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Detection, Molecular Typing and Phylogenetic Analysis
Of *Leishmania* Isolated From Cases of Leishmaniasis
among Syrian Refugees in Lebanon

by

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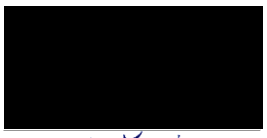
Signature: 

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I dedicate this work to my loving husband and son.

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Detection, Molecular Typing and Phylogenetic Analysis of Leishmania Isolated From Cases of Leishmaniasis among Syrian Refugees in Lebanon

By Tamara Salloum

ABSTRACT

Leishmania is a parasitic protozoan with more than two-dozen species causing the disease leishmaniasis. It is transmitted to humans through the bite of an infected female phlebotomine sand-fly. In the past two years the incidence of leishmaniasis has been drastically increasing in Lebanon. This was in parallel with the deterioration of the security in Syria forcing thousands to flee seeking shelter at refugee camps in Lebanon. Water, sanitation and hygiene facilities in those collective shelters are far from adequate, increasing the risk and turning those camps into fertile grounds for the spread of diseases. Cutaneous leishmaniasis (CL) is now considered a public health problem, but its epidemiology has not been fully elucidated. A crucial point in containing the disease is first *Leishmania* species identification and then correlation with clinical parameters. In this study, two molecular typing methods for *Leishmania* speciation were used along with sequencing and phylogenetic analysis. The parasites identified were all *Leishmania tropica* as determined by both the ITS1-PCR RFLP and the nested ITS1-5.8S rDNA gene amplification. ITS1-5.8S rDNA gene based typing proved to be more sensitive in the detection of parasites (positive in 70% of the isolates) as opposed to the ITS1-PCR RFLP

method that was successful in identifying *Leishmania* in only 47.4% of the isolates. Sequencing and phylogenetic analysis revealed high levels of heterogeneity among the sequenced isolates. To our knowledge, this is the first study using two different molecular methods for the detection and identification of *L. tropica* in Lebanon. The obtained results could be used for accurate detection and diagnosis of *Leishmania* in CL and for tracing possible changes in the population structure of *L. tropica*.

Key words: *Leishmania*, Cutaneous leishmaniasis, ITS1, 5.8S rDNA gene, PCR, RFLP, Sequence analysis, Lebanon.

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LIST OF ABBREVIATIONS

Bp: Base pair

CDC: Center for Disease Control and Prevention

CL: Cutaneous leishmaniasis

FFPE: formalin-fixed paraffin embedded

ITS: Internal transcribed spacer

L. tropica: *Leishmania tropica*

PCR: Polymerase Chain reaction

rDNA: Ribosomal DNA

WHO: World Health Organization

Chapter One

INTRODUCTION

Leishmania is a digenetic parasitic protozoan of the *Leishmania* genus, family Trypanosomatidae and Kinetoplastida order (Ramos et al, 2013). *Leishmania* parasites are transmitted to humans by phlebotomine female sand fly vectors (Teixeira et al, 2013) and are the causative agents of leishmaniasis. At least 21 *Leishmania* species have showed to cause disease in humans (WHO, 1990). The disease ranges from localized cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) to widespread visceral leishmaniasis infections (VL), also known as kala-azar, which is fatal if left untreated (Tsukamaya et al, 2008; Zhang et al, 2013). It is reported that the disease can be both anthroponotic and zoonotic (Ajouad et al, 2013). Signs of localized cutaneous leishmaniasis (CL) include papules, crusted nodules, plaques, or nodulo-ulcerative lesions. Transepidermal elimination (a skin perforating disorder) is often associated with cases of CL (Bolognia et al, 2008), especially with *L. donovani* (Karram et al, 2012). Atypical CL usually does not show these features and thus may be overlooked (Shirian et al, 2014). Leishmaniasis is especially aggravated in immune-deficient individuals (Nuwayri-Salti et al, 2012). CL is far from being a recent infection. In fact, the occurrence of CL has been detected since 2000 BC in Egypt (Oumeish, 1999) and 650 BC in Assyria (Bray, 1987). VL has been even detected in Egyptian mummies dating 2050-1650 BC (Zink et al, 2006).

The life cycle of *Leishmania* parasites is fairly complex and usually involves both vertebrate and invertebrate hosts. It can be divided into two developmental stages: During the first developmental form, *Leishmania* proliferate in the lumen of a female sandfly vector. At this stage they are referred to as promastigotes. During the second developmental form, *Leishmania* occurs as amastigotes and they proliferate inside several types of mammalian host cells, including humans. The details of the *Leishmania* life cycle are illustrated in Figure 1 (Teixeira et al, 2013).

Apart from the usual mode of transmission by the sand fly vector, it has been reported that *Leishmania* parasites can also be transmitted through the placenta (Meinecke et al, 1999), through sexual intercourse (Symmers et al, 1960), sharing needles (Magill, 1995), laboratory acquired infections (Herwaldt et al,1993) and blood transfusions (Dey et al, 2006).

All *Leishmania* species that are pathogenic to humans are able to cause cutaneous disease, though with varying severities (Van der Auwera & Dujardin, 2015). CL is the most common form of leishmanisis with more than 95% of the cases occurring in the Americas, the Mediterranean basin, the Middle East and Central Asia especially in the following countries: Afghanistan, Algeria, Brazil, Colombia, Iran (Islamic Republic of) and the Syrian Arab Republic. In fact, leishmaniasis is sometimes referred to as "Aleppo boil" in the medical literature (Hayani et al, 2014). According to the WHO, it is estimated that more than 0.7 million to 1.3 million new cases of CL occur worldwide each year whereas an estimated of 200,000 to 400,000 new cases of VL occur worldwide annually (WHO, 2014). Despite these numbers, leishmaniasis is still considered one of the

world's most neglected diseases with more than 350 million people at risk of contracting it (Ramos et al, 2013).

Some species, such as *L. major*, generally cause localized self-healing skin lesions. They are found in the Mediterranean region, Africa, the Middle East, and further, as far as India. Others, such as *L. braziliensis*, cause severe mutilating mucocutaneous leishmaniasis (MCL). They are usually endemic in South America (Reithinger et al, 2007). *L. donovani* can also lead to CL. It is mostly endemic in Sri Lanka (Karunaweera, 2009; Siriwardana et al, 2007). *L. infantum* (syn., *L. chagasi*), on the other hand, can lead to both CL and VL. It is usually found in Mediterranean countries and Brazil (Singh et al, 2011). The endemic appearance of *L. donovani* and *L. gerbilli* has been recently reported in China (Zhang et al, 2013). Similar outbreaks of *L. braziliensis* have been documented as well in South and Central America (Tsukamaya et al, 2008). However, CL cannot be always attributed to specific species. For instance, *L. braziliensis* have been also reported to cause mucosal leishmaniasis (ML) in addition to CL (Guerra et al, 2011). Since different *Leishmania* species have been shown to cause CL and due to population travel and migration, unexpected *Leishmania* species can appear in unexpected regions (Dujardin, 2006). What's more alarming, is that different species show different susceptibility to drugs (Blum et al, 2004). Moreover, the parasite has shown primary and secondary resistance due to low drug doses, insufficient treatment duration and substandard drugs (Desjeux, 2004). It is thus evident that efficient control of leishmaniasis, especially CL, requires accurate and fast species typing.

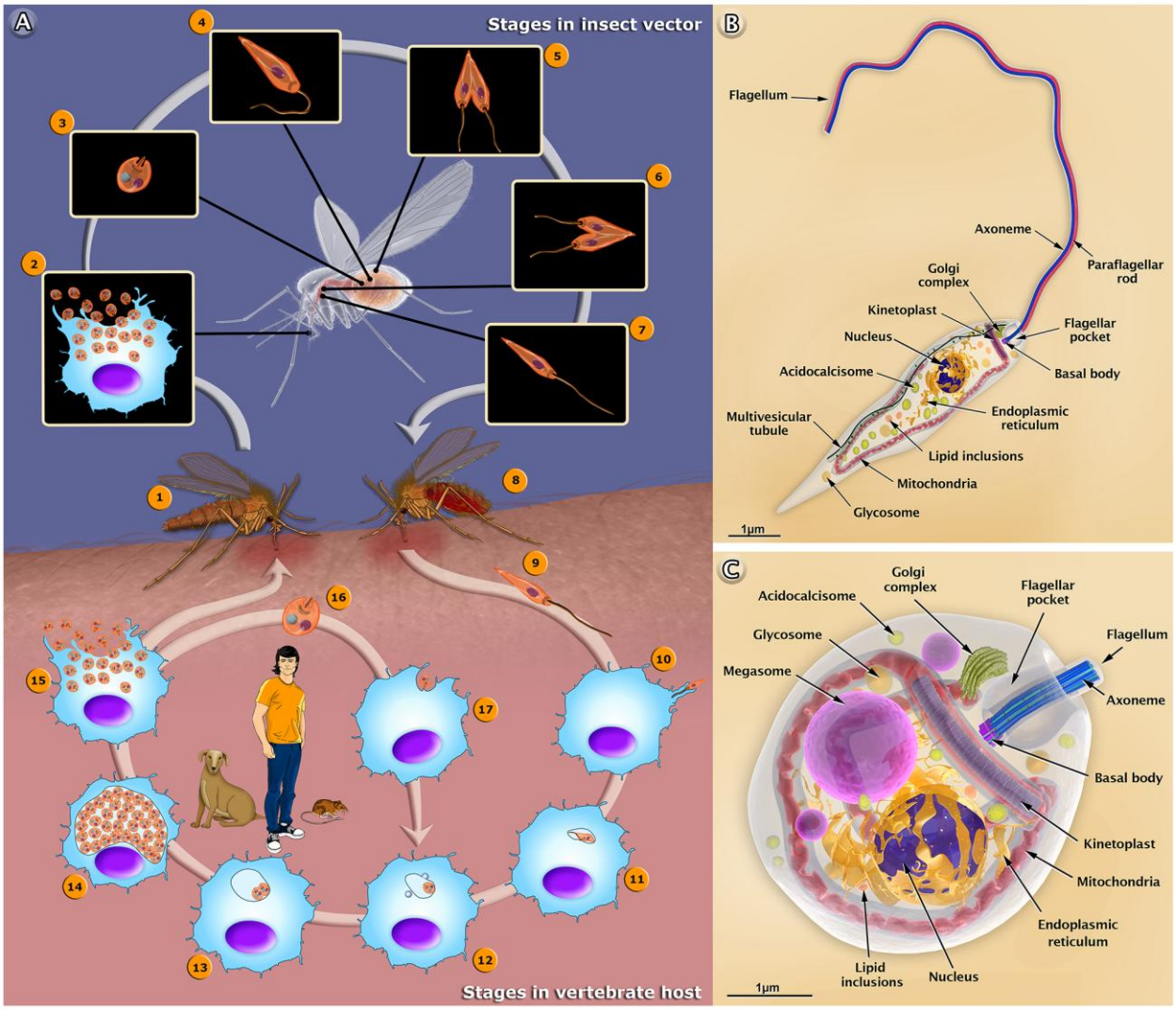

Figure 1. Illustration of the life cycle of *Leishmania* spp. (A), and a detailed structure of the promastigote (B) and amastigote (C). In (A), a female sandfly (1) bites an infected person during the blood meal. Macrophages become infected and harbor the amastigote form of the parasite (2). Infected macrophages (2) with amastigote forms (3). Amastigotes are transformed into procyclic promastigotes (4) that proliferate in the midgut of the sandfly (5). Then, they move into the stomodeal valve in the anterior midgut and activate cell division (6). Promastigotes now become infective metacyclic promastigotes (7). These are then released into a new host following a sandfly bite (8). Metacyclic promastigotes (9) infect macrophages (10) and transform into amastigotes (11). These attach to the membrane of the parasitophorous vacuole (12) and multiply (13). Following intense amastigote multiplication (14), the cell bursts (15). Some of the amastigotes (16) infect macrophages (17). In the central portion of the figure, the most important reservoirs involved in the maintenance of the parasite are illustrated. In (B) and (C), the schematic 3D representations of the organelles found in the *Leishmania* promastigote and amastigote are illustrated, respectively (Teixeira et al, 2013).

The currently available *Leishmania* classification is not entirely consistent. The main problem is due to the fact that some species are so closely related to each other that they are often considered to be the same. For instance, *L. infantum*, *L. peruviana*, and *L. panamensis* are considered to be subgroups of *L. donovani*, *L. braziliensis*, and *L. guyanensis* (Van der Auwera & Dujardin, 2015).

Another problem that makes species discrimination more difficult is the rare but clinically significant occurrence of natural interspecies hybrids (Tojal da Silva et al, 2006; Nolder et al, 2007; Odiwuor et al, 2011). That is in addition to sexual recombination that might be frequent at the intraspecies or population levels (Ravel et al, 2006). Therefore, proper discrimination at the species level, and sometimes at the sub-species level as well, is critical for both clinical and epidemiological reasons

Most of the CL *Leishmania* isolates in the Mediterranean area usually belong to *L. tropica* and *L. major* (Schonian et al, 2008; Karram et al, 2012; Yehia et al, 2012). Other clinical samples have also shown to be positive for *L. infantum* (Nakkash-Chmaisse et al, 2011; Yehia et al, 2012). Recently, leishmaniasis outbreaks have been reported in a number of Lebanese regions (Nakkash-Chmaisse et al, 2011). These coincide with the increase in the number of Syrian refugees in several rural areas. Since the beginning of the Syrian crisis in 2011, many Syrians have taken refuge in a number of Middle East and Gulf countries (UNHCR, 2014). An estimated 1,167,242 Syrian citizens have been registered in Lebanon since the beginning of the Syrian war in March 2011 making up over a quarter of Lebanon's population (UNHCR, 2015). Unofficial numbers suggest a count in excess of 2 million people making around 45% of the Lebanese population

(WHO, 2014). In 2010, it has been reported that more than 25,000 cases of CL occur in Syria yearly (WHO, 2010). Shockingly, these numbers increased to 53,000 cases in 2012 and 41,000 cases in the first two quarters of 2013 mainly due to the war related living conditions, the accompanying migrations of civilians and the lack of appropriate control measures (Ministry of Health, Syrian Arab Republic, 2013). Recently, Saroufim et al (2014) identified *L. tropica* in 85% and *L. major* in 15% out of 948 patients. Other studies conducted in Lebanon on 162 patients with CL proved that CL is associated in about 30% of the cases with systemic leishmaniasis, which was considered previously to be extremely rare (Nakkash-Chmaisse et al, 2011). The number of reported cases of leishmaniasis in Lebanon during the years of 2013-2014 and the distribution of *Leishmania* cases in Lebanon are depicted in figures 2 and 3, respectively. The current conditions of the affected individuals include poverty, malnutrition, population migration, weakened immunity, poor housing and unsanitary living conditions. All of these factors favor the spread of leishmaniasis (Desjeux, 2001).

