the sneaking of considerable number of false positive cases into the definition (gold standard) causing inflation of the denominator. The sensitivity will be even lower under the clinical gold standard if there is another disease in the area that mimics

CL like Impetigo caused by *Streptococcus pyogens* which formed 10.8% of the skin diseases reported in Palestine (Ministry of Health-Palestine, 2004).

In all three gold standards, US as a sampling method was shown to give a higher sensitivity than using FP (Table 3.4). The reason for this is that the US has more clinical material which was completely used for the test, compared to two disks taken from FP. Therefore, taking more disks i.e. 4, may increase the sensitivity of PCR-ITS1. Despite the fact that the FPs and USs were stored under the same conditions, collected by the same technician and usually at the same time and that all Giemsa-stained slides that contained numerous amastigotes were always positive when tested by PCR, striking outcomes were noticed. Amongst the 64 cases tested by the 4 methods, 3 *Leishmania* cases were culture and microscopy positive but PCR negative by FP and US and 10 cases were positive by one PCR method and negatives by the other. This discrepancy causing false negative by PCR may be due to many factors such as the stage of the lesion (active, healing, new or chronic) which affects the number of parasites as the older the lesion the less is the probability of recovering amastigotes (Weigele *et al*., 1987; Weigle *et al*., 2002), the uneven distribution or unequal apportionment of amastigotes in the material sampled for different diagnostic approaches (Mathis A & Deplazes, 1995), lack of experience and severe secondary infection with enormous pus cells which may come on the expense of amastigotes, thus impairing diagnostic capability, uneven sampling from one part of the lesion and inhibition, which was minimal (<1%) in this study.

Sampling is a very critical issue, therefore, it is recommended to sample from more than one site in the lesion and to test different spots of the filter paper. Tissue containing the amastigotes should be included, while the oozing blood should be minimized, as it may have PCR inhibitors such as haemoglobin. Whenever clinical samples are involved, it is very important to have a stringent control panel to avoid discrepancies as false positives or false negatives. Furthermore, a defined diagnostic scheme like the one we propose in this study (Figure 3.14) which depends mainly on one primer pair and RFLP should be set and explained to the operating staff. Another scheme was suggested by Schonian *et al*. (2003) which depends on two primer pairs targeting two different DNA sequences for the sake of increasing sensitivity.

Unlike the USs, FPs have been used or proposed long ago as a sampling method for the diagnosis of different diseases such as trypanosomiasis (Katakura *et al.,* 1997), leprosy (Tomimori-Yamashita *et al.,* 1999), HIV (Nielsen *et al*., 1987), hepatitis B (Farzadgan *et al*., 1978) and the famous Guthrie's test for phenylketonuria (PKU) (Guthrie and Susi, 1963). For leishmaniasis, it

was introduced in1997 for diagnosing VL (Osman *et al.,* 1997) and PKDL (Osman *et al.,* 1998), and from 1997 on, we started to collect FPs for diagnostic purposes (Schonian *et al.,* 2003, Al-Jawabreh *et al.,* 2004).

An optimal FP sample (Figure 3.3) is no less than 3 mm thick (Whitman No. 3), sterilized, and properly labeled. Each filter paper close to the standard size of a glass slide (67x26 mm) is preferred to have 3-5 blood/tissue drops that are equal to the diameter of a euro cent, fully immersed in blood and tissue that can be seen from both sides of the FP. On the other hand, an optimal US sample (Figure 3.3) must be properly labeled, clean and have enough material. Both FP and US should be air dried, wrapped in aluminum foil and kept at room temperature till use.

There are several advantages to using FP; first they are light which is very important for international transport and sample exchange. Secondly, they have a long shelf-life at room temperature in which the earliest FP (+) in this study dates back to June 1997. The aluminum foil-wrapped FPs were left on the shelves at temperatures ranging from 12°C in winter to 37°C in summer. Campino *et al.,* (2000) recommended freezing filter paper samples in humid climates. FPs can be used more than once if enough drops have been sampled. Finally, they can be easily processed, as they need only punching of disks. But on the other side, one would note at least one disadvantage for FPs as they require sterilization (UV or steam autoclaving) before use. Although USs also have a long-shelf life, they are heavy, need larger storing area, are difficult to be used more than once, are fragile and scraping material off the slides is time-consuming. The length of storage period, time elapsing from collection to testing, for FPs and USs, depends on the nature of the target molecule. For example, hot and humid storage conditions for 20 weeks caused progressive decline in HIV-1 antibodies (Behets *et al.,* 1992), while in our case, samples were stored for 7 years at a temperature range of 12-37$^{°}$C as the target molecule was DNA. Therefore, low-temperature storage at temperatures between 4°C and -20$^{°}$C is recommended when testing for antibodies (Tomimori-Yamashita *et al.,* 1999), while DNA can be stored for a long time at room temperature.

Filter papers (FPs) and unstained smears (USs) are secure tools for an optimized and well-controlled molecular-based assay for direct clinical diagnosis of leishmaniases as well as for strain genotyping in terms of long shelf-life, relative non-invasiveness compared to culture and histopathology. PCR diagnosis using these specimens is simple and relatively quick, not requiring prior cultivation of the parasites. *In-vitro* culture is prone to contamination (4.8%) and can be higher for field work (El-Tai *et al*., 2001) as well as the need for different fastidious

ingredients depending on the species as some *Leishmania* species grow faster than others (Schuster & Sullivan 2002 and references therein).

