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MOLECULAR DIAGNOSIS AND GENOTYPING OF CANINE VISCERAL LEISHMANIASIS IN THE NORTHERN PART OF THE WEST BANK, PALESTINE

By

Suhair Ibrahim Mohammad Ereqat

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By
Suhair Ibrahim Mohammad Ereqat

B. Sc: Medical Technology, Al-Quds University, Jerusalem- Palestine

Supervisor

Prof. Ziad Abdeen

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By

Student name: Suhair Ibrahim Ereqat
Registration No: 20111023
Supervisor : prof. Ziad Abdeen
Master thesis submitted and accepted, Date----------

The names and signatures of the examining committee members are as follows:

1- Prof. Ziad Abdeen (Supervisor) Signature……

2- Dr. Moaen Kanan (External Examiner) Signature……..

3- Dr. Robin Abu-Ghazaleh (Internal Examiner) Signature……..

Al-Quds University
2005

Dedication

This thesis is dedicated to my parents, husband and friends

GENERAL CONCLUSIONS

This study gave an idea about the role of domestic dogs in transmitting the disease and also points to the importance of the diagnosis and genotyping of CVL in the West Bank. ITS1 PCR-RFLP used for species typing with no intra species variation, while the kDNA PCR-RFLP, utilizing the primer pair Uni 21/Lmj4, was useful to check the polymorphism, in this study the microheterogenity was evident among the strains tested. The one strain that appeared more different from others was IPT1, a human VL isolated from Tunisia. Thus, parasite polymorphism may be attributed to variations among parasites from different geographical locations or clusters.

The data presented in this study indicate a successful attempt to satisfy the need for a more sensitive, specific, and rapid test for the diagnosis of CVL.

Serological techniques (e.g. ELISA) alone, which are used to estimate the infected dog population (Cardoso et al., 1998), are not a suitable tools for diagnosis of CVL, while PCR offers more accurate results. Indeed, the sensitivity and specificity of the PCR assay depends on several factors, including PCR primers, DNA extraction methods, source of clinical materials (Reithinger et al., 2000) PCR target and PCR target molecular size as we proved. The advantage of using blood sampling that it is less invasive than bone marrow, spleen, or lymph node aspirate, and samples

can be processed readily. This approach on blood samples has proven useful for the diagnosis and monitoring of VL in adults and children in the Mediterranean region (Sundar and Rai, 2002).

Further studies are under way in other VL endemic regions, particularly in the southern part of the West Bank, to validate the methodology and to evaluate the ability of PCR-RFLP to identify other *Leishmania* species.

Recommendation points

- We recommend to keep screening the potential reservoirs in the Jenin area, in order to eradicate and break the leishmaniasis cycle in this region, and to prevent or decrease the HVL incidence in the area.
- Continue spraying insecticides to eradicate the vector.
- Educate the locals by widening their knowledge of the disease using leaflets, seminars, and distributing education materials targeting the schools, so the people should know more about the disease and prevention methods.
- Establish a database on the disease for planning and evaluation.
- Regional clinicians must keep in their mind all infectious agents as potentials for abdominal enlargement and fever of unknown origin, as VL suspected referring them for Al-Quds University, Leishmania Laboratory Center for diagnosis as a reference laboratory.
- Palestinian Ministry of health should consider the *Leishmania* test at referred laboratory and implement appropriate control measures in endemic areas.

CHAPTER ONE

INTRODUCTION AND LITREATURE REVIEW

1.1 GENERAL INTRODUCTION

Leishmaniasis is a parasitic disease caused by obligatory intracellular protozoa of the genus *Leishmania* family *trypanomastidae*. These parasites are transmitted by the bite of infected female sand flies. Leishmaniasis is considered the most divers and complex of all vector borne diseases in their ecology and epidemiology since they include more than two dozen species of parasite that may infect humans, several reservoirs, several vector species, and different foci (Aransay et al., 2000). Currently, leishmaniasis threatens 350 million people in 88 countries around the world (Jacobson et al., 2003; Gatti et al., 2004) throughout Africa, Asia, Europe, North and South America (Markle and Makhoul, 2004). There are an estimated 12 million new cases of Leishmaniasis reported each year; 1.5 to 2 million cases of them are coetaneous Leishmaniasis and 500,000 are visceral Leishmaniasis (Jaffe et al., 2004). The major clinical forms of Leishmaniasis range from self healing cutaneous lesions through metastasizing muco-cutaneous forms to potentially lethal visceral forms (Bhattacharyya et al., 1993; Schonian

et al., 1996; Cardoso et al., 1998). The severity of clinical forms in immune competent persons depends mainly on the species or subspecies of the parasite (Dedet et al., 1999), the immunologic status of the host and his genotype (Blackwell et al., 2004).

1.2 THE LEISHMANIASIS AS HUMAN DISEASES

1.2.1 Cutaneous Leishmaniasis (CL)

Cutaneous Leishmaniasis (CL), or oriental sore, is usually caused by *L. tropica, L. major, L. aethiopica* in the old world (Berman, 1997) and *L. braziliesis* complex and *L. mexicana* in the new world (Oumeish, 1999; Reithniger et al., 2003). CL may also, however, be caused by any of the *Leishmania* species which infect humans. *L. infantum,* which causes visceral leishmaniasis, has been isolated from skin lesions of 'atypical cases of cutaneous leishmaniasis'(Carreira et al., 1995; Martin-sanches, 2001). The vector for leishmaniasis is the sand fly and the oriental sore first appears as a persistent sand fly bite. After the sand fly bite the incubation period varies from one week to several months. Gradually the lesion enlarges, remaining red, but without heat or pain, furthermore complications like secondary bacterial infection can occur, and can affect the style of the lesion (Jawabreh, 2001). The lesion resolution involves immigration of leucocytes, which isolate the infected area leading to necrosis of the infected tissues, and formation of a healing granuloma in

the floor of the lesion (Ashford, 2000). Self healing without treatment is the rule, but the time taken for healing varies greatly according to the species of the parasite and the site of the lesion. Cured patients remain immune to homologous infection for many years. Rarely, leishmaniasis cell-mediated immunity fails to develop to the parasite a severe form of this disease will develop as a diffused cutaneous leishmaniasis (DFL) that may it caused by *L. mexicana amazonensis* (Salman et al., 1999). This form usually starts with primary lesion that progress to numerous lesions resembling leprosy, which do not heal spontaneously (Desjeux, 1999).

1.2.2 Mucocutaneous Leishmaniasis (MCL)

Mucocutaneous Leishmaniasis is mostly found in Latin America (Aviles et al., 1999), but is occasionally reported from Sudan and other old world foci (Ashford, 2000). This form occurs when *Leishmania* invades the mucosal membrane of the mouth, nose and throat, causing severe destruction and permanent disfiguring of these organs. The causative agents of this leishmaniasis are *L. aethiopica* in the old world and *L. brasilliensis* in the new world (Mehregan et al., 1999).

1.2.3 Visceral Leishmaniasis (VL)

Visceral Leishmaniasis, also known in Asia as black fever or kala-azar, is the most severe form of leishmaniasis and is lethal if not promptly diagnosed and treated (Adini et al., 2003; Tintaya et al., 2004). Several *Leishmania* species are present in the foci of VL in the old and new worlds such as *L. infantum* in the Mediterranean region and in Asia (Obzel et al., 2000), *L. donovani* in India and East Africa (Dereure et al., 2003, b; Elnaiem et al., 2003), and *L. chagasi* in the new world (Vexenat et al., 1998). The parasite invades the reticuloendothelial system of internal organ particularly the liver, spleen, bone marrow and lymph nodes; the incubation period is ten days to over one year from the sand fly bite and the clinical manifestations include fever, malaise, loss of weight hypergamaglobulinaemia, hepatosplenomegaly, lymphoadenopathy and panacytopenia (Abranches et al., 1991; Rhalem et al., 1999; Gavagani et al., 2002). Occurrences of asymptomatic cases of kala-azar have been reported from Kenya, Brazil and India. These persons never give any history of symptoms or show any clinical sign of infection (Sharma et al., 2000). Asymptomatic phases and relapses suggest that the parasite can exist in the tissues for a long time before and /or after clinical onset of the disease. Two cases of VL have been described in a patient with lymphoma and in a pregnant woman (Dereure et al. 2003, a). In both

cases, parasites remained present in the lymph nodes after clinical cure for a few months with no parasite clearance.

1.2.4 Post-Kala-azar Dermal Leishmaniasis (PKDL)

This form is endemic in Africa and India, and is primarily caused by *L. donovani*. PKDL is an important sequel seen in 10 to 20% of kala-azar patients in India (Salman et al., 1999). It appears to be a result of kala-azar (Kumar et al., 2001), but occasional cases are reported with no history of kala-azar (Ashford, 2000). The clinical picture is characterized by multiple non ulcerating nodules on the skin of the face, limbs and/or trunk (El-Hassan et al., 1995).

1.3 CANINE VISCERAL LEISHMANIASIS

Canine Visceral Leishmaniasis (CVL) is a severe systemic disease of dogs, generally with a long asymptomatic period followed by a clinical period which leads to animal death (Santos-Gomes et al., 2003). Clinically, old and new world CVL gives similar symptoms to those of human VL (Rhalem et al., 1999). Additional symptoms typicaly found in infected dogs are depilation, onychogryposis, and emaciation (Abranches et al., 1991). The prevalence of CVL in the Mediterranean region varies between 10-37% (Obzel et al., 2000). However, there is evidence that infection prevalence rates may be higher than those given by serological

studies (Lachaud et al., 2002, b). Reliable diagnostic tests are therefore required for the detection of CVL in both clinically ill and asymptomatic dogs.

1.4 THE PARASITIC LIFE CYCLE

The parasite exists in two morphological forms, the first is as non flagellated intracellular spherical amastigote found in its vertebrate host and the second is flagellated promastigote found in sand fly species (Ashford, 2000; Cunningham, 2002). Throughout their life cycle, *Leishmania* parasites encounter a wide range of environmental conditions such as temperature and pH. Promastigotes survive at an average temperature of 26 °C in a mildly basic environment (> 8) in the intestinal tract of the sand fly vector, while amastigotes survive temperatures typical to mammalian hosts and a pH range of 4.5-6.0 within macrophages. (Garlapati et al., 1999). Sand fly females become infected during blood meal from infected hosts, they ingest infected macrophages. These parasites transform in to sand fly midgut to procyclic promastigotes, which then differentiate into infective flagellated metacyclic promastigotes. During the next blood meal, the infective metacyclic promastigotes are passed in to the vertebrate hosts through the puncture wound and are phagocytosed by macrophages which they transform back to intracellular amastigotes (Cunningham, 2002).

Amastigotes multiply in infected cells which soon lyse releasing large number of amastigotes. Those affect different tissues depending on the *Leishmania* species, thereby accounting for the many different clinical manifestations of leishmaniasis (see fig. 1.1).

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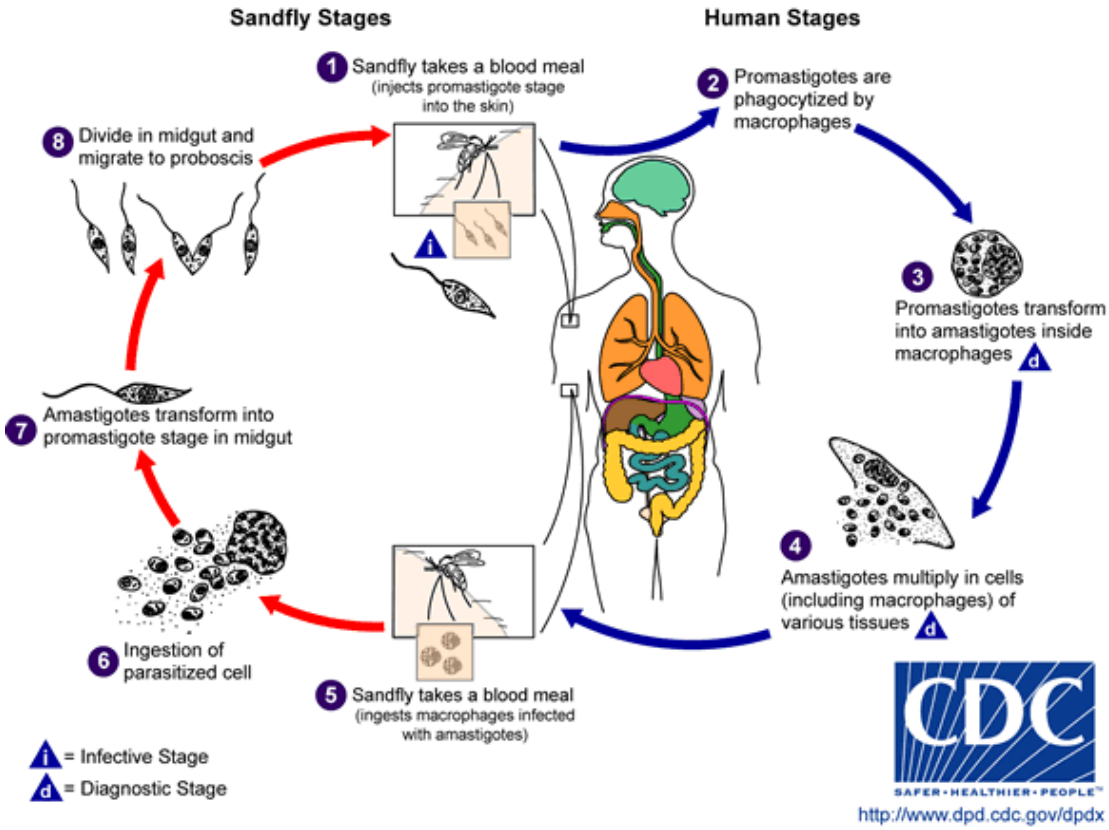

Figure 1.1 shows the Life cycle of *Leishmania*

1.5 VECTORS

Sand fly vectors belong to the family *Psychodidae*, subfamily *phelobotominae* (Killic-Kendric, 1999) and are blood sucking insects that can be distinguished from other insects by their brownish color, small size (1.5-2.5 mm), hairy appearance, long legs, jerk flight pattern (Sawalha, 2001) These flies have public health importance since they

serve as vectors of etiologic agents of leishmaniasis. The subfamily *phlebotominae* comprises six genera *:Worileya, Plebotomus, Sergentomyia, Chinius, Brumptomyia* and *Lutzomyia* (Aransay et al., 1999). The most medically important species belong to the genus *phlebotomus* in the old world and *lutzomyia* in the new world. Sawalha et al. (2003) described the Phlebotomine sand flies as the potential vector of leishmaniasis in the Jenin district, West Bank. Killick-Kendric, (1990) noted that of eighty one suspected vector species and sub species only nineteen were confirmed. A further fourteen are considered to be strong candidates for transmission to humans.