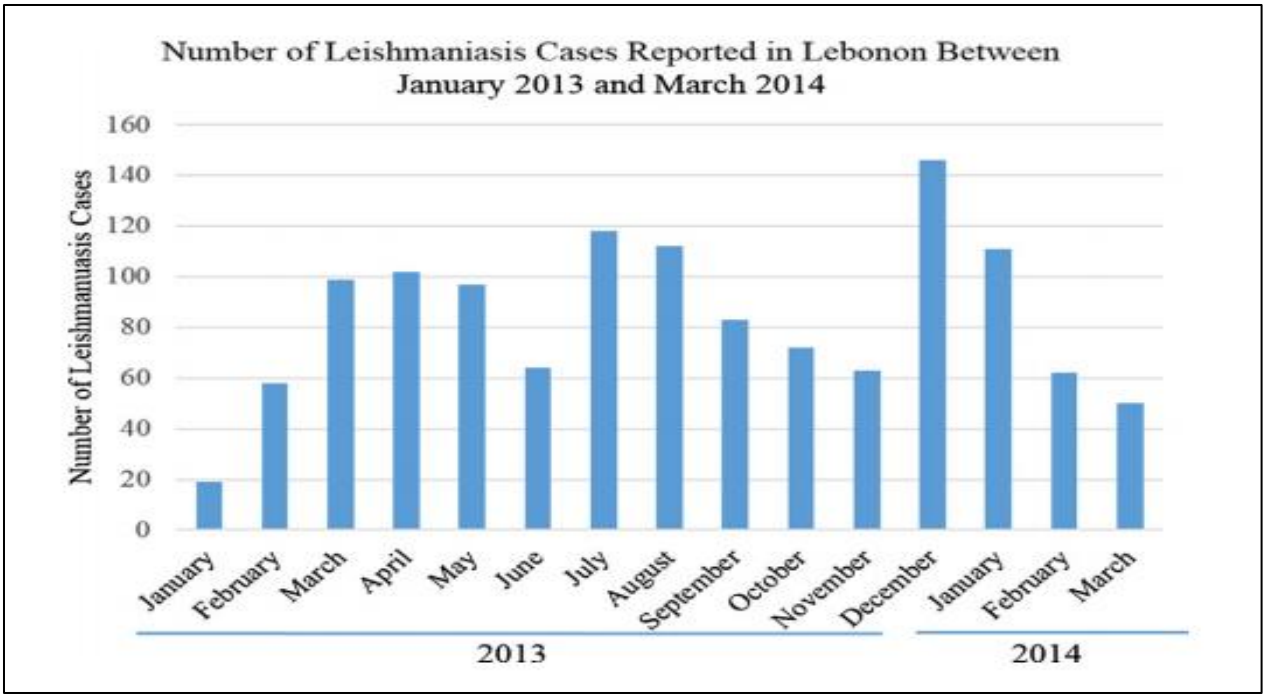

Figure 2. Reported cases of leishmaniasis in Lebanon per month from January 2013 through March 2014. Cases have continued to occur in 2014 at a high rate relative to the previous years (2001–2012) (Alawieh et al, 2014).

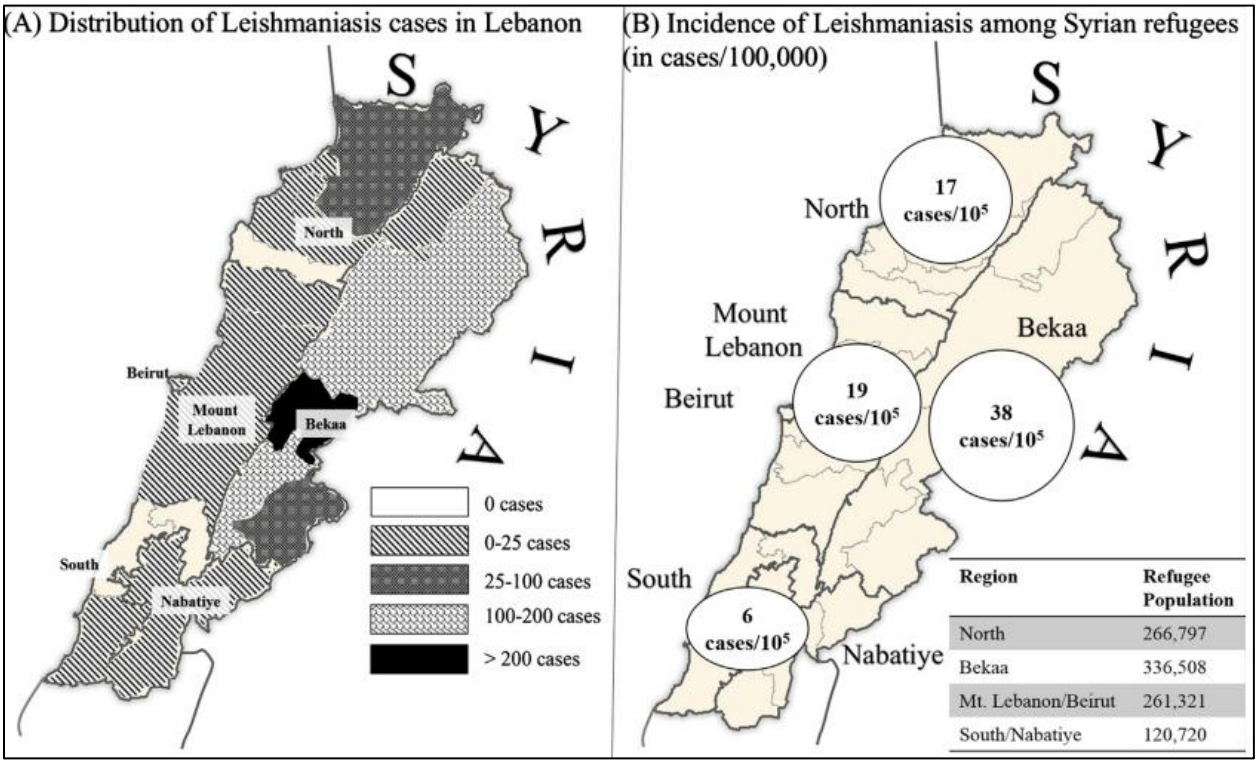

Figure 3. Map showing the distribution of *Leishmania* cases in Lebanon (A). Areas along the Syrian–Lebanese are the most affected. Map showing the incidence of leishmaniasis among Syrian refugees in different regions of Lebanon (B). Bekaa area had the highest incidence (Alawieh et al, 2014).

No absolute correlation between the clinical manifestation of the disease and the causative species has been confirmed (Lainson and Shaw, 1998). Thus, accurate differentiation of *Leishmania* and the species and sub-species levels is critical for treatment. Detection of *Leishmania* parasites by PCR amplification has been proven to be a rapid, highly sensitive and specific identification method (Garcia et al, 2005). Several PCR-based methods have been employed for typing *Leishmania* parasites. MLEE (Multilocus Enzyme Electrophoresis) is considered by many the gold standard for *Leishmania* identification (Banuls et al, 2007; Schonian et al, 2010). DNA sequencing, PCR-RFLP (Restriction Fragment Length polymorphism) and MLST (Multilocus Sequence Typing) have all been employed for this purpose (da Silva et al, 2010; Montalvo et al, 2010; Maiden et al, 1998). However, some genes such as *hsp70*, proved to be too conserved for intra-specific diversity discrimination (da Silva et al, 2010; Montalvo et al, 2010). Highly polymorphic markers, such as microsatellites are often problematic with taxonomic levels higher than the species, while most other methods target multi-copy genes that are often difficult to analyze (Boite et al, 2012). Moreover, PCR based methods used for the discrimination of *Leishmania* parasites are often hindered by low sensitivity results that fail to detect amastigotes in samples that proved to be positive by microscopy. This caveat in molecular typing methods is often due to a low parasite load in the original sample (Shahbazi et al, 2008).

The long term developmental benefit of typing of the obtained *Leishmania* species in CL is to identify the corresponding isolates with rapid and accurate PCR based methods and

trace their epidemiological and evolutionary origins. This allows us to get a better understanding of this parasite and have sufficient information for the subsequent development of new vaccines or other more efficient preventative methods for the control and prevention of leishmaniasis outbreaks.

The objectives of this study are:

- Identification and typing of *Leishmania* isolates obtained from Syrian refugees in Lebanon using two molecular typing techniques: the ITS1-PCR-RFLP and the nested ITS1-5.8S rDNA gene PCR both followed by sequencing and phylogenetic tree analysis.
- Comparing the efficiency of these two techniques in *Leishmania* identification.
- Assessing the evolutionary and phylogenetic relationships among these isolates.
- Assessing the DNA storage in formalin-fixed paraffin embedded (FFPE) tissues by evaluating the DNA quality and quantity of the extracted DNA.

Chapter Two

LITERATURE REVIEW

Different *Leishmania* species are known to display specific transmission patterns, to cause different clinical manifestations and might even have different reactions to administered drugs (Arévalo et al, 2007; Romero et al, 2001; Modabber et al, 2007; Buffet, 2010). The clinical manifestations of CL are broad and may mimic other inflammatory and neoplastic diseases (Saab et al, 2011). Transepidermal elimination was also identified in CL (Karram et al, 2012). The immune microenvironment in CL was recently evaluated (Jabbour et al, 2014). The choice of treatment is often guided by a combination of factors such as the clinical presentation of the disease, the host's genetic background and immunity, and other confounding factors (Reithinger et al, 2007). Thus, typing and correct identification of the infecting species is critical for accurate and rapid diagnosis (Gonzalez et al, 2010).

2.1. Leishmaniasis in Lebanon

Only few studies were conducted to evaluate the epidemiology of leishmaniasis in Lebanon. Nuwayri-Salti et al (2000) studied the prevalence of leishmaniasis in Lebanon between the years of 1993 and 1997 on a population sample of about 81,000 subjects (60% rural and 40% urban). They concluded that the prevalence of cutaneous leishmaniasis was only 0.18% in the rural versus 0.41% in the urban areas (Nuwayri-Salti et al, 2000). Visceral leishmaniasis was practically non-existent during that time period. Moreover, a significant difference was observed between the prevalence of clinical

disease and positive skin testing and/or antibodies. It was suggested that this difference is due to the presence of past or present subclinical disease. The prevalent *Leishmania* species belonged to the *L. donovani* complex. All the affected populations were also living under the burden of poverty (Nuwayri-Salti et al, 2000). Between 1962 and 1964, sporadic cases of VL were recorded in western Lebanon among children under the age of 10 years (Desjeux, 1991). Later on, parasites escaping the skin and invading the blood were detected by Nakkash-Chmaisse et al (2011) where *L. infantum* was identified in most of the studied cases.

The crisis in the Syrian Arab Republic that started in March 2011 has resulted in outbreaks of a large number of previously overlooked diseases in the country itself, as well as in neighboring countries including Iraq, Jordan and Lebanon (WHO, 2012). It is now certain that environmental changes, including the consequences of climate change, agricultural development projects or disasters, and population migrations may affect distribution of vector and rodent populations. These changes may favor the parasite transmission to new populations, and thus leading to outbreaks (WHO, 2014). It is worth mentioning that about 12% of the population in the Eastern Mediterranean countries, including Lebanon is living on less than 1 US dollar per day (WHO, 2012).

Syria is known to have the highest prevalence of CL in the Middle East and North Africa (MENA) region (WHO, 2013). In the literature, it is even referred to as 'Aleppo boil' after the Syrian city Aleppo (McDowell et al, 2011). Recently, due to the massive population migrations caused by the ongoing Syrian crisis, new leishmaniasis outbreaks have been reported in countries bordering Syria including Turkey, Iraq and Lebanon (Sharara & Kanj, 2014; Caglaravci et al, 2013; Alawieh et al, 2014). The United Nations

Refugee Agency (UNHCR) has registered around 985 346 Syrian refugees in Lebanon with the majority living in the Bekaa and North provinces (62%) (UNHCR; 2013) (Figure 3). However, it is reported that the true number of Syrian refugees in the country has exceeded one million (Burky, 2013). Facing the increasing risk of the leishmaniasis spread, the Lebanese Ministry of Public Health (LMOPH) has implemented active surveillance for leishmaniasis since the beginning of the Syrian conflict (Berry et al, 2013).

The current leishmaniasis situation in Lebanon in light of the increasing numbers of Syrian refugees in the country was evaluated by Alawieh et al (2014). The reported cases of infection in Lebanon per month from January 2013 through March 2014 were measured as depicted in figure 2 (Alawieh et al, 2014). Another study by Saroufim et al (2014) identified *L. tropica* in 85% (a species endemic to the Aleppo region in Syria) and *L. major* in 15% of 948 cases from a CL outbreak that began in September 2012 among Syrian refugees in Lebanon (Saroufim et al, 2014). Saab et al (2014) optimized a novel method for diagnosis of CL based on drop scrapings. Yehia et al (2012) identified *L. tropica* in 122 skin biopsies using conventional and nested ITS1-PCR.

An initial cure rate of 82% after 3 months of megulamine antimonite therapy was reported among CL patients (Saroufim et al, 2014). So far, no effective vaccine is available for *Leishmania*. Preventation is mainly achieved by limiting exposure to the vector through vector control, the use of insect repellents, insecticide-impregnated bed nets, and other modalities (Clem et al, 2010; Wang et al, 2012). A lack of evidence in the literature for treatment of several leishmaniasis infections is clearly observed (Hodiamont et al, 2014).

2.2. FFPE tissue storage

Biopsies of skin lesions that show cutaneous infections, such as leishmaniasis, are usually stored in formalin-fixed paraffin-embedded (FFPE) blocks (Ludyga et al, 2012). It is generally thought that FFPE tissues can be stable for long periods of time (Abrahamsen et al, 2003). They are relatively easy to handle and require a low cost (Kayser et al, 1988; Perlmutter et al, 2004). However, the main problem with FFPE is the obvious decrease in PCR efficiency due to cross-linking and nucleic acid degradation that increases significantly with time, thus yielding low DNA concentrations (Gilbert et al, 2007). Oftentimes, the biopsies are taken for histological staining and analysis. The cellular composition and features of the pathogen are stable enough to suite this purpose. However, DNA starts to degrade the moment the biopsy is taken until it is paraffin fixed in a laboratory. This is especially true for *Leishmania* DNA that has been shown to degrade quickly after the death of the parasite (Prina et al, 2007). Moreover, if crosslinks are present, negative PCR results can still be obtained despite a high DNA yield, simply because the polymerase "falls off" the DNA strand at every crosslink. Standardized techniques for DNA extraction from FFPE are yet to be determined (Ludyga et al, 2012).

2.3. Criteria for species typing methods

The search for a universally accepted typing method for *Leishmania* discrimination has been going on for decades (Kirk, 1949). Recently, seven criteria have been designed for the purpose of finding an ideal typing tool for the clinical purposes of *Leishmania* identification (Van der Auwera & Dujardin, 2015). First, a species typing tool has to be able to discriminate all the variety of *Leishmania* species available in the studied region.

