The Impact of Syrian Immigration on the Prevalence of
Cutaneous Leishmaniasis in Jordan

By

Kamal Jehad Hijjawi

Supervisor
Dr. Nawal Sameeh Hijjawi
Associate Professor

Co-Supervisor
Dr. Jwan Hussien Ibbini
Assistant Professor

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I would like to dedicate this thesis to my beloved wife Islam Hussien Al-Zyoud. She will eternally remain my source of inspiration and strength.
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<td>ACL</td>
<td>Anthroponotic cutaneous leishmaniasis</td>
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<tr>
<td>CL</td>
<td>Cutaneous leishmaniasis</td>
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<tr>
<td>ITS</td>
<td>Internal transcribed spacer</td>
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<tr>
<td>kDNA</td>
<td>Kinetoplast DNA</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
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<tr>
<td>LST</td>
<td>Leishmanin skin test</td>
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<tr>
<td>MCL</td>
<td>Mucocutaneous leishmanias</td>
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<tr>
<td>NWCL</td>
<td>New World cutaneous leishmanias</td>
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<tr>
<td>OWCL</td>
<td>Old World cutaneous leishmanias</td>
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<tr>
<td>Rcf</td>
<td>Relative centrifugal force</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<tr>
<td>RPM</td>
<td>Round Per Minute</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA</td>
</tr>
<tr>
<td>VL</td>
<td>Visceral leishmanias</td>
</tr>
<tr>
<td>ZCL</td>
<td>Zoonotic cutaneous leishmanias</td>
</tr>
<tr>
<td>ºC</td>
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<td>µl</td>
<td>Microliter</td>
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Abstract

The Impact of Syrian Immigration on the Prevalence of Cutaneous Leishmaniasis in Jordan

By
Kamal Jehad Hijjawi

Supervisor
Dr. Nawal Sameeh Hijjawi
Associate Professor

Co-Supervisor
Dr. Jwan Hussien Ibbini
Assistant Professor

Leishmania is a parasitic protozoan with more than two-dozen species causing disease known as leishmaniasis. Leishmaniasis is transmitted to human through the bite of an infected female Phlebotomine or Lutzomyia sand flies. The crisis in the Syrian Arab Republic that started in March 2011 has resulted in the immigration of a large number of refugees into Jordan where they reside in camps near the Syrian boarders. Water, sanitation and hygiene facilities in those camps are far from adequate, increasing the risk and turning those camps into fertile grounds for the spread of many infectious diseases. Among the important diseases which might be carried by the refugees into Jordan is cutaneous leishmaniasis (CL). CL is endemic in many areas in Jordan and is considered as a public health problem, but its epidemiology has not been fully elucidated in this country. A crucial point in containing the disease and controlling its spread is to identify the existing Leishmania species and then correlate it with clinical parameters. In this study, three molecular typing methods for the discrimination of different Leishmania species were used along with sequencing and phylogenetic analysis. The Leishmania species was inspected from 66 samples which were collected from scrapings from lesions of Jordanian and Syrian suspected to suffer from CL. Results indicated that 20 and 9 of the inspected 66 patients were infected with Leishmania major and Leishmania tropica respectively as determined by the ITS1-PCR, RFLP and Kinetoplast DNA (Lmj4 and Uni 21 gene). The Nested ITS1-5.8S rDNA gene amplification was used to identify L. tropica species which was further confirmed by the sequencing and phylogenetic analysis which revealed high levels of heterogeneity among the sequenced isolates. To our knowledge, this is the first study in Jordan which combined three different molecular methods for the successful detection and identification of L. tropica and L. major from clinical isolates of Jordanian and Syrian patients who suffer from cutaneous leishmaniasis. The results from the present study confirmed that the above mentioned molecular techniques could be used as
standard methods for the accurate detection and diagnosis of CL in Jordan and for the tracking of possible changes in the population structure of *L. tropica* due to the insertion of new isolates / strains by the Syrian refugees. Therefore, the application of the above mentioned molecular based assays might facilitate the CL diagnosis especially during outbreaks in Jordan and to determine the route of transmission as zoonotic or anthroponotic.

In this study, it was observed that the majority of CL cases is caused by *L. major* (20 cases) in both Jordanians (14) and Syrians (6). There were (9) cases of CL caused by *L. tropica* (3) of them are Syrian refugees and the other (6) are Jordanians.

Key words: *Leishmania*, Cutaneous leishmaniasis (CL), ITS1, 5.8S rDNA gene, PCR, RFLP, Sequence analysis, Jordan, Syria
CHAPTER ONE
INTRODUCTION

1. Introduction

Leishmaniasis is a vector-borne disease which is caused by the obligate intracellular protozoan parasites of the genus *Leishmania*. *Leishmania* belong to the Kingdom Protista, Class Kinetoplastea, Subclass Metakinetoplastina, Order Trypanosomatida, Family Trypanosomatidae, Subfamily Leishmaniinae, and Genus *Leishmania* [1]. *Leishmania* species are heterogeneous, which means that they are able to infect two hosts animal and human. At least 21 *Leishmania* species were confirmed to cause disease in humans known as leishmaniasis [2]. Leishmaniasis ranges from localized cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) to a widespread visceral leishmaniasis (VL) known as kala-azar, which is a fatal disease if left untreated [3]. Leishmaniasis can possess two means of transmission either anthroponotic (from human to human) or zoonotic (from animals to human) [4, 5, 6]. *Leishmania* parasites are dimorphic, existing as intracellular amastigotes within the macrophage of the mammalian host, and as flagellated promastigotes in the midgut of *Phlebotomine* and *Lutzomyia* sand flies [7, 8, 9] Leishmaniasis is a global disease in its distribution, where it has been reported in 98 countries worldwide with approximately 0.4 to 0.2 million cases and 1.2 to 0.7 million CL and VL cases, respectively, occurring every year [10]. CL is more widely distributed than the other two forms of leishmaniasis (MCL and VL), with about one-third of cases occurring in three epidemiological regions, the Americas, the Mediterranean basin, and Western Asia from the Middle East to Central Asia [11]. The ten countries with the highest estimated case counts for leishmaniasis is Syria, Afghanistan, Brazil, Algeria, Colombia, Iran, Ethiopia, North Sudan, Costa Rica and Peru where these regions together account for 70 to 75% of the global CL incidence [12,13]. In Jordan CL the only form which exists in the country is regarded as an endemic disease. Two species (*L. major* and *L. tropica*) were reported from many regions all over Jordan where the transmission of *L. major* is regarded as zoonotic which occurs through the bite of infected female sand flies most likely *Phlebotomus papatasi* [14], which gets the infection with *Leishmania* parasites from reservoir host animals such as *Psammomys obesus* [15]. The other species exist in Jordan is *L. tropica* which is transmitted by *Phlebotomus sergenti* species of sand flies that acquires the
Infection from suspected reservoir hosts (canines and possibly hyrax) [16, 17]. The life cycle of *Leishmania* parasites is complex and usually involves both vertebrate and invertebrate hosts. It can be divided into two developmental stages: the first developmental form which is known as promastigote, the form which proliferate in the midgut of a female sand fly vector, and the second developmental form known as amastigote form is found in the mononuclear phagocytes and circulatory systems of humans. Amastigotes are taken up from the blood of an infected host when the female sand fly bites, and in the sand fly midgut they develop into promastigotes where they multiply by binary fission; promastigotes are introduced into the vertebrate host when the sand fly bites again. The promastigotes injected by the sand fly during feeding are phagocytized and developed into intracellular amastigotes.

### 1.1 Statement of the problem

In Jordan, CL is an endemic disease and mainly caused by *L. major*, utilizing *P. papatasi* as a vector and *Psammomys obesus* as its major reservoir host and the transmission is believed to be zoonotic. Historically, there has been little evidence of anthropoponic transmission of CL (ACL) caused by *L. tropica* in Jordan [139]. Nonetheless, Jordan, like Lebanon and Turkey, which also host large numbers of Syrian refugees, is at high risk of ACL to be introduced, firstly among the refugee population and subsequently among Jordanian residents [43].

The war in Syria has greatly increased the risk for CL and reports have indicated sharp increase in the number of CL cases in Syria and in the surrounding areas of the Middle East [18]. The annual incidence of CL in Syria between 2004 and 2008 was estimated to be 23,000 cases per year, however in 2012, 53,000 cases were reported, and in the first half of 2013 alone, 41,000 cases were reported [19]. Therefore, there is a concern from the current influx of Syrian refugees that are actually infected with CL with the possibility for outbreaks occurrence in camps areas that are reported to be free of the disease but confirmed to have the sand flies vector such as Al-Azraq [71]. In addition the possibility of anthropoponic transmission might be increased in the crowded camps [140].