1.6 RESERVOIR HOSTS

A reservoir and vectors should share the same habitat in order for the disease to be transmitted. In zoonotic CL, the parasite is transmitted from animal to man; the primary reservoir host of *L. major* is *Psammomys obesus* in north and West Africa and western Asia including Jordan, Palestine, Israel and Syria (Schlein et al., 1982). The reservoir for *L. tropica* which causes anthroponotic CL and spreads from person to person is not well defined. However, *L. tropica* could also be zoonotic since parasites of this species have been isolated from dogs in Kabul, Iran, and Morocco (Dereure et al., 1991). Hyraxes are now well established as the main reservoir host of *L. aethiopica* in Ethiopia and

Kenya (Ashford, 2000). Hyraxes have also been demonstrated to contain the DNA of *L. tropica* which was detected in the skin and blood of hyraxes captured in northern Israel (Jacobson et al. 2003). In general, wild and domestic canids should therefore be considered as possible reservoirs for *L. infantum*, the causative agent of human VL. Humans become infected accidentally and do not act as a reservoir hosts for *L. infantum*, except in cases where contaminated syringes are shared among intravenous drug addicts (moreno and Alvar, 2002). Among domestic animals the dog is the principal reservoir of *L. infantum* in most countries bordering the Mediterranean Sea and through out the Middle East (Qubain et al., 1997; Baneth et al., 1998; Dereure et al., 2000), in addition, the red fox *Vulpes vulpes* and the common rat *Rattus rattus* have been infected (Ashford, 1996; Ashford, 1999). On the other hand, there is a growing belief that dogs may also be the main reservoir host of American cutaneous leishmaniasis (Reithinger et al., 2003; Ryan et al., 2003). In the case of anthroponotic VL due to *L. donovani* such as in India, no animal reservoirs have been identified (Elnaiem et al., 2003). It is presumed that lesions from PKDL patients act as reservoirs (Singh and Sivakumar, 2003).

1.7 HIV-LEISHMANIA COINFECTION

The co-infection of *Leishmania* and HIV is emerging as a new and frightful disease. Most of the co-infection cases have been reported in south –western European countries (Cortes et al., 2004). Where 50% of adult VL cases are associated with HIV infection (reviewed by Adini et al., 2003). In Israel, several cases of *Leishmania*–HIV co-infection have been reported (Ya'ari et al., 2004). Visceral leishmaniasis accelerates the onset of AIDS and shortens the life –expectancy of HIV-infected people, while HIV spurs the spread of VL since the *Leishmania* parasites and HIV destroy the same immune response cells, leading to increase in the severity and fatality of the disease (Ya'ari et al., 2004; Desjeux, 1999).

1.8 VISCERAL LEISHMANIASIS IN PALESTINE AND ISRAEL

The first case of VL in Israel was described in 1929 in a 3 years old child from Ein Harod in northern Israel. Since then sporadic cases have been reported, primarily among Arab communities in Jaffa, Jerusalem, Haifa and Nablus districts. Before 1960, most of the patients were not hospitalized or their cases not reported, which causes underestimation for the prevalence of the disease (Ya'ari et al., 2004). During the 1960s, 45 cases of VL were diagnosed from different Arab villages in Gallilee (Adini et al., 2003). During the following few decades, however, clinical human VL in the Gallilee has declined significantly and where only seven

cases were reported from northern Israel in the 1990s (Jaffe et al., 2004). Abdeen et al., (2002) have shown that, 50 cases of human VL were diagnosed from Jenin district, and 5.5% of the domestic dogs were seropositive during 1989-1998. In the same ten year period, 32, 17 and 15 cases of human VL were also diagnosed in the Hebron, Tulkarm and Ramallah districts, respectively. In addition, four cases of human VL were reported from Al-muhtasib and Alia hospital, in Hebron during 2004 (Ahmad Amro, unpublished data).

1.9 CONTROL OF VISCERAL LEISHMANIASIS

Some countries where VL is endemic control programs are designed to prevent infections in human beings by controlling the vector and reservoir. One of the approaches to reduce the incidence of human VL is to cull infected dogs but a Brazilian study showed that killing of seropositive dogs is insufficient as a measure for eradicating VL in dogs (Ashford et al., 1998). The dog culling programs failed for several reasons: first, necessary proportion of infected dogs are not culled either because of miss diagnosis, or non compliance of dog owners (Borja-Cabrera et al., 2004) second, long delays between sample collection, sample analysis, and control implementation, this delay may be as long as 80 days with infected dogs remaining infectious to sand fly vectors (Reithinger et al., 2002) Third, owners quickly replace their dogs with

puppies, which are more susceptible to infection (Gavgani et al., 2002). Treatment of canine leishmaniasis has been an important issue since its causative agents are the same as in humans offering the possibility of treatment with the same or related drugs; most often pentavalent antimonials are used. Treatment of infected dogs with anti-*Leishmania* drugs has proved, however, not to be a practical control policy because of the high cost and the very high relapse rates of up to 80% (Vexenat et al., 1998; Moreno and Alvar, 2002; Gavgani et al., 2002). Protection of dogs by means of topical persistent insecticides in collars or by dipping might be effective (Ashford, 1996) and house spraying with insecticides used in vector control may also prevent infections in human (Sawalha et al., 2001). Vaccines against canine VL represent the main tool for controlling leishmaniasis, in that successful immunization of dogs could significantly reduce the incidence of human visceral leishmaniasis (Dye, 1996). Borja-Cabrera et al. (2004) reported that Fucose Mannose Ligand (FML) vaccine was effective in immunotherapy against VL of asymptomatic infected dogs. However, an effective vaccine against human and canine- VL is not yet available.

1.10 DIAGNOSIS OF LEISHMANIASIS

The diagnosis of leishmaniasis is difficult because the clinical signs are variable can be caused by a number of other etiological agents leading to a potential missdiagnosis (Harris et al., 1998). The need for correct diagnosis is the more important because treatment for leishmaniasis is very expensive, very lengthy and is associated with toxic side effects (Baneth and Shaw, 2002).

1.10.1 Traditional Diagnostic Methods:

1.10.1.1 Microscopic and Culture Techniques

The parasitological diagnosis is the simplest and most commonly performed procedure. The diagnosis in VL is based on observation of amastigotes in giemsa stained smears of bone marrow, lymph nodes or splenic aspirates. Though microscopic examination is rapid, cheap and easy to perform, it lacks sensitivity due to the low number of parasites in tissue samples and does not distinguish between species (Singh et al., 1999; Reale et al., 1999; Aransay et al., 2000; Silva et al., 2001). Parasite culture can improve the sensitivity in parasite detection, but tissue samples from visceral organs are required to initiate cultures and obtaining these samples requires a traumatic procedure that has been associated with risk of hemorrhage, In Israel, however, *Leishmania* culture was applied in routine clinical practice (Abed Nasereddin,

personal communication). They are mainly required for obtaining a sufficient number of organisms to use as an antigen for immunologic diagnosis (Sundar and Rai, 2002; Iniesta et al., 2002) or obtaining parasites to be used in inoculating susceptible experimental animals (Abranches et al., 1991; Santos –Gomes et al., 2003; Moreno and Alvar, 2002) and finally, to provide a diagnosis when routine methods have failed. In vitro cultivation needs a sophisticated laboratory setup, are time-consuming (Lachaud et al., 2000; Marfurt et al., 2003), harbor the risk of contamination and does not allow for species identification.

1.10.1.2 Skin Testing

Leishmania skin test is a test for delayed type hypersensitivity specific to leishmaniasis. This method uses 0.5 ml of phenol-killed whole parasites ($5x10^7$) that are injected on the vola forearm and the iduration size is measured after 48-72 hours. A positive test measures induration with 5 mm diameter or greater. It is not, however, a species specific test and cannot distinguish between active and past infection (Rodriguez et al., 1994 ; Delgado et al 1996).

1.10.2 Current Diagnostic Methods:

1.10.2.1 Serodiagnosis

The human body makes an attempt to fight VL by producing some of the highest levels of antibodies found in response to any disease. The

presence of high levels of antibodies against parasitic antigens can simplify diagnosis of VL by serological techniques that detect these antibodies. Enzyme-linked immunosorbent assay (ELISA) has been used as a potential serodiagnostic tool for almost all infectious diseases, including leishmaniasis. The sensitivity and the specificity of ELISA is greatly influenced by the antigen used (Singh and Sivakumar, 2003). Several antigens have been tested; the common one is a crude soluble antigen (CSA). Cross reactions with sera of patients with other trypanosomiasis, tuberculosis, and toxoplasmosis, have been recorded (Belli et al., 1998; Reithinger et al., 2003). Furthermore, ELISA sensitivity and specificity could be increased by the use of soluble antigens derived from promastigotes cultivated in a protein free medium (Romero et al., 2004). A recombinant antigen, rK39, derived from *L. chagasi* has also been shown to be specific for antibodies in a patient with VL caused by members of the *L. donovani* complex (Sundar and Rai, 2002). It was previously shown that anti-rK39-titers were 59-fold higher than those of antibody against CSA at the time of diagnosis, and with successful therapy, it fell sharply at the end of treatment and during follow-up monitoring. (Kumar et al., 2001). Practically, because of the condition prevailing in areas of high endemicity, there is a need for a simple, rapid and accurate test with high sensitivity and specificity, which can be applied by any laboratory technician. Immune chromatographic

dipstick test based on rK39 antigen has been developed as a rapid test for use in difficult field condition. Carvalho et al. (2003) confirmed the accuracy of antigen strip test in the diagnosis of VL in Brazil, the sensitivity and specificity were 90% and 100%, respectively. Finally, specific antibodies can also be detected by western blotting, promastigotes of *Leishmania* parasites were grown to log phase and lysed, the soluble protein is subjected to electrophoresis, the separated proteins are blotted onto nitrocellulose sheets and probed with serum from the patient, western blotting find minor antigenic differences among various organism (Aisa et al., 1998) but the process is time consuming and expensive.