The sensitivity of the conventional diagnostic methods, microscopy and *in-vitro* culture is lower (Table 3.4) than that of PCR techniques, 78% for FP 86% for US and 87% for stained smears. This runs parallel with other studies (Table 1.1). On the other hand, some studies produced conflicting results as they showed higher sensitivity of smear than PCR. This discrepancy (Table 1.1) is due to different gold standards being used to define a case of CL. In a study by Romero *et al* (2001), *in-vitro* culture was the gold standard. Such a poor gold standard produced a smear sensitivity of 95%. Belli *et al* (1998) used microscopy as a gold standard to obtain a sensitivity and specificity of 100%. Due to the lack of a good gold standard, Weigle *et al* (2002) used laboratory and clinical criteria to define a case of CL, which proved the higher sensitivity of PCR diagnosis. Another source of discrepancy is the high prevalence of CL in the study area, which may result in an increase of the sensitivity of all types of tests (Sharquie, *et al*., 2002). To prevent this, control groups of patients with non-leishmania lesions according to the gold standard used, which unfortunately most studies lack, should be involved. In this part of our study, the control group (15 Germans) was dropped as the ratio of case-to-control was less than 1 (Gordis, 1996) which leads to lowering others statistics like specificity and predictive values. The sampling method plays also a role in the comparisons by impacting overall sensitivity.

The comparison between traditional diagnostic methods and PCR with its various types was the object of several studies in the last decade (Hernandez-Montes *et al.,* 1998; Katakura *et al.,* 1998;; Breniere *et al.,* 1999; Pirmez *et al.,* 1999). In these studies, many types of specimens, sampling techniques, DNA targets and primers were proposed as candidates for diagnosing leishmaniasis. The clinical specimens and sampling methods used were: skin/lesion biopsies and syringe-sucked lesion aspirates for culture (Mathis & Deplazes, 1995, Matsumoto *et al*., 1999), blood and tissue spotted on filter papers (Osman *et al.,* 1997; Harris *et al* ., 1998; Färnert *et al* ., 1999; Campino *et al.,* 2000; Schonian *et al.,* 2003; Al-Jawabreh *et al.,* 2004), cotton swabs (Mimori *et al.,* 2002), wooden toothpick (Belli *et al.,* 1998), serum (Fissore *et al.,* 2004), formalin-fixed biopsies obtained from u1cer lesions (Mimori *et al.,* 1998), Giemsa-stained smears (Motazedian *et al.,* 2002), formalin-fixed and paraffin-embedded tissue specimens (Laskay *et al.,* 1995; Momeni *et al.,* 1996) and even conjuctival samples from animals for PCR diagnosis of VL were proposed (Strauss-Ayali et al., 2004). The DNA targets ranged from conserved and variable regions of major classes of kinetoplast minicircle DNA (kDNA)

(Bhattacharyya *et al.,* 1993; Lachaud *et al.,* 2002), sequenced whole kinetoplast DNA minicircles (de Bruijn and Barker, 1992; de Bruijn *et al.,* 1993; Lopez *et al.,* 1993, Anders *et al.,* 2002); partial sequence, representing the most variable part of the small subunit ribosomal RNA (SSU rRNA gene (Van Eys *et al.,* 1992), mini-exon gene (Brecelj *et al.,* 2000), and ribosomal internal transcribed spacer 1 (ITS1) separating the genes coding for ssu rRNA and 5.8S rRNA (El Tai *et al.,* 2000; Schonian *et al.,* 2003). This wide range of alternatives may reflect enormous activity in scientific research for improving diagnostic capabilities; yet, this has brought us into such a puzzling situation that one would not be able to tell which is the correct or the reference method for diagnosing different types of leishmaniasis. In a sense and in addition to sensitivity and specificity, an ideal PCR setup for an endemic area should be reproducible, a parameter rarely tackled by studies. The reproducibility should be with-in run, between-runs and between laboratories. One-step PCR for differentiation of the prevalent species in areas such *L. tropica* and *L. major* in Jericho would be useful with the possibility of further extending characterization i.e by RFLP when needed. A screening field-PCR that can be applied outside the laboratory during field visits for rapid diagnosis and differentiation with minimum use of instrumentation and procedures is greatly desirable. The aim of such rapid PCR is to minimize the number of samples for further investigation and to give crucial decisions related to treatment.

It is very unfortunate that the researchers world-wide are still unable to introduce PCR as part of the operational case definition of leishmaniasis. For this reason, microscopy and culture, despite their low sensitivities, are still the reference methods for diagnosis of CL, and serology for MCL and VL (WHO Recommended Surveillance Standards, 1997).