The accurate diagnosis of CL is highly necessary for therapeutic purposes and for epidemiological surveillance. In Jordan the diagnosis of CL depends mainly on the clinical picture of the lesion and the confirmation of the presence of the parasite stage (amastigotes) by direct microscopy of Giemsa-stained smears prepared from scrapings
taken from the margins of the suspected skin lesions. PCR-based methods for the
diagnosis of CL have provided the ability to identify Leishmania species, but currently
are not employed in Jordan. PCR amplification has been proven to be a rapid, highly
sensitive and specific for *Leishmania* diagnosis and at the same time it can discriminate
the different *Leishmania* species, and sometimes at the sub-species level as well, which
is critical for both clinical diagnosis and epidemiological reasons mainly to track the
source of infection especially during outbreaks and also to facilitate and help in the
implementation of control programme.

1.2 Objectives

1.2.1 Identification and typing of *Leishmania* isolates obtained from Jordanian CL
patients (from years, 2009, 2015 and 2016) and Syrian refugees CL patients (who
entered Jordan in 2016 and suffering from CL) using three molecular typing
techniques: the ITS1-PCR-RFLP, the nested ITS1-5.8S rDNA gene PCR and kDNA
Lmj4 and Uni 21 gene PCR- RFLP.

1.2.2 Comparing the efficiency of the above mentioned three techniques in *Leishmania*
identification and speciation.

1.2.3 Assessing the evolutionary and phylogenetic relationships among Jordanian and
Syrian isolates by sequencing and phylogenetic analysis.
2. Literature review

2.1. Leishmaniasis epidemiology and distribution

2.1.1 Global prevalence and distribution of leishmaniasis

Leishmaniasis is a parasitic infection which is transmitted by the female of the infected sand flies. According to recent reports, leishmaniasis is endemic in 98 countries, and around 1.3 million new cases are reported annually, with an estimated 20,000 to 40,000 deaths every year (Figure 1) [20]. Leishmaniasis was found to be prevalent in poor countries in the world and clinically manifest itself in three different clinical forms; depending upon the infecting *Leishmania* species and the disease manifestation and is usually classified as cutaneous, mucocutaneous and visceral leishmaniasis [21]. Cutaneous leishmaniasis (CL) is the most common and the least lethal form of the disease which manifests itself in infected patients as ulcerative skin lesions, and it is caused by several *Leishmania* species: *L. major*, *L. aethiopica*, *L. tropica*, *L. mexicana*, *L. panamensis*, *L. guyanensis*, *L. braziliensis*, *L. peruviana*, and *L. amazonensis* [22]. Almost two-thirds of CL cases are reported from six countries: Afghanistan, Algeria, Brazil, Colombia, Iran, and Syria [23]. Mucocutaneous leishmaniasis (MCL) results in complete or partial destruction of mucous membranes of the nose and mouth, and is caused by *L. braziliensis* and *L. panamensis* species and restricted to South America [24]. Visceral leishmaniasis (VL) is caused by the two species *L. donovani* in East Africa and the Indian subcontinent and *Leishmania infantum* in Europe, North Africa and Latin America [25]. VL is regarded as the worst type of leishmaniasis and is considered to be the most life-threatening disease which is highly endemic in the South Asia, East Africa and Latin America [26]. An estimated 200,000 to 400,000 new cases of VL occur worldwide each year and about 90% of new cases occur in six countries: Bangladesh, Ethiopia, Brazil, India, and Sudan [27].
2.1.2. Regional prevalence and distribution of cutaneous leishmaniasis

Cutaneous leishmaniasis is an endemic disease in many Middle East countries including Jordan and despite the establishment of national control programs for eradicating and diminishing the vector (sand flies) and the treatment of the infected patients; however the disease continues to spread [29]. In addition to the endemcity of leishmaniasis in the Middle East, the region experienced excessive human migration and displacement in recent years due to the political revolution and instability in many Middle East countries such as Iraq, Syria, and Lebanon which might in part contributed to the spread and amplification of the causative species *Leishmania* and its sand fly vector.

Most of the CL isolates obtained from human patients in the Mediterranean area usually belong to two species *L. tropica* and *L. major* [30]. Other clinical samples have also shown to be positive for *L. infantum* [31]. Both zoonotic CL, caused predominantly by *L. major*, and anthroponotic CL, caused by *L. tropica*, are widespread in the Middle East and North Africa (MENA region) [32]. The largest number of *L. major* cases occurs in the arid areas of Iran, Saudi Arabia, Morocco, Tunisia, Syria, Libya, and Iraq, with most of the cases transmitted by the sand fly vector *Phlebotomus papatasi* or closely related species [33]. The largest numbers of *L. tropica* cases occur in Syria, Iran, Morocco, and Yemen, in addition to Algeria, where they are transmitted by *Phlebotomus sergenti*, especially in urban areas [34]. Syria is known to have the highest
prevalence of CL in the MENA region [35]. CL lesions referred to as Aleppo boil after the Syrian city Aleppo [36]. The incidence rate of CL has increased in Syria during the last fifteen years from 12,027 cases in 1997 to 58,156 cases in 2011 (Figure 2). Recently, many outbreaks have been observed to occur inside Syria due to the ongoing war and the lack of measures to combat the disease, particularly in the besieged and medically underserved areas [37]. New publications from the Ministry of Health in Syria revealed shocking statistic regarding CL recent incidence. In 2012, the incidence rate was 53,000 cases and jumped to 41,000 cases in the first 2 quarters of 2013 [38].

CL caused by *L. tropica* is still endemic in its traditional home of Aleppo, but also in Edlib, Lattakia, Tortous, Hama and the city of Damascus [39]. CL caused by *L. tropica* represents about 90% of all CL cases and is one of the most important public health problems in the Syria, especially in Aleppo [40]. CL caused by *L. major* is less common in Syria and occurs in rural areas close to Damascus, Deir al Zour and Al Hasakeh [41].

Recently, due to the massive population migrations caused by the ongoing Syrian crisis, new CL outbreaks have been reported in countries bordering Syria including Turkey, Iraq and Lebanon [42].

Figure 2. Year-wise trend of CL cases reported in some Middle Eastern Countries including Jordan [43]
2.1.3 Local prevalence and distribution of cutaneous leishmaniasis

Cutaneous leishmaniasis caused by *L. major* is an endemic disease in Jordan which is known as “Jericho boil.” Since 1985, outbreaks were reported in areas where CL was previously unknown to occur in these areas [44, 45]. The Jordan valley especially Sweimeh is the well-known endemic area for CL with a very high infection rates [46]. Sweimeh is a village near the Dead Sea and is regarded as the hyper-endemic area for CL, where about 100% of individuals over 5 years old were found to be seropositive in the leishmanin skin test in 1992. Higher infection rates were recorded in males (72.4%) than females (27.6%) [47]. Outbreaks of CL have been reported in Aqaba, north Agwar, and south Shuneh, with 100–200 cases being reported from 2004 to 2005. However, this number is probably skewed due to a spike in 2007 where 354 cases were reported. The most recent Jordanian annual report confirmed a total of 2,560 CL cases throughout Jordan between 1994 and 2014 (Figure 3) [48]. In Jordan the reservoir host has been identified as *Psammomys obesus*, and the vector, *Phlebotomus papatasi*. [49] However, it has been recognized in recent years that a particular CL form due to a variant of *L. tropica* also occurs in widely separated foci [50]. Reliable evidence was found that *Phlebotomus sergenti* might act as a vector and the hyrax as reservoir hosts, present in all these *L. tropica* foci [51, 52]. Historically, there has been little evidence of anthropontic CL (ACL) transmission caused by *L. tropica* in Jordan. Nonetheless, Jordan, like Turkey and Lebanon, which also host large numbers of Syrian refugees, is at high risk for the introduction of ACL which might be established first among the refugee population and subsequently transmitted into the host communities [53].

Severe underreporting of CL is suspected in Jordan, which impacts its eradication [54]. Many factors can lead to this underestimation, some of which include the self-healing nature of the disease, the lack of awareness of the physicians of the importance of disease notification, and the occurrence of the majority of the CL cases in endemic rural areas which have limited resources for treatment due to the scarcity of clinics in these areas [55].
Figure 3. Cutaneous leishmaniasis trends in Jordan (Number of confirmed cases 1997-2015) [56].

2.2. The life cycle of the *Leishmania* parasite

Leishmaniasis is spread by the bite of female sand flies of the genus *Phlebotomine* and *Lutzomyia*, and is caused by more than 20 recognized flagellated unicellular protozoa belonging to the genus *Leishmania* which are responsible for different clinical forms of leishmaniasis [57]. The *Leishmania* parasite may follow two different transmission cycles: a zoonotic cycle, with mammals as reservoir hosts; and a strictly anthroponotic cycle, with humans as the only host, which is typically observed in densely inhibited urban areas [58, 59].