1.10.2.2 Polymerase Chain Reaction (PCR)

Serology is an inefficient method to differentiate active and past cases and the fact that culture of *Leishmania* cells is laborious method with low sensitivity (De Brujin et al.,1993) creates a diagnostic obstacle that can be, perhaps solved by the application of the DNA detection method. PCR development has provided a powerful approach to the application of molecular biology techniques to the diagnosis of leishmaniasis, and is a simple tool that has proven to be more specific and sensitive than conventional diagnostic methods (Ashford et al., 1995). Primers have been designed to amplify several DNA targets for *Leishmania*, such as; the minicircle Kinetoplast DNA (kDNA) (Smyth et

al., 1992; Noyes et al., 1998), the small subunit rRNA gene (ssu rRNA) (Pizzuto et al., 2001; Lemarni et al., 2002), the mini exon gene sequences (Harris et al., 1998; Marfurt et al., 2003), a repetitive genomic sequences (Piarroux et al., 1995), internal transcribed spacer (ITS) (Schonian et al., 2003; Strauss-Ayali et al., 2004) and the *Leishmania* antigens gene locus e.g. surface metalloprotease glycoprotein 63 (gp63) encoding gene (Guerbouj et al., 2001; Mauricio et al., 2001; Tintaya et al., 2004). Mitochondrial Kinetoplast DNA is one of the most unusual DNA structures with double stranded DNA minicircles and maxicircles, catenated together into a single giant network of DNA situated adjacent the basal body of flagellum. The minicircle Kinetoplast DNA (0.8 to 1Kb in length) is an ideal target since it is present in 10,000 copies per cell. In addition, its sequence is known for most *Leishmania* species; also it possesses a variable region that offers accurate discrimination between species (Bozza et al., 1995). A variety of clinical samples, including bone marrow, spleen, lymph nodes, skin and conjunctiva biopsy specimen as well as whole blood samples have been used for diagnosis. PCR done from blood spots on filter paper can also be used as a screening test to identify *Leishmania* infection in humans or dogs (Campio et al., 2000; Franca –silva et al.,2003; Da-silva et al., 2004; Cortes et al., 2004). Moreover, PCR with peripheral blood was considered as a more reliable method for diagnosis of VL in HIV-infected patients (Lachaud et al.,

2000; Pizzuto et al., 2001) since the sensitivity of antibody detection method in these patients is very low (Vilaplana et al., 2004). In a study reported from Israel, PCR using non invasive conjunctiva samples was found to be more sensitive (92%) than either PCR using other tissues (17%-77%) or detection of parasites in culture (74%) (Strauss-ayali et al., 2004). Several PCR methods with a wide range of sensitivities and specificities have been described (Piarroux et al., 1994; Osman et al., 1997). A PCR- (ELISA) technique using a primer that was able to identify 33 *L. infantum* strains from 19 different zymodeme has been developed. It has sensitivity higher than that of other traditional diagnostic techniques (Martin-Sanches et al., 2001). This PCR-ELISA technique can be used for diagnosis of VL from peripheral blood samples.

1.10.2.3 Hybridization using kDNA probes

DNA-DNA hybridization technique is very useful in the classification of *Leishmania* parasites. The molecular basis for DNA hybridization is based on the use of a labeled single standard DNA sequence, selected from well-characterized reference species, to hybridize with homologous DNA from, unknown isolates of parasites. The first study of a DNA sequence identification was reported from India, in which a specific probe cloned from minicircle DNA of *Leishmania* strain UR 6 (MHOM/IN/1978/UR6) reacted only with a strain of PKDL but not

with strains or isolates of VL (Gupta et al., 1991). Also a species – specific DNA probe from *L. infantum* has been generated (Reale et al., 1999). DNA hybridization has been employed to detect and identify leishmanial infection in sand fly vectors (Barker, 1989). Da Silva et al. (2004) showed that the sensitivity of PCR was increased from 71.7% to 83.5% when the technique was combined with molecular hybridization; all the PCR products were confirmed as *L. chagasi* after hybridization with a minicircle probe cloned from the same species.

1.11 SPECIES AND STRAIN IDENTIFICATION

Species identification is important because different species require distinct treatment (Marfurt et al., 2003). Furthermore, Characterization of *Leishmania* parasite is also necessary for epidemiological objectives, and for designing appropriate control measures (Volpini et al., 2004). Different degrees of intraspecies variations were observed in *Leishmania* parasites. However, these variations range from small or lacking in *L. infantum* to large highly observed in *L. tropica* (Schonian et al., 2001; Jacobson et al., 2003; Schnur et al., 2004; Schwenkenbecher et al., 2004).

1.11.1 Excreted Factor (EF)

Excreted factor (EF), which is a negatively charged carbohydrate-like substance obtained from in vitro cultures, was shown to precipitate by double diffusion in contact with homologous sera of promastigote-infected rabbits (Romero et al., 2004). Three serotypes exist A, B and AB, and *L. infantum* gives only the B-serotype reaction (Schnur and Zuckerman, 1977). These serotypes are divided in to subserotypes and are specific identifiers for different species, and thus this method permits the minimal characterization of strains.

1.11.2 Multilocus Enzyme Electrophoresis (MLEE).

Characterization of *Leishmania* is usually based on multilocus enzyme electrophoresis of cultured promastigotes. It is classically considered that variation due to isoenzymes reflect neutral mutation (Guerbouj et al., 2001) thus the enzyme profiles produced by different migration rates allow for species identification. MLEE revealed a broad enzymatic polymorphism within the species *L. infantum*. So far, more than 20 different Zymodemes have been described. Some of them have a large geographic distribution such as MON-1 which is the most frequent zymodeme in VL patients and infected dogs in the Mediterranean region (Martin-Sanchez et al., 2004). All *L. infantum* strains isolated in Israel and Palestine resemble MON-1 zymodemes (Lee Schnur, Personal communication) except for one human strain isolated from El-yamon in

Jenin district, which showed a new zymodeme profile (MON-281) (Bader et al., 2005 in press). Although several studies used isoenzyme analysis to identify *Leishmania* parasites (Romero et al., 2001; Lemrani et al., 2002; Franca-silva et al., 2003; Schnur et al., 2004), this technique requires prior cultivation and is time consuming since each isolate must be examined by multiple enzyme reaction (Andersen et al., 1996).

1.11.3 Advanced Molecular Technique:

1.11.3.1 Permissively Primed Intergenic Polymorphic-PCR

Eisenberger and Jaffe (1999) have developed a permissively primed intergenic polymorphic (PPIP)-PCR, which uses the specific intergenic *Leishmania* primer 2B and the non leishmanial primer 2A. This technique can distinguish between the old world *Leishmania* complexes. Moreover it discriminates between strains of the same species isolated from distant geographical regions.

1.11.3.2 Random Amplified Polymorphic -PCR

Random Amplified Polymorphic DNA (RAPD), based on fragment – length polymorphism, has the advantage that it does not require prior knowledge of specific DNA sequence for primer design. This method gives a variety of amplification products that are sensitive enough to show *Leishmania* species specificity and to identify isolates derived from geographically diverse regions (Schonian et al., 1996; Noyes et al., 1998; Mauricio et al., 1999). The disadvantage is that non specific primers are

able to amplify non target DNA from host and/or other contaminating organisms present in the sample (Eisenberger and Jaffe, 1999) and result in sensitivity much lower than that of standard PCR (Harris et al., 1998).

1.11.3.3 PCR- Restriction Fragment Length Polymorphism (PCR-RFLP) :

Parasite identification by RFLP is based on digestion of amplified kinetoplast or nuclear DNA with restriction endonuclease followed by visualization of the resulting pattern using gel electrophoresis. Recently, several PCR-RFLP methods that differentiate *Leishmania* species by the digestion of different amplified targets have just been described (Schonian et al., 2003; Marfurt et al., 2003;Volpini et al., 2004; Tintaya et al., 2004). Schonian et al. (2003) distinguished all medically relevant *Leishmania* species by digestion of the ITS1 amplicon with the restriction enzyme *Hea III.* In another study reported from Switzerland, in which miniexon PCR-RFLP assay was used, this new genotyping method was able to differentiate new and old world leishmanial species (Marfurt et al., 2003). Tintaya et al. (2004) developed two new PCR-RFLP assays that target the gene locus of an important *Leishmania* antigen cycteine proteinase B (cpb). The assays allowed direct genotyping of parasites in bone marrow aspirates and venous blood samples obtained from patients with VL.

STUDY OBJECTIVES AND SIGNIFICANCE:

The main objectives of the study are:

1. Evaluate the use of PCR as a diagnostic tool for canine leishmaniasis.

2. Isolate and identify *Leishmania* parasites from potential reservoirs present in the northern part of the West Bank.

3. Detect polymorphism between local and regional isolates of *L. infantum* by PCR-RFLP based genotyping assay.

4. Evaluate the sero-prevalence of CVL in the northern part of the West Bank.

Significance

Domestic dogs have been suspected to be potential reservoirs for VL in the northern part of the West Bank. It is hoped that this study will:

- Provide empirical evidence about the correlation between dogs as reservoirs and humans as accidental hosts by employing traditional and advanced techniques;
- Detect genetic polymorphism between different *L. infantum* strains which are may be attributable to different geographical location;
- Investigate the utility of PCR as a diagnostic tool for CVL specifically, on canine blood and to compare serological analysis with 3 types of PCR assays which use different DNA targets.

In addition analyze their advantages and drawbacks for routine diagnosis or epidemiological use.

CHAPTER TWO

MATERIALS AND METHODS

2.1 STUDY AREA

2.1.1 West Bank

The west Bank is bordered by Israel from the north, west , south, and the Jordan River and the Dead sea form a natural border with Jordan from the east. It is a hilly region, divided in to 10 districts with a mediterrnean climate. Approximately 27% of the inhabitants lived in urban communities with the remaining people present in rural areas and involved in agriculture. Most of residents live in houses built of concrete and or/ stone, many of these houses were constructed in the peripheral areas, and domestic animals like sheep, goats, chickens, and dogs are common. Four districts in the northern part of the West Bank including Tubas, Tulkarm, Nablus and Jenin districts were chosen for sample collction in this study (fig. 2.1).

2.2 PARASITES

Most of the *Leishmania* strains used in this study were obtained from dogs and humans from different regions in Israel and the West Bank (table 2.1).

Figure 2.1 show the main cities in dark spots that samples collected. http://www.arij.org/atlas.

Table 2.1 Details of the strain used in this study

WHO LRC Designation	**Species**	**International Code**	**Origin**
LD1	*L. infantum*	MCAN/PS/2004/LQU-D1	Domestic, Jenin, Palestine
L773		MHOM/PS/1999/LRC-L773	Human , IVL*, Jenin, Palestine
L806		MCAN/PS/2000/SawalhaK56	Domestic dog, Jenin, Palestine
L693		MCAN/IL/1996/LRC-L693	Domestic dog, Nili, Israel
IPT1		MHOM/TN/1980/IPT1	[a]Human IVL, Tunisia
L782	*L. tropica*	MHOM/PS/2000/GOKS17	Human CL*, Jenin, Palestine
L36		MHOM/IQ/1966/BRAYL75	Human CL, Baghdad, Iraq
L747		ISER/IL/1998/LRC-L747	*Ph. Sergenti*, Kfar Adumim
L137	*L. major*	MHOM/PS/1967/Jericho II	Human CL, Jericho, Palestine
5ASKH		MHOM/IM/1973/5ASKH	Human CL,WHO*

*IVL= infantile visceral Leishmaniasis: CL=Cutaneous Leishmaniasis.

[a]WHO international reference strain.

2.3 DOG POPULATION

Domestic dogs were surveyed by house-to-house visits. None of the dogs belong to a recognizable breed and all were guard dogs. The dog's age ranges from six months to seven years. 148 dogs were sampled, 20% (30) of them were females, and 80% (118) were males.

2.4 CANINE SAMPLES

Peripheral blood samples (10 ml) from 148 domestic dogs, were taken by venipuncture, 5 ml into sterile EDTA- tubes for buffy coat preparation, and 5 ml in plain tubes for ELISA testing, EDTA-blood samples were also spotted on filter paper and preserved at 4ºC as a back up samples. The blood was centrifuged at 3000 rpm for 15 min; the buffy coat layer (~300 µl) separated, and washed with an equal volume of phosphate buffered saline (PBS) (pH 7.4). Sera and buffy coat were stored at –20 until use. Seropositive dogs were re-visited, both bone marrow and splenic aspirates were obtained and cultured for parasites.

2.4.1 Direct Examination of Smears

Smears of peripheral blood were fixed with absolute methanol for five minutes, stained with giemsa stain, and the slides were examined under high power (100X) with a standard light microscope. Approximately 50 microscopic fields were examined for the presence of *Leishmania* like amastigots.

2.4.2 Culturing, Freezing, and Parasites Maintenance

Bone marrow aspirates and biopsy samples from spleens were cultured, immediately after aspiration; into a semi solid blood-agar medium Supplemented with 10% rabbit blood. Promastigotes from positive cultures were grown in Schneider's *Drosophila* medium (SDM) supplemented with 10% inactivated fetal calf serum (FCS), 2mM L-glutamin, 200µg/ml penecillin and 200 µg/ml streptomycin (Schnur and Jacobson, 1987). These cultures were incubated at 26 ºC and checked microscopically every week until promastigotes were observed. A culture was considered negative after five weeks of observation. Cultured parasites were centrifuged at 3000 rpm for 15 min, washed three times with PBS pH 7.2, and then resuspended in 1 ml SDM containing 15% glycerol, cooled for one hour on ice, and then frozen at –70 ºC until use.