This basic criterion is often overlooked when using new techniques or markers, because these are often validated on reference strains not belonging to the studied area. Thus, they often give unreliable results (Van der Auwera & Dujardin, 2015). Second, the employed typing method should preferably be globally applicable and that is to accommodate all the species that might unexpectedly appear in one or more regions around the world (Hashiguchi et al, 1991; Katakura et al, 1993; de Oliveira et al, 2009). Third, the assay must provide enough sensitivity, especially for typing that starts with culture-independent steps. Fourth, the applied testing methods have to be *Leishmania* specific. This aims to avoid any unwanted cross-reactions with other microorganisms that might be co-infecting a single patient. This is particularly important in HIV patients in whom several trypanosomatids can be encountered (Chicharro and Alvar, 2003) as well as co-infections such as mycobacteria and *Schistosoma* (Bañuls et al, 2007). Fifth, the employed technique has to be standardizable. It is easier to standardize a PCR based method than, for instance, RFLP where small size differences and incomplete digests may interfere with the results analysis. This also proves especially difficult in species-specific PCRs, hybridization assays, and melting assays (Van der Auwera & Dujardin, 2015). Sixth, the used method has to be only applicable in the setting where it provides the most optimal results. And finally, the seventh criterion emphasizes on the fact that the assays used has to be validated. This quality check point has to be applied on both *Leishmania* and non-*Leishmania* parasites (Van der Auwera & Dujardin, 2015).

2.4. An overview of the most commonly used *Leishmania* typing methods

During the time period of 1989 to 2007, more than 700 articles on PCR diagnosis of *Leishmania* species have been published in the Pub-Med database. These report a variety of gene targets, protocols, and applications and including genus and/or species-specific PCR, ranging from low-tech to high-tech approaches (Reithinger & Dujardin, 2007**)**. Now, only eight years later, these numbers have almost doubled with more than 1300 articles available on Pub-Med database. PCR-based assays currently constitute the main molecular diagnostic approach for the detection and identification of *Leishmania* parasites in clinical samples (Reithinger & Dujardin, 2007**).**

Several methods have been described for *Leishmania* discrimination in clinical, environmental, or cultured samples (Van der Auwera & Dujardin, 2015). Serological based testing does not provide enough sensitivity because of the strong Th1 bias. Therefore, molecular methods are superior, especially when the parasite load is low (Reithinger & Dujardin, 2007). PCR based methods, have targeted different genes to suite this purpose. However, in terms of sensitivity and validation, the ITS1 region offers the best resolution for *Leishmania* discrimination in the Old World (Odiwuor et al, 2011). The *hsp70* and the cytochrome *b* genes have been found to be globally acceptable as well (Folgueira et al, 2007; Alves da Silva et al, 2010; Luyo-Acero et al, 2004). Also, the miniexon has shown a sufficient resolution globally speaking, but has a cumbersome downstream sequencing (Marfurt et al, 2003). MLEE (Multilocus enzyme electrophoresis) is the only method currently available that has been applied to all species in epidemiological and environmental studies (Bañuls et al, 2000). So far, no other method has been used on the entire *Leishmania* genus. However, further testing is needed

to include species exclusively found in sand fly vectors or animals (Van der Auwera & Dujardin, 2015).

2.4.1. PCR-based typing techniques

Given the fact that microscopically identifiable parasites are absent in up to 47% of the cases, molecular testing for *Leishmania* has become a reliable diagnostic tool in clinical microbiology (Swick, 2012). PCR amplification methods are thought to be highly efficient techniques for identification of *Leishmania* species and these include PCR-RFLP, PCR-ITS, and RAPD (Random amplified polymorphic DNA) (Ajouad et al, 2013). Other methods are also available such as multilocus enzyme electrophoresis (MLEE), which is thought to be costly and time consuming (Zemanova et al 2006), multilocus microsatellite typing (MLMT) and multilocus sequencing typing (MLST) (Tsukamaya et al, 2008). When applying any of the above mentioned methods, it is important to consider the level of resolution targeted by each of these techniques. If a specific marker detects too much variation, an accurate representation of distantly related species will not be achieved and useful traits might be overlooked. On the other hand, if a marker does not provide enough variability, it will fail to discriminate closely related isolates (Schonian et al, 2011).

Leishmania parasites contain only 10^{-13} g of DNA, meaning that 10 million parasites would be required to generate 1 ug of workable DNA (Barker, 1989). This makes gene detection in *Leishmania* isolates extremely difficult. In order to obtain sufficient amount of DNA for further analysis, the process of DNA extraction has to be critically efficient.

Mouttaki et al (2014) demonstrated that 13A/13B primers targeting kinetoplast DNA (kDNA) were able to detect 0.1 pg of leishmanial promastigote cells per PCR tube and thus were significantly more efficient than other reported primers. This may be explained by the fact that *Leishmania* promastigotes contain approximately 10,000 copies of the kDNA target which is 50 and 250 fold higher than ITS1 target regions (40 to 200 copies) (Mouttaki et al, 2014). Anders et al (2002) found that the Lmj4/Uni21 PCR, also targeting kinetoplast DNA (kDNA), could detect 0.25 parasites. Ajouad et al (2013) reported that nested PCR of the ITS1-5.8S rDNA region allowed the detection of 1pg/uL of DNA as opposed to the ITS1-PCR method that detected 10 pg/uL of extracted DNA. Hence, the observed differences in sensitivity can be due several variable parameters within the PCR assays such as the quality of the clinical samples, the DNA extraction method that largely determines the obtained DNA quality and quantity and finally, the Taq polymerase used as well (Anders et al, 2002).

2.4.2. rDNA array

Ribosomal DNA (rDNA) array has been found to be one of the most efficient species typing targets. In fact, 10 to 20 copies of rDNA are tandemly repeated per haploid genome of *Leishmania*. Additionally, each repeat unit consists of several genes and spacers (Martinez-Calvillo et al, 2001; Inga et al, 1998). Within the array, primarily the spacer regions provide enough variability for species discrimination. They have also been reported to be used for discrimination at the subspecies level (Schönian et al, 2000; Schönian et al, 2001; el Tai et al, 2000). Thus, ITS-PCR is often used in molecular typing because ITS (rRNA internal transcribed spacers) are subjected to less evolutionary

pressure and thus show more sequence divergence than other coding regions in the genome (Almeida et al, 2011).

Large amount of evidence has proven that the ITS region in *Leishmania* consists of at least two alleles. A study conducted by Ghatee et al (2013) revealed a 100 bp gap in the ITS sequences of *L. tropica* isolated from Iran. The entire ITS1-5.8S-ITS2 sequence in rDNA was amplified using LITSV and LITSR primers. The additional 100 bp fragment was then excluded by designing new primers: LITSR and LITS-MG (Ghatee et al, 2013). Also, Mauricio et al (2004) reported at least two alleles in ITS of *L. tropica* based on RFLP fragments size that showed to be larger than the actual amplification product.

The difference in the alleles of the ITS sequence proposes possible recombination in *L. tropica*. In fact, recombination has been previously detected in the ITS region. For instance, the enzyme (N-Acetyl Glucosamine-1-Phospate Transferase) was subjected to recombination in *L. tropica* (Waki et al, 2004). The presence of significant heterogeneity and genetic variation in some *Leishmania* species, mainly in *L. tropica* (Shonian et al, 2001) strengthens the hypothesis of both recombination and sexual reproduction. This might have a major influence on drug resistance and pathogenicity in these isolates.

2.4.3. Available primers

Different studies use different sets of primers that flank various regions within the ITS. A list of the used primers that target the ITS region in *Leishmania* is summarized in table 1(Guerbouj et al, 2014). For instance, the primer pair L5.8S and LITSR has been used to target the entire ITS-1 region (Schonian et al, 2003) while the primers IR1 and IR2 followed by nested PCR using ITS1F and ITS2R4 target the ITS1-5.8S rDNA gene

region (Figure 4) (Ajouad et al, 2013; Parvizi et al, 2005; Parvizi et al, 2008; Cupollilo et a, 1995).

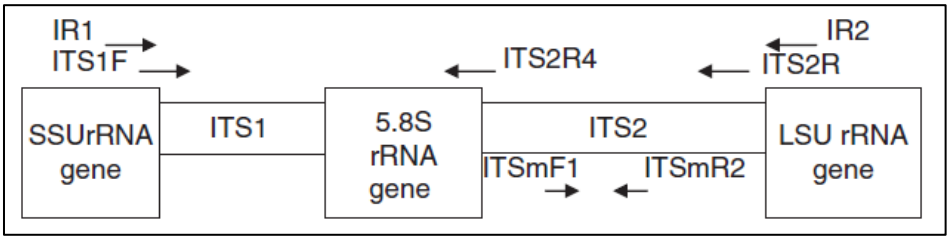

Figure 4. Schematic representation of the different ITS regions covered by primers in nested-PCR in *Leishmania***.** SSU=small subunit gene, LSU= large subunit gene (Parvizi et al, 2008).

2.4.4. ITS1-PCR

ITS1 region is located between the 18S rRNA and 5.8S rRNA genes (Cupollilo et al, 1995). The major advantage of using the ITS1-PCR typing method for species identification is that the obtained PCR amplicons can be further digested and distinguished by RFLP. However, several studies have concluded that ITS1-PCR followed by RFLP does not provide enough sensitivity to accurately diagnose cases of leishmaniasis (Schonian et al, 2003; Bensoussan et al, 2006; Azmi et al, 2011). Thus, nested ITS1-5.8S rDNA gene PCR showed to provide better means for species discrimination (da Silva et al, 2013; Ajouad et al, 2013; Parvizi et al, 2008; Yehia et al, 2012). Also, high-resolution melting analysis has been developed based on a 265- to 288-bp fragment within ITS1 (Talmi-Frank et al, 2010). Following PCR, the temperature at which both DNA strands separate was recorded, and this temperature was found to be species specific. Similar to the results obtained from RFLP analysis, the Old World species were all distinguishable, with the exception of *L. infantum* from *L. donovani*. Spanakos et al (2008) depicted the use of sequencing, RFLP, and single-strand conformation polymorphisms for species discrimination in the Old World. They focused

on a specific ITS1 fragment that largely overlaps the LITSR-L5.8S region. They found the same level of species discrimination as provided by the LITSR-L5.8S region but these methods were not as extensively validated (Spanakos et al, 2008**)**. Other examples based on ITS1 sequencing include the studies performed by Parvizi and Ready. They also used primers covering the LITSR-L5.8S target. But their fragment extended into the 5.8S rDNA gene. However, this did not show any advantage for species identification (Parvizi and Ready, 2008). Others further extended this region into the small-subunit (SSU) rRNA. This strategy achieved typing of *Leishmania* species in the New World by RFLP analysis, but failed to discriminate all species and all complexes (Rotureau et al, 2006).

2.4.5. ITS2 typing

Several studies covering the either the ITS2 region or both the ITS1 and the ITS2 regions have been reported (Cupolillo et al, 1995; de Almeida et al, 2011). PCR-RFLP analysis of a fragment covering both ITS1 and ITS2 proved to be successful in identifying all tested species of the *Leishmania* (*Viannia*) (Cupolillo et al, 1995). However, the main problem with this strategy is that it requires 10 enzymes for DNA product digestion and sequencing proved to be cumbersome due to microsatellite regions in ITS2 (Parvizi and Ready, 2008). Recently, Almeida et al reported an ITS2-based assay that could successfully separate three tested *Leishmania* (*Viannia*) species (de Almeida et al, 2011). Since targeting the ITS2 region did not show any noticeable advantage over ITS1, it has been less used and evaluated. However, it might be useful in complementing the ITS1 region for discriminating species of the *Leishmania* (*Viannia*) subgenus (Van der Auwera & Dujardin, 2015).

2.4.6. Nested PCR

Nested PCR is a modification of conventional PCR that aims to reduce non-specific binding on the DNA template that might arise due to the erroneous amplification of unexpected primer binding sites. For this purpose, nested PCR involves two sets of primers, used on two successive PCR runs, where the second set of primers binds to and amplifies a more specific region located internally to the first run product (Figure 4) (Parvizi et al, 2008). Nested PCR provides a sensitive and specific alternative to traditional techniques used for *Leishmania* identification (Akahavan et al, 2010). Although often tedious and time-consuming, nested PCR has been widely employed in *Leishmania* identification methods (Parvizi et al, 2008; Schonian et al, 2006). This aims to overcome the problems of low DNA concentrations usually observed in *Leishmania* parasite samples and to enhance the sensitivity of subsequent PCR-based analysis (Ajouad et al, 2013). Nested ITS1-5.8S rDNA gene PCR provided enough sensitivity for the discrimination of most clinically relevant *Leishmania* species (da Silva et al, 2013; Ajouad et al, 2013; Parvizi et al, 2008; Yehia et al, 2012).

2.4.7. RFLP

One of the most widely used techniques for speciation and sub-speciation of *Leishmania* isolates is RFLP (restriction fragment length polymorphism) analysis. Here, the PCR product is digested with one or several restriction endonucleases and generates differently sized DNA fragments. The sizes of the obtained bands allow identification of the parasite (Van der Auwera & Dujardin, 2015). *HaeIII* restriction enzyme is the most commonly used. However, it does not allow the discrimination of members of the *L. donovani*

complex (*L. donovani* and *L. infantum*) nor the *L. braziliensis* complex (*L. braziliensis*, *L. guyanensis* etc.) because these show almost identical RFLP profiles (Schonian et al, 2003; Morales et al, 2001). In the New World, the gene encoding the heat shock protein of 70 kDa (*hsp70*) has been used as a target for PCR-RFLP (Garcia et al, 2004). Also, *Hsp70* PCR-RFLP with different restriction endonucleases has been employed for identification of Old World and New World *Leishmania* species (Fraga et al, 2010; Montalvo et al, 2010; Khanra et al, 2012).

2.4.8. Sequencing analysis

A more informative method is the sequence analysis of the PCR amplicon. The subsequent identification of single-nucleotide polymorphisms (SNPs) or comparison of the obtained sequence with available reference sequences allows for species identification and tracking of evolutionary relationships among the sequenced isolates (Van der Auwera & Dujardin, 2015). In comparative sequence analysis, it is crucial to select appropriate reference sequences which are often difficult to find because recently it has been shown that several species designations in the public databases are in fact erroneous (Van der Auwera et al, 2013). Thus, as it was performed in this study, a combination of different assays provides more information for species discrimination and thus decreases the chance of errors.

In this study two molecular typing methods are performed for the detection and identification of *Leishmania* parasites in cases of cutaneous leishmaniasis. First the FFPE storage method is assessed and then for species identification the ITS1-PCR RFLP and the nested ITS1-5.8S rDNA gene PCR followed by sequencing and phylogenetic analysis

are performed. Finally, to rule out that the observed variability between the two techniques is not due to DNA quality, DNA quality assessment is conducted.

Chapter Three

MATERIALS AND METHODS

3.1. Sample collection and description:

A total of 40 FFPE blocks were obtained from the American University of Beirut Medical Center in Lebanon. FFPE blocks contained skin biopsies from patients suspected of having cutaneous leishmaniasis during the years of 2013-2014. Each patient had one biopsy performed, and one FFPE block per biopsy was available. Cases included in the study were restricted to cutaneous lesions of patients who did not receive treatment prior to the biopsy. Clinical information pertaining to the lesion was also collected including: number, duration, location and dermatologic appearance. In addition, the patient's age, gender and country of residency were tabulated (Figure 5, Figure 6, Annex I). Organisms collected from cultures were used as positive controls to validate the analysis. IRB approval was granted prior to the initiation of this study (PALM I.K.01).