Many mammalian species could act as a reservoir host for *Leishmania* parasite, such as rodents and dogs [60] where the parasite amplify and proliferate in their macrophages as amastigote stage which is ingested by the sand fly vector. Amastigotes are round non-motile, intracellular sages (3–7 µm in diameter), which are ingested by the sand flies vector where they are released into the posterior abdominal midgut of the sand fly, and transform into the infective promastigotes to begin their extracellular life cycle in the vector. Promastigote is the motile, elongated (10–20 µm) stage of *Leishmania* parasite and possess a single flagellum usually migrate to the anterior part of the alimentary tract of the sand fly where they multiply by binary fission. Approximately 7 days after the sand fly feeding on *Leishmania* infected mammalian host; the promastigotes undergo a gametacyclogenesis process and become infectious (metacyclic promastigotes). Metacyclic promastigotes are released into the salivary
glands of the sand flies and injected along with the fly saliva into the skin of the mammalian host during feeding [61]. Thereafter the metacyclic promastigotes are taken up by host macrophages, where they are transformed into amastigotes form which increases in number by binary fission until the cell eventually bursts and the amastigotes are released to re-infect other phagocytic cells and continue the cycle. In case of visceral leishmaniasis, all organs, containing macrophages and phagocytes, can be infected, especially the lymph nodes, liver, spleen, and bone marrow [62].

Figure 4. Illustration of the life cycle of Leishmania spp. (A) [63], a detailed structure of the promastigotes (B) and amastigotes (C). In (A), a female sand fly (1) bites an infected person during the blood meal. Macrophages become infected and harbor the amastigotes form of the parasite (2). Infected macrophages with amastigotes forms (3). Amastigotes are transformed into procyclicpromastigotes (4) that proliferate in the midgut of the sand fly (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 sand fly 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.

2.3. Vector and Reservoir hosts
2.3.1. Vector

Sand flies are the vector of Leishmania parasite which belongs to the order Diptera, family Psychodidae, and subfamily Phlebotominae of the genus Phlebotomus (Old World), and genus Lutzomyia (New World) [64]. Sand flies are generally no more than 3.5 mm in length and covered with dense hair, holding their wings in a characteristic V-shaped position [65]. They are generally active during the night and early morning. Both male and female adults survive on sugary secretions from plants; females, though, require blood meal for development of egg batches [66]. There are different modes of transmission of leishmaniasis in the Middle East; zoonotic cutaneous leishmaniasis (ZCL), caused by L. major, transmitted through P. papatasi, with rodent species [67]. Zoonotic visceral leishmaniasis (ZVL) which is caused by L. infantum, and spread through P. galilaeus, P. syriacus, P. tobbi, P. halepensis [68]. Anthroponotic cutaneous leishmaniasis (ACL) which circulates exclusively in human. Anthroponotic visceral leishmaniasis (AVL) caused by L. donovani spreads through P. alexandri without any non-human reservoir [69].

In Jordan, the genus Phlebotomus includes 11 species (P. alexandri, P. arabicus, P. halepensis, P. jacusiel, P. kazeruni, P. major P. syriacus, P. mascittii, P. papatasi, P. perfiliewi galilaeus, P. sergenti and P. tobbi) [141]. P. papatasi was reported in the majority of sites in Jordan, being the dominant species in Jordan especially in (Sweimeh) where CL caused by L. major is common. It was more abundant around houses than in areas away from human habitation [70]. P. sergenti, the vector of ACL caused by L. tropica was found in several areas in Jordan including Awajan, Mowoqqar, Barha, Ras el Naqb, Bushra and Azraq. The Azraq Syrian refugee camp,
located near Azraq where the *P. sergenti* vector was found which can increase the ability of ACL transmission [71, 72].

### 2.3.2 Reservoir hosts

The transmission of leishmaniasis is either anthroponotic, where infection is transmitted from human to human through the sand fly vector, or zoonotic, where an animal reservoir host is involved. In the Middle East ZCL, caused by *L. major*, with rodent species such as *Psammomys obesus*, *Meriones libycus*, *Nesokia indica*, and *Rhombomys opimus* serving as non-human reservoirs [73]. ZVL is caused by *L. infantum*, and the dog species *Canis familiaris* is suspected to act as a non-human reservoir [74].

In Jordan, *L. major* infection is maintained by the reservoir host *Psammomys obesus* and the sand fly *P. papatasi* as a vector [75, 76]. *P. obesus* rodent (also called gerbil) possess a specific habitat requirements since it feed exclusively on leaves and stems of succulent plants of the family: Chenopodiaceae. The incidence of CL was reported to be similar to the distribution of *P. obesus* colonies in the region [77, 78]. The density of *P. papatasi* population in uncultivated areas was correlated with the soil conditions favoring high humidity in *P. obesus* burrows [79]. Gerbil plays a major rule in the epidemiology of leishmaniasis (ZCL), where they live in underground burrows that dug in soft soil close to vegetation with many openings, passages and chambers, which maintain high humidity and controlled temperature regardless of the sever heat and dryness of the external environment [80]. The underground burrows provide sand flies with shelter and, presumably, breeding grounds.

### 2.4 Clinical forms of leishmaniasis

Leishmaniasis can manifest itself in different clinical forms; depending upon the infecting species of *Leishmania*, the disease could emerge as cutaneous, mucocutaneous, or visceral leishmaniasis. CL is characterized merely by skin manifestations including papules and nodules that may ulcerate. Although this skin papule tend to heal spontaneously, but at the same time might result in disfiguring and stigmatizing scars with a considerable impact on the quality of life [81].
2.4.1 Cutaneous leishmaniasis

Cutaneous leishmaniasis is the most common form of leishmaniasis which is characterized by the presence of skin lesions, mainly ulcers, on exposed parts of the body, causing bad manifestation as scars and some times serious disability. CL often infects only the skin, and may be characterized by one to dozens of lesions depending on the number and location of the sand fly bites [82]. According to the species of *Leishmania*, ulcers, smooth nodules, flat plaques or hyperkeratotic wart-like lesions may be seen. The initial skin lesions, which occur after sand fly bite, are usually papules. Many lesions remain localized, but in some cases, the parasites may transfer via the lymphatics and produce secondary lesions on the skin, or other parts of the body causing sometimes local lymphadenopathy [83]. CL is usually painless unless the lesions become secondarily infected, and except in the ear, the ulcers tend to remain close to the skin and do not affect the subcutaneous tissues. Most skin lesions recover spontaneously; however, the speed of healing varies with the species of *Leishmania*. In some cases, it may take several months to a year or longer [84].

*Leishmania major* tends to cause ‘wet’ exudative lesions that are less chronic than the “dry” lesions with central crusting that are caused by *L. tropica*. The main difference appears to be the duration of the *L. tropica* lesion, which can persist as an erythematous papule unchanged for more than three years and become more difficult to treat [85]. In hyperendemic regions multiple lesions can be frequently observed mainly on the exposed parts of the body. Beside CL, *L. tropica* has been incriminated as the causative agent of either a systemic illness or classical visceral leishmaniasis which can be fatal [86].

Figure 5. Appearance of lesions from confirmed cases of human cutaneous leishmaniasis, the photo taken by researcher in Al-Azraq camp.
2.4.2 Visceral leishmaniasis

Visceral leishmaniasis is a fatal disease if left untreated in over 95% of the cases. It is characterized by fever, weight loss, enlargement of the liver and spleen, and anemia [87]. The most severe and potentially fatal form of leishmaniasis is VL, which affects internal organs including the bone marrow, liver, and spleen, and may exhibit a latent form reviving during states of immunodeficiency, where its extreme pathogenicity comes from the ability of this species to disseminate to visceral organs like the liver, bone marrow, and spleen. The general clinical symptoms of VL include prolonged and atypical fever associated with hepatosplenomegaly, chills, lymphadenopathy, progressive anemia, weight loss, and hypergammaglobulinemia [88].

2.4.3 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis causes partial or total destruction of oral-nasal and pharyngeal cavities with hideous disfiguring lesions, mutilation of the face and great suffering for life [89]. MCL is typically found in the Americas and caused by different species (*L. braziliensis*, *L. amazonensis*, *L. panamensis*, and *L. guyanensis*) and mainly transmitted by *Lutzomyia* species of sand flies [90].

2.5. Prevention and Treatment

No vaccines or preventive medications are available for CL so far and the best prevention is to avoid sand fly bites by using protective clothing, insecticides, and insect repellants. Bed nets impregnated with permethrin give good protection, but ordinary screening my not keep out sand flies because of their small size (about 2 mm). Outdoor activities between dusk and dawn should be avoided when possible. Selected cases of CL can be followed carefully without treatment as long as the infected individuals are healthy and have small stable lesions. For others, currently, Pentostam represent the treatment of choice. It is given by injection over a three or four week period for CL patients, and much longer for VL. Anti-fungal treatment such as amphotericin B and ketoconazole, and the aminoglycoside, paromomycin can also be used in some cases [91, 92].
2.6 Methods of leishmaniasis diagnosis

Diagnosis of leishmaniasis is based mainly on clinical presentation, and the results of laboratory tests. Careful differential diagnosis is very critical for epidemiological surveys and therapeutic purposes [93]. In Jordan microscopic examination of Giemsa-stained parasites from skin lesion and aspirates from spleen, bone marrow, and lymph nodes for CL and VL, respectively, are commonly used. Tests based on the serological detection of anti-Leishmania antibodies in patient’s serum by ELISA and direct agglutination test is also used. More specific and sensitive techniques such as the PCR-based methods are considered to be highly specific and can detect current infection, unlike immunological tests that cannot distinguish between current and past infections. Moreover, PCR based methods was repeatedly used for the specific diagnosis and discrimination of Leishmania species [94]. The PCR-RFLP method was found to be highly sensitive and specific in detecting samples that have Leishmania when compared to the Giemsa-stain microscopy method. A major drawback for the use of PCR-based methods is that it requires a certain setup of instruments and expertise which is not available usually outside research settings. Apart from the problems with availability and cost-effectiveness of the PCR techniques, the lack of standardized protocols makes it a challenge to use this technique especially in the field [95].