PCR for clinical diagnostics has proven more sensitive than the other methods, is well-controlled (Table 3.5), and applicable for direct clinical diagnosis as well as genotyping of strains. Yet precautions should be taken as Reithinger *et al* (2003a) and others concluded that ELISA (81%) is more sensitive than PCR (31%) for active canine leishmaniasis. Calls by Schallig and Oskam (2002) and others to standardize diagnosis for leishmaniasis should be heeded as they come in parallel to the idea of incorporating PCR in the operational case definition for leishmaniasis in the future. The adaptation of PCR in the WHO operational case definition of leishmaniasis, exactly as in the case of typhus, smallpox, SARS or Anthrax (WHO, 1999), will free us from adopting the low sensitive smear and/or culture methods or clinical manifestation as 'gold standard' for method comparison, treatment clinical trials, control assessment studies, etc. Some researchers

also favour approaching the gold standard by PCR (Vega-Lopez, 2003) whereas others argue against PCR being the only diagnostic 'gold standard' (Reithinger and Davies, 2002).

4.2 Molecular epidemiology

Restriction analysis of the ITS1-PCR positive samples revealed the co-appearance of *L. major* and *L. tropica* in Jericho and its immediate vicinity (Figure 3.2). The existence of *L. major* as causative agent of zoonotic CL in the lower Jordan Valley, including the margins of Jericho, has been well-documented (Schlein *et al*. 1982 and 1984; Al-Jawabreh *et al*. 2004). The presence of *L. tropica* in the human population of the Jericho area is a novel finding. This species has been, however, recorded as causing human cases of CL in a focus at a higher altitude halfway between Jerusalem (Al-Quds) and Jericho (A'riha) (Klaus *et al*. 1994; Jawabreh *et al*., 2001). There is clear overlap of the two species in almost all the populated areas included in this study, particularly in Jericho and the closely adjacent areas, i.e., the refugee camps. This change in the distribution of leishmanial species in the study area probably started more than a decade ago after the withdrawal of the Israeli Army when there was an extensive movement of the Palestinian population to and from the Jericho area. This included security forces, internal tourists, workers, farmers and Bedouin shepherds from all the other Palestinian districts, including the Gaza Strip. Although 92% of the individuals with *L. tropica* claimed that they did not leave Jericho during 3 months prior to the appearance of their lesion; for the present, one has to assume that the cases of CL caused by *L. tropica* are imported. Because of the significant difference in climatic conditions between the Jordan Valley and the mountains to the west of the Jordan Valley in both summer and winter, many of the people live in Jericho area during the winter and spend the summer in the hills surrounding Ramallah, Nablus, Jenin and Hebron (Al-khalil). Normally, they are in the hills when there are sand flies and transmission is occurring in both the mountains and the Jordan Valley. They arrive in the Jericho area only after the transmission period has ended. *L. major* does not circulate in the hills, but in the Jordan Valley where sand rats, the animal reservoir are found. Infections occurring in the migrant population are therefore most probably incurred in the hills, and caused by *L. tropica*. The parasites are then brought by the infected people to Jericho. With the absence of sand flies during the winter, these patients are, however, unlikely to be a source of CL caused by *L. tropica* in the Jericho area. To prove whether at least some of these cases were autochthonous, it should be confirmed that these people had not travelled out of the area for at least one sand fly season. Also one has to consider that the incubation period of CL

caused by *L. tropica* seems to be much longer than that caused by *L. major*. It has to be emphasized that people always travelled between the hills and the Jordan Valley, yet in the past cases of CL, caused by *L. tropica* were extremely rare throughout the whole District of Jericho, including the mountainous regions. Now, more and more cases caused by *L. tropica* are being seen in many foci of this part of the Eastern Mediterranean region (Klaus *et al*. 1994; Anis *et al*. 2001; Nimri *et al*. 2002; Jacobson *et al*. 2003; Schnur *et al*. 2004). This indicates that there has definitely been a change in the epidemiology of CL in this area which appears to have started in the hills and spread down to the Jordan Valley.

Alternatively, one should search in the Jericho area for the presence of sand fly vectors, such as *Ph. sergenti*, which was found to transmit *L. tropica* in a focus at higher altitude just east of Jerusalem (Schnur *et al*. 2004) and *Ph. arabicus* which was found to harbour *L. tropica* in Tiberias (Jacobson *et al.,* 2003). Detection of sand flies infected by *L. tropica* parasites and, if there is one, also animal reservoir hosts would provide evidence for endemicity of the parasite in Jericho area. A pilot study in September 2004 revealed the presence of *Ph. sergenti* in Jericho City and outside it (unpublished data), supporting the idea of autochthonous *L. tropica* cases. Schlein *et al*. (1984) also found this vector at Ein-Gedi, 40-50 km south of Jericho, on the western shore of the Dead Sea and in the Arava, which is even further south. The presence and increasing numbers of human cases in Jericho area caused by *L. tropica* are a definite change compared with the past when all CL cases from this area, from which parasites were isolated and identified, were shown to be due to *L. major* only.

