

## Endorsement

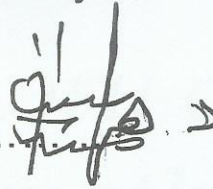
### Title of thesis:

*Diagnosis of Visceral Leishmaniasis Using the Polymerase Chain Reaction and Restriction Fragment Length Polymorphic analysis of the Kinetoplast DNA Minicircles*

Supervisor  
Dr. Haroon Khanfar  
Parasitology

Associate Professor  
Al Quds University

Signature .....

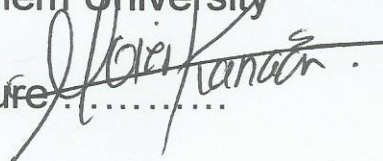


Date: 30 / 9 / 2001

External Examiner  
Dr. Moien Kanan  
Molecular Genetics

Associate Professor  
Bethlehem University

Signature .....

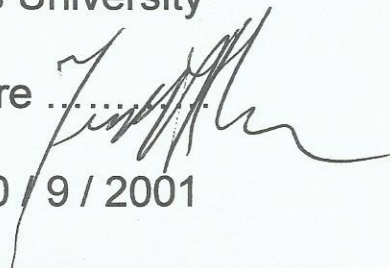


Date: 30 / 9 / 2001

Internal Examiner  
Dr. Ziad Abdeen  
Epidemiology

Associate Professor  
Al-Quds University

Signature .....



Date: 30 / 9 / 2001

## List of contents

	Page
Endorsement	I
Dedication	II
Acknowledgements	III
List of tables	X
List of figures	X
Abstract	XII

### Chapter One

1.1 General Introduction on leishmaniasis	1
1.1.1 Clinical forms	2
1.1.1.2 Cutaneous leishmaniasis	3
1.1.1.3 Post Kala-azar dermal leishmaniasis	4
1.1.1.4 Mucocutaneous leishmaniasis	4
1.1.2 Vectors	4
1.1.3 Reservoir host	5
1.1.4 Treatment	6
1.2 Visceral leishmaniasis in the West Bank and Israel	7
1.3 Diagnosis of leishmaniasis	8
1.3.1 Microscopy	9

	Page
1.3.2 Culture	9
1.3.3 Skin test	10
1.3.4 Serodiagnosis	10
1.3.4.1 The enzyme-linked immunosorbent assay	11
1.3.4.2 Excreted factor serotyping	12
1.3.5 Polymerase chain reaction	12
1.4 Species and strain identification	14
1.4.1 Isoenzyme analysis	14
1.4.2 Molecular biology	15
1.4.2.1 Hybridization using kDNA probes	15
1.4.2.2 Nucleotide sequence analysis of the internal transcribed spacer	17
1.4.2.3 Permissiveley Primed Intergenic-Polymerase Chain Reaction (PIPP-PCR)	17
1.4.2.4 use of species-specific primers in PCR	18
1.4.2.5 Single-strand conformation polymorphism	18
1.4.2.6 Restriction fragment length polymorphic analysis of the amplified region	19
Goals of the study	21

## Chapter two

	Page
2.1 Field specimen collection and preparation	22
2.1.2 Buffy coat preparation	22
2.2 Parasites	22
2.2.1 Culturing parasites	23
2.2.2 Freezing and maintenance of parasites	23
2.3 Enzyme linked immunosorbent assay	26
2.4 DNA extraction methods	26
2.4.1 Preparation of parasites for DNA extraction	26
2.4.2 Phenol-chlorophorm extraction	27
2.4.3 Guanidine extraction	27
2.5 polymerase chain reaction (PCR)	28
2.5.1 13A/13B primer pair	28
2.5.1.1 Design	28
2.5.1.2 PCR conditions	29
2.5.2 Lmj4/Uni21 primer pair	30
2.5.2.1 Design	30
2.5.3 PCR conditions using the Uni21/Lmj4 primer pair with different taq polymerases	30
2.5.3.1 Promega taq PCR	30
2.5.3.2. Takara ex taq PCR	31
2.5.3.3 Fermentas recombinant taq polymerase	31
2.6 Column purification	31
2.7 Photodensitometry	32