2.7. Criteria for species typing methods

The search for a universally acceptable typing method for Leishmania discrimination has been going on for decades. Species typing tool has to be able to discriminate all 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. The employed typing method should preferably be globally applicable and be able to discriminate all the species that might unexpectedly appear in one or more regions around the world. The assay must provide enough sensitivity, especially for typing that starts with culture-independent steps. The applied testing methods have to be Leishmania specific. It is easier to standardize a PCR based method than, for instance, RFLP where small size differences and incomplete digests may interfere with the analysis of results. This also proves difficulty in species-specific PCRs, hybridization assays, and melting assays [142, 143].
2.7.1. PCR-based typing techniques

Molecular testing for *Leishmania* has become a reliable diagnostic tool in many clinical microbiology labs worldwide. PCR amplification methods are thought to be highly efficient for the identification of *Leishmania* species and the most widely used ones are the PCR-RFLP and PCR-ITS [96, 97]. *Leishmania* parasites contain only \(10^{-13}\) g of DNA, which means that 10 million parasites would be required to generate 1 µg of workable DNA. This makes gene detection in *Leishmania* isolates extremely difficult [98]. In order to obtain sufficient amount of DNA for further analysis, the process of DNA extraction has to be critically efficient. *Leishmania* promastigotes contain approximately 10,000 copies of the kinetoplast DNA (kDNA) target which is 50 and 250 fold higher than ITS1 target regions (40 to 200 copies) [99]. The Lmj4/Uni21 PCR, also targeting (kDNA), could detect 0.25 parasites. Nested PCR of the ITS1-5.8S rDNA region allowed the detection of 1 pg/µL of DNA as opposed to the ITS1-PCR method that detects 10 pg/µL of extracted DNA [100]. Hence, the observed differences in sensitivity can be due to several variable parameters within the PCR assays such as the quality of the clinical samples, the DNA extraction method that largely determines the extracted DNA quality and quantity and finally, the *Taq* polymerase used as well [101]. 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. L5.8S and LITSR has been used to target the entire ITS-1 region while the primers IR1 and IR2 followed by nested PCR using ITS1F and ITS2R4 target the ITS1-5.8S rDNA gene region. ITS1 region is located between the 18S rRNA and 5.8S rRNA genes. 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 [102, 103].

2.7.2. 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. Nested PCR provides a sensitive and specific alternative to traditional techniques used for *Leishmania* identification. Although often tedious and time-consuming, nested PCR
has been widely employed in *Leishmania* identification methods. 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. Nested ITS1-5.8S rDNA gene PCR provided enough sensitivity for the discrimination of most clinically relevant *Leishmania* species [104].

### 2.7.3. Restriction fragment length polymorphism (RFLP) analysis

One of the most widely used techniques for speciation and sub-speciation of *Leishmania* isolates is RFLP (restriction fragment length polymorphism) analysis. 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. *HaeIII* restriction enzyme is the most commonly used since it allow the discrimination of members of the *L. major* and *L. tropica*. Also *RsaI* was also used to discriminate between the *L. tropica* subspecies [105, 106, 107].

### 2.7.4. Sequencing and phylogenetic analysis

A more informative method which can further confirm and discriminate *Leishmania* species is the sequence analysis of the PCR amplicon. The subsequent identification of single-nucleotide polymorphisms (SNPs) or the comparison of the obtained sequence with available reference sequences facilitates species identification and the tracking of evolutionary relationships among the sequenced isolates [114]. 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. 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 [108, 109].
CHAPTER THREE
MATERIALS AND METHODS

3.0 Materials and Methods:

3.1 Ethical approval:

This study was approved by the Hashemite University Institutional Review Board, the Ministry of Health Institutional Review Board and the Ministry of Interior approval for AL-Azraq camp entrance.

3.2 Sample collection:

The study participants from Jordan were suspected patients with CL, 39 specimens were collected from patients who were admitted to the Ministry of Health and Royal Medical Services clinics. For Syrian 27 specimens were collected from Syrian refugees in Al-Azraq Camp who were CL suspected patients. All patients gave their informed consent to participate in the study, which was reviewed by the institutional review board and approved by the Research Ethics Committee at Hashemite University and the Ministry of Health. Therefore, a total of 66 patients thought to be infected with CL based on clinical examination only (i.e., size, number, location, and type of lesion) were recruited in the present study. Demographic data from the recruited patients were collected after filling a designed questionnaire and thereafter, skin scrapings were saved on NucleoSave Cards from CL suspected lesions (Figure 6).

Figure 6. NucleoSave cards were the samples saved for DNA extraction.
Figure 7. Chart showing a summary of the procedures conducted and a summary of the results obtained.

3.3 DNA extraction from NucleoSave Cards:

The DNA was extracted from the clinical samples using QIAamp® DNA Mini kit. Briefly, three discs (3 mm in diameter) were punched out from lesion of each clinical case was taken on the NucleoSave cards using a sterile blade and transferred to a sterile Eppendorf tubes containing 180 µl cell lysis buffer (ATL), (incubated at 85°C for 10 min), 20 µl of proteinase K were added to the test tube (incubated at 56°C for 1 h), after its incubation with 200 µl buffer (AL) which was added with thoroughly mixing (incubated at 70°C for 10 min). 200 µl ethanol (96-100%) was added to the sample then the mixture was applied to the QIAamp Mini spin column (centrifuged at 8000 rpm for 1 min), then the samples were washed using QIAamp Mini spin column and a washing solution was used to purify the extracted DNA, finally the elution buffer was added to each sample to get a 50 µl volume containing the extracted DNA.
3.4 ITS1 PCR of *Leishmania* isolates

The extracted DNA was identified by *Leishmania*-specific ribosomal ITS1 region by PCR amplification using L5.8S and LITSR primers (Table 1) followed by RFLP analysis. 2 µl of DNA was used for ITS1 gene amplification in a 20 µl total reaction volume. 4 µl of master mix (0.4 M Tris-HCl, 0.1 M (NH₄)₂SO₄, 2.5 mM MgCl₂, 1 mM dNTPs, blue dye and yellow dye) were added to the reaction with 0.5 µl of (L5.8S and LITSR) primers and 13 µl of nuclease free water were added to get 20 µl final volume, the cycling conditions were 95 °C for 12 min followed by 44 amplification cycles, each consisting of three steps: 94 °C for 20 s, 53 °C for 30 s and 72 °C for 1 minute, followed by a final extension at 72 °C for 6 min. All PCR assays were performed on (Bio-Rad C1000 Touch™) thermal cycler. PCR amplicons were analyzed by 2.0 % agarose gel electrophoresis. 5 µl of PCR products were separated by electrophoresis at 100 V in 1X TBE buffer and compared to a standard 50 bp DNA ladder. A DNA band of 300-350 bp was considered as a positive indicator for the presence of *Leishmania*.

3.5 ITS1-PCR-RFLP analysis

Ten microliters of the PCR products were digested with 2 µl of the restriction endonuclease (*HaeIII*) in 2 µl of 10 X Buffer (Promega) (1 X of buffer consist of 10 mM tris HCL (pH 8.5 at 37 °C, 10 mM MgCl₂, 100 mM KCl, 0.1 mg/ml BSA) and 18 µl nuclease free water H₂O. Digestion was performed in a thermal cycler, in a total reaction volume of 32 µl, with the following conditions: 6 h at 37 °C followed by 20 min at 80 °C. 10 µl of the PCR product were run on 3.0 % agarose gel in 1 X TBE for 1 h. The obtained bands were compared to 50 bp DNA ladder.

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 MgCl₂, 60 µM of each dNTP, 1 µl of IR1 and 1 µl 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 MgCl2, 60 μM of each dNTP, 1 μl of ITS1F and 1 μl 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 1st stage of amplification. The obtained bands were subjected to electrophoresis on a 1.5% agarose gel in 1X TBE buffer and compared to a 100 bp ladder.

Table (1) the primers used in this study, their corresponding sequences and target amplicon size.