Although the determinants of *Leishmania* infection such as the parasite, the vector, the reservoir and environment, are multi-factorial, the two demographic variables age and sex have been reported to be a source of variation in exposure to CL. Host behavioural/cultural factors were thought to make males more exposed than females, and lack of immunity to make children more exposed than adults (Arda and Kamal; 1983; Greenblatt *et al.,* 1985; Al-Jawabreh *et al*., 2003). This gender-age pattern was noticed in CL in other parts of the world like Brazil (Jones *et al.,* 1987) and, moreover, also in VL (Abdeen *et al*., 2002; Shiddo *et al.,* 1995). In the current study, where causative agents of the disease were identified at species level by molecular techniques, it was attempted to find out if the patterns of sex-age distribution were different between CL cases caused by either *L. major* or L. *tropica*. The total incidence of CL was higher in males than in females and this tendency was even more pronounced in CL caused by *L. major* (M: F ~2:1) (Figure 3.7) rather than those caused by L. tropica (M: F 1:1). In one study conducted by

Reithinger *et al*. (2003b) in Kabul-Afghanistan, *L. tropica* cases were more frequent in females and with increasing age. Although it has been reported that boys often developed VL threefold more than girls (Shiddo *et al.,* 1995); in our study no differences were observed between male and female children (<14). In this age group both genders had the same risks of infection. The gender-age difference was clearer in adults between 20-29 years. These results favour the explanation that differences in exposure to the disease is based mainly on host behaviour for adults in their 20s. For children, it may be due to naivety of the immune system and to genetic factors which have been suggested to explain the sex-age variance in other parasitic infections such as malaria and schistomiasis (Cooke *et al*., 2003; Henri *et al*., 2002). Immune regulatory mediators have been shown to develop with age (Sack *et al*., 1998; Tsaknaridis *et al*., 2003). Using hamsters infected in the laboratory it was found that the burden of *Leishmania* infection was more on males than females and this was attributed to different levels of sex hormones and of cytokines known to promote experimental leishmaniasis: interleukin 4 and transforming growth factor (TGF) (Travi *et al*., 2002).

4.2.1 Seasonality of CL

The seasonal distribution of CL from October to April mentioned earlier by Al-Jawabreh *et al* (2003) has been confirmed in this study (Figure 3. 9). Another study by Anis *et al* 2001 on patients from 1971-2000 showed some difference in seasonality, in which cases start to peak from June till December. This study does not, however, mention the geographical origin of the cases, making it difficult to explain the discrepancy.

The reasons for seasonality are, in general, the activity of sand fly vectors and the incubation period of the infection. *L. major* has an incubation period that ranges from a few weeks to a few months, averaging from 1 week to 3 months. It is longer for *L. tropica* which could range from 2 -24 months (Harrison's Principles of Internal Medicine, 1991). *Ph. papatasi*, vector of *L. major*, has its activity and abundance peaks in the spring (April) and the autumn (October), and was found to be not active in mid-summer (July) (Wasserberg *et al*., 2002). Nevertheless, the seasonality pattern in our study suggests a continuous sand fly activity from April to October. Using molecular methods that allowed identification of *Leishmania* parasites into *L. major* and *L. tropica* did not add much to the overall picture of seasonality patterns (Figure 3.9).

Over the ten-year-study period, three peaks were recorded (Figure 3. 8): 1995, 2001 and 2004. Several explanations can be put forward to explain surges of CL (Neoumine, 1996). In 1994-

1995, non-immune population was introduced into an active focus like the deployment of Palestinian soldiers. Urbanization process, which is believed to put the host closer to the reservoir that had taken place in the late 1990s, would be a plausible explanation for the 2001 peak. The dramatically increased prevalence of iron deficiency anaemia among Palestinian children and women (Abdeen *et al.,* 2002; Al-Rai, 2005) and changing in farming patterns related to decreased water resources (Neoumine, 1996) can be presented as other possible explanations in addition to urbanization.

Climatic changes and global warming is another important factor that plays a role in the sand fly infections (Kuhn, 1999) which needs to be further studied in the district of Jericho.

In this study, the correlation between the number of cases and annual rainfall was very weak, yet Anis *et al* (2001) concluded the opposite**.**

PCR/RFLP has improved the epidemiological understanding by showing *L. tropica* peak taking place in 2001 while 2004 for *L. major (*Figure 3.8); thus, once again, proving the necessity of using PCR/RFLP or similar methods for species diagnosis.

4. 3 Public health surveillance and cluster analysis

Remarkably, both purely spatial and space-time statistics, proved the necessity of molecular diagnosis in the epidemiology of leishmaniasis as a parasitic infection**.** The spatial and /or space-time scan statistics have been successfully applied to both retrospective and prospective surveillance of various diseases including breast cancer (Gregorio *et al*., 2001; Sheehan *et al.,* 2004), Creutzfeldt-Jakob disease (d'Aignaux *et al.,* 2002), and systemic sclerosis (15), sudden infant death syndrome (George *et al.,* 2001), West Nile virus (WNV) (Mostashari *et al.,* 2003), bovine spongiform encephalopathy in France (Abrial *et al.,* 2003) and prostate cancer (Klassen *et al.,* 2005). In the current study, *SaTScan* statistic is used for the first time for spatial and spatial-temporal clustering of CL and, facilitated by the use of molecular methods, PCR and RFLP.