2.5 ENZYME LINKED –IMMUONOSORBENT ASSAY (ELISA)

2.5.1 Crude Soluble Antigen and ELISA Plates Preparation

Promastigotes of *L. infantum* isolated from Jenin district were used as antigen . These promastigotes were cultured in 40 ml SDM then harvested by centrifugation (3000 rpm for 15 min) at a concentration of about $5x10^7$ promastigote/ml. The pellet was washed three times with PBS and maintained on ice, then resuspended in ice cooled lysis buffer (0.2M EDTA ,pH 8.0, 1 M Tris-base,(pH 7.3), 2 M NaCl, X1 Protease

inhibitor cocktail (sigma, USA cat. P2714)) 1 ml lysis buffer /8 ml parasite culture, and kept at –70ºC as aliquots. The relative protein concentration of the soluble antigens used for coating ELISA-micro-titer well was estimated by measuring the optical density (OD) at 280 nm (Peterson, 1983) using the lysis buffer as blank. The antigen protein concentration adjusted to 100 µg/ml PBS containing 0.1% thimerosal with a 50 µl dispensed per well (5 µg/well), then the plates were incubated at 4C^{o} for 8-16 hours, washed four times with washing solution (1% Tween-20, 0.01% thimerosal in X1 PBS, pH 7.2). The washing solution was completely discarded by blotting on tissue paper, 200 µl of blocking solution (2% FCS, 0.01% thimerosal in PBS, pH 7.2) added. Again the plates were incubated for 3-8 hours at 4C°, washed three times with washing solution and stored at –20 until use (Baneth et al., 1998) .

2.5.2 Determination of the Cut-off Value

One test serum from a microscopically confirmed human case of leishmaniasis from Hebron, one positive control serum from a dog confirmed with VL microscopically from Israel and 20 negative human reference sera, collected from Abu-Deis village presumed to be non endemic for leishmaniasis (disease has not been reported), were tested at two dilutions (1:100 and 1:1000) to determine the cut-off value and its corresponding dilution (1: 100 dilution was chosen in order to increase the sensitivity of the test). 50 µl of each diluted serum were added in

duplicate to the coated wells. The plates were incubated for 1 hour at 37ºC, washed three times with 0.1% Tween –20 in 50 mM PBS (pH 7.2), incubated with protein A conjugated to horseradish peroxidase (1:8000 dilution; Zymed Laboratories, Inc., San Francisco, CA) for one hour at 37C^{o}. Excess conjugate was removed by extensive washing (four times) in PBS-Tween and the plates were developed by addition of 1X 100 µl of substrate 2,2'-azino-di-3-Ethylbenzthiazolihne sulfonate (ABTS) (Boerhinger Manneheim, Germany). After incubation for 10 minutes at room temperature in the dark, optical density was measured at 405 nm by a Rosys anthos ELISA reader (Wals, Austria).

2.5.3 ELISA Sero-Survey

148 study dogs' sera were diluted 1:100 with PBS, the pre-determined dilution that corresponded to the cut-off value and assayed in duplicate for positivity as described in section 2.5.2., negative blank(PBS), known negative and positive controls were included in each run to exclude any plate variation.

2.6 DNA EXTRACTION METHOD

phenol chloroform DNA extraction method was done as described by Schonian et al., (1996). The parasite cultures were pelleted at 2500 rpm for 15 min, washed and resuspended three times with PBS (pH 7.4),

centrifuged at 3000 rpm for 10 min, and finally frozen at -70C° until processing. Frozen or newly prepared parasites were re-suspended in lysis buffer (50 mM NaCl, 10mM EDTA, 50mM Tris –HCl (pH 7.4)). Sodium dodecyl sulfate (SDS) and ribonuclease (RNase) were added to a final concentration of 0.5 % and 100 µg/ml, respectively. The mixture was incubated for 30 min at 37C°. Then proteniase K was added to a concentration of 100 µg/ml, the incubation was continued over night at 60C^{o} with gentle mixing. The DNA solution was extracted with an equal volume of buffered phenol (Sigma, USA), and centrifuged at 3000 rpm for 15 min. The upper phase was transferred to fresh tubes, followed by addition of a phenol-chloroform-isoamyl alcohol mix (v/v 25:24:1), mixed gently and centrifuged for 5 min at 3000 rpm . The upper phase was again transferred to fresh tubes, and one volume of chloroform – isoamyl alcohol (v/v, 24:1) was added, the mixture was vortexed and centrifuged at 3000 rpm for 10 min. DNA was precipitated with a final concentration of 0.3M sodium acetate (pH 6.0) and 100% isopropyl alcohol (Finkelman LTD, Israel)(1:1 cold), mixed gently, and placed on ice for 15 min, centrifuged for 30 min at 14,000 rpm. The isopropanol was removed and the DNA pellets were washed with cold 70% ethyl alcohol (Finkelman LTD, Israel) and centrifuged at 14,000 rpm for 15 min. The ethyl alcohol was removed and the DNA was dried in a vacuum pump (eppendorf concentrator 5301) and dissolved in 100µl TE

buffer (10mM Tris-HCL (pH 8), 1mM EDTA) then stored at -20ºC until use.

2.7 STRAIN CHARACTERIZATION AND IDENTIFICATION

2.7.1 Molecular Characterization

PCR-based DNA analysis was done by targeting both nuclear and kinetoplast DNA.

2.7.1.1 The internal transcribed spacer 1 (ITS1)-PCR

Primers LITSR (5'-CTG GAT CAT TTT CCG ATG-3') and L5.8S (5'-TGA TAC CAC TTA TCG CAC TT-3') are both used to target the ribosomal internal transcribed spacer 1 (ITS1) region lying between the genes coding for the ssu rRNA and 5.8srRNA gene (El Tai et al., 2001; Schonian et al., 2003). Amplification reactions were performed in volumes of 50 µl, containing ammonium sulfate buffer X1 (75 mM Tris HCl, pH 8.8, 20mM $(NH4)_2So4$, 0.01% Tween 20) 1.5 mM $MgCl_2$, 1µM of each primer, 0.25mM of each deoxy ribonucleoside triphosphates, 2U of fermantas recombinant Taq DNA polymerase (MBI Fermantas ,Germany), 2µl of isolated DNA were added to a reaction mixture. Samples overlaid with 50µl mineral oil and amplified in a thermocycler (Gene Amp PCR-system 9700) programmed for denaturing step, 5 min at 95 ºC followed by 35 cycles; denaturing step 1 min at 95ºC; annealing step: 1 min at 53ºC and primer extension at 72 ºC for 1 min followed by a final extension of 72 ºC

for 6 min. Amplification products were added to Bromophenol blue (BPB) (1mM EDTA pH 8.0, 50% glycerol) loading dye, then subjected to electrophoresis in 1.2% LE Seakem agarose at 120 V in X1 TAE (1.0 M Tris acetate, 0.5M EDTA, pH8.0) buffer. 100 bp DNA ladder was used as a molecular size marker (Promega, Madison, WI). The gels were stained with ethidium bromide (0.25 µg/ml) and visualized under UV light for analysis.

2.7.1.2 RFLP analysis of the ITS1-PCR

This was done according to (Schonian et al., 2003). PCR products were digested with *Hae*III endonuclease restriction enzyme (Promega, Madison, WI) without purification. 10 µl final volume of reaction containing: 1µl of X1 enzyme buffer (100 mM Tris-Hcl pH 7.9, 500 nM NaCl, 100mM $MgCl_2$ and 10 nM DTT), 0.1 µl Bovine serum albumin (BSA), 1 µl of *Hae* III enzyme and 8 µl of PCR product used. Samples were incubated at 37ºC for 2-3 hours. The restriction fragments were mixed with BPB and subjected to electrophoresis in 2.5 % metaphore agarose (FMC BioProduct, Rockland, MN), containing 0.005% gel star stain, at 100v in X1 TAE buffer.

2.7.1.3 Kinetoplast DNA-PCR using Uni21/Lmj4 primers

Oligonucleotide primers Uni21 (5'-GGG GTT GTT GTA AAA TAG GCC-3') and Lmj4 (5'-CTA GTT TCC CGC CTC CGAG-3'), based on a minicircle sequence of *L. major*, were used to amplify kDNA from

cultured promastigotes (Anders et al., 2002). Reaction mixtures (50 µl) contained X1 buffer (10mM Tris-HCl pH 8.8, 50 mM KCl, 0.08% Nonidet P 40), 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleoside triphosphate, 1µM of each primer, 2U of Ampli Taq DNA polymerase (Perkin-Elmer-cetus, Norwall, CT), 2µl of DNA, amplification was performed in a thermocycler (Gene Amp PCR-system 9700) programmed for first denaturing step of 5 min at 95ºC, and 35 cycles of: 30 sec at 94 ºC 50 sec at 60ºC , and 1.0 min at 72ºC followed by 7 min at 72ºC as final extension. The products were added to BPB loading dye and analyzed by electrophoresis in 1.2% agarose gels. 100 bp DNA ladder was used as size marker.

2.7.1.4 RFLP analysis of KDNA-PCR products

The generation of RFLP profiles of kDNA was done as described by (Abdeen et al., 2002). 17µl of PCR product were subjected to restriction with *Rsa1* and *HpaII* enzymes (promega, madison, WI). Digestion conditions and documentation was done as described in section 2.7.2.2 except for, an extra 1µl of the enzyme was added for extra 30 minutes as boost to insure a complete digestion.

2.7.1.5 Data analysis

The PCR and RFLP for the leishmanial strains were analyzed using the RAPDistance Package ver. 1.04 (**http://WWW.anu.edu/BoZo/software/**). All Bands obtained were numbered using the Binary system scoring 1 for presence and 0 for absence of bands. Similarities representing the ratio of shared bands among the total bands of all the *L. infantum* strain tested. A tree was built using distance-matrix (Neighbor-joining), based on pair wise comparisons and using DIC Coefficient. The Evolutionary tree constructed based on kDNA- RFLP analysis using 2 different restriction enzymes.

2.8 VALIDATION OF PCR ASSAYS FOR CVL DIAGNOSIS

2.8.1 Kinetoplast DNA –PCR using 13A/13B primers:

The primer pair 13A (5'-GTG GGG GAG GGG CGT TCT- 3') and 13B (5'-ATT TTA CAC CAA CCC CCA GTT-3'), based on the conserved region of the leishmanial kDNA minicircle yeilding 120 bp product (Reale et al., 1999). Reactions were performed in 25µl containing X1 buffer (10mM Tris-HCl, pH 8.3, 1.5mM $MgCl_2$, 25mM KCl), 1µM of each primer, 0.4mM of each dNTP, 2Uof Taq DNA (MBI Fermantas, Germany) and 2.5 µl of DNA extraction product. The following conditions were applied: initial denaturation at 95 ºC for 5 min followed by 35 cycles consisting of denaturation at 95 ºC for 1 min, annealing at 60 ºC for 1 min and extension at 72ºC for 1.5 min followed by 7 min of

72 ºC for final extension. PCR samples were electrophoresed, using 2.5% LE Seakem agarose and visualized under ultraviolet light after staining in eithdium bromide.

2.8.2 Modified internal transcribed spacer 1 (mITS1)

In an attempt to enhance the sensitivity of ITS1-PCR, we recently modified the ITS1- PCR assay; this was done by choosing a new 3' Primer 100 bp up stream of the old 3'- primer site, there by shortening the target sequence to about 200 bp instead of 300 bp. (fig. 2.3).

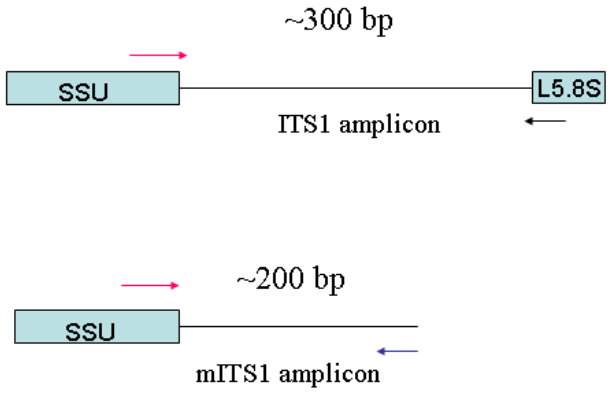

Fig. 2.3 Schematic representation of the internal transcribed space (ITS1) in the ribosomal operon and the position of newly designed primer (blue arrow) used in this study to amplify the mITS1 sequence. Primer sequences are given in the text .

2.8.2.1 Alignment and primer design

DNA sequences of ITS1 region of several *Leishmania* species were obtained from Gene Bank (www.ncbi.nlm.nih.gov/Blast/GeneBank). Using nucleotide to nucleotide BLAST search the ITS1 sequences were aligned to each other, we focus on *L. infantum*, *L. major*, *L. tropica* since our target area was the northern part of the West Bank, where these three species coexisted. For that, three ITS1 sequences from three different regional *Leishmania* species were chosen (*L. infantum* AJ000289.1, *L. tropica* AJ000301.1, and *L. major* AJ000310.1) and aligned to each other using BCM Search Launcher: Multiple Sequence Alignments web site (searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The new primer sequence was chosen due to its presence in the 3 species, absence from the polymorphic region of each species, and the presence of *Hae* III restriction sites between opposing primers that are important to provide different profile on the gel for each *leishmania* speceis. A theoretical cut was applied to modified ITS1 sequences of all three *Leishmania* species using NEB cutter. Ver. 2 program (http://tools.neb.com/NEBcutter2/index.php.).