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Primer sequences</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>LITSR</td>
<td>5′-CTGGATCATTTTCCGATG-3′</td>
<td>300–350 bp</td>
</tr>
<tr>
<td>L5.8S</td>
<td>5′-TGATACCATTTATCGCATT-3′</td>
<td>300–350 bp</td>
</tr>
<tr>
<td>IR1</td>
<td>5′-GCTGTAGGTAACCTGACAGCTGGATCATT-3′</td>
<td>320 bp</td>
</tr>
<tr>
<td>IR2</td>
<td>5′-GCGGGTAGTCTCGCAACACTCAGGTCTG-3′</td>
<td>320 bp</td>
</tr>
<tr>
<td>ITS1F</td>
<td>5′-GCAGCTGGATCATTTC-C-3′</td>
<td>400 bp</td>
</tr>
<tr>
<td>ITS2R4</td>
<td>5′-ATATGCAGAAGAGGAGAGGC-3′</td>
<td>400 bp</td>
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<td>Lmj4</td>
<td>5′-CTAGTACCCGCCTCGAGGAGG-3′</td>
<td>650-800 bp</td>
</tr>
<tr>
<td>Uni21</td>
<td>5′-GGGGTTGATGTAACATAGGCC-3′</td>
<td>600 bp</td>
</tr>
<tr>
<td>Actin</td>
<td>5′-CGCTTGCTGGTCACTCAGCTGAGC-3′</td>
<td>600 bp</td>
</tr>
</tbody>
</table>

3.7 RFLP analysis of the kinetoplast minicircle DNA (kDNA)

Polymerase chain reaction amplification of the leishmanial kDNA minicircle for the *L. tropica* was performed using the primer pair Uni 21 and Lmj4 (Table 1). The cycling conditions were 94 °C for 4 min followed by 35 amplification cycles, each consisting of three steps: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. the PCR products were digested with endonuclease *RsaI*.

Six microliters of the PCR products were digested with 1 μl of the restriction endonuclease (*RsaI*) in 2.5 μl of 10 X Buffer (Biolabs) (1 X of buffer consist of 50 mM
Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, and 100 μg/ml BSA, pH 7.9 at 25°C). Digestion was performed in a thermal cycler, in a total reaction volume of 9.5 μl, with the following conditions: 3 h at 37 °C. 10 μl of the PCR product were run on 3.0 % agarose gel in 1 X TBE for 1 h. The obtained bands were compared to 50 bp DNA ladder.

3.8 DNA sequencing reaction

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). The amplicons were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA). 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 (Table 1). All sequencing reactions were performed with 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 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 7 (CLC BIO, Denmark) software. Sequences were aligned and a phylogenetic tree was constructed using the Neighbor-Joining algorithm in CLC Main Workbench 7 and bootstrap values for 1000 replicates were indicated.

3.10 DNA quality assessment

In order to assess the quality of the extracted DNA, actin amplification was performed under the following conditions: A total reaction volume of 20 μL was prepared consisting of 0.2 mM dNTPs, 1 X Taq buffer, 1.5 mM MgCl₂, 0.5 ml of 10 pM of both forward and reverse primers and 2 U of Taq DNA polymerase. For actin, 5 μL 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. 5 μL of the obtained PCR products were loaded on a 2.0% agarose gel and compared to a 100 bp DNA ladder.
CHAPTER FOUR
RESULTS

4.1 Sampling sites and demographic data

The collected samples from patients suspected to be infected with CL were obtained from different locations in both Syria and Jordan (Figure 8). The locations are extend from the North to the South of Jordan these locations include North Ghor, Irbid, AL-mashare’a, Jarash, Ajloun, Zarqa, Amman, Sahab, Arabah, Iraq Al-Amir, Dair alla, Dead Sea, Sowimeh, Ghor Alsaffe, South Shouna, Karak. The Syrian samples were obtained from Azraq camp; all Syrian suspected CL patients were infected inside Syria before they arrive to the camp from the following areas (Deir ez-zor, Aleppo, Palmyra, Hims, Idlib).

The age of the suspected CL patients who participated in the present study varied from 1 to 63 years (mean: Syrian 20.1 and Jordanian 29.3 years). Among the Jordanian patients 66.7 % of them were males and 33.3% were females, however, among the Syrian patients 59% were males and 41% were females. All suspected CL patients were diagnosed based on the clinical presentation by a dermatologist, however just the diagnosis of CL for two Jordanian patients was confirmed by culturing the parasite in Schneider’s Drosophila Media at Jordanian Ministry of Health laboratory. The number of skin lesions which were observed in the 66 recruited patients ranged from 1 to 15 lesions in Syrian patients and 1 to 6 lesions in Jordanian patients, most of the inspected lesions appeared as papules or ulcers (Table 2 and 3).

4.2 DNA Extraction

Skin scraping from the 66 suspected CL patients were spotted on NucleoSave Cards and were further inspected and diagnosed using molecular based assays. The obtained DNA concentrations ranged from (7.3 ng/μl to 30.7 ng/μl). DNA extraction was successful for 29 (44 %) of the spotted skin scrapings samples, 9 (30%) of the positive DNA samples were *L. tropica* and 20 (70 %) were *L. major*. 
Figure 8: Map of Jordan and Syria, showing the foci of the 66 suspected CL cases and their distribution; the red dots represent the sample location.
Table 2: Major characteristics of the study participants (Syrian) (N = 27)

<table>
<thead>
<tr>
<th>Characteristic (N)</th>
<th>M (16) 59%</th>
<th>F (11) 41%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>M (16) 59%</td>
<td>F (11) 41%</td>
</tr>
<tr>
<td>Age (years)</td>
<td>3 – 62</td>
<td>Average 20.11</td>
</tr>
<tr>
<td>Nationality</td>
<td>Syrian (27)</td>
<td></td>
</tr>
<tr>
<td>Infected body parts</td>
<td>Facial (20)</td>
<td>Upper Limbs (15)</td>
</tr>
<tr>
<td>Stage of infection</td>
<td>Papule (20)</td>
<td>Ulcerated (6)</td>
</tr>
<tr>
<td>Number of lesions</td>
<td>Average 2.0</td>
<td>Minimum 1</td>
</tr>
<tr>
<td>Type of diagnosis</td>
<td>Clinical (27)</td>
<td>Laboratory (0)</td>
</tr>
<tr>
<td>PCR results (Leishmania species)</td>
<td>L. major (6)</td>
<td>L. tropica (3)</td>
</tr>
<tr>
<td>Treatment</td>
<td>Yes (27)</td>
<td>No (0)</td>
</tr>
<tr>
<td>Jordan Border Entry Date</td>
<td>From (1/1/2016)</td>
<td>To (1/5/2016)</td>
</tr>
<tr>
<td>Origin of Infection</td>
<td>Aleppo (4)</td>
<td>Hims (18)</td>
</tr>
<tr>
<td>Treatment Pentostam Injection</td>
<td>Yes (27)</td>
<td>NO (0)</td>
</tr>
</tbody>
</table>

N: Number; M: male; F: female.

Table 3: Major characteristics of the study participants (Jordanian resident) (N = 39)

<table>
<thead>
<tr>
<th>Characteristic (N)</th>
<th>M (26) 66.7%</th>
<th>F (13) 33.3%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>M (26) 66.7%</td>
<td>F (13) 33.3%</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1 – 63</td>
<td>Average 29.3</td>
</tr>
<tr>
<td>Nationality</td>
<td>Jordanian (32)</td>
<td>Other (7)</td>
</tr>
<tr>
<td>Infected body parts</td>
<td>Facial (15)</td>
<td>Upper Limbs (27)</td>
</tr>
<tr>
<td>Stage of infection</td>
<td>Cured (2)</td>
<td>Papule (17)</td>
</tr>
<tr>
<td>Number of lesions</td>
<td>Average 1.56</td>
<td>Minimum 1</td>
</tr>
<tr>
<td>Type of diagnosis</td>
<td>Clinical (37)</td>
<td>Laboratory (2)</td>
</tr>
<tr>
<td>PCR results (Leishmania species)</td>
<td>L. major (14)</td>
<td>L. tropica (6)</td>
</tr>
<tr>
<td>Treatment</td>
<td>Yes (10)</td>
<td>No (29)</td>
</tr>
<tr>
<td>Origin of Infection</td>
<td>South Shouna (6)</td>
<td>AL-mashare’a (4)</td>
</tr>
</tbody>
</table>

N: Number; M: male; F: female.

4.3 Leishmania genotyping

The first step of genotyping was to identify the Leishmania species. For this purpose, ITS1 PCR-RFLP was performed. In order to enhance and confirm the sensitivity of the results, second type of PCR was applied which is the nested PCR of ITS1-5.8S rDNA gene region. Finally RFLP analysis of the Kinetoplast minicircle DNA was performed for the characterization of isolates infected with L. tropica (Table 4 and 5).
4.4 ITS1 and kDNA PCR of *Leishmania* isolates

LITSR and L5.8S primers were used to detect the ITS1 gene, after performing PCR on all isolates using the previous primers, (29) out of (66) isolates (44%) produced a band at around 350 bp which confirmed the presence of *Leishmania* species (Figure 9). Also Lmj4 and Uni 21 primers were used for the identification of *Leishmania*, (24) out of (66) isolates (36.4 %) produced 650 bp band for *L. major* and 800 bp band for *L. tropica* (Figure 10 A).