Each of the two leishmanial species had different clustering patterns. In purely spatial statistic, the most likely clusters for *L. major* were in Fasayil and A'uja villages (RR 4.607 and 2.438, respectively) while that of *L. tropica* was in Zubaidat village (RR 14.866). Also, the clustering of total CL, (CL= genotyped and non-genotyped cases), revealed more and different secondary clusters (4) than for *L. major* (2) and *L. tropica* (2) alone (Table 3.8, Figure 3.11). However, purely spatial analysis has the drawback that the power of detecting recently emerging clusters is

effected by the length of time period analyzed (Kulldorff *et al.,* 2001).The space-time statistic was put into effect to overcome this dilemma.

The space-time statistic also presented clearer differences in distribution between the two *Leishmania* species. The most likely cluster for *L. major* were Fasayil and A'uja villages in the years 2003-2004 (RR 4.587) which differed from *L. tropica* alone and from total CL (Table 3.9, Figure 3.12). Zubaidat village was the most likely cluster for both *L. tropica* and total CL. The time of appearance was, however, different; it was the period 2001-2004 for total CL in contrast to 2001-2002 for *L. tropica* alone. The temporal length of the Nabi-Musa cluster for CL and *L. tropica* was relatively long (2000-2004) and the same applied for the *L. major* clusters in Jericho city and the adjacent village of Nuaimeh (Figure 3.12). The plausible explanation for this is the endemicity of CL and *L. major* in these areas and that ideal determinants existing there support the flourishing of leishmaniasis for long periods. The following factors have been reported in the literature: i) climatic and topographical factors like surface temperature, soil type, vegetation and rainfall, although the latter lacks high correlation; ii) human activity and behaviour such as farming (Schlein *et al*., 1984; Neoumine, 1996; Ashford, 1996; Wasserberg *et al*., 2003); iii) the people's utilization of available health resource which insures good reporting of cases; and iv) demographic factors like age and sex. The latter two variables are, however, most unlikely to have a great input in our study as they are even and uniform all over the district of Jericho (Booth and Dunne, 2004). Another factor is genetic predisposition leading to different host immune responses to parasitic infection, (Blackwell, 1996; Chang and McGwire, 2002; Desjeux, 2004), malaria (Stirnadel *et al*., 2000; Aucan *et al*., 2003; Cooke *et al.,* 2003) and schistosomiasis (Bethony *et al*., 1999; Henri *et al*., 2002; Rodrigues *et al*., 1996). The long-term clusters identified in our study are, however, of less importance than the emerging or disappearing cluster like the *L. tropica* cluster in Aqbat-Jaber refugee camp in 2001-2002 (Fig 3.6-c) because new foci need new, prompt and timely action plan for therapy, control and health education.

It is worth noting that 1995 witnessed a surge in cases as many Palestinian soldiers and their families returned from the Diaspora or came down to Jericho from other Palestinian districts following the 1994 Oslo agreement (Al-Jawabreh *et al*., 2003). The protective relative risk of 0.686 was, however, non-significant (p=0.7620), as only 200 cases were observed instead of the expected 245 cases. The reason for this was that not all cases were reported in the relatively large city of Jericho as the surveillance program had been just newly initiated. Also, in Jericho City the

L. major clustering showed significant protectiveness (RR 0.867, P=0.0140). We have to bear in mind that Jericho has the largest population in the Jordan Valley compared to the surrounding villages, refugee camps and Bedouin encampments, and *SaTScan* considers population size (40000) as background in its calculation.

The two spatial and space-time statistics show that *L. tropica* clusters are in the far north and far south of Jericho district. The Zubaidat village lies on rocky foot hills with sporadic caves ideal for sand fly vectors. Also in 2001, packs of rocky hyraxes were seen around the village and sometimes invading it. Nabi-Musa in the south, the other *L. tropica* focus, has the same topography mixed with semi-aridity as it is mid way between Jericho and Jerusalem. On the other hand both statistics indicate that *L. major* clusters occur in Fasayil and A'uja villages, and areas in and around the city of Jericho. These foci are characterized by soft alluvial soil and agricultural land suitable for the reservoir *Psammomys obesus*. An interesting secondary cluster in the *L. tropica* space-time statistic is the Aqbat-Jaber refugee camp which was recorded only during the period 2001-2002. The relative risk is not high and close to 1 (RR 1.029, p=0.015), but the refugee camp is adjacent to Jericho city, meaning that *L. tropica* is closing to the city, known as a classical focus of *L. major*. Early signs of the presence of *L. tropica* adjacent to the city gained support later as we have been able to catch *Ph. sergenti*, vector for *L. tropica,* in Wadi-Al-qelt (unpublished data). This wadi is a steep valley full of small caves and a relatively high plant coverage providing the city with water coming from the hills above. This valley which is very close to Aqbat-Jaber refugee camp could be the platform for *L. tropica* to enter the city, particularly as *L. tropica* has been isolated near the springs of the wadi 10 km away (Schnur *et al*., 2004). On the other hand, it is most unlikely that the northern focus of *L. tropica* at Zubaidat Village will affect the city in the near future because there are 60 km of wilderness between them.