3.2. Histopathology

Sections from each of the 40 FFPE tissue blocks were previously stained for hematoxylin and eosin, Giemsa, Acid Fast bacilli, Gomori Methylamine Silver and Periodic Acid-Schiff. All cases were reviewed by four pathologists (JS, RK, FF and IK) and classified according to the modified Ridley's parasitic index (Table 1 and Figure 7).

Table 1. Modified Ridley's parasitic index.

1+	1 or more amastigotes per standard section
2+	10 or more amastigotes per standard section
3+	100 or more amastigotes per standard section
4+	1000 or more amastigotes per standard section
5+	10,000 or more amastigotes per standard section
6+	100,000 or more amastigotes per standard section

3.3. DNA Extraction

Eight 10 μm ribbons were obtained from each FFPE block. Then 400 μL of cell lysis solution (Qiagen Gmbh, Hilden, Germany) and 1.5 μL of proteinase K were added (Fermentas, Canada). Excess paraffin was trimmed off. After brief vortexing, the samples were incubated overnight at 65°C. After incubation, the tubes were centrifuged at 13,000 g for 10 min. A paraffin layer formed at the top of the tube. With a pipette, the layer was gently punctured and the liquid aspirated and transferred to a new autoclaved 1.5 ml microcentrifuge tube. The samples were then cooled on ice for 1 min and 133 μL of protein precipitation solution (Qiagen Gmbh, Hilden, Germany) were added. This was followed by vortexing for 20 sec and leaving the samples on ice for 30 min. The samples were then centrifuged at 20,000 g for 10 min. Supernatant was transferred to a new 1.5 mL microcentrifuge tube and 400 μL of 100% isopropanol and 1 μL of glycogen solution (Qiagen Gmbh, Hilden, Germany) were added. After centrifugation for 15 min at 20,000 g, the DNA appeared as a white pellet. 200 μL of 70% ethanol were added to the pellet and then centrifuged at 20,000 g for 10 min.

The supernatant was poured off and the sample dried at room temperature for approximately 10 min. Finally, 30 μL of Elution Buffer (Qiagen Gmbh, Hilden,

Germany) were added and the tube incubated at 50°C for 30 min with vigorous vortexing every 10 min. The resultant DNA was then quantified with NanoDrop ND-100 spectrophotometer and Qubit fluorometer (Thermo Fisher Scientific, USA) and stored at 4°C for subsequent PCR analysis.

3.4. ITS1 PCR of *Leishmania* isolates

The extracted DNA from FFPE blocks were examined for the *Leishmania*-specific ribosomal ITS1 region by PCR amplification using L5.8S and LITSR primers followed by RFLP analysis. The volume of DNA to be amplified was calculated to normalize the quantity among all isolates. 5 uL of DNA was used for ITS1 gene amplification in a 50 uL total reaction volume. A master mix was prepared consisting of 0.2 mM dNTPs, 1 X Taq buffer, 1.5 mM $MgCl_2$, 500 nM of both the forward and the reverse primers and 2 U of Hot-Start Taq Gold DNA polymerase. The cycling conditions were 95°C for 12 min followed by 32 amplification cycles, each consisting of three steps: denaturation at 94°C for 20 sec, annealing at 53°C for 30 sec and extension at 72°C for 1 min, followed by a final extension at 72°C for 6 min in the thermocycler. All polymerase chain reaction (PCR) assays were performed on a PerkinElmer GeneAmp 9700 thermal cycler (PerkinElmer, Wellesly, Massachusetts).

Nested PCR was performed on all isolates that were negative by conventional PCR. 10 uL of the ITS1 PCR amplicon were amplified in a 50 uL total reaction volume using the same primers and the same cycling conditions.

PCR amplicons were analyzed by 1.5% agarose gel electrophoresis (1.5% TAE gels stained with ethidium bromide). 25 uL of loaded PCR products were electrophoresed at

90 V in 1X TAE buffer (400mM Tris-acetate, 10mM EDTA, pH: 8.2–8.4) and compared to a standard 100 bp DNA ladder. A DNA band of 300-350 bp was considered as a positive indicator for the presence of *Leishmania*.

Table 2. The primers used in this study, their corresponding sequences and target amplicon size**.** AS, antisense primer; ITS1, internal transcribed spacer 1; PCR, polymerase chain reaction; S, sense primer.

Targeted Gene	**Primer Sequences**	**Amplicon size**
LITSR	5'-CTGGATCATTTTCCGATG-3'	300-350 bp
L5.8S	5'-TGATACCACTTATCGCACTT-3'	
IR1	5'-GCTGTAGGTGAACCTGCAGCAGCTGGATCATT-3'	320 bp
IR2	5'-GCGGGTAGTCCTGCCAAACACTCAGGTCTG-3'	
ITS1F	5'-GCAGCTGGATCATTTTC-C-3'	400 bp
ITS2R4	5'-ATATGCAGAAGAGAGGAGGC-3'	
Actin	S 5'-CGC TGC GCT GGT CGT CGA CA-3'	600 bp
	AS 5'-GTC ACG CAC GAT TTC CCG CT-3'	
GAPDH	S 5'-TGGTGCTCAGTGTAGCCCAG-3'	110 bp
	AS 5'-GGACCTGACCTGCCGTCTAG-3'	

3.5. RFLP

10 uL of the obtained PCR products were digested with 2 uL of the restriction endonuclease BsuRI (HaeIII) in 2 uL of 10X Buffer R (Thermo Fisher Scientific, USA) (1X of buffer R consists of 10 mM Tris HCl (pH 8.5 at 37°C), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA) and 18 uL H2O. The samples were mixed gently and spun down.

Digestion was performed in a thermal cycler, in a total reaction volume of 32 uL, with the following conditions: 6h at 37° C followed by 20 min at 80°C. 25 uL of the PCR product were run on 2.5% agarose gel in 1X TAE (0.04M Tris-acetate, 1mM EDTA, pH=8) for 1 h. The obtained bands were compared to 100bp and 20 bp ladders.

3.6. Nested PCR of ITS1-5.8S rDNA genes of *Leishmania* isolates

The previously extracted DNA was further screened for infection with *Leishmania* by targeting the ITS1-5.8S rDNA gene region. This step consisted of two stages of amplification: The first was performed using the forward IR1 and the reverse IR2 primers, while the second was performed with the nested-forward ITS1F and the nested-reverse ITS2R4 primers (Table 1). In the first stage of amplification: A total reaction volume of 20 µl was prepared of 1X PCR reaction buffer, 1.5 mM of $MgCl_2$, 60 µM of each dNTP, 1 µM of IR1 and 1 µM of IR2 primers, 1 U of Taq polymerase and 2 uL of the template DNA. PCR was performed under the following conditions: initial denaturation at 95°C for 12 min followed by 39 cycles consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 90 sec. The extension step was further continued for 10 min after the last cycle.

In the second stage: The products obtained from the first PCR were further amplified by the nested-PCR technique. A total reaction volume of 20 µl was prepared consisting of 1X PCR reaction buffer, 1.5 mM of $MgCl_2$, 60 µM of each dNTP, 1 µM of ITS1F and 1 µM of ITS2R4 primers, 1 U of Taq polymerase and 2 µL of the previously obtained PCR product. PCR was performed under the same conditions as the 1^{st} stage of amplification.

The obtained band were electrophoresed on a 1.5% agarose gel in 1X TAE buffer and compared to a 100 bp ladder.

3.7. PCR Product Purification

The obtained PCR products of both PCR reactions, targeting the entire ITS1 and the ITS1-5.8S rDNA gene regions, were purified using ExoSAP-IT (Thermo Fisher Scientific, USA).

3.8. DNA Sequencing Reaction

The amplicons were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA). Two sequencing reactions were performed for each isolate. The sequencing reaction consisted of the BigDye premix, 0.2 pmol of either forward or reverse primer, and the cleaned PCR product in a total volume of 10 µl. The same primers used in the PCR were used for sequencing. All sequencing reactions were performed with 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min. PCR products were sequenced by Genetic Analyzer 3500 (Life Technologies, USA) using the BigDye XTerminator purification kit (Applied Biosystems, USA).

3.9. Sequence Analysis and Phylogenetic Tree

Sequences obtained were analyzed on CLC Main Workbench V3.1 (CLC BIO, Denmark) software. Sequences were aligned and a phylogenetic tree was constructed using the Neighbor-Joining algorithm in CLC Main Workbench v.7.0.3 and bootstrap values for 100 replicates were indicated.

Sequences obtained from both procedures were compared to seven references extracted from NCBI BLAST (Accession numbers: KJ567476, KJ567477, KJ567478, KJ567479, KJ567480, KJ567481 and KJ567482). References used cover the ITS 1, partial sequence; 5.8S rRNA gene, complete sequence and the ITS 2, partial sequence (Table 4).

Table 3. The sizes and G+C content of the reference samples included in the phylogenetic trees. References were extracted from NCBI BLAST. References used cover the ITS 1, partial sequence; 5.8S rRNA gene, complete sequence and ITS 2, partial sequence.

Reference	**Size (bp)**	**G+C content (%)**	**GenBank accession no.**
L. tropica	390	44.10	KJ567476
L. tropica	390	44.36	KJ567477
L. tropica	390	44.10	KJ567478
L. tropica	390	45.90	KJ567479
L. infantum	391	43.73	KJ567480
L. infantum	392	43.62	KJ567481
L. infantum	392	43.88	KJ567482

3.10. DNA quality assessment

In order to assess the DNA quality of the extracted DNA, Actin and GAPDH amplification were performed under the following conditions: A total reaction volume of 50 uL was prepared consisting of 0.2 mM dNTPS, 1 X Taq buffer, 1.5 mM $MgCl_2$, 500 nM of both forward and reverse primers and 2 U of Taq DNA polymerase. For actin, 10 uL of DNA were amplified under the following cycling conditions: initial denaturation at

95°C for 12 min followed by 35 cycles consisting of denaturation at 94°C for 20 sec, annealing at 56°C for 30 sec and extension at 72°C for 1 min. Followed by a final extension step of 72°C for 10 min. For actin, 10 uL of DNA were amplified under the following cycling conditions: initial denaturation at 95°C for 12 min followed by 40 cycles consisting of denaturation at 94°C for 20 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. Followed by a final extension step of 72°C for 10 min.

25 uL of the obtained PCR product were loaded on a 1.5% agarose gel and compared to a 100 bp and a 20 bp ladders.

Chapter Four

RESULTS

4.1. DNA Extraction

Skin biopsies from 40 patients from Lebanon and Syria with clinical diagnosis of suspected cutaneous leishmaniasis were assessed. The specimen was obtained by either shave or punch biopsy, and the duration of the lesions ranged from 1 to 12 months (mean: 4.61 ± 2.36 months).

Ages of patients varied from 1 to 83 years (mean: 25.90 ± 23.22 years). 57.5% of patients were females and 42.5% were males (Figure 5). Most of the samples (37.5 %) were from the 0-10 years old age group followed by the above 60 years old age group (22.5 %) (Figure 6).

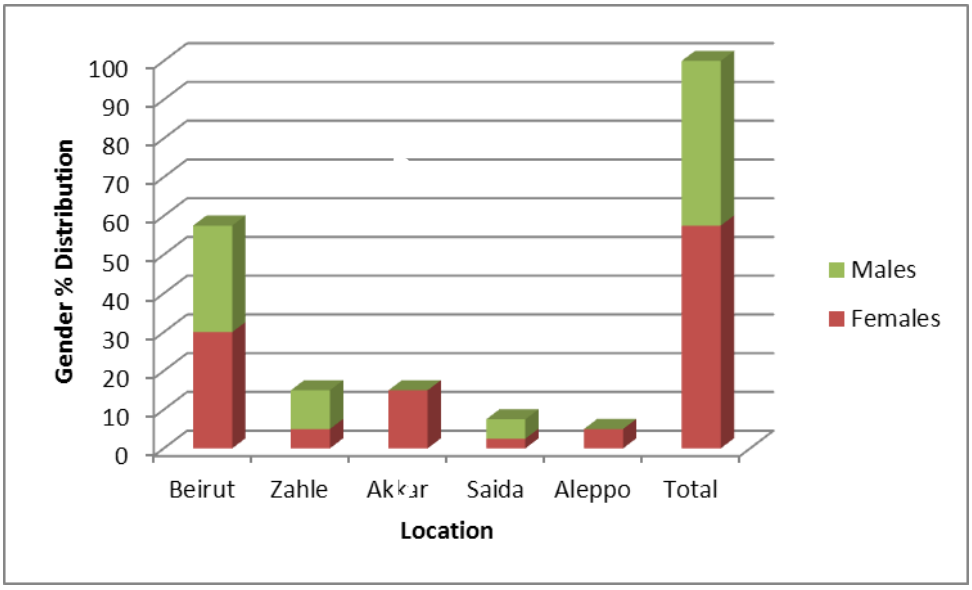

Figure 5. Distribution of male and female cases of leishmaniasis among different locations of sample collection. Location include Beirut, Zahle, Akkar, Saida and Aleppo. The highest number of leishmaniasis cases was reported in Beirut; Male and female cases had an equal occurrence.

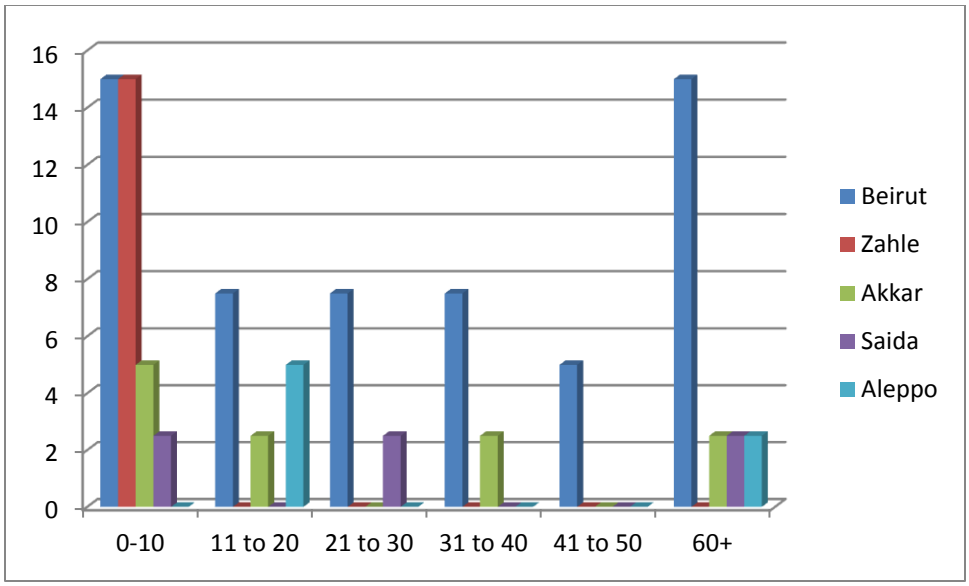

Figure 6. Distribution of cases of leishmaniasis among different age groups and locations. The graph shows that the highest frequency of cases occurred in the younger age groups (<10 years old) followed by the oldest age group (> 60 years old).