4.5 ITS1-PCR-RFLP analysis

RFLP analysis of the 29 PCR positive clinical specimens with the endonuclease *HaeIII* restriction enzyme identified 20 samples infected with *L. major* consisting of two bands (203 bp and 140 bp) and 9 were infected with *L. tropica* consisting of two bands (185 bp and 60 bp) (Figure 9).

![Figure 9: Representative picture showing agarose gel electrophoresis (3%) of random RFLP results which were extracted from the positive *Leishmania* samples. Lane M: 50bp DNA ladder. Lanes 1-9 showing the digestion of amplified ITS1 regions for different *Leishmania* species with the restriction endonuclease *HaeIII*. Lane 3: negative control. Lane. Lane 1: *L. major* positive control showing two bands (203 bp and 140 bp). Lane 2: *L. tropica* positive control showing three bands (185 bp, 60 bp). Lanes 4 and 8: random samples for *L. major* detected in clinical samples. Lane 5, 6, 7 and 9: *L. tropica* detected in clinical samples.](image)

4.6 Characterization of *L. tropica* PCR-RFLP analysis of kDNA

Amplification of the kDNA gene of the 7 *L. tropica* isolates gave the same- PCR product size (800 bp), which differ from the smaller-size (650 bp) PCR product of *L. major* (Figure 10 A). RFLP analysis of all 7 PCR products of *L. tropica* after their digestion with *RsaI* produced different kDNA RFLP profiles that consisted of two basic
kinds: *Ltro*-kD1 and *Ltro*-kD2, which can be differentiated mainly by the presence of a 417 bp component only in the *L. tro*-kD2 profiles (Figure 10, B).

(Figure 10 A)

(Figure 10 B)

Figure 10. The kDNA PCR and RFLP products of the 7 isolates of *L. tropica*. A, The kDNA PCR products: Lane 3 *L. tropica* positive control, lane 4, 5 and 6, *L. tropica* (800 bp), Lane 1 *L. major* positive control (650 bp). M: molecular weight marker 100 bp. B, The RFLP patterns of the 5 different genotypes resulting from digestion of kDNA PCR products with *RsaI*. M: molecular weight marker 50 bp; Lane 1, 4 and 5 *L. tro*-kD2, Lane 2 and 3 *L. tro*-kD1.
4.7 Nested PCR for amplifying the ITS1-5.8S rDNA gene of *Leishmania* isolates

Seven isolates were screened for *Leishmania* infection by nested PCR. The first PCR reaction using the IR1 and IRs primers followed by the second primers ITS1F and ITS2R4. The nested PCR reaction produced bands at around 400 bp (Figure 11).

![Figure 11. Nested-PCR of ITS1-5.8S rDNA gene of *Leishmania* DNA. Agarose gel electrophoresis of representative isolates positive for *Leishmania* produced a DNA band around 400 bp characteristic of *L. tropica*. M 100 bp DNA ladder was used as a molecular marker. N: Negative Control.](image)

Table 4. List of *L. tropica*, origin, and database accession numbers

<table>
<thead>
<tr>
<th>Species</th>
<th>Actin PCR</th>
<th>ITS1 PCR</th>
<th>Nested PCR</th>
<th>Lmj4 uni21 PCR</th>
<th>Rsal RFLP</th>
<th>Gene Bank Accession number</th>
<th>Geographical origin of patient</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. tropica</em></td>
<td>+</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td><em>Ltro-kD1</em> KT363779</td>
<td>Jordan-Ajloun</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
<td>+</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td><em>Ltro-kD2</em> KT363794</td>
<td>Jordan-Amman</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
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<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td><em>Ltro-kD1</em> KT363797</td>
<td>Jordan-Zarqa</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
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<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td><em>Ltro-kD2</em> KT363795</td>
<td>Jordan-Dair alla</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
<td></td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td><em>Ltro-kD1</em> N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
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<td>+</td>
<td>++</td>
<td></td>
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<td><em>Ltro-kD2</em> KT363795</td>
<td>Syria - Homs</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
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<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
<td>+</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td><em>Ltro-kD1</em> KT363796</td>
<td>Jordan-Karak</td>
</tr>
</tbody>
</table>

* sequencing was done for all positive nested-PCR.
N/A: not applicable
Alignment showed several polymorphisms and variations between the 7 L. tropica sequences 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. The phylogenetic tree showed two different Clades. These two Clades included: (Clade I) L. tropica (27) sequence which was compared to four references extracted from NCBI BLAST (KT363779, KT363778, and KT363786, KT363785), (Clade II) sequences L. tropica (33), L. tropica (32), L. tropica (31), L. tropica (28), L. tropica (30) and L. tropica (26) in Clade I showed significant similarity between (L. tropica (27) and KT363779) but with few point mutations. Also similarity was observed between (L. tropica (26) and L. tropica (30)) and (L. tropica (32) and L. tropica (33)) but with several point mutations (Figure 12 and 13). It was noticed that the samples (L. tropica (32) and L. tropica (33)) belongs to old collection date from a previous study in Jordan (2009).
Figure 12. 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 7. Bootstrap values are based on 1,000 replicates.
Figure 13: ITS1-5.8S rDNA gene sequences alignment of *L. tropica* isolates using CLC Main Workbench 7. Matching residues are depicted as dot and highlighted with the same color. Gaps are denoted as dashes.
4.9 Sequencing and Phylogenetic tree analysis of ITS1 gene for *L. major*

The phylogenetic tree showed two different Clades. These two Clades included: (Clade I) sequences (*L. major* (9), *L. major* (1), *L. major* (11), *L. major* (6), *L. major* (7) and *L. major* (8)) and (Clade II) sequences (*L. major* (5), *L. major* (4), *L. major* (2), *L. major* (12), *L. major* (10) and *L. major* (3)) where every Clade showed significant similarity but with several point mutations (Figure 14). It was noticed that samples (*L. major* (5), *L. major* (4), *L. major* (2), and *L. major* (3)) belongs to old collection date from a previous study in Jordan (2009).

Assemble sequences analysis showed several polymorphisms and variations between the 12 sequences. The significant conflicts were observed at position 195 and 200 with G→A change in (*L. major* (10), *L. major* (12), *L. major* (4), and *L. major* (2)), position 273 A→G change in (*L. major* (1), *L. major* (9), *L. major* (11), *L. major* (6), *L. major* (7) and *L. major* (8)), and position 291 and 293 T→A and A→G change respectively in *L. major* (8) and *L. major* (9). *L. major* (5) showed higher variability than other sequences (Figure 15 and 16). Regarding the G+C content, all sequences appeared to have G+C contents ranged from (41.1 % to 44.7 %).

![Figure 14. Neighbor-joining tree showing the relationships of the *L. major* isolates based on the ITS1 gene sequences, using the CLC Main Workbench 7. Bootstrap values are based on 1,000 replicates.](image)
Figure 15: ITS1 gene sequences alignment of *L. major* isolates using CLC Main Workbench 7. Matching residues are depicted as dot and highlighted with the same color. Gaps are denoted as dashes.
Figure 16: ITS1 gene sequencing for *L. major* assemble sequences revealed a high level of conservation with the presence of single point mutations using CLC Main Workbench 7.
CHAPTER FIVE