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2.8.2.2 mITS1-PCR

Amplification reactions were performed in volume of 50 µl using the previously described LITSR primer as a forward primer and our newly

designed primer RmITS1 (5' AAA ACC GAA ACG CCG TA) as a reverse primer. Specific amplification and digestion conditions were as described by Schonian et al., (2003).

2.8.3 Comparison of Diagnostic Tools and Statiatical Analysis

Of the 97 dog sample collected from Jenin District, 60 samples were randomly chosen for detection of leishmanial infection by ELISA and 3 different PCR methods: kDNA, ITS1, and mITS1 PCR, in order to evaluate the use of PCR as a diagnostic tool for CVL, which would allow the reliable detection of *Leishmania* DNA from peripheral blood samples. ELISA test , kDNA, ITS1, and mITS1-PCR were done as described above. Statistical analysis was done using SPSS program ver. 10. (Chicago, IL, USA), and the on-line statistics calculator at (http://www.graphpad.com/quickcalcs/index.cfm). The agreement between diagnostic methods was assessed; also the sensitivity and specificity of each method were compared with the golden standard test.

CHAPTER THREE

RESULTS

3.1 PARASITE CULTURES

Ten seropositive dogs were reexamined for clinical signs of the disease and subject to biopsy to check for the presence of parasites. Among them 3 (30%) were symptomatic, with the external signs of leishmaniasis (hair and weight loss and onychogryposis), and 7 were asymptomatic (70%) without any external signs. Out of seven spleen cultures, two were positive (28.6%), one was isolated from Qabatiya village in Jenin district, then identified, the other being contaminated by fungi, while three seropositve dogs did not undergo a biopsy due to refusal of the dogs' owners.

3.2 MICROSCOPY RESULTS

Microscopic examination of peripheral blood smears showed that only one out of 60 smears (1.7%) have amastigotes. The amastigotes appeared as pale blue oval bodies with dark blue nucleus, and a small point shaped kinetoplast (see fig. 3.1).

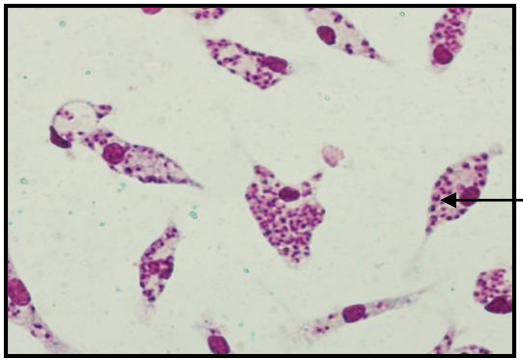

Fig. 3.1 Experimentally amastigotes infected macrophages, Photo by A. Nasereddin

3.3 ELISA-SEROSURVEY

3.3.1 Determination of the cutoff value

Using the crude soluble antigen, the cutoff value, the lower limit of positivity, was established as the mean OD + 3 SD of the negative controls this was o.4 OD, values above this were positive. The cutoff value was established with a serum dilution of 1:100 in PBS.

3.3.2 Sero-Survey

The sera of 148 dogs from Tubas, Nablus, Tulkarm, and Jenin districts, in the northern part of the west Bank, were tested. Ten of them were ELISA positive, (fig. 3.2) indicating a sero prevalence of (6.8%). Nine out of 97 dogs sera collected from six villages in the Jenin district were sero positive dogs indicating a sero prevalence of (9.2%). One dog out of 17 (5.9%) was seropositive from Tulkarm district. 15 and 19 dogs were seronegative from Tubas and Nablus respectively. Figure 3.3 shows

the correlation of HVL cases in northern West Bank reported by the Palestinian Ministry of Health over 9 years (1990-1999) to canine VL according to districts seroprevalence, where Jenin had 39.3% of HVL with our dog seroprevalence 9.2%, followed by Tulkarm with human cases 13.3% and our dog seroprevalence was 5.9%, lasting with 0% dog seroprevalence in both Tubas and Nablus, where human cases 2.4% in both areas.

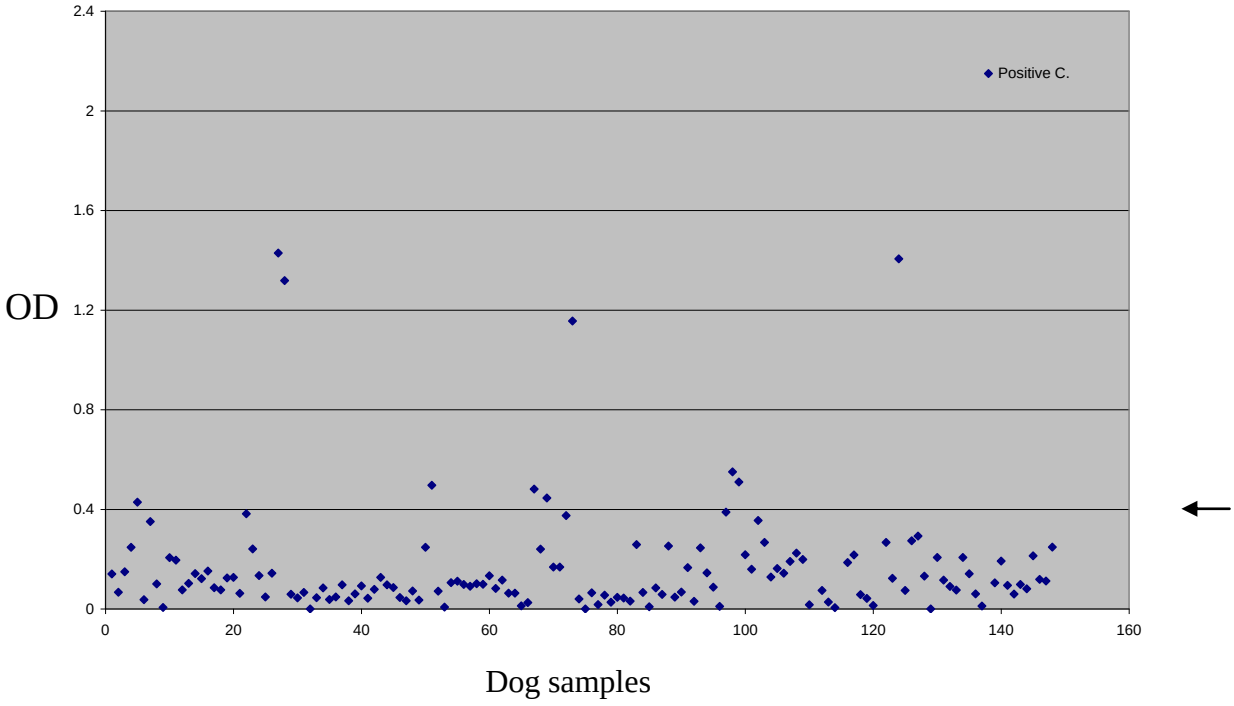

Fig. 3.2 ELISA survey for anti-*Leishmania* anti bodies of domestic dogs, the arrow indicates the cut off value.

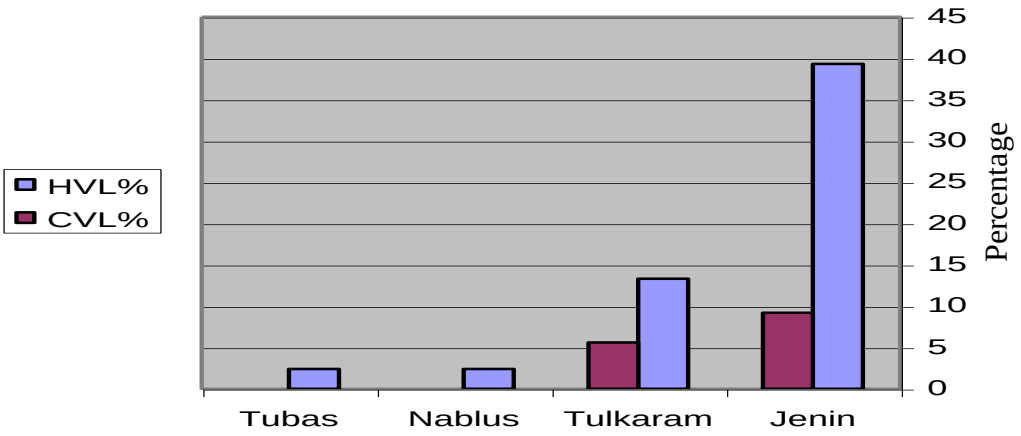

Fig 3.3 Show the association of HVL cases with respect to seropositives dogs in the four districts.

3.4 STRAIN IDENTIFICATION AND GENOTYPING

3.4.1 Serological and Biochemical Characterization

The Excreted factor (EF) and the Zymodeme analysis were done by Schnur L.F. (Hebrew University-Hadassah Medical school, Israel) and Dedet J.P (Universite' de Montpellier, France) respectively. The strain Li-QUD1 (LD1) was EF sub-serotype B_2 in compatibility with the serotyping of WHO *L. infantum* reference strain (MHOM / TN/80/IPT1). Zymodeme analysis profile of strain (LD1) using fifteen enzymes showed it was MON-1 and identical to IPT1 (table 3.1). Together these analyses indicate that strain (LD1) is *L. infantum* With profiles identical to all HVL and CVL strains isolated and analyzed in the region, except for one strain isolated from a human (Bader et al., 2005, in press) show faster MDH mobility with zymodeme MON-281.

Table 3.1 The enzyme profiles of strain MCAN/PS/2004/LQU-D1.(LD1)

Enzyme Profiles															
Zymodeme	MDH	ME	ICD	PGD	G6PD	GLUD	DIA	NP_1	NP_2	GOT_1	GOT_2	PGM	FH	MPI	GPI
MON-001	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

3.4.2 ITS1-PCR analysis

Fig. 3.4.A show the result of ITS1-PCR analysis, This PCR assay identifies *Leishmania* at the genus level. All *Leishmania* strains in this study produced a single band approximately 300 base pair (bp) as expected by ITS1-PCR.

3.4.3 ITS1-PCR-RFLP

The major advantage of the ITS1-PCR is that species identification can be achieved by digestion of the PCR product with restriction enzyme *Hae III*. Thus, all clinically important species can be distinguished by their RFLP-pattern. When ITS1-PCR products were digested with Hae *III*, the banding patterns of all *L. infantum* strains differed from those of *L. major* (L173) and *L. tropica* (L36, L782) strains. While they were identical to *L. infantum* pattern of the WHO reference strain MHOM/TN/80/IPT1, *L. tropica* (L36, L782) strains have identical patterns, but its profile was easily distinguishable from *L. major* (Fig

3.4.B). Both *L. infantum* and *L. tropica* are coexisting in Jenin district (Sawalha et al., 2003), for that *L. tropica* included in this study, also because *L. tropica* ability to infect dogs reported in Morocco and other localities (Dereure et al., 1991). L693 proved to be *L. infantum* from previous study (Abdeen et al., 2002).

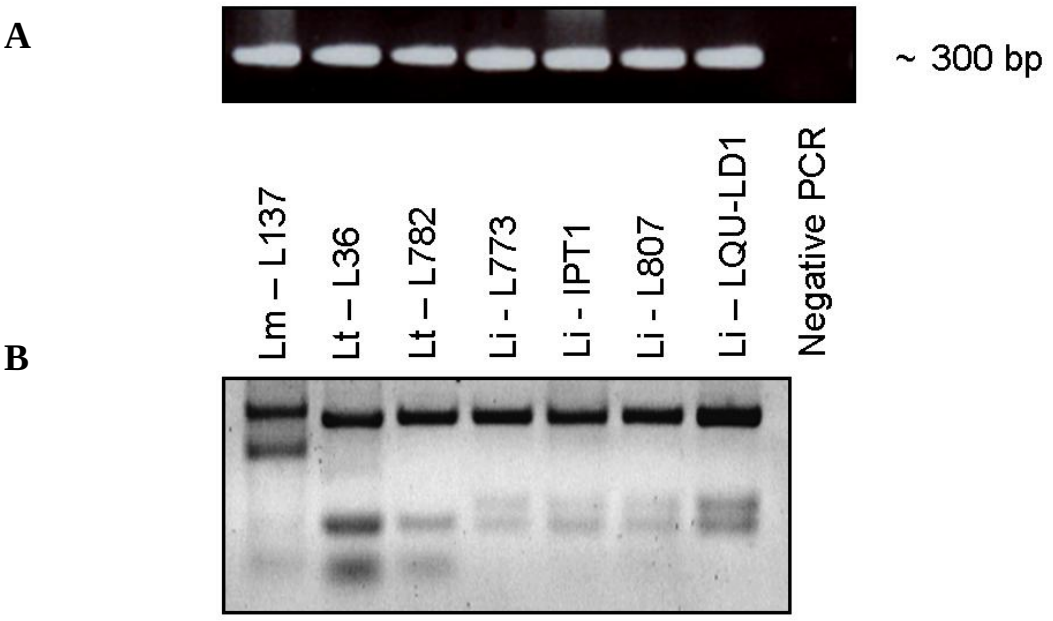

Fig. 3.4 Comparison of the ITS1-PCR amplification products using the primers L5.8S and LITSR (A) ITS1-PCR digestion with the endonuclease *HaeIII* (B). Negative PCR: PCR with water added instead of Leishmanial DNA.