Of the 40 skin biopsies, microscopic evaluation of stained slides identified about 17 cases (42.5%) showing a parasitic index of 0–1+ and labeled as microscopically negative (with no unequivocal amastigotes detected) (Figure 7).

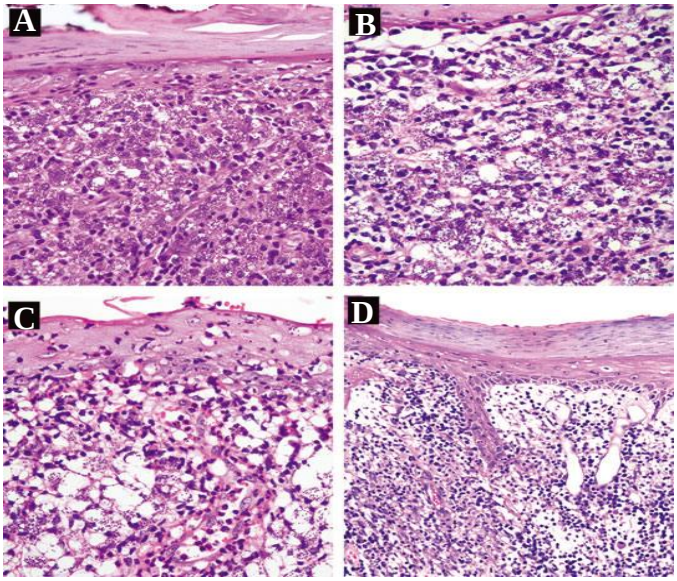

Figure 7. An illustration of (A) parasitic index 6, (B) parasitic index 5, (C) parasitic index 4 and (D) parasitic index 0 (Yehia et al, 2012).

Figure 8. Chart showing a summary of the procedures conducted and a summary of the results obtained.

Following extraction, the obtained DNA concentrations ranged from 8.5 ng/uL to 462.3 ng/uL (66.16 ± 99.62 ng/uL) as measured with the NanoDrop ND-100 spectrophotometer and with a purity ranging from 1.25 ng/uL to 7.24 ng/uL (2.03 ± 1.11). The Qubit fluorometer (Thermo Fisher Scientific, USA) revealed DNA concentrations ranging from 0.2 ng/uL to 28.5 ng/uL (4.34 ± 0.29 ng/uL) (Annex II).

4.2. *Leishmania* genotyping

The first step of genotyping was to identify the *Leishmania* species. For this purpose, ITS1 PCR-RFLP was performed. The second step by nested PCR of ITS1-5.8S rDNA gene region allowed enhancing the sensitivity of the results. A comparison of these two methods was performed accordingly.

4.2.1. ITS1 PCR of *Leishmania* isolates

None of the isolates were positive for the first PCR step targeting the ITS1 region, using the LITSR and L5.8S primers (Table 5). After performing nested PCR on all isolates using the same primers, 18 out of 38 isolates (47.4%) produced a band at around 350 bp. Figure 9 shows a comparison between loading volumes of either 10 uL or 25 uL below for two isolates followed by a negative control. Loading 25 uL of the PCR product yielded significantly sharper bands.

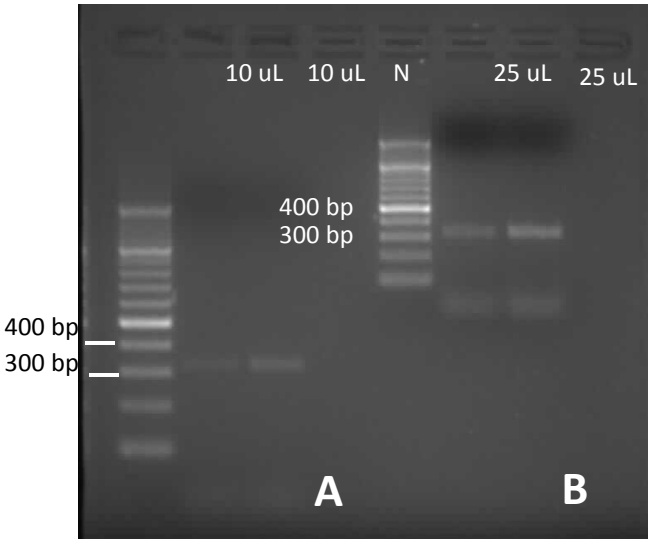

Figure 9. Nested internal transcribed spacer 1 polymerase chain reaction (ITS1-PCR). Agarose gel electrophoresis of the amplicons of ITS1-PCR for two representative isolates obtained after nested PCR

using the same primers (A). 10 uL of the PCR amplicon were loaded on the gel. The gel revealed two positive specimens that showed a DNA band between 300 and 350 bp. Agarose gel electrophoresis of the same two isolates positive for *Leishmania*. 25 uL of DNA were loaded on the gel (B). Thicker bands were observed between 300 and 350 bp. A 100 bp ladder was used as a molecular marker. N: negative control.

Table 4. A list of the isolates that gave a positive ITS1-PCR band and that were subsequently used for RFLP. Sequencing resulted in the following sizes and G+C content.

	Biotype	Isolate Name	Size (bp)	G+C Content (%)
1	HH-13-10	L1	279	53.05
2	HH-13-11	L2	289	38.06
3	HH-13-12	L3	262	41.22
4	HH-13-14	L4	138	44.93
5	HH-13-16	L5	185	53.43
6	HH-13-17	L6	284	39.44
7	ZA-13-5	L7	91	52.75
8	ZA-13-6	L8	141	46.10
9	AK5	L15	289	40.48
10	1-HH	L16	278	44.60
11	4-HH	L17	289	41.87
12	S13-60-1A	L18	277	40.07
13	S13-193-1A	L19	287	35.54
14	AK13-13	L20	273	38.46
15	D02-727A	L24	287	42.51
16	D04-228	L26	90	52.22
17	D10-533	L34	123	50.4
18	D94-270	L43	90	40

4.2.2. RFLP

The digestion of the PCR product with endonuclease *HaeIII* revealed the profiles of *L .tropica* consisting of two bands (200 bp and 60 bp). Most of the bands obtained appeared faint.

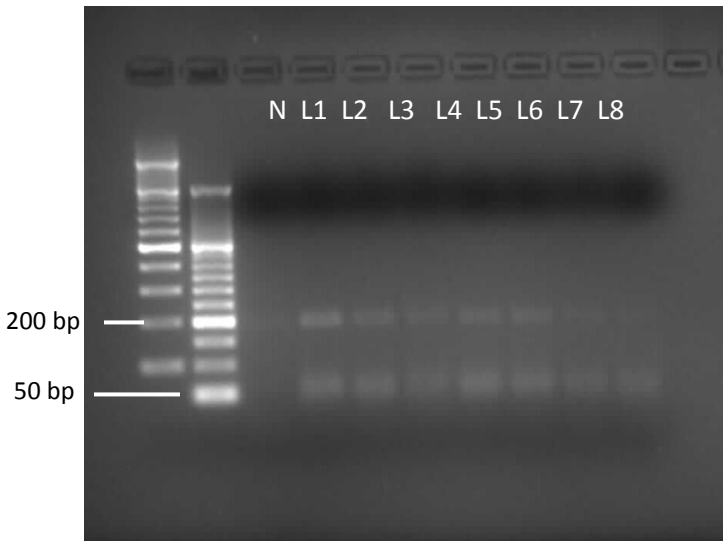

Figure 10. Results of Restriction Fragment Length Polymorphism (RFLP). RFLP pattern typical for *Leishmania tropica* showing two restriction fragments of 200 and 60 basepair (bp) obtained after digestion of representative internal transcribed spacer 1 polymerase chain reaction (ITS1-PCR) with the restriction enzyme *HaeIII*. 100 bp and 50 bp ladders were used as molecular marker. N: negative control.

4.2.3. Sequencing and Phylogenetic tree analysis

Sequences showed a size range of 90 to 289 bp (average: 219.55 ± 82.62 bp) and a G+C content ranging from 35.54% to 53.43% (average: 44.17 ± 5.83%). Alignment of the 18 sequences revealed a percent conservation of 6% to 94% (average: 50.85 ± 19%) per nucleotide. Alignment showed three different sequence types. These three types included: sequences L1-L6, L8, L15-L20 and L24 that showed significant similarity but with

several point mutations; sequences L7, L26 and L34 that appeared to have several common regions and L43 that showed to be distantly related to the other sequences. The phylogenetic tree revealed that L7, L26 and L43 clustered together (Clade II) while L34 appeared to be more closely associated to the references (Clade III) than to the other isolates. Regarding the G+C content, L7, L26 and L34 appeared to have significantly higher G+C contents than the other isolates (52.75%, 52.22% and 50.4% respectively) while the G+C content of L43 was 40%.

The aligned sequences showed several blocks of conserved nucleotides in addition to a high occurrence of polymorphisms and variations (Figure 11). Variation was reflected by the low bootstrap values obtained. For instance, a block of 13 conserved nucleotides (GCTTTATGTGAGC) was observed at position 40 with T→G change at position 43 in L5 and A→G at position 51 in L8. Another 8 consecutive conserved nucleotides (ACCCCCCC) were observed in all the obtained sequences, except for L1, L4, L5, L34 and L43, at position 70. In L26, AC→GG at position 70 was observed. Another 17 nucleotides were conserved at position 230 (TATAAAAAGTCTCCGCA) in all isolates except for L4, L5, L7, L8, L34 and L43. T→A was observed in L17 at position 230 and AAA→TTT in L18 at position 233.

Phylogenetic analysis based on the partial sequence of the ITS1 rDNA gene separated the isolates into four distinct clusters (Figure 12). Sequences were compared to *L. tropica* and *L. infantum* references retrieved from GenBank with the following accession numbers: KJ567478, KJ567477, KJ567476, KJ567479, KJ567481, KJ567480 and KJ567482. The references covered ITS1 partial sequence, 5.8S rDNA gene complete sequence and ITS2 partial sequence. Cluster I, included L3, L6 and L18, cluster II was

composed of L7, L5, L26, L4, L8, L43 and L1, cluster III consisted of all the above mentioned references, in addition to L34 and L20 and finally, cluster IV consisted of L2, L16, L24, L15, L19 and L17.

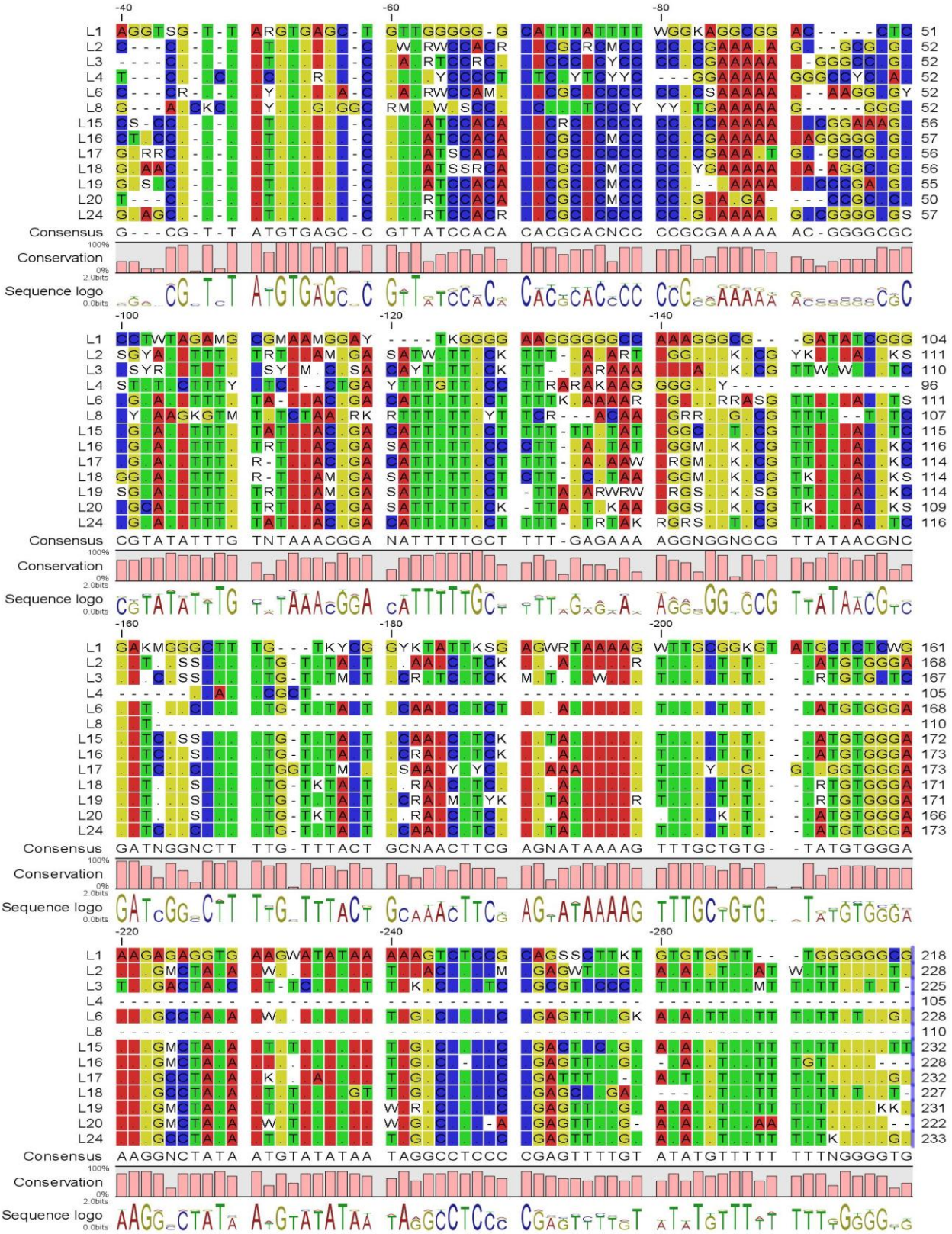

Figure 11. ITS1-PCR RFLP gene sequences alignment of *L. tropica* isolates using CLC Main Workbench v3.1. Matching residues are depicted as dot and highlighted with the same color. Gaps are denoted as dashes.