DISCUSSION

The present study was conducted in order to optimize and validate a sensitive diagnostic PCR method for the proper diagnosis of CL in Jordan, also to investigate whether the Syrian crises and the flow of refugees could influence the epidemiology and enhance the transmission of this devastating disease. The possibility for the occurrence of future outbreaks of CL among different areas of Jordan is high mainly around the Syrian refugee camps. Leishmaniasis is an endemic disease in Jordan which is always identified to be cutaneous in all previous reported cases in the country [118]. Throughout Jordan, Jordan Valley "especially Swaimeh village in the dead sea reported to have the highest infection rates and is regarded as a hyperendemic area for CL where 100% of individuals over 5 years old were found positive in a Leishmania skin test survey in 1992 [48]. Currently in Jordan CL is believed to be zoonotic in its transmission and is caused by two Leishmania species; L. major and L. tropica where the most prevalent among them is L. major which is responsible for 75% of the CL cases and always regarded as zoonotic which is maintained between the reservoir host Psammomys obesus and the sand fly P. papatasi [119]. L. tropica species is also present in some areas in the North of Jordan but at a lower prevalence than L. major and is thought to be maintained by canines or hyrax as reservoir hosts and the sand fly P. sergenti as a vector [120]. The establishment of Syrian refugee camps possesses a high risk for the introduction of leishmaniasis into areas which were considered to be free of the infection and mainly the ACL due to L. tropica since this is the predominant species in Syria which might be carried by the infected Syrian refugees who reside in camps inside Jordan. This might be a real risk since the vector of L. tropica (P. sergenti) was reported to exist in several areas in Jordan including Awajan, Mowoqar, Barha, Ras el Naqb, Bushra and Azraq [71, 72]. In the year 2012 recent study reported 52,982 confirmed cases of CL in Syria [144]. With the political instability in Syria, millions of Syrian refugees who might be infected with CL entered the neighboring countries of Lebanon, Turkey, and Jordan. In Jordan, there are about 1.4 million Syrian refugees, only 20 percent are living in the Za’atari, Marjeeb Al-Fahood, Cyber City and Al-Azraq refugee camps while 80% reside within the Jordanian population in major cities.
Worldwide there are several methods which are currently used for the diagnosis of leishmaniasis; these include parasite detection by microscopic examination of Giemsa-stained smears prepared from the suspected lesions, *Leishmania* culture to confirm the morphological stages of the parasite, and the molecular-based assays (PCR) for detecting the parasite DNA [121]. The molecular based assays are more sensitive than microscopic examination and parasite culture, but their use remain restricted to referral hospitals and research centers. The serodiagnostic tests such as IFA, ELISA and DAT are limited to be used for CL due to their low sensitivity and variable specificity [122]. Recently, two studies evaluated the use of PCR technique for the diagnosis of CL in Jordan [48, 123]; however, the PCR based assays are not a readily available diagnostic tool in our medical laboratories. The use of PCR based assays can confirm the diagnosis with CL and at the same time can discriminate the infecting species which is important for epidemiological and clinical reasons; therefore the characterization of *Leishmania* species in clinical samples is important, as different species may require different treatment regimens. Furthermore, such information is also valuable in epidemiologic studies where the identification and the understanding of the distribution of different *Leishmania* species is regarded as a prerequisite for designing appropriate control measures and combating the spread of the disease. Therefore, three molecular techniques included: ITS1 PCR-RFLP, kDNA minicircle PCR-RFLP and nested ITS1-5.8S rDNA gene PCR were used in the present study for diagnosis as well as for species discrimination and tracking the source of infection. Furthermore, phylogenetic tree analysis based on the PCR products of both ITS1 PCR and nested ITS1-5.8S rDNA gene PCR methods was also performed during the present study.

5.1 Sampling and demographic data

The distribution and epidemiology of CL is not fully understood among different areas of Jordan. Severe underestimations of the burden of the disease among Jordanians have been reported by a recent study in 2008 [124]. The underestimation of the CL burden in Jordan might be attributed to many factors such as the insufficient epidemiological information on CL, the poor reporting system and disease surveillance in different parts of the country. During the present study, only 39 samples were collected from suspected CL patients from several dermatology clinics throughout Jordan, there were many factors which resulted in just having the present small sample
size, first reason might be due to the poor awareness and lack of knowledge of the Jordanian CL patients about the shape and appearance of the CL papule which might appear as a normal acne therefore visiting a dermatologist and seeking a clinical care is not necessary. Second obstacle was the complexity in obtaining samples from CL lesions especially if the lesion is dry and the patient is receiving treatment. In one visit to Al-Azraq camp 27 samples were collected from Syrian suspected CL patients, however, lots of problems faced the researchers here to obtain more samples due to political issues and the fact that all suspected patients receive a fast treatment few hours after their arrival.

In the present study males both Jordanians and Syrians were infected more with CL than females and this might be explained by the fact that males are usually more engaged with outdoor activities and their work required to sleep at night such as the soldiers and lots of them are engaged with agricultural activities with a higher risk to sand fly bites than females who stay home. In this study the lesions number recovered from the Syrian patients were higher (1 to 15 lesions) compared to those observed in CL Jordanian patients (1-6 lesions) which indicated that the exposure and the number of sand fly bites is more among the Syrians and this might be due to the active war in Syria and the instability which often leads to poor living conditions, thus further exacerbate the risk for rapid transmission of infectious diseases. The war in Syria has greatly increased the risk for CL due to a combination of factors such as the collapsed health care infrastructures and population displacement from endemic and non-endemic regions and the poor control of the sand flies vector.

5.2. DNA extraction

DNA extraction from Leishmania-suspected skin scraping spotted on NucleoSave Cards was performed based on an optimized protocol. Since Leishmania parasites only contain few ng of DNA. DNA extraction has to be efficient to obtain enough concentration for further analysis. Several methods have been reported previously for DNA extraction from clinical leishmaniasis samples. Crude DNA can be extracted from NucleoSave Cards. DNA extraction from NucleoSave Cards was reported in several studies [48]. Of the 66 samples, PCR amplicons could only be successfully extracted from 29 samples and all of these were positive by PCR. The other 37 samples did not have enough DNA concentration on the cards (<5ng/μL), hence the
negative results, which indicate the need for more sample quantity during collection to recover enough stages (amastigotes) for DNA extraction. Most of the Syrian patients receive medical treatment inside Al Azraq camp directly when the papule is suspected clinically to be CL; the dermatologist treat the patient by pentostam injection therefore quick treatment of the papule prohibited the development of CL ulcers, thus minimizing the chances of getting enough \textit{Leishmania} DNA for further molecular analysis and characterization. However, if the papule was not observed by the dermatologist and transformed into ulcer then the chances to obtain \textit{Leishmania} DNA becomes higher and this might explain why only (9) out of (27) samples were positive for DNA extraction among Syrian patients and therefore enough DNA from the samples were used for molecular characterization during the present study.

In Jordan when the patients become infected with \textit{Leishmania} they are often undiagnosed patients and when they admitted to their local medical center, there are insufficient diagnostic tools and expertise. It was evident from this study and another previous study that impression smears from CL lesions spotted and stored on NucleoCards filter paper is considered as a successful tool for \textit{Leishmania} DNA isolation [125]. Moreover, the collected material on the NucleoCards can be easily mailed via normal local mail to the central diagnostic facility where molecular tools and skilled specialist are available to properly diagnose the specimen in a relatively short period of time, allowing better patient management and treatment. In order to assess whether the observed PCR problems were linked to DNA quality, Actin housekeeping gene, was amplified. Only two isolates proved to be negative for the gene.

5.3. \textit{Leishmania} typing using ITS1-PCR-RFLP

The ITS1 sequence (300-350 bp depending on the species) was chosen in the present study as the target for PCR assay of \textit{Leishmania} species. The ITS1-PCR-RFLP method was found to be highly sensitive and specific in detecting samples that have \textit{Leishmania} when compared to nested PCR and kDNA PCR, which is congruent with previous reports [16, 23, 24]. Previously, several studies exploited the ITS1 region amplification technique for the discrimination of \textit{Leishmania} parasites [110]. 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. A previous study identified size polymorphisms in PCR
amplified ITS regions that proved to be specific for certain *Leishmania* species \[111,112\]. In the above-mentioned study, the bands obtained upon amplification of the ITS1 region were greatly observed and (29) samples showed positive results for ITS1. Many recent studies have shown that ITS1-PCR followed by restriction fragment length polymorphism analysis is a suitable tool for diagnosing and identifying *Leishmania* species \[137, 138\]. The major advantage of ITS1-PCR is that species identification can be achieved by digesting the PCR product by just one restriction enzyme (*Hae III*) and this is sufficient to distinguish almost all medically important *Leishmania* species \[137\]. Using ITS1-PCR to identify the infecting *Leishmania* species in clinical samples could be the future routine test that needs to be adopted in Jordan. Moreover, ITS1 PCR-RFLP is the most widely used assay for direct detection and identification of *Leishmania* species in the Old World \[48\]. In this study, RFLP of the ITS1 PCR product revealed that the isolates belonged to *L. major* and *L. tropica* \[63\]. 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 \[64\]. In this study we found that the majority of CL is caused by *L. major* (20 cases) in a mixture of nationalities of Jordanians (14) and Syrians (6). There were (9) cases of CL that were caused by *L. tropica*. (3) Of them were Syrians and the other (6) were Jordanians.

### 5.4 *Leishmania* typing using ITS1-5.8S rDNA gene

Nested ITS1-5.8S rDNA gene PCR enhanced the sensitivity of the diagnostic tool by targeting two fragments in the ITS-rDNA region one region consisting of ITS1 with the 5.8S rDNA gene \[115\]. In this study, the first PCR reaction targeted by the first primers IR1 and IR2 followed by the second primers ITS1F and ITS2R4. 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 sensitive for *L. tropica* identification \[116, 117\]. One sample (27) of *L. tropica* which was from Ajloun showed high similarity with *L. tropica* sample isolated from a Syrian patient in Lebanon refugee camp (KT363779), this sample showed high similarity with *L. tropica* ITS1-5.8S rDNA gene sequences in GenBank at 98% identity, therefore indicating the probability of ACL.
5.5. Sequencing and phylogenetic tree analysis of haplotype sequences of *Leishmania* ITS1-5.8S rDNA gene and ITS1 gene.