3.4.4 kDNA-PCR analysis using LMJ4/UNI21 primers

Under the experimental conditions described in materials and methods, the kDNA amplification products from the human isolate L773 and three canine VL strains LD1, L807, and L693 all were aligned with one another and with the reference Strain of IPT1 (fig. 3.5). L137 and

L36 strains, both were excluded from this kDNA-PCR since the ITS1-PCR proved that they are not correlated with *L. infantum* as described above .These strains produced a clear band at the level of 780 bp.

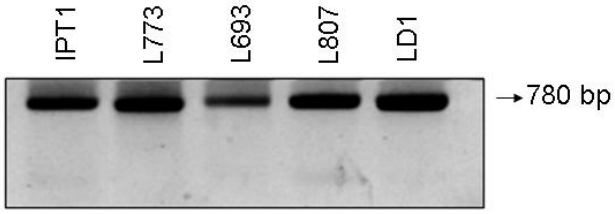

Fig. 3.5 The PCR product of the kDNA, run in 1.5% LE Seakem agarose and visualized under ultraviolet light after staining in ethidium bromide.

3.4.5 kDNA-PCR-RFLP analysis

The RLFP pattern using the *Hpa II* endonuclease enzyme shows variation between all of the *L. infantum* strains, significant difference pattern was observed between the IPT1 strain from Tunisia and the 4 samples from Palestine and Israel regions (fig. 3.6)

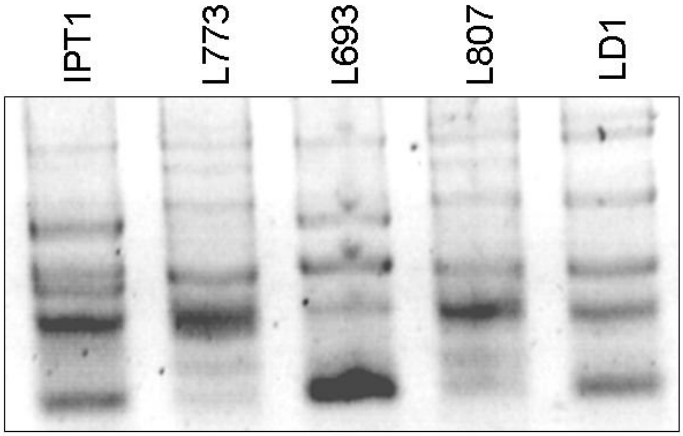

Fig. 3.6 shows the kDNA-RFLP patterns of several *L. infantum* strains following digestion with *Hpa II*

The generated profile of the *Rsa I* endonuclease enzyme also showed obvious variation between the all *L. infantum* strains. LD1, L773 and L807 show 82% shared bands. L693 (data not shown) show high homology with the Palestinian strains.

The total same bands shared between all the strains were 45%. Three extra bands appeared in the Tunisian strain and show major differences while only minor differences noticed in the rest of the strains (fig. 3.7).

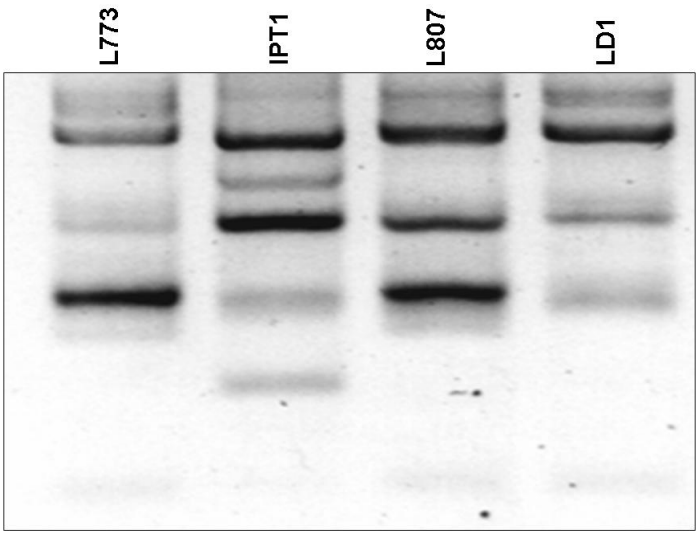

Fig. 3.7 the kDNA-RFLP patterns of (L807, IPT1, L773, and LD1) following digestion with *Rsa I.*

3.4.6 The dendrogram analysis

The dendrogram in (fig. 3.8) presents the intra-relationship of various strains of *L. infantum* according to their RFLP patterns using two different restriction enzymes. Two main clusters were observed and the strains within each cluster showed further intra-specific micro

heterogeneity. The IPT1 strain from Tunisia shows about 80% homology with the others. While the strains from Palestine together display 95% homology.

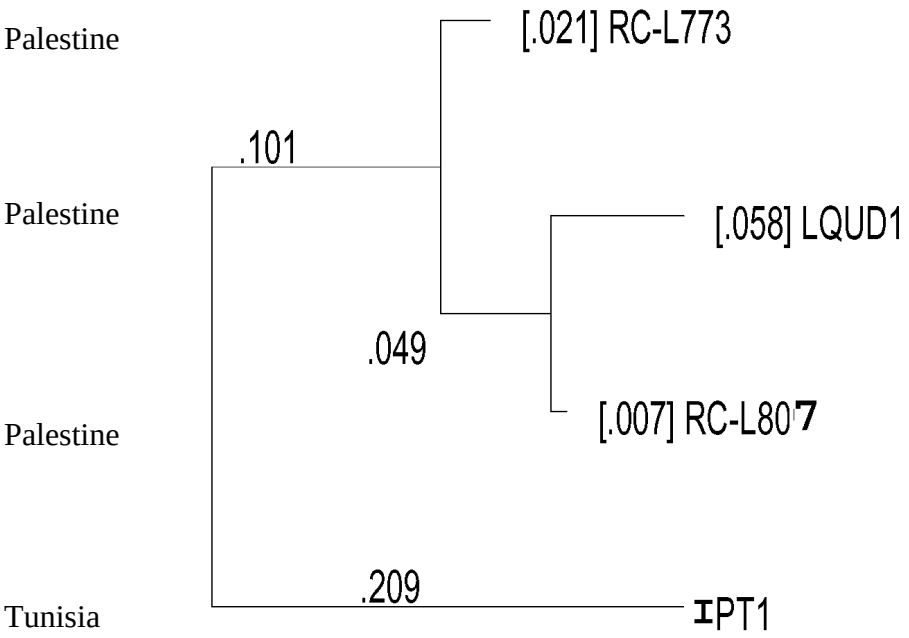

Fig. 3.8 Evolutionary tree constructed based on KDNA- RFLP analysis using 2 different restriction enzymes). The geographic origin of the strains is indicated. The numbers on the branches indicate patristic distances while in square brackets indicate patristic distances to individual out group.

3.5 VALIDATION OF THE PCR ASSAYS FOR CVL DIAGNOSIS

3.5.1 Sequence analysis of mITS1 region and reverse primer design

Sequence analysis of the mITS1 region of *L. major* shows high identity with mITS1 region from both *L. infantum* and *L. tropica*. The most significant homology was observed between 190 to 210 bp and part of this sequence was therefore selected as the modified primer for the 3'

region for the mITS1-PCR. The identified sequence was (5’TAC GGC GTT TCG GTT TT) and its reverse complementary sequence was RmITS1 (5'AAA ACC GAA ACG CCG TA), which was used for PCR reaction (fig. 3.9).

The primer was 17 bases in length with a content of 50% GC, the annealing temperature fits that of the forward primer LITSR and both primers lack any complementarities to each other. The primer allowed generation of a PCR product ~200 bp when it was applied on purified DNA from cultured promastigotes of different *Leishmania* species (fig. 3.10), as well as on peripheral blood samples obtained from domestic dogs. On the other hand, the amplified mITS1 sequence showed polymorphic regions according to *Leishmania* species, which allows species identification by RFLP analysis. The obtained RFLP pattern by *Hae III* digestion of mITS1 amplicon of all *Leishmania* species, used in this study, revealed distinct RFLP profile for each species with no geographical differences observed in the same species, for that we included local strains *L. infantum* (LD1), *L. major* (L137), *L. tropica* (L747) and *L. tropica* (Goks17), also WHO references strains were included as *L. major* (5ASKH) and *L. infantum* (IPT1) (fig. 3. 11. A).

```
LTITSB297     1 CTGGATCATTTTCCGATGATTACACC--AAAAAACATATACAAACTCGGGGAGGCCTAT
LMITSASKH     1 CTGGATCATTTTCCGATGCTTACACCCCAAAAAACATATACA--ACTCGGGGAGGCTTAT
LIITSIPT1     1 CTGGATCATTTTCCGATGATTACACCC-AAAAAACATATACA--ACTCGGGGAGACCTAT

LTITSB297    59 A-TAT-TATACATTATATAGGCCTTTCCCACACATACACAGCAAACTTGTATACTCGAAG
LMITSASKH    59 TCTATATATATATAGTATAGGCTTTTCCCACA--TACACAGCAAACTTTTATACTCAAAA
LIITSIPT1    58 G-TATATATA--TG---TAGGCCTTTCCCACA--TACACAGCAAAGTTTTGTACTCAAAA

LTITSB297   117 TTTGCAGTAAACAAAAGGCCGATCGACGTTATA--ACGCACCGCCTATACACAAAAGCAA
LMITSASKH   117 TTTGCAGTAAA--AAAGGCCGATCGACGTTGTAGAACGCACCGCCTATACACAAAAGCAA
LIITSIPT1   110 TTTGCAGTAAAAAAAAGGCCGATCGACGTTATA--ACGCACCGCCTATACA--AAAGCAA

LTITSB297   175 AAATGTCCGTTTATACAAA----TATACGGCGTTTCGGTTTT----------GTTGGC
LMITSASKH   175 AAATGTCCGTTTATACAAAAAAATAGACGGCGTTTCGGTTTTTGGCGGGAGGGAGAGAGA
LIITSIPT1   166 AAATGTCCGTTTATACAAAAAA-TATACGGCGTTTCGGTTTT----------TGGC
```

Fig. 3.9 Multiple alignment of 200bp of the ITS1 (mITS1) for old world *Leishmania* species

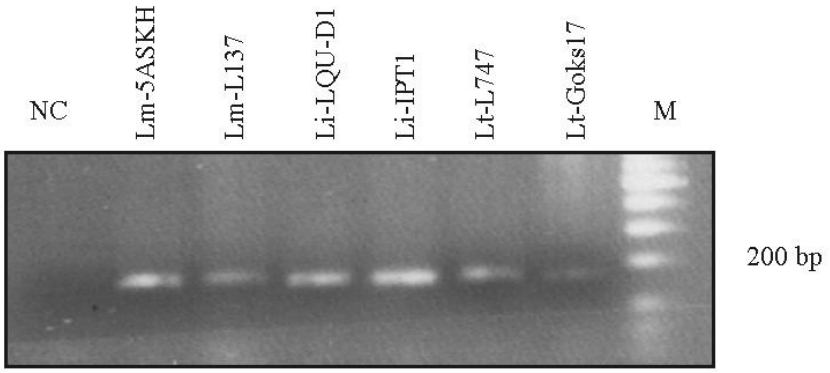

Fig. 3.10 200bp product of mITS1-PCR, applied on local and WHO leishmanial strains

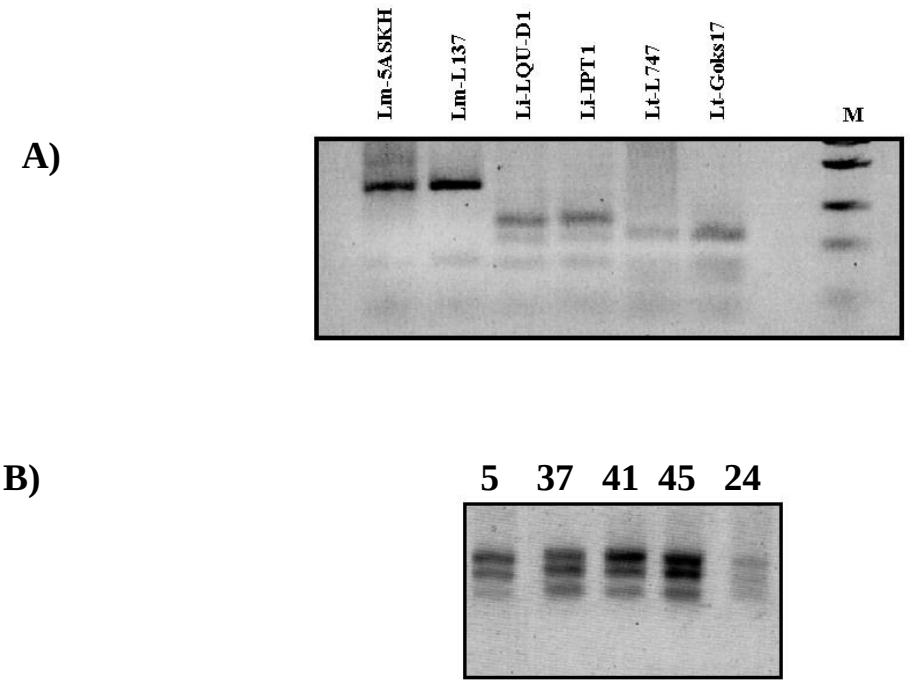

Fig. 3.11 The mITS1-RFLP patterns of (5ASKH, L137, LD1, IPT1, L747, Gocks17) (A) and dog's blood samples (B) following digestion with *Hae* III restriction endonuclease