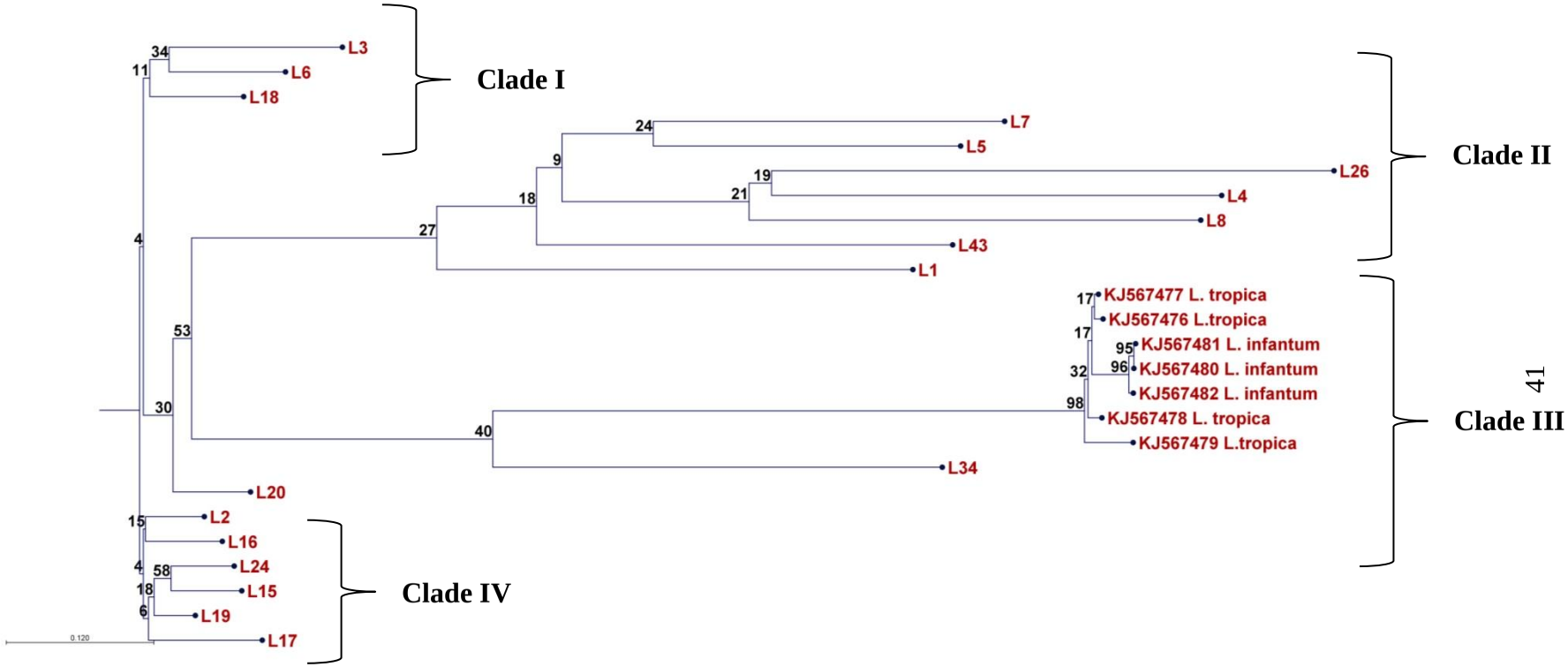

Figure 12. Neighbor-joining tree showing the relationships of the *L. tropica* isolates based on the ITS1-PCR RFLP sequences, using the CLC Main Workbench v3.1. Bootstrap values are based on 1,000 replicates.

4.3. Nested PCR for amplifying the ITS1-5.8S rDNA gene of *Leishmania* isolates

40 isolates were screened for *Leishmania* infection by nested PCR. The first PCR reaction using the ITS1 and ITS2 primers showed no bands on gel electrophoresis. The nested PCR reaction produced bands at around 400 bp for 28 isolates (70%).

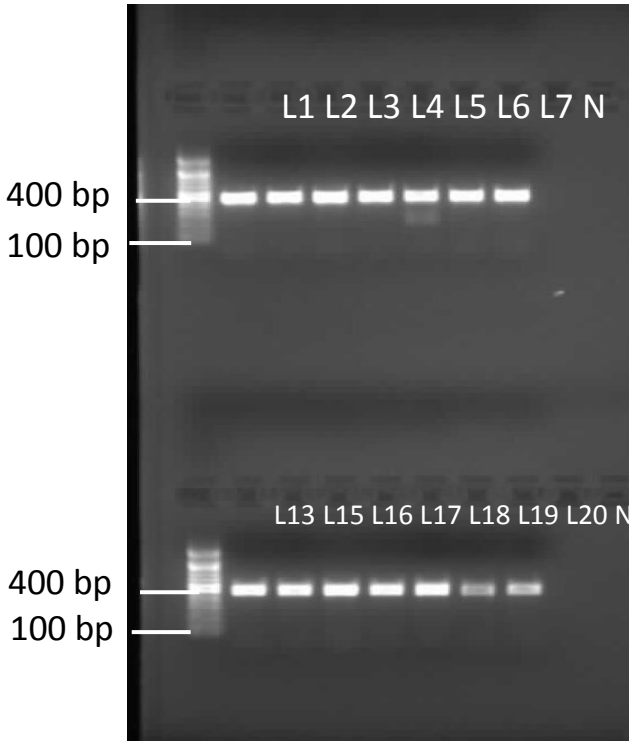

Figure 13. Nested-PCR of ITS1-5.8S rDNA gene of *Leishmania* DNA. Agarose gel electrophoresis of representative isolates positive for *Leishmania* give a DNA band around 400 bp characteristic of *L. tropica*. 100 bp ladder was used as a molecular marker. N: negative control.

Table 5. A list of the isolates that gave a positive band after nested ITS1-5.8S rDNA gene PCR. Sequencing resulted in the following sizes and G+C content. GenBank accession numbers were obtained.

	Biotype	Isolate Name	Size (bp)	G+C Content (%)

1	HH-13-10	L1	440	22.5
2	HH-13-11	L2	435	34.47
3	HH-13-12	L3	438	37.67
4	HH-13-14	L4	436	38.30
5	HH-13-16	L5	439	39.86
6	HH-13-17	L6	436	42.89
7	ZA-13-5	L7	435	40.69
8	ZA-13-6	L8	512	49.41
9	ZA-13-8	L9	429	45.22
10	ZA-13-10	L10	435	42.76
11	ZA-13-11	L11	441	38.55
12	ZA-13-13	L12	405	45.43
13	AK1	L13	428	39.72
14	AK2	L14	410	39.27
15	AK5	L15	439	39.18
16	1-HH	L16	435	40.23
17	4-HH	L17	436	39.68
18	S13-60-1A	L18	430	35.11
19	S13-193-1A	L19	429	52.11
20	AK13-13	L20	254	46.46
21	D02-727A	L24	439	37.13
22	D02-727B	L25	434	39.17
23	D04-228	L26	432	40.05

24	D05-536	L27	435	41.61
25	D08-154	L29	440	41.82
26	D08-1029	L30	422	37.68
27	D08-1030	L31	439	36.90
28	D10-533	L34	436	41.28

4.3.1. Sequencing and Phylogenetic tree analysis

Alignment revealed a high level of conservation with the presence of single point mutations. Analysis of the ITS1-5.8S rDNA gene sequences further confirmed that they all belonged to *L. tropica*. Sizes ranged from 71 bp to 512 bp (average: 416.46 ± 77.83 bp) and the G+C content ranged from 22.5% to 52.11% (average: 40.18 ± 5.28%).

In general a high level of conserved nucleotides was observed between the sequenced isolates and the reference strains. Percent conservation ranged from 4% to 100% (as per every nucleotide) (mean= 62.5% ± 29.75%).

Alignment revealed that blocks of conserved nucleotides start appearing at around position 60. Thirty-one conserved nucleotides appear in all the 25 sequences, except for L8 and L19, (CTTCTCCCATGCGCCGTTTGCGTTCAAAGAT). SNPs are observed on this region as well such as at position 65, C→A in L1 and L14 and C→G in L18, L24 and L30. At position 66, C→A in L18, L24 and L30 and, at position 83, T→G in L9 and T→A in L18 and L24 were also observed.

A block of 68 conserved nucleotides was observed between positions 107-174 in all of the 28 sequences. Single point variations were observed here as well. For instance, at position 114 T→C in L8 and L30 and T→G in L9 and at position121, A→G in L8, L9,

L18, L24 and A→T in L30. Furthermore, TTT →CAT at position 151 to 153 and TTT→AAG and AAA in L12 and L13, respectively were observed.

Moreover, 34 conserved nucleotides (GGAAGCCAAGTCATCCATCGCGACACGTTATGTG) were also observed in 27 of the sequences with the exception of L19 between 181-214. Similar SNPs were also in this region. For instance, G→C in L8 and L9, G→A in L18 and L24 and G→T in L30 at position 181. Also at 188-189 AA→CA in L9, and GG in L18 and L24. Also, at position 216-218, ACA→AAA in L18 and L24.

GCACCCCCC was found in all the sequences at 242-250 and GTATAAAC at 284-291 and TTTTGTATATGTTTTTTTT in all the sequences at position 1167-1185 except for L8, L19 and L20.

The sequences were compared to the same references as the first phylogenetic tree retrieved from Genbank (KJ567478, KJ567477, KJ567476, KJ567479, KJ567481, KJ567480 and KJ567482). The phylogenetic tree revealed the presence of four different clusters. Cluster I' consisted of L34, L7, L17, L25, L20, L13 and L31. Cluster II' consisted of all the references, in addition to L8, L1, L2, L3, L4 and L16. Cluster III' was composed of L6, L14, L9, L12, L10, L19, L30, L24 and L18. And finally, cluster IV' consisted of L15, L5, L11, L29, L26 and L27. Low boot strap values were correlated to a high level of heterogeneity.

Alignment of the obtained 28 isolates also revealed three different sequence types. Based on the alignment, sequences L1-L7, L9-L18, L30-L31 and L34 showed significant similarity but with several point mutations as indicated above. Sequences L8 and L19 showed to be distantly related from each other and from the other sequences. L8 clustered

distantly from the other sequences and appeared to be closely related to *L. tropica* and *L. infantum* reference strains (bootstrap value 98). L19 appeared to be part of the cluster III'. Regarding the G+C content, both L8 and L19 revealed a significantly higher G+C content than the other iolates (49.41% and 52.11%, respectively).

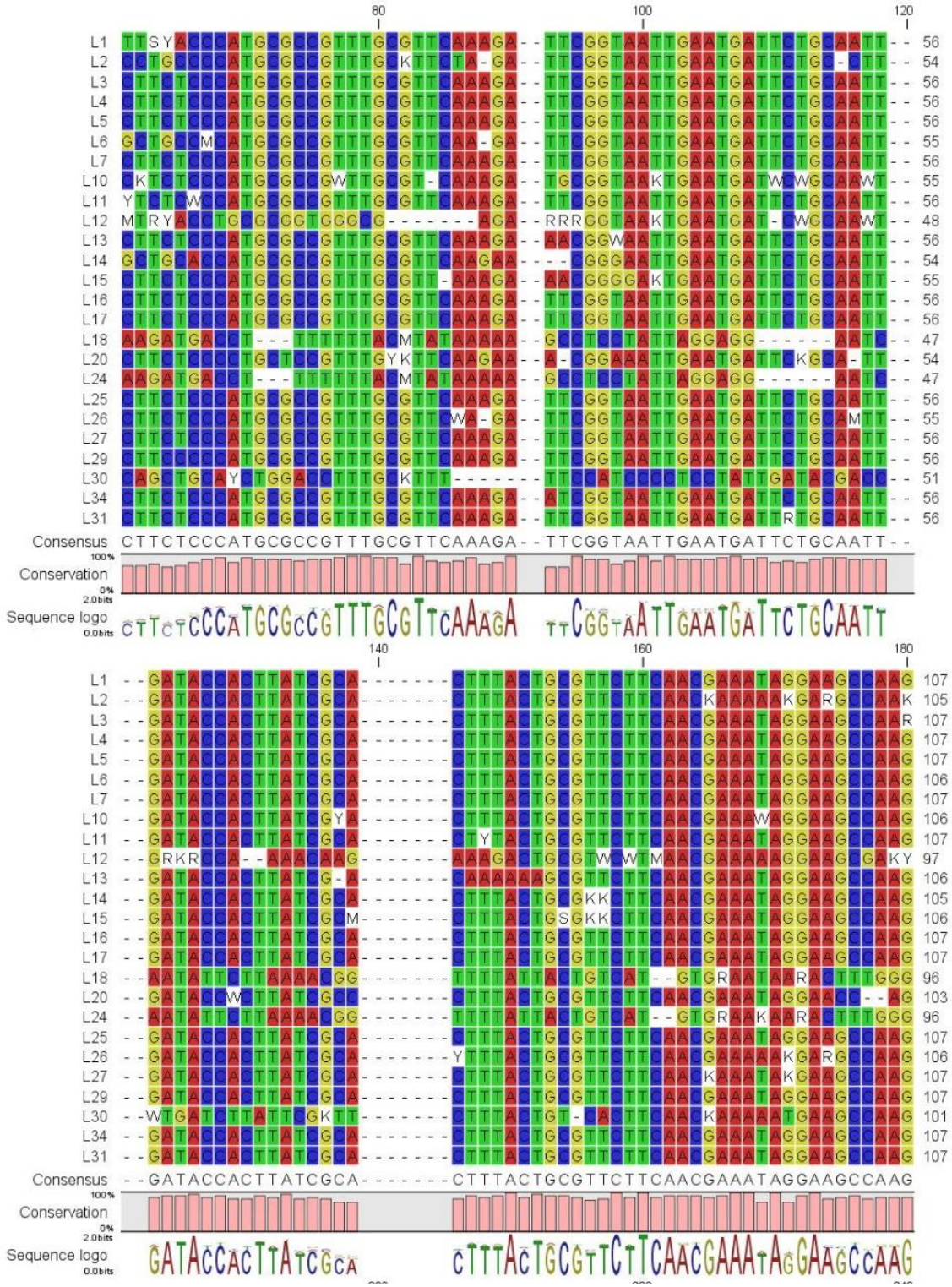

Figure 14. ITS1-5.8S rDNA gene sequences alignment of *L. tropica* isolates using CLC Main Workbench v3.1. Matching residues are depicted as dot and highlighted with the same color. Gaps are denoted as dashes.