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* and *L. major* isolates [126,127,128,129]. In this study, sequencing of the obtained PCR products of both PCR reactions, targeting the ITS1 in *L. major* and the ITS1-5.8S rDNA gene regions in *L. tropica* was performed [130]. *Leishmania tropica* is known to be a very heterogeneous species [131, 132]. Alignment based on the ITS1-5.8S rDNA gene revealed a significantly higher level of variation. It is commonly reported that samples positive for leishmaniasis often show unreadable sequences upon ITS1-5.8S rDNA gene examination which was observed in two Syrian samples, this can be explained by the extremely high heterogeneity levels or sometimes may be due to mixed infections of two or more *Leishmania* species [133]. The ITS1-5.8S rDNA PCR proved to be a sensitive identification method for *L. tropica*. A high level of heterogeneity was revealed in the obtained sequences [66].

Sequencing based on the ITS1 gene was used for the analysis and genotypic variations of *L. major* since this method showed high sensitivity for the identification of *L. major* and to study the molecular variation between *L. major* isolates [134, 135]. In this study, *L. major* sequencing showed significant differences between Clade I and Clade II by using CLC Main Workbench 7 for phylogenetic analysis. Clade I include Jordanian and Syrian sequences which were collected during 2015 and 2016 and they showed high similarity with few point mutations, (Clade II) sequences included just Jordanian sequences which were collected from a previous study in 2009 and these samples showed significant difference from the samples obtained recently from Syrian and Jordanian patients. ITS1 sequencing proved to be successful for the identification of cases of CL due to Syrian immigrations. These results indicated that new isolates /clades of CL might be introduced and imported to areas surrounding the refugee camp such as in Al-Azraq, and possible outbreaks might occur in areas which were free from CL in Jordan.
5.6 *Leishmania* typing using kDNA minicircle gene

Kinetoplast DNA minicircle can be highly sensitive for detecting *Leishmania* parasites because of the abundance of minicircles in each kinetoplast. However, high-level sequence polymorphism among minicircles is an impediment for species identification with protocols based solely on kDNA PCR [136].

Amplification of the kDNA minicircle sequence by using Lmj4 and Uni 21 primers successfully identified the two *Leishmania* species (*L. tropica* and *L. major*) except for 5 samples who showed negative results that make ITS1 more sensitive than this gene loci. The RFLP analysis of the PCR products of the 7 samples after their digestion with *RsaI* also identified them as *L. tropica*, and enabled their separation into the kDNA Clades L.tro-kD1 and L.tro-kD2, respectively. The analysis of the kDNA RFLP profiles also exposed a further level of micro-heterogeneity [130].
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 NucleoSave Cards clinical samples. ITS1-5.8S rDNA gene PCR proved to be a more sensitive method for *Leishmania tropica* discrimination. ITS1-PCR RFLP showed high sensitivity to identify *Leishmania* species and highly sensitive for *L. major* sequencing. kDNA minicircle gene showed less sensitivity compared to the methods which were mentioned before. The obtained results highlighted 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* and *L. major* are the causative agent of leishmaniasis observed among Syrian and Jordanian population. 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. The risk of transmitting this “flesh-eating” parasite into the Jordanian 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 [69].
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. [67]. Thus, it can be used for further in-depth study of the epidemiology, evolution and pathogenomics of *Leishmania* [68]. Also, it would be of interest to type *Leishmania* isolated from sand fly vectors collected from the area surrounding Azraq Camp. 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 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 due to the political instability in the Middle East and the influx of refugees mainly from Syria to Jordan.
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APPENDICES

Appendices.1 The Ministry of Interior approval
Appendices.2 The Ministry of Health Institutional Review Board approval
وصف الدراسة:
أنت ستكون جزء من هذه الدراسة البحثية لأننا نحاول دراسة مدى تأثير الهجرة السورية على انتشار مرض الليشمانيا في الأردن. ودراسة استجابة جسم الإنسان المناعية الناجم عن التعرض للسعة ذبابة الرمل (السويكيته أو القروديحة). إن مرض الليشمانيا هو أحد الأمراض المعدية التي تؤثر على الإفراد الذين يعيشون في الأردن. خلال إجراء هذه الدراسة سيتم إجراء تحليلات للجراثيم المختلفة من الطفيل الذي يتقلّب عبر ذبابة الرمل، وتحديد الإصابة بمرض الليشمانيا، ودراسة التعرض للسعة ذبابة الرمل أثناء تنقل المرضى، ودراسة نسبة الإصابة في سوريا عالية جدا، وطريقة انتقال المرض، حيث أن توقعات من هذه الدراسة أنه من المحتمل أن المرضى السوريين سيؤديون على نسبة ومعدل انتشار المرض في الأردن.

المشاركة في هذه الدراسة تطوعية تماماً، وإذا وافقت المشاركة في هذه الدراسة، سيتم سحب 10 مل (حوالي ملعقتين من الشاي) دم من الذراع ومسحة جلدية من المصابين. سيتم ترتيب أنواع الدم والمسحة الجلدية في أنابيب مميزة. سيتم إرسال العينات إلى المختبر في الجامعة الهاشمية، سيتم في هذه المختبرات وبشكل منتظم، واعتماداً على ما سيجنيه المتطوع من المشاركة في هذه الدراسة.

المدة الزمنية المتطلبة لإكمال هذا البحث:
الوقت المطلوب لإنجاز هذا الجزء من الدراسة إضافة إلى الوقت اللازم لقراءة هذا النموذج وجمع العينات قد يستغرق حوالي 10 دقائق.

ما سيجنيه المتطوع من المشاركة في هذه الدراسة:
إنك لن تستفيد بشكل مباشر من كونك في هذه الدراسة، بيد أن المعلومات المكتسبة من إجراء هذا البحث قد تساعد على حماية الآخرين من الإصابة بالليشمانيا في المستقبل.
الرجاء التوقيع أدناه إذا كنت مستعدا للمشاركة بهذه الدراسة وتمت الإجابة على كافة أسئلتك المتعلقة بهذه الدراسة بشكل كامل. إن أي أسئلة أُطلقت بشأن مشاركتكم في هذه الدراسة أو أي أصابع ذات صلة بالدراسة، يمكنكم الاتصال بالدكتورة نوال حجاوي أستاذ مساعد / علم الطفيليات / الجامعة الهاشمية في الجامعة الهاشمية.

توقيع المتطوع
التاريخ

اشهد إن هذا النموذج قد تم توضيحه وقراءته للشخص أعلاه وان أي أسئلة حول هذه الدراسة قد تم الإجابة عليها.

توقيع الباحث
التاريخ

Appendices.3 Consent form
Appendices.4 The study Questionnaire
ملخص
دراسة تأثير الهجرة السورية على تفشي مرض اللشمانيات الجلدية في الأردن
إعداد
كمال جهاد كمال الفراحنة حجاوي
المشرف
الدكتورة نوال سميح حجاوي
أستاذ مشارك
داء اللشمانيا الجلدي (CL) من الأمراض المتوطنة ويشكل إحدى المشاكل الصحية الرئيسية في سوريا. هي السلالة الأكثر شيوعاً و المسؤولة عن اللشمانيات الجلدية في سوريا وsand fly التي تنتقل غالباً من إنسان إلى إنسان عن طريق نفق الرمل، وفي الأردن هناك حالات قليلة من داء اللشمانيات مقارنة بسوريا. L. major هي السلالة الأكثر شيوعاً و المسؤولة عن اللشمانيات الجلدية في الأردن، وهي تحتاج إلى sand fly و حيوان محتضن مثل الجرذان لنقلها. الطرق المصلية والمجهرية التقليدية في تشخيص داء اللشمانيات هي طرق قليلة الحساسية خاصة بالنسبة للإجثام الفرعية إضافة إلى أنها مستهلكة للوقت. وفي هذه الدراسة قمنا باستخدام ثلاث طرق للكشف عن اللشمانيات وهي:

ITS1-PCR RFLP, Kinetoplast DNA and Nested ITS1-5.8S rDNA.

و أظهرت ببشرة التطور و قمنا بفحص بادئين أثنين أحدهما خاص بجنس اللشمانيات والآخر خاص بنوع L. tropica. تم في هذه الدراسة للكشف عنه بطريقة تفاعل البلمرة المتسلسل (PCR) جمع العينات عن مرضى أردنيين من مناطق الشمال وحتى الجنوب ومرضى سورون في مخيم الأزرق في الأردن، إذ تم استخلاص الحامض النووي DNA من الطور اللاستوطي للطفيل و بعدها خضع للتنشط باستخدام تفاعل البلمرة المتسلسل (PCR). أظهرت نتائج تفاعل البلمرة المنتج بواسطة المهرجان الكهربائي 20 نوع كان من L. tropica و 9 أنواع من L. major. كما أظهرت النتائج عن وجود تشابه بين عينة معزولة من مريض أردني مع عينة معزولة من مريض سوري في مخيم اللاجئين في لبنان وذلك نوصي باستخدام هذه التقنيات في المستشفيات في حالات تفشي داء اللشمانيات الجلدية في مناطق المملكة الأردنية الهاشمية.