2.8 Restriction	33
2.8.1 Restriction enzymes	33
2.8.1.2 Digestion conditions	33
Chapter three	
3.1 Sensitivity	35
3.1.1 Lmj4/ uni21	35
3.1.2 13A/13B	36
3.1.3 Agarose and stain	37
3.2 Screening of field samples for <i>leishmania</i> parasites	38
3.2.1 ELISA	38
3.2.2 13A/13B PCR	41
3.3 Uni21/Lmj4 PCR	42
3.4 Restriction of promega PCR products using RsaI, BanII, MboI, and HpaI	43
3.5 Restriction of TaKaRa PCR products	46
3.5.1 RsaI	46
3.5.2 HpaI	50
3.5.3 MboI	54
3.5.4 BanII	58
Chapter four	
4.1 DNA extraction methods	62
4.2 PCR	63
4.2.1 13A/13B primer pair	63

	Page
4.2.2 Lmj4/Uni21 primer pair	65
4.3 Restriction of Promega taq PCR products	65
4.3.1 Differentiation between <i>L. tropica</i> and <i>L. infantum</i>	65
4.3.2 Differentiation between local and distant regional <i>L. infantum</i> isolates using the enzymes: Mbol, HpaII, and RsaI	66
4.3.3 HaeIII- the only enzyme that could differentiate between <i>L. donovani</i> isolates	66
4.4 Restriction of PCR products of High fidelity DNA polymerases	67
4.4.1 Differentiation between local <i>L. infantum</i> isolates that originate from different local foci	67
4.4.2 RsaI endonuclease gives restriction profiles representative of the local <i>L. infantum</i> species	69
4.5 The distribution of the Leishmania positive cases of dogs corresponds with the distribution of human VL cases in El Yamun	69
General conclusion	70
References	72
Abstract in Arabic	83

## ABSTRACT

In the Old World, Visceral Leishmaniasis (VL) is caused by the *Leishmania donovani* (*L. donovani*) complex. In the Middle East, VL is mostly caused by the sub-species *Leishmania donovani infantum* (*L. infantum*) with the host reservoir being dogs and wild canids. A kinetoplast DNA based polymerase chain reaction (PCR) technique was employed for the diagnosis of *Leishmania* parasites, utilizing the Uni21/Lmj4 primer pair, originally designed to amplify the full *Leishmania major* (*L. major*) minicircle DNA. This primer pair amplifies the minicircles of all the Old World *Leishmania* species, including *Leishmania tropica* (*L. tropica*) and *Leishmania aethiopica* (*L. aethiopica*) as well as the *L. donovani* complex. The minicircles of the different species have similar sizes, ranging between 650-900 bp. An investigation utilizing the restriction fragment length polymorphic analysis (RFLP) was carried out to known minicircle kDNA sequences using a group of different restriction endonucleases (*Rsa*I, *Mbo*I, *Ban*II, *Hpa*II) which cut the DNA PCR products of *L. infantum*, and *L. donovani* isolates. In this project discrimination between local and distant regional isolates based on different restriction profiles was achieved successfully. The same group of endonucleases gave more complicated patterns showing variation even between the isolates from the same region, when amplified by a high fidelity DNA polymerase. A field survey was conducted in the village of El -Yamun located in a visceral leishmaniasis focus in the Jenin district of the West Bank. Fifty-nine blood samples were collected from domestic dogs, during a time interval of one-month including two samples from Ramallah region. All samples were screened for anti -leishmanial antibodies by the

enzyme - linked immunosorbent assay (ELISA). Five out of 59 (8.5%) were seropositive, and one borderline. All the seropositive and borderline dogs were biopsied. Parasites were successfully cultured from three dogs out of four seropositive dogs biopsied and both RFLP of kDNA and the excretion factor serology characterized the strains as *L. infantum*. The PCR method utilized in this study will be of great value if optimized to work directly on clinical samples of any kind this will provide fast and reproducible diagnostic tool.