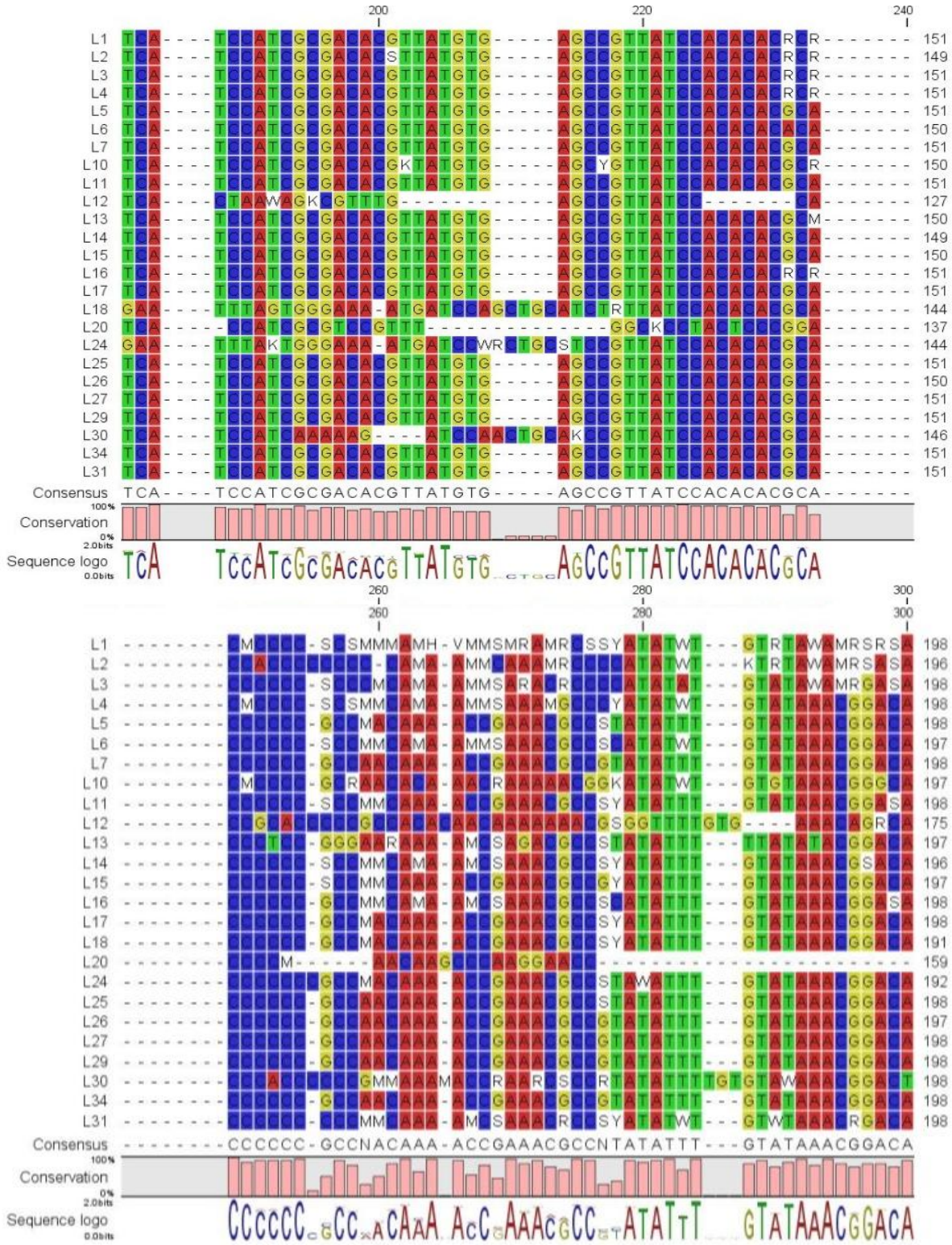

Figure 14. ITS1-5.8S rDNA gene sequences alignment of *L. tropica* isolates using CLC Main Workbench v3.1. Matching residues are depicted as dot and highlighted with the same color. Gaps are denoted as dashes. Continued

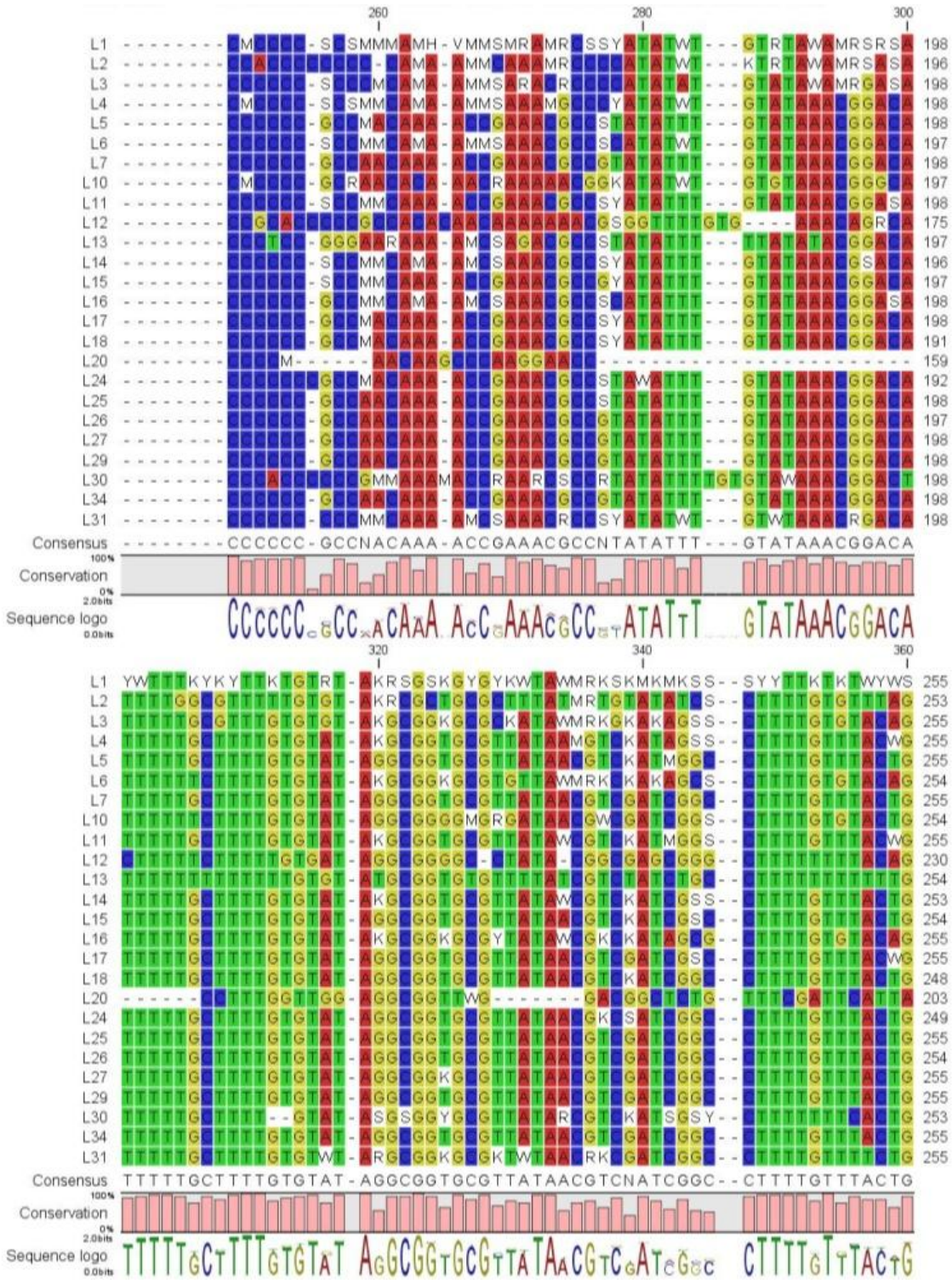

Figure 14. ITS1-5.8S rDNA gene sequences alignment of *L. tropica* isolates using CLC Main Workbench v3.1. Matching residues are depicted as dot and highlighted with the same color. Gaps are denoted as dashes. Continued

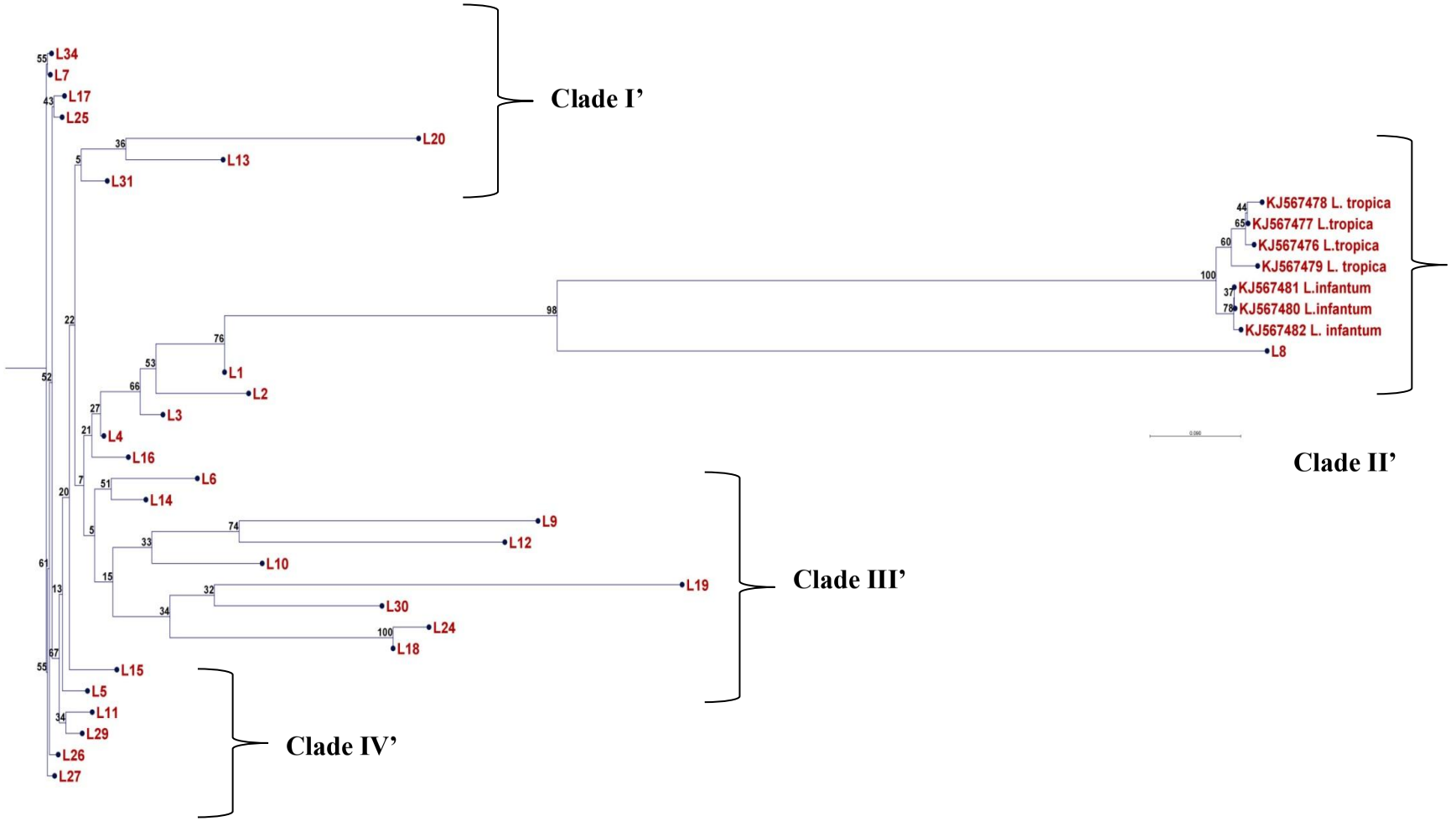

Figure 15. Neighbor-joining tree showing the relationships of the *L. tropica* isolates based on the ITS1-5.8S rDNA gene sequences**,** using the CLC Main Workbench v3.1. Bootstrap values are based on 1,000 replicates.

4.4. Assessing DNA quality through Actin & GAPDH amplification

In order to rule out possible DNA quality reasons that might have caused the observed differences in results, two housekeeping genes, Actin and GAPDH, were amplified.

The first PCR amplification yielded no bands. Nested PCR resulted in bands at around 60 bp and 150 bp for Actin and GAPDH, respectively, after loading 25 uL of the PCR amplicon on the gel.

All the isolates used in this study were positive for Actin and GAPDH after performing nested PCR. Two isolates (data not shown) proved to be negative for both of these genes and hence were not included in this study.

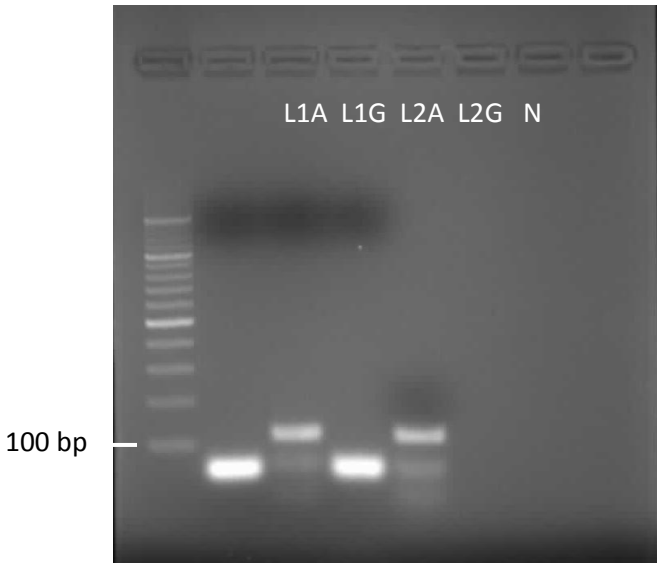

Figure 16. Agarose gel electrophoresis of two DNA isolates (labeled L1 and L2) for the housekeeping genes, GAPDH and actin**.** Actin depicted in lanes 2 and 4 give a band size of around 50 bp; GAPDH depicted in lanes 3 and 5 give a band size of around 120 bp. L1A, actin for isolate 1; L2A, actin for isolate 2; L1G, GAPDH for isolate 1; L2G, GAPDH for isolate 2. A 100 bp ladder was used as a molecular marker. N: negative control.

Chapter Five

DISCUSSION

The main objectives of this study were to identify the *Leishmania* parasites responsible for leishmaniasis outbreaks observed recently in Syrian refugee camps and collective shelters in Lebanon. Two molecular techniques were used for this purpose, ITS1 PCR-RFLP and nested ITS1-5.8S rDNA gene PCR. Phylogenetic tree analysis based on the PCR products of both methods was then performed.

5.1. DNA extraction from FFPE tissue

DNA extraction from *Leishmania*-suspected FFPE skin biopsies was performed based on an optimized protocol according to Yehia et al (Yehia et al, 2012). Since *Leishmania* parasites only contain few ng of DNA, DNA extraction has to be highly efficient to obtain enough concentration for further analysis (Barker, 1989). In this study, commercial reagents (Qiagen GmbH) were used to extract purified DNA from FFPE skin biopsies. However, this method was not sufficiently efficient. Although the obtained DNA concentrations ranged from 8.5 ng/uL to 462.3 ng/uL (66.16 ± 99.62 ng/uL), all the conducted PCR reactions, including the isolates having a DNA concentration as high as 400 ng/uL, required a second PCR to obtain a visible band, making the analysis process more costly, tedious and time consuming.

Several methods have been reported previously for DNA extraction from clinical leishmaniasis samples. Crude DNA can be extracted from FFPE using the direct boiling method by incubating at 56°C, then boiling for few minutes (Safaei et al, 2002; Laskay et al, 1995). DNA extraction can also be performed from stained slides (Schonian et al,

2003; Meredith et al, 1993; Osman et al, 1998), lesion scraping on filters (Schonian et al, 2003; Bensoussan et al, 2006; Boggild et al, 2010), promastigote cultures (van Eys et al, 1992), fresh skin punch biopsies (Mugasa et al, 2010), bone marrow aspirates (Minodier et al, 1997) and blood (Boom et al, 1990; Spanakos et al, 2008).