One of the limitations of the current study is related to a village in the northern part of Jericho called in Jiftlik with a population of 4127 (PCBS, 2005) spread over a relatively large area. The people in this village seek their health service in a city other than Jericho causing under-reporting of cases of CL in our database. This explains why this village never appeared as a cluster and puts forward that aggressively diligent surveillance and good diagnostic facilities are pre-requisite to successful cluster analysis. The other limitation is the southern focus of *L. tropica* in Nabi Musa and Khan-al-ahmar area between Jericho and Jerusalem which is a semi-arid and

desert place with few sporadic nomadic Bedouin encampments scattered over a relatively wide area. The population of these encampments is very small and this lead to considerably higher number of observed cases (17) than expected (0.73) unlike the 'dilution effect' seen in the City.

PCR as a molecular–based diagnostic method have had an eye-apparent effect on the epidemiological picture of leishmaniasis in Jericho. The classical epidemiology represented by the scan statistics of CL cases diagnosed by non-molecular (conventional) methods like microscopy and/or culture, that cannot distinguish between the two species, lead to different clustering patterns compared to the cases where molecular species identification by PCR was employed. This difference in epidemiology proves that genotyping of leishmaniasis in the district of Jericho is crucial, as we are dealing with two aetiologies having probably different life cycles and may need also different control measures.

The present study emphasizes the importance of utilizing *SaTScan* together with molecular epidemiology of leishmaniasis. It can be run annually as in this study or monthly and even weekly depending on the efflux of cases to monitor the development of CL over small periods of time. And furthermore, cluster analysis parameters can be adopted and calibrated depending on the situation, total population and disease prevalence like for example the meningococcal study conducted by Vogel and Elias at the University of Wuerzburg-Germany (personal communication) in which the maximum cluster size was 10% and the maximum temporal cluster size is 30 days, rather than 50% and one year used in this study, respectively. *SaTScan* is recommended as an early warning tool for systematic and periodic geographical disease surveillance (Kulldorff *et al.,* 2001) for the following reasons:

1. Detection of new foci, disappearance of an existing one or any increase in rates of CL infection.
2. Clustering can give hints towards understanding the life cycle and behaviour of infection especially when bearing in mind that *L. tropica* is still ambiguous in Jericho.
3. On a larger scale, cluster maps help to direct control campaigns.

The other method for public health surveillance, Shewhart's Chart, has proved to be a simple and quick method for detecting trends, shifts and outbreaks in CL. On the other hand, this method has a few limitations when used for public health and epidemiological purposes. A major limitation is the need for relatively large numbers of "historical'' data to develop a reliable target value, or

centre line, and subsequently the in-control limits. A minimum of 20-30 numbers is required (Handbook *of Statistical Methods*; Vermaat *et al*., 2003). This limitation can cause false alarms and results different from what is assumed (Handbook *of Statistical Methods*; Quesenberry, 1993). To minimize this limitation, seasons instead of years can be used as units of time. Another problem is the failure to predict future behaviour of CL. The plotted chart would provide information only for the present situation depending on 'good' historical data. Shewhart's Chart is a simple informative tool that can be readily applied in the field of molecular epidemiology for evaluating the historical and current status of CL in terms of trends, shifts and outbreaks. The same argument applies to the moving average technique. *SaTScan*, Schewhart's Chart and moving average are useful early warning systems.

4. 4 Multilocus microsatellite analysis and population structure

Ten microsatellite markers located on five chromosomes were used to analyse genetic variation in 106 strains of *L. major* from 19 countries in Asia and Africa. Distance-based method such as DPS and Ddm using NJ and UPGMA and model-based methods such as *Structure* which is based on an admixture model were used for the analyses.

4.4.1 Optimal number of markers and isolates

Two problems related to the study setup itself have to be mentioned: sample size and number and nature of microsatellite markers in-use.

Population size is critical in population genetic studies; whether it is either total samples size or sample size from each geographical area. In studies on chicken and humans, 600 individuals were tested with 27 markers and over 1000 individuals from 52 populations by 377 microsatellite loci, respectively (Rosenberg *et al*., 2001, Rosenberg *et al*., 2002). It was found that using 10 individuals instead of 5 per population improved clustering by more than 90% and that accuracy of clustering decreases with decreasing number of samples (Rosenberg *et al*., 2001 and 2002). The question of how many individuals should be analysed per population and how many markers should be used is always raised in population studies. The presence of genetically atypical isolates or drastic genetic drifts or recombination will prevent having a 100% accurate clustering no matter how many markers or individuals are used. In general, the more samples from more areas collected and analyzed, the more accurate clustering will become. Yet, collecting samples of *Leishmania* is not an easy task.

In our study the number of collected isolates of *L. major* from Asia and Africa was 106. However Strains from Middle East (Southwest Asia) and Central Asia formed, 65% (69/106) of the total sample, and the rest are strains from 16 countries with some area having one isolate only. Pritchard *et al* (2000a) hints that, unlike Dps-based phenetic analysis (NJ, UPGMA) which is not affected by the population size (Mountain *et al.,* 1997), *Structure* improves with larger samples sizes in each population. In our study the analysis by *Structure* was consistent and in agreement with both NJ and UPGMA bootstrap-supported Dps-based phenetic analysis, meaning that latter supported the results of the former. However, Ddm-based tree did not match *Structure* analysis which may be due to robustness of Ddm to population size fluctuations (Takezaki and Nei, 1996) and its weakness in cases of closely related populations (Goldstein *et al*., 1995b).