3.5.2 Sensitivity of ITS1and the mITS1-PCR assays

Following the DNA titration of WHO *Leishmania* reference strain (LRC-L137) from 200ng-0.2fg DNA concentration measured by the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Delaware, USA) we succeeded to amplify the ITS1 product using purified *Leishmania* DNA down to 200 pg DNA concentration in a 50 µl PCR reaction using LITSR/L5.8S primer pair,

while LITSR/RmITS1 primer pair was found to be more sensitive with sensitivity of a bout 200 fg. (fig. 3.12)

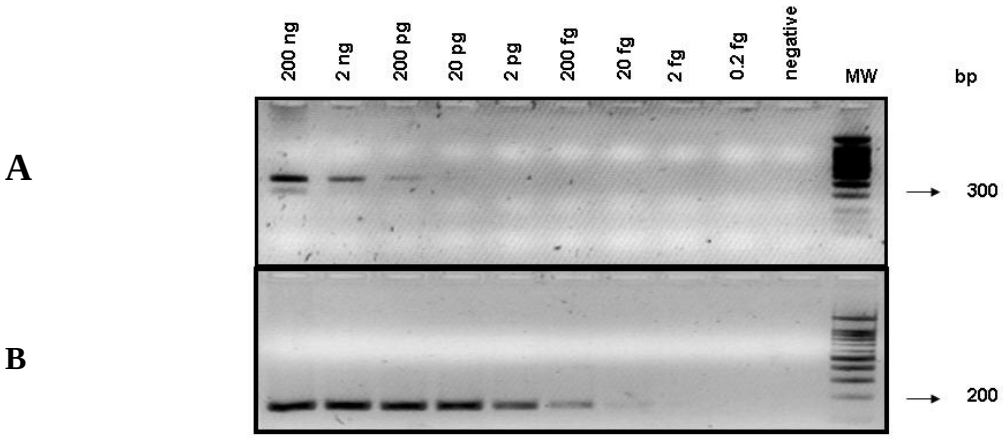

Fig. 3.12 Purified DNA titration of *L. major* (LRC-137) from 200 ng down to 0.2 fg using LITSR/L5.8S primers (A) and LITSR/RmITS1 primes (B)

3.5.3 Comparison of several diagnostic tools for CVL

Out of 60 suspected cases, 13 infections were detected by kDNA-PCR (21.7%)(fig. 3.13), 9 (15%) were positive by mITS1, 5(12%) were seropositive by ELISA, and 3(5%) were detected by ITS1-PCR (fig. 3.14), to identify the infecting species, amplified ITS1 and mITS1 were cut by restriction enzyme *Hae III*, all three ITS1- and nine mITS1-PCR positives proved to be belong to *L. infantum* species (fig. 3.15 and fig. 3.11.B). Because no single test serve as gold standard, an ideal test that would identifiy all infected dogs, for *Leishmania* test from peripheral blood against, which the different assays could be evaluated. Therefore, assessment of test performance was based on the assumption that: a dog

was confirmed positive, when one assay was positive for *Leishmania*, and considered as confirmed negative if all the four assays were negative (Marfurt et al., 2003). These confirmed values were used as the "gold standard" against which each individual diagnostic assay was measured.

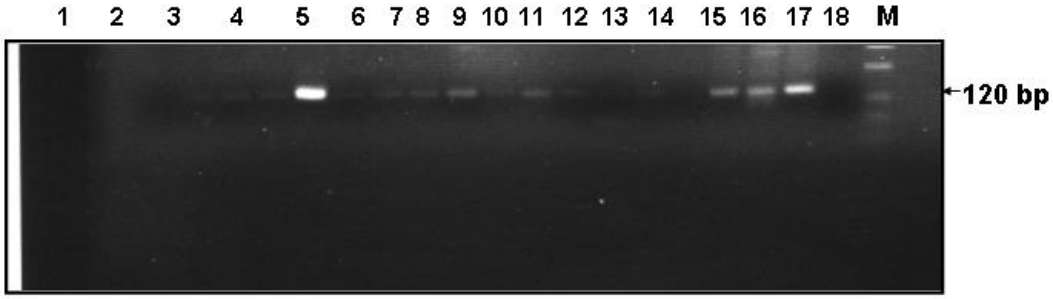

Fig 3.13 kDNA-PCR products using the primer pair 13A/13B (1-16, dogs samples) Lane 5,7-9,11,15,16, +ve samples; Lane 17, +ve control; Lane 18, –ve control; M, 100 pb DNA ladder.

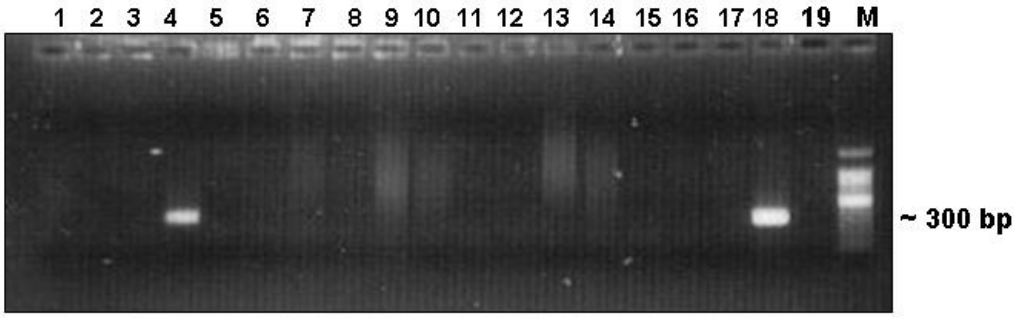

Fig. 3.14 The ITS1-PCR products using LITSR / L5.8S primers (Lane 1-17, 42-58 dog samples) Lane 4, +ve sample (dog 45); Lane 18, +ve control; Lane 19, -ve control; M, 100pb DNA ladder.

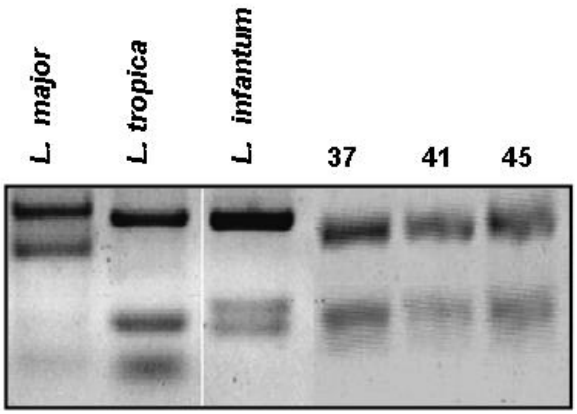

Fig 3.15 Digestion of ITS1 amplicon with restriction endonuclease *Hae III*: 37,41,45, *Leishmania* infected dogs samples characterized as *L. infantum.*

Results obtained from each assay were compared, as expected the entire diagnostic assay were highly specific for the diagnosis of VL. Using kDNA-PCR (table 3.2) 13 and 45 samples were identified as true positives and negatives, respectively. kDNA-PCR had the highest sensitivity of any individual assay, while missing 2 samples identified as positives by ELISA. The positive predictive value (PPV) was 97% indicates that, there was 97% chance that the result was true positive leaving 3% that it was false positive, the negative predictive value (NPV) was 96% indicates that, there was 96% chance that the result was true negative, leaving 4% chance that it was false negative. The level of agreement between kDNA-PCR and the confirmed results, K± standard

error (SE), k=0.907±0.064 was excellent, kappa coefficient is the agreement between two tests where 0 is chance agreement and 1 is perfect agreement. The mITS1-PCR correctly diagnosed 9 true positives and 45 true negatives with 6(10%) false negatives. it has a good sensitivity with PPV 95%, and the NPV was 88%. The overall agreement was good (k±SE= 0.692± 0.110).

ELISA serology was somewhat better than the ITS1-PCR giving 45 true negative, 5 true positive and 10 (16.6%) false negatives that were identified as positive by at least one PCR assay. The PPV for the ELISA assay was 92% and the NPV was 82%. Agreement between the confirmed results and ELISA was moderate, (k±SE=0.429±0.135). The ITS1-PCR gave the poorest result of any of the assays used (table 3.2) only 3/15(20%) were correctly diagnosed by this PCR with 12 false negatives. The sensitivity was very low, PPV and NPV, 87% and 79% respectively. The agreement between the ITS1-PCR and the confirmed golden standard was only slight, (k±SE=0.273±0.129). No DNA product was observed in any assay of the negative control PCRs.

Table (3.2): show ITS1-, mITS1-, kDNA-PCR and ELISA results against the confirmed results.

		Confirmed Results		Total	PPV%	NPV%	Kappa*
		Negative	Positive				
ITS1	Negative	45	12	57	87	79	0.273±0.129
	Positive	0	3	3			
Total		45	15	60			
ELISA	Negative	45	10	55	92	82	0.429±0.135
	Positive	0	5	5			
Total		45	15	60			
mITS1	Negative	45	6	51	95	88	0.692±0.110
	Positive	0	9	9			
Total		45	15	60			
kDNA	Negative	45	2	47	97	96	0.907±0.064
	Positive	0	13	13			
Total		45	15	60			

*All kappa calculations show highly significant (P<0.001).

CHAPTER FOUR

DISSCUSION

4.1 PARASITES CULTURE AND MICROSCOPY

In this study, of 7 culture attempts in the field only one was successful. This was due to problems with contamination (aseptic techniques in the field were not ideal as in the equipped laboratory) other problems were concerned with the conservation of adequate media, and with the maintenance of the optimal temperature under field conditions. Although the microscopic technique is rapid and very simple it has low sensitivity, probably due to low concentration of parasites in peripheral blood (Le Fichoux et al., 1999; Reithinger et al., 2002). Only one out of 60 smears confirmed positive for amastigotes for that, we exclude the microscopic examination from the comparison of diagnostic tools. However, neither direct examination of smears nor cultures may offer adequate sensitivity (Gatti et al., 2004)

4.2 SCREENING OF THE DOGS' SAMPLES BY ELISA

To investigate the role of domestic dogs as a potential reservoir for *Leishmania* parasites in the northern part of the West Bank, We sampled 148 dogs residing in four districts northern Palestine (Tubas, Jenin, Tulkarm, and Nablus). ELISA data presented in this study demonstrated that (6.8%) of domestic dogs residing in these areas have been infected

with *Leishmania*, this seroprevalence is identical to Baneth study (1998), where his total seroprevalence was 6.8% of his tested dogs in Central Israel. It must be stressed that the positive result from ELISA are not related with active parasitic infection (Iniesta et al., 2002). This technique detects host-produced antibody to the parasite antigens. Indeed antibody response in the host can be extremely variable and can depend on immunological status of the host being sampled. However, the meaning of asymptomatic but seropositive dogs undoubtedly, indicates previous contact with the parasite, but we don't know whether these dogs will subsequently develop the disease, or if they are only carriers. We know from other studies that when host inhabit disturbed environments where a natural cycle of *Leishmania* transmission takes place it can result in dramatic increase in disease incidence (Desjeux et al., 2001), it was clear from our work in these districts, particularly in Jenin where 9.2% of domestic dogs were seropositive , that nearly every household has at least one dog, the sero prevalence presented here was higher than 5.3% obtained in Jenin using a smaller numbers of dogs (Abdeen et al., 2002). However, the prevalence of CVL in the Mediterranean region varies from 1-37% with the infection rates increasing in some foci and disappearing in others (Baneth et al., 1998), so the total seroprevalence (6.8%) obtained in our study fits within this range. Those exposed dogs may present a source of living parasite to sand fly vector, and the close

association between human and dogs may be a risk factor for the increase in HVL in those areas. Since the annual prevalence of CVL in endemic regions frequently fluctuates and can vary among adjacent villages, additional surveys are needed in order to establish the trend of CVL in this region. However, this study suggests that there may be a correlation between the percentage of HVL cases in northern Palestine and the prevalence of CVL. Jenin district had the highest percentage of both HVL and CVL cases followed by Tulkaram, Tubas and Nablus. While more work is needed to confirm this correlation. This study demonstrates that the prevalence of *Leishmania* infection in Jenin district is higher than assumed and 70% of seropositive dogs were asymptomatic while 30% of them were symptomatic with external signs of leishmaniasis (hair and weight loss and onychogryposis) and may act as reservoirs for parasite transmission to sand flies. This information is essential to keep screening in this area for designing and implementing appropriate control measures since the inhabitants are predominantly with a low –income rate who can't depend on expensive treatment for their infected dogs.