DNA extraction from FFPE tissue blocks was first reported in 1985 (Goelz et al, 1985). Since then, this method has been extensively optimized. Laskay et al (1995) extracted DNA from FFPE tissues using a direct boiling technique. They concluded that a DNA concentration corresponding to 0.1 pg of *Leishmania* promastigotes was sufficient for subsequent PCR analysis of *L. aethiopica*. However, this method only allowed the identification of 32% (7 out of 22 cases) of the suspected leishmaniasis cases (Laskay et al, 1995). In 2002, Safaei et al (2002) could identify 92% of suspected leishmaniasis cases using the same method.

Despite these promising results, FFPE tissue storage faces several limitations. It has been reported that DNA often gets degraded during the process of formalin fixation as well as during the extraction process itself (Huijsmans et al, 2010; Momeni et al, 1996). In order to assess whether the observed PCR problems were linked to DNA quality, two housekeeping genes, Actin and GAPDH, were amplified. Similarly, the first PCR amplification yielded no bands. Nested PCR using the same set of primers yielded positive results for all the isolates used in this study. Only two isolates (data not shown) proved to be negative for both of these genes and hence were excluded. Therefore, the need to conduct nested PCR was most probably due to low DNA quality in the samples which can be linked to the presence of crosslinks in the extracted DNA (Ludyga et al, 2012).

5.2. *Leishmania* typing using ITS1-PCR-RFLP and ITS1-5.8S rDNA gene

Previously several studies exploited the ITS1 region amplification technique for the discrimination of *Leishmania* parasites (Shonian et al, 2003; Roelfsema et al, 2011; Amro et al, 2009; Hamarsheh et al, 2012). In prokaryotes, the genes coding for ribosomal RNAs are arranged in an operon in the following order 5'-16S-23S-5S-3'. These are separated by two spacer regions known as the Intergenic Transcribed Spacer (ITS) (Condon et al. 1995). The ITS1 is the sequence in between the 18S rRNA and 5.8S rRNA genes. It contains enough conservation to serve as a PCR target but sufficient polymorphisms to facilitate species typing and identification (Roelfsema et al, 2011). In eukaryotes, rRNA genes are organized in tandem repeat units separated by a non-transcribed spacer. As opposed to these non-transcribed spacer regions, the combined internal transcribed spacers of rDNA (ITS-rDNA) in *Leishmania* species are considered to be relatively small. Also, these are often flanked by highly conserved gene segments that can be successfully targeted by PCR primers (Cupolillo et al., 1995). Schönian et al (2001) identified size polymorphisms in PCR amplified ITS regions that proved to be specific for certain *Leishmania* species. In this study, the bands obtained upon amplification of the ITS1 region were often faint even after nested PCR. The first PCR reaction targeted DNA concentration of around 0.1 pg/uL. The nested PCR reaction required a minimum of 10 uL of the previously obtained amplicon. Considering that previous studies reported a minimum of 10 pg/uL for visualization (Ajouad et al, 2014; Bensoussan et al, 2006), the obtained results explain the reason why the first PCR of the ITS1 region did not yield an amplification product. On the other hand, it was previously reported that ITS1 PCR

could detect 0.2 parasites per PCR assay of leishmanial DNA equivalent, when using filter paper spotted with peripheral blood mixed with culture promastigotes (Schonian et al, 2003). However, these results indicate that performing nested PCR amplicon partially solved the problem of poor initial DNA concentration and that initial amounts as little as 0.1 pg of DNA can in fact be detected by PCR- based molecular typing techniques (Schonian et al, 2003).

Nested ITS1-5.8S rDNA gene PCR enhanced the sensitivity of this diagnostic tool by targeting two fragments in the ITS-rDNA region one region consisting of ITS1 with the 5.8S rDNA gene (Parvizi and Ready, 2008). In this study, the first PCR reaction similarly targeted a DNA concentration of around 0.1 pg/uL. Also, no bands were obtained during the first PCR reaction. However, for the nested PCR reaction, only a minimum of 2 uL of the original PCR amplicon were needed to obtain a good yield. These results suggest that the nested PCR of ITS1-5.8S rDNA gene is more sensitive for *Leishmania* species identification. Indeed, the ITS1-5.8S rDNA gene PCR that was able to identify *L. tropica* in 70% of the isolates as opposed to only 47.4 % identified by the ITS1-PCR method. This proved to be consistent with a number of previously obtained results (el Tai et al, 2000; el Tai et al, 2001; Spanakos et al, 2008; Schallig et al, 2002; Parvizi et al, 2008; Es-Sette et al, 2014). In fact, this PCR approach has been employed in the detection of *Leishmania* in entomological surveys (Ajouad et al, 2013). No clear-cut correlation was observed between the results of the PCR assays and the time of evolution of the cutaneous lesions.

Moreover, ITS1 PCR-RFLP is the most widely used assay for direct detection and identification of *Leishmania* species in the Old World (Schonian et al, 2011). In this

study, RFLP of the ITS1 PCR product revealed that the isolates belonged to the *L. tropica* (Schonian et al, 2001; Schonian et al, 2003; Doudi et al, 2010; Rotureau et al, 2006; Al Nahhas and Kalda, 2013). It is worth noting that previous attempts of extending the RFLP fragment to include the 5.8S rDNA gene region did not show any advantage (Parvizi and Ready, 2008). RFLP proved to be successful in the distinction of sympatric species, especially in the Mediterranean region, as well as for the identification of imported cases of leishmaniasis due to population migrations such as those associated with war (Harms et al, 2003; Al-Jawabreh et al, 2004; Rhajaoui et al. 2007).

5.3. Sequencing and phylogenetic tree analysis of haplotype sequences of *Leishmania* ITS-rDNA

Although the ITS region serves as a marker for the differentiation of *Leishmania* at both the species and the strain levels, only few studies employed ITS sequence analysis to compare *L. tropica* isolates (Schonian et al, 2001; Khanra et al, 2011; Talmi-Frank et al, 2010; Mahdy et al, 2010; Bosslimi et al, 2012; Ghatee et al, 2014). In this study, sequencing of the obtained PCR products of both PCR reactions, targeting the ITS1 and the ITS1-5.8S rDNA gene regions was performed. Sequences were compared to four *L. tropica* and three *L. infantum* reference strains (Es Sette et al, 2014).

L. tropica is known to be a very heterogenous species (Azmi et al, 2012; Shonian et al, 2001; Le Blancq et al, 1986) and this was clearly observed in the alignment of the ITS1-PCR amplification products and the low bootstrap values obtained on the phylogenetic tree. The reason behind this is the occurrence of at least two alleles for ITS in ribosomal DNA in *Leishmania* spp. (Ghatee et al, 2013; Mauricio et al, 2004). Alignment based on the ITS1-5.8S rDNA gene revealed a significantly higher level of conservation than the

ITS1 based alignment. In both cases, the sequences were separated into three different sequence types though accommodating different isolates. Also, the topology of both trees revealed that references were joined in a single clade. The separate clustering of the references was also reported in previous studies (Mahdy et al, 2010; Es-Sette et al, 2014). It is commonly reported that samples positive for leishmaniasis often show unreadable sequences upon ITS-rDNA fragment examination (Mirzaei et al, 2013). This can be explained by extremely high heterogeneity levels or sometimes may be due to mixed infections of two or more *Leishmania* species (Mirzaei et al, 2013; Parvizi et al, 2008; Parvizi et al, 2008; Strelkova et al, 2001)..

In this study, two techniques for the molecular detection of *Leishmania* isolates were used: ITS1-PCR RFLP and ITS1-5.8S rDNA gene PCR amplification. ITS1-PCR RFLP profiles proved that the obtained isolates were all *L. tropica*. This was further validated by sequencing the ITS1-PCR product as well as the ITS1-5.8S rDNA gene product. ITS1-5.8S rDNA PCR proved to be a more sensitive identification method for *Leishmania* isolates. A high level of heterogeneity was revealed in the obtained sequences. The outbreaks of leishmaniasis in Lebanon are hypothesized to be caused by the migration of Syrian refugees. This hypothesis is confirmed by the fact that Syria shows high levels of CL infections. Leishmaniasis outbreaks were reported in many Syrian cities, including Aleppo (Desjeux, 2004). Unfortunately, vaccination against *Leishmania* has shown many drawbacks (Murray et al, 2005). So far, no vaccines or drugs to prevent infection are available (CDC, 2013).

The molecular typing of *Leishmania* will continue using accurate clinical microbiology typing techniques in order to get a better understanding of this parasite and have sufficient information for the subsequent development of new vaccines or other more efficient preventative methods.

For the differentiation of *Leishmania* at the sub-species level, it is recommended to perform multi-locus sequence typing (MLST) method. Moreover, whole-genome sequencing (WGS), also referred to as next-generation sequencing (NGS), provides a greater potential to identify genetic component of health problems and infectious diseases. The massive amount of data generated from WGS can reveal disease-causing alleles that could not be detected otherwise such as virulence drug-resistance, etc. (Gvan et al, 2013). Thus, it can be used for further in-depth study of the epidemiology, evolution and pathogenomics of *Leishmania* (Schonian et al, 2011).

To achieve better DNA quality and quantity it is recommended to try a commercial FFPE DNA extraction kit designed to remove cross links. Also, it would be of interest to type *Leishmania* isolated from sand fly vectors. Furthermore, accessible international databases for cases of leishmaniasis should be created for a better epidemiological assessment of these infectious agents and for tracing their patterns of migration between countries and continents. Better cooperation between laboratory scientists, epidemiologists, clinicians, veterinarians and public health authorities is needed, as well, in order to create a surveillance program of leishmaniasis. Finally, international collaboration under the supervision of the World Health Organization (WHO) is necessary for controlling the spread of leishmaniasis.

Chapter Six

CONCLUSION

In conclusion, the use of ITS1-PCR RFLP and ITS1-5.8S rDNA gene PCR enabled the accurate detection and identification of *L. tropica* in FFPE clinical samples. ITS1-5.8S rDNA gene PCR proved to be a more sensitive method for *Leishmania* spp. discrimination. The obtained results highlight the need to find a universally accepted diagnostic tool for *Leishmania* typing, that is specific, sensitive, rapid and capable of identifying all clinically significant *Leishmania* species. Also, it was demonstrated that *L. tropica* is the causative agent of leishmaniasis outbreaks observed among Syrian refugees that are seeking shelter in the Lebanon. The living conditions in refugee's camps and collective shelters are characterized by poor sanitary conditions and inadequate waste disposal, thus increasing the risk of *Leishmania* transmission through the sand-fly vector. Unfortunately, children of less than 10 years old are the most affected (Alawiyeh et al, 2014). The risk of transmitting this "flesh-eating" parasite into the Lebanese community is high. Thus, effective prevention methods and appropriate therapy is critical. Prevention can be as simple as using nets treated with insecticide or spraying insecticides to kill sand-fly vectors (Barret et al, 2012).

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ANNEX I

Table. Biotype numbers, isolate names, location where the samples were collected, the duration of the lesions (in months), patient age (in years) and gender (M: male; F: female) of all the tissue samples assessed in this study.

	Biotype	**Isolate Name**	**Location**	**Date of Lesion (months)**	**Age**	**Gender**
1	HH-13-10	L1	Beirut	4	6	F
2	HH-13-11	L2	Beirut	4	21	M
3	HH-13-12	L3	Beirut	4	31	F
4	HH-13-14	L4	Beirut	5	25	F
5	HH-13-16	L5	Beirut	6	25	M
6	HH-13-17	L6	Beirut	5	5	M
7	ZA-13-5	L7	Zahle	2	10	F
8	ZA-13-6	L8	Zahle	5	5	M
9	ZA-13-8	L9	Zahle	2	9	M
10	ZA-13-10	L10	Zahle	5.5	5	M
11	ZA-13-11	L11	Zahle	6	1	M
12	ZA-13-13	L12	Zahle	7	3	F
13	AK1	L13	Akkar	5	9	F
14	AK2	L14	Akkar	4	12	F
15	AK5	L15	Akkar	3	4	F
16	1-HH	L16	Beirut	4	34	F
17	4-HH	L17	Beirut	4	4	M

18	S13-60-1A	L18	Akkar	3	11	F
19	S13-193-1A	L19	Akkar	2.5	74	F
20	AK13-13	L20	Akkar	5	42	F
21	D02-727A	L24	Beirut	3	37	M
22	D02-727B	L25	Beirut	4	9	F
23	D04-228	L26	Beirut	3	54	F
24	D05-536	L27	Beirut	1	13	F
25	D08-154	L29	Beirut	4	18	M
26	D08-1029	L30	Beirut	3	83	M
27	D08-1030	L31	Beirut	3	83	F
28	D10-533	L34	Beirut	6	83	F
29	D93-194	L39	Beirut	3	60	F
30	D93-470	L41	Beirut	4	53	F
31	D94-247	L42	Beirut	12	16	M
32	D94-270	L43	Beirut	3	46	F
33	D95-157	L44	Beirut	4	46	M
34	D95-494	L45	Beirut	12	5	M
35	10345	L62	Aleppo	3	11	F
36	11469	L63	Aleppo	2	65	F
37	28636	L67	Saida (HA)	3	4	M
38	52536	L68	Saida (HA)	7	23	M
39	P11-26000	L69	Saida (HA)	9	72	F

40	4HH	L70	Beirut	4	4	M

ANNEX II

Table. DNA concentrations and purities obtained after performing DNA extraction on the 40 extracted *Leishmania* isolates (as measured by the NanoDrop Spectrophotometer and by Qubit).

	Isolate Name	NanoDrop (ng/uL)	Purity	Qubit(ng/uL)
1	**L1**	37.6	1.39	Too low
2	**L2**	41.7	1.77	1.76
3	**L3**	31.2	1.34	2.38
4	**L4**	48.3	1.43	1.63
5	**L5**	31.7	1.47	2.24
6	**L6**	35.2	1.66	2.23
7	**L7**	45.3	1.65	1.19
8	**L8**	82.7	1.45	2.93
9	**L9**	53.7	1.25	1.34
10	**L10**	37.7	1.63	1.27
11	**L11**	40.6	1.93	1.69
12	**L12**	71	1.91	12
13	**L13**	46	1.93	1.94
14	**L14**	41.5	1.8	Too low
15	**L15**	34.5	1.41	3.5
16	**L16**	21.9	1.36	Too low
17	**L17**	34.8	1.37	3.74
18	**L18**	61.1	1.37	1.83
19	**L19**	26.1	1.3	Too low
20	**L20**	20.2	1.57	1.37
21	**L24**	41.6	1.76	?
22	**L25**	462.3	1.83	15.9
23	**L26**	16.5	.	Too low
24	**L27**	8.5	.	1.24
25	**L29**	8.8	2.01	Too low
26	**L30.1**	129.1	2.14	?
27	**L30.2**	19.1	2.36	4.92
28	**L34**	460.7	1.98	7.2
29	**L39**	31.4	3.74	0.97
30	**L41**	14.4	.	0.2
31	**L42**	86.4	2.14	1.17
32	**L43**	137.8	2.46	4.44
33	**L44**	35	3.92	10.11

34	**L45**	48.7	2.58	12.13
35	**L62**	28.6	7.24	2
36	**L63**	209.1	2.05	28.5
37	**L67**	16.9	.	Too low
38	**L68**	12.6	.	0.992
39	**L69**	13.8	.	0.572
40	**L70**	22.3	.	1.06