The proper selection of the right type and number of microsatellite marker plays a crucial role for their use in genetically structuring populations. As a rule of thumb, the higher the cross-population the less within-population variability (heterozygous) and the greater within-population homogeneity and across-population polymorphism is for genetic markers the more accurate and powerful genetic population structuring becomes (Reed 1973; Shriver *et al*. 1997; Rosenberg *et al*., 2001). Rosenberg *et al*. (2001) found that clustering was more accurate when 12-15 highly variable and informative markers were used rather than 6-7 markers. Therefore, it becomes challenging for such genetic studies to find the smallest optimal amount of genetic microsatellite markers for differentiating populations. To infer the genetic diversity of CL caused by *L. major,* a set of 10 independent microsatellite markers was developed which represented 5 chromosomes and proved to be sufficient for population analysis.

The main criteria for establishing the optimal clustering potential of the markers were: 1) highest number of alleles provided, 2) highest expected heterozygosity, and 3) highest Fst, which quantifies the between-population component of genetic variation. However, the former two were superior to the third. In general, if extremely informative markers (highly heterozygous) are available, a minimized number of loci will be needed (Rosenberg *et al*., 2001).

If we are able, using the above mentioned criteria, to utilize the most variable markers, this will minimize the laboratory work, in terms of resources, human and financial, thus increasing efficiency and cost-effectiveness. Yet over-using markers with maximum variability may lead to inflation of divergence times estimated by using genetic distance (Goldstein *et al*., 1995) and will bias population growth statistics (*e.g.*, Zhivotovsky *et al*., 2000) and, hence, should be used

cautiously. Using few numbers of markers will maximize the effect of homoplasy. This means that optimal number of samples and markers will always be an active debate.

4.4.2 Distance and model-based methods: congruence and contradiction

Both distance-based methods using Dps and model-based methods using admixture model represented by the trees (Figure 3.18) and the plots of Q by *Structure* (Figure 3.16), respectively, gave comparable results proving that clustering did not follow geographical pattern. This was confirmed by the great genetic isolation between the populations identified. The clearest evidence of this, are the two genetically distinct and geographically proximal clusters, Jordan Valley (ME1) and Negev (ME2) that are separated by less than 100 km.

A peculiar case is the Central Asian cluster (n=39) which represented a very homogenous subgroup that remained solid after several runs and began to split off only at K=6 and re-joined again at K=7 in the *Structure* analysis. In addition, it had the lowest mean number of alleles (Figure 3.19). Many strains share identical genotypes. Eleven strains from Termiz foci in UZ isolated at the same time (year) shared the same number of alleles. Nine isolates from PS and IL also shared the same genotype, but the time elapsing between isolation and analysis ranged from 1 year to 36 years. The most plausible explanation for this is that this population emerged only recently and its evolutionary process is short. The isolation of Central Asian samples might be due to the fact that deserts create a barrier to genetic exchange by reducing the probability of contacts with other subgroups leading to a reproductive isolation, thus, gene flow is restricted to within-population leaving heterozygosity extremely low. Also, large population size may minimize the effect of genetic drift.

A probable explanation to the peculiarity of the central Asian foci lies in more than just genetic factors, but rather in the mechanical system supporting *L. major*. The life cycle is different from those in Middle East and African. The Reservoir in Central Asia is *Rhombomys opimus* while in the other two areas it is mainly *P. obesus*. In almost all cases, *R. opimus* was found to host, two types of leishmania species i.e. *L. turanica* and *L. major,* with the former being non-pathogenic for humans while the latter is. Even three leishmania species including *L. gerbelli* can co-exist the *R. opimus* (Strelkova *et al*., 1997).   This phenomenon of mixed infections in the *R. opimus* is not seen in *P. obesus*. Furthermore, the vector in the Middle East is *P. papatasi* while its existence is not definite in Central Asia.

The African strains are the most genetically variable, as shown by the highest number of alleles found for these strains (Figure 3.19). This supports the theory of an African origin for *L. major* (see below: Origin of *L. major* and bottleneck theory). Two Turkish, one Iraqi and one Iranian strain grouped with African isolates which was displayed both in predefined (K=5) and non-predefined (K=7) genetic *Structure* analyses. The African clusters were more inter-mixed compared to the large representative samples such as those from Middle East and Central Asia. Thus, the difficulty of clustering in Africa may have been exacerbated by small sample sizes and the fact that little information can be drawn from a few isolates. Nonetheless, if it is agreed that phenetic analyses, in general, are less prone to the under-sampling effect, this would support the *Structure* results as they agree with cladograms.

Using S*tructure,* it was possible to determine the membership of strains of unknown origin. Using population identification by relying on geographical origin of sampling which were 5 areas (K=5)*, Structure* efficiently assigned the unknown strain of *L. major* MHOM/WA?/87/NEL2 isolated from a traveller to east rather than West Africa. This proves that predefined labels such as geographical origin of sample were highly informative about membership in genetic clusters (Figure 3.17).