4.3 CLINICAL SAMPLES

Our primary objective was to evaluate the use of PCR assay using peripheral blood to detect *Leishmania* in dogs, indeed this type of sampling has the advantages of being much less invasive, easily repeatable, and more acceptable to dog owners than bone marrow or lymph node collection. We also chose to work with the buffy coat portion since the *Leishmania* is an intracellular parasite of monocytes and macrophages and this approach has been validated in humans (Lachuad et al., 2000). Nevertheless, the DNA extraction methods from peripheral blood had to be optimized in order to reduce inhibition of PCR, which was probably due to certain factors such as EDTA and hemoglobin that remained after DNA extraction and blocked the PCR reaction (Hartman et al., 2005). In our study the results of kDNA-PCR-based testing of peripheral blood samples (using 13A/13B) were particularly encouraging (table 3.2), and allowed diagnosis based on routine samples of peripheral blood. We found that blood samples are a reliable clinical material type for the detection of *Leishmania* DNA by kDNA-PCR and this observation is in agreement with previous reports (Reale et al., 1999; Ikonomopoules et al., 2003).

4.4 SPECIES IDENTIFICATION AND GENOTYPING

L. infantum has been recognized as the most homogenous *Leishmania* species, while in our study the micro-heterogenity was readily demonstrable despite their very small number and restricted geographical region studied. However, all Old World CL strains (*L. major* and *L. tropica*) proved different from the *L. infantum* by using the ITS1-PCR RFLP, so we excluded them from kDNA analysis. At the same time ITS1-PCR RFLP showed no differences on the species level, while it was useful for *Leishmania* species typing. Micro heterogeneity was identified by kDNA-RFLP analysis by using 2 restriction enzymes (*RsaI, Hpa II*). Enzyme boost dose and two enzymes used on the same kDNA-PCR template proved stability and reproducibility of the cuts, in order to avoid inhibition or incomplete digestion. However, the kDNA PCR using Lmj4/Uni21 primers (fig. 3.5) and ITS1-PCR/RFLP (fig 3.4) analysis did not detect differences between *L. infantum* strains tested, so they were excluded from the genotyping analysis. Using *Rsa I* kDNA-RFLP analysis, the IVL isolate L773 from El-Yamon was also notably different from LD1 strain, which was from the next village Qabatiya and more similar to L807 isolated a few years ago from the same village El-yamon, this results give explanation that both originating from the same locality, and excluding the time effect. The dog strains themselves were not identical. The difference between the 2 strains from the same village is

noteworthy because they were collected from the same village with reservoir differences (L773 strain from infantile VL, L807 strain from Canine VL). The local strains, show high similarity (95%) by the pattern of the bands, while the Tunisian strain showed plus /minus bands on the gel, with high percentage of differences (20%) in comparison to the local ones. All above data was analyzed by constructing the evolutionary tree, heterogeneity and homology was obvious on that tree. The dendogram divided the strains in to 2 clades, placed L807, LD1, and L773 in one cluster, separating them from the Tunisian one, within the local strains LD1 and L807 show higher homology. The IPT1 appeared more different from the other strains used in this study, since it was isolated from Tunisia, the very far geographical range. The kDNA variation in RFLP analysis show lots of bands due to high number of kDNA minicircles. This study focus on VL disease in Palestine and uncover the disease dynamic, explaining the activation of latent foci with specific genotype due to human integration into the *Leishmania* life cycle (human-host-vector), this happen as a result of human civilization, building new settlements (Desjeux et al., 2001), and invading new areas unknown to be endemic. In conclusion, this study identifies the causative agent of CVL and compares it with local and reference WHO strains. CVL causative agent proved to be *L. infantum* by several traditional and advanced techniques. EF, zymodeme analysis and ITS1-PCR proved it is

the same species of parasite circulating in the Mediterranean area and responsible for CVL and HVL.

4.5 EVALUATION OF THE mITS1-PCR FOR DETECTION LEISHMANIAL DNA

We turned our attention to the ITS1-PCR results since several laboratories depend on this assay for VL diagnosis, for that we recently developed the mITS1-PCR by shortening the PCR product to ~200 bp using our newly designed 3' RmITS1 primer as described previously in materials and methods. The results of mITS1-PCR came as a surprise when applied on pre measured different concentrations of purified leishmanial DNA since we could detect PCR product down to 200 fg, corresponding to one parasite (Vitale et al., 2004), while the sensitivity of ITS1-PCR was only about 200 pg. Thus, by decreasing the DNA template to 200 bp instead of 300 bp we successfully enhanced the sensitivity by a factor 10^3, these results supported by application of our approach on peripheral blood samples obtained from domestic dogs, nine CVL cases were detected by the mITS1-PCR, while three cases only detected by ITS1-PCR this increased the sensitivity three fold. Our assay has proven to be highly specific, with a sufficient sensitivity, also has ability to differentiate between *Leishmania* species by RFLP analysis. Therefore, we recommend the use of mITS1 in laboratories that rely on ITS1-PCR

for VL diagnosis and species identification, since our newly developed assay may detect additional cases that otherwise remain undetected by ITS1-PCR.

4.6 COMPARISON OF DIAGNOSTIC METHODS

With the aim of finding the most efficient method for detecting the leishmanial DNA, we compared three PCR assays with a serological assay (ELISA), and assessed their sensitivities and specificities. A number of studies have compared PCR diagnosis, with conventional diagnostic techniques for VL. These studies have shown that kDNA-PCR is significantly more sensitive than the conventional parasitological methods (Reale et al., 1999; Ikonomopoules et al., 2003). However assay sensitivity varies considerably depending on the source of the DNA used for PCR. The fact that the density of *Leishmania* in the peripheral blood is low (Le Fichoux et al., 1999) may lessen the sensitivity of PCR detection. ITS1-PCR was shown to be significantly less sensitive in diagnosing CVL when DNA used for amplification was purified from either buffy coat or whole blood of seropositive dogs, as opposed to conjunctiva, spleen and lymph node biopsies (Strauss-Ayali et al., 2004). kDNA-PCR is especially useful in regions were only one species, *L. infantum*, causes leishmaniasis in dogs. PCR allowed the identification of dogs with both symptomatic, as well as asymptomatic disease, and was

useful in identifying infected dogs even before the appearance of serum antibodies to leishmanial antigen by ELISA.

In our hands, as expected kDNA-PCR using 13A/13B was found to be highly sensitive, identifying additional cases missed by either the ITS1-and / or mITS1 PCRs (table 3.2), the extremely high sensitivity of this PCR due to the presence per parasite, of multiple copies of kinetoplast DNA (~10,000 copies/cell) in comparison to nuclear DNA targets (a few hundred copies for the ITS1 region) (Reale et al., 1999; Marfurt et al., 2003; Pravizi et al., 2005). Also the 13A/13B primers, that we used, can detect a single or even less than one parasite (Reale et al., 1999; Vitale et al., 2004). However, nested PCR was shown to improve the sensitivity of ITS1 - PCR (Parvizi et al., 2005; Schonian et al., 2003). Other parameters in addition to copy number, such as secondary structure and amplicon size, can also affect PCR sensitivity (Marchetti et al., 1998). As we proved shortening the ITS1 fragment amplified from ~300 bp (ITS1-PCR) to ~200 bp (mITS1-PCR) increased the CVL assay sensitivity by three fold from 3 to 9 positive dogs, respectively. One advantage that both the mITS1- and ITS1-PCR share is that digestion of the amplicon by restriction enzymes, such as *Hae*III, is sufficient to distinguish between *Leishmania* species (Schonian et al., 2003). Using this PCR-RFLP analysis we successfully identified three and nine positive cases detected by ITS1-PCR and mITS1 respectively, they all

belonged to *L. infantum* species (fig. 3.15 and fig. 3.11.B). Using kDNA-PCR we could detect *Leishmania* DNA in eight seronegative dogs. This observation is in agreement with previous studies showing that PCR can detect parasite DNA in the tissues and/or blood of seronegative naturally infected (Solano-Gallego et al., 2001) or experimentally infected dogs (Strauss-Ayali et al., 2004). Experimental CVL shows that a 2-4 months delay occurs between parasite infection and ELISA seroconversion (Abranches et al., 1991; Strauss-Ayali et al., 2004). This delay also occurs in natural infections where dogs, parasite positive by culture, remain seronegative during long pre-patent periods (Quinnell et al., 2001). We are therefore confident that the 10 PCR positive / ELISA negative dogs were infected, but had not yet developed a humoral immune response against the parasite. Another explanation is that some dogs never seroconvert (Silva et al., 2001) and can be considered as asymptomatic carriers. The findings that two ELISA positive dogs were PCR negative suggests that both assays may be required to accurately assess CVL prevalence in endemic areas. Cross-reactions for this ELISA using sera from patients with Chagas's disease or African sleeping sickness are seen, however these diseases are not endemic to our region, therefore these two samples can not be considered false positives. This discrepancy is most probably that ELISA technique still detects only a humoral response to previous contact with the pathogen, so the antibody

levels that were detected by ELISA may be due to immunological memory rather than to the presence of the parasites in the host, especially in areas of endimicity. Another possible explanation for the negative PCR reactions in seropositive dogs is that the amount of parasites in peripheral blood is low and may fluctuate dramatically over time, especially in asymptomatic or oligosymptomatic dogs. ITS1-PCR using buffy coat DNA isolated from experimentally infected oligosymptomatic dogs showed significant fluctuations in the number of PCR positive dogs over a 30 day period following seroconversion (Strauss-Ayali et al., 2004). Quinnell et al. (2001) showed that the number of positive parasite cultures from bone-marrow peaked in naturally infected dogs two months post-seroconversion and then decreased. Similarly real-time PCR analysis of parasite load in the spleens of experimentally infected dogs showed them to peak one month post-infection and then decline (Strauss-Ayali et al., unpublished). On the other hand anti-leishmanial IgG antibody titers tend to remain high following seroconversion and may take years to decrease to negative levels even following drug treatment and cure.

Although a lot of difficulties are associated with the interpretation of the sero prevalence data, the main advantage of ELISA test are that large numbers of samples can be processed readily and inexpensively, so it may provide a standard tool for screening surveys.

4.7 GENERAL CONCLUSIONS

This study gave an idea about the role of domestic dogs in transmitting the disease and also points to the importance of the diagnosis and genotyping of CVL in the West Bank. ITS1 PCR-RFLP used for species typing with no intra species variation, while the kDNA PCR-RFLP, utilizing the primer pair Uni 21/Lmj4, was useful to check the polymorphism, in this study the microheterogenity was evident among the strains tested. The one strain that appeared more different from others was IPT1, a human VL isolated from Tunisia. Thus, parasite polymorphism may be attributed to variations among parasites from different geographical locations or clusters.

The data presented in this study indicate a successful attempt to satisfy the need for a more sensitive, specific, and rapid test for the diagnosis of CVL.

Serological techniques (e.g. ELISA) alone, which are used to estimate the infected dog population (Cardoso et al., 1998), are not a suitable tools for diagnosis of CVL, while PCR offers more accurate results. Indeed, the sensitivity and specificity of the PCR assay depends on several factors, including PCR primers, DNA extraction methods, source of clinical materials (Reithinger et al., 2000) PCR target and PCR target molecular size as we proved. The advantage of using blood sampling that it is less invasive than bone marrow, spleen, or lymph node aspirate, and samples

can be processed readily. This approach on blood samples has proven useful for the diagnosis and monitoring of VL in adults and children in the Mediterranean region (Sundar and Rai, 2002).

Further studies are under way in other VL endemic regions, particularly in the southern part of the West Bank, to validate the methodology and to evaluate the ability of PCR-RFLP to identify other *Leishmania* species.

Recommendation points

- We recommend to keep screening the potential reservoirs in the Jenin area, in order to eradicate and break the leishmaniasis cycle in this region, and to prevent or decrease the HVL incidence in the area.
- Continue spraying insecticides to eradicate the vector.
- Educate the locals by widening their knowledge of the disease using leaflets, seminars, and distributing education materials targeting the schools, so the people should know more about the disease and prevention methods.
- Establish a database on the disease for planning and evaluation.
- Regional clinicians must keep in their mind all infectious agents as potentials for abdominal enlargement and fever of unknown origin, as VL suspected referring them for Al-Quds University, Leishmania Laboratory Center for diagnosis as a reference laboratory.
- Palestinian Ministry of health should consider the *Leishmania* test at referred laboratory and implement appropriate control measures in endemic areas.

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