4.4.3 Origin of *L. major* and bottleneck theory

The decreasing pattern of genetic diversity of strains of *Leishmania* across Africa to Middle East and, finally, Central Asia can be explained by the occurrence of a population bottleneck (or genetic bottleneck) which is the reduction of a population by more than 50% due to an evolutionary event. In this case, a population suffers from immediate and transient increase in heterozygosity as a result of rapidly loosing rare alleles (Cornuet & Luikart, 1996; Hoezel, 2002). In the long run, after generations of bottlenecking, the genetic diversity or polymorphism which will deprive this particular population of facing environmental changes is extremely reduced, this leads to extermination (Hoelzel, 1999). The bottleneck phenomenon is bound by the size and growth rate of the population which are inversely proportional to the bottleneck. The time it took for change to take place, usually very long, is another factor impacting a bottleneck. Based on this, the high degree of heterogeneity may indicate that Africa is the origin of *L. major*. The highest number of alleles amongst African genotypes may indicate that *L. major* stemmed from Africa and poured, geographically, into the Middle East, which has a lower number of alleles, until it reached Central Asia, with the lowest number of alleles. This process had an effect on a

certain population of *L. major* in time and place dimensions. Hence, the low genetic variation in Central Asia may be due to i) an ancient genetic bottleneck event indicating long-term effective population size. ii). Demographic decline process a long time after a bottleneck event had taken place, followed by inbreeding in an isolated environment, and/or iii) several bottlenecks that exterminated the original *L. major* population leaving a small pocket that survived and continued its in-breeding with a certain degree of isolation (O'Brien *et al.,*1987; Gottelli *et al., 1994*).

Finally, the results of the studies on genetic variation can be viewed in a molecular epidemiological and public health sense. Information on the distribution of *L. major* genotypes, their movement patterns and their origin could influence treatment and chemically-based control methods. The short term challenges are to correlate differences in genotypes with different issues like the appearance and persistence of variants escaping immunity or the emergence of drug-resistance, pathogencity and susceptibility to CL. Although microsatellite analysis has proved to be a superior tool for genetic diversity studies, it did not reduce cost or labour even when it became fully automated, and this is another challenge.

4.5 Recommendations

The success of this study, and any study, is measured by its concrete commitment to the goals and objectives originally set. The conclusions and recommendations in the light of the study objectives are as follows:

1. Molecular clinical diagnosis

Molecular-based techniques, especially ITS1-PCR and RFLP, are essential tools for clinical diagnosis and strain genotyping of leishmaniasis. Basically, knowledge of the infecting species will provide a guide to appropriate treatment and improve control measures.

a. Continuous use of classical methods like culture is also vital for collecting isolates that may form the basis for a future Palestinian cryo-bank in Jericho.

b. Sampling methods as unstained tissue scrapings, and blood and tissue scrapings on filter paper can be equally used for the instantaneous diagnosis of leishmaniasis by PCR or for long-term archiving for future studies.

c. Although PCR is a supreme sensitive and specific diagnostic technique, yet conventional methods should remain to be used for reason like isolation and archiving.

d. The study that evaluated graded microscopy with ITS1-PCR using Giemsa-stained clinical samples showed that it is possible to use stained smears for both microscopy and PCR.

e. It is highly encouraged to exert an international effort to include PCR to the WHO operational case-definition.

2. **Molecular epidemiology**

a. Molecular-based methods i. e ITS1-PCR or ssu-rDNA-PCR and RFLP are extremely important tools for epidemiological studies of leishmaniasis (molecular epidemiology). It was only possible by PCR to unravel the presence of two different *Leishmania* species in Jericho, *L. major* and *L. tropica*. These species have different epidemiology, different vector and different reservoir. This may require different control measures, e.g. possible need to spray pesticides of different types, at different dosages and sites. More importantly, leishmaniasis caused by *L. tropica* is more refractory to the antileishmanial chemotherapy currently in use in Jericho as compared to *L. major*. Lengthy and invasive treatment is thus required, draining financial resources. It is imperative to elucidate the molecular epidemiology of leishmaniasis due to *L. tropica* in Jericho by conducting entomological surveys to establish and to study the vector for attempting to improve the control measures.

b. It is highly recommended to conduct active public health surveillance for leishmaniasis in the district of Jericho and including continuous statistical spatial and space-time analysis of the collected data by *SaTScan* software. Also, moving average and Shewhart's Chart should be used to as early warning systems to pinpoint epidemiological trends.

c. A national committee should be established that glues together all expertise and decision makers. Ministry of Health, Academic institutions and NGOs are the recommended targets for such a committee.

3. **Molecular strain typing and genetic diversity**

For research purposes it is advised to carry out multilocus microsatellite analysis for studying genetic heterogeneity in strains of both *L. major*, as mentioned in this study, and *L. tropica*. However, for this to succeed, mutual cooperation between countries of the area should exist. The

current political turmoil in the Middle East makes the interference of a third party to act as a facilitator in such studies something unpreventable.

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Erklärung

„Ich, **Amer, Al-Jawabreh**, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: **Molecular Epidemiology, Clinical Molecular Diagnosis and Genetic Diversity of Cutaneous Leishmaniasis in Jericho, Palestine** 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."

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