Deanship of Graduate Studies

Al-Quds University

Identification and Analysis of Cutaneous Leishmaniasis in Northeastern Libya from Giemsa-Stained Tissue Smears between 2014 and 2020

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Identification and Analysis of Cutaneous Leishmaniasis in Northeastern Libya from Giemsa-Stained Tissue Smears between 2014 and 2020

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Thesis Approval

Identification and Analysis of Cutaneous Leishmaniasis in Northeastern Libya from Giemsa-Stained Tissue Smears between 2014 and 2020

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Declaration

I certify that this thesis submitted for the degree of the master is the result of our research, and the content of the thesis is the result of work that has been carried out since the date of approval of the research program. All ethics procedures and guidelines have been followed properly while preparing a thesis.

Signed: 

Hemam Adel Doudin

Date: 5-8-2023

DEDICATION

To my mother and father

To my brothers, to my lovely sister

To all my family

To my teachers

To my loyal friends

To everyone who supported me in my graduate study

Hemam Adel AbdulAziz Doudin

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I am grateful to Allah, who granted me the power and patience to finish this study. I would like to deeply thank and appreciate Dr. Kifaya Azmi, my supervisor for her skillful help and support. Special thanks to my colleagues in my Master's journey and my colleague Yafa Melhem for her kind help with the analysis

I would like to thank all my teachers in Biochemistry and Molecular Biology Department

And finally, for my Family my mother, father, my beloved brothers, and my great sister thank you for your support. Your cheers always pushed me towards success Thank you so much, my great family.

Abstract:

Cutaneous *leishmaniasis* (CL) is a prevalent skin infection caused by the transmission of a protozoan parasite through the bite of a phlebotomine sandfly. This study aimed to evaluate the epidemiological aspects of CL in patients attending the dermatology clinic of the main referral hospital in Zliten, Libya. Data from 355 patients diagnosed with CL between 2014 and 2020 were analyzed to determine the incidence of CL and its distribution based on age, sex, residence, season, and affected body sites. In addition, this study aimed to identify the species of *Leishmania* using the ITS1-RFLP PCR technique and to compare the sensitivity and specificity of different PCR-based assays targeting the Ribosomal Internal Transcribed Spacer 1(ITS-1), Hexokinase (HK), and Phosphoglucomutase (PGM) genes on Gimza stained tissue smears. Furthermore, the study assessed the current spatiotemporal distribution of CL cases and projected the future incidence of the disease. The findings revealed a higher risk of CL in the coastal regions of Libya. The projected trends until 2060 indicated an increasing incidence of CL in the north-western part of Libya, a spread along the coastal region, and pthe otential emergence of new endemic areas in the north-eastern districts. These findings highlight the need for health authorities to develop appropriate effective control programs. The majority of patients in this study came from Zliten and its suburban areas, with a minority from neighboring cities.

Keywords: cutaneous *leishmaniasis*, epidemiology, PCR, RFLP, spatiotemporal distribution, Zliten, Libya.

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العنوان: تحديد وتحليل داء الليشمانيا الجلدي في شمال شرق ليبيا من شرائح الأنسجة المصبوغة بالغيمزا بين عامي 2014 و 2020

إعداد: همام عادل عبد العزيز دودين

إشراف: د. كفاية عزمي

الملخص : داء الليشمانيا الجلدي (CL) هو عدوى جلدية منتشرة ناجمة عن انتقال طفيل أولي من خلال لدغة ذبابة الرمل الفاصدة. هدفت هذه الدراسة إلى تقييم الجوانب الوبائية لمرض اللشمانيا الجلدية لدى المرضى الذين يراجعون عيادة الأمراض الجلدية في مستشفى الإحالة الرئيسي في زليتن- ليبيا. تم تحليل بيانات 355 مريضًا تم تشخيص إصابتهم باللشمانيا الجلدية بين عامي 2014 و 2020 لتحديد معدل الإصابة باللشمانيا الجلدية وتوزيعه على أساس العمر والجنس والإقامة والموسم ومواقع الجسم المصابة. بالإضافة إلى ذلك، تهدف هذه الدراسة إلى التعرف على أنواع الليشمانيا باستخدام تقنية ITS1-RFLP PCR ومقارنة حساسية ونوعية فحوصات مختلفة تعتمد على PCR والتي تستهدف الريبوسوم الداخلي المكتوب 1(ITS-1)، الهيكسوكيناز (HK)، وجينات الفوسفوجلوكوموتاز (PGM) في شرائح الأنسجة المصبوغة بجيمزا. علاوة على ذلك، قامت الدراسة بتقييم التوزيع الزماني المكاني الحالي لحالات اللشمانيا الجلدية وتوقعت حدوث المرض في المستقبل. كشفت النتائج عن ارتفاع خطر الإصابة باللشمانيا الجلدية في المناطق الساحلية في ليبيا. تشير الاتجاهات المتوقعة حتى عام 2060 إلى تزايد حالات الإصابة بمرض اللشمانيا الجلدية في الجزء الشمالي الغربي من ليبيا، وانتشاره على طول المنطقة الساحلية، واحتمال ظهور مناطق موبوءة جديدة في المناطق الشمالية الشرقية. تسلط هذه النتائج الضوء على حاجة السلطات الصحية إلى تطوير برامج مكافحة فعالة مناسبة. غالبية المرضى في هذه الدراسة جاءوا من زليتن وضواحيها، مع أقلية من المدن المجاورة.

الكلمات المفتاحية: داء الليشمانيا الجلدي، علم الأوبئة، PCR، RFLP، التوزيع الزماني المكاني، زليتن، ليبيا.

القدس - فلسطين

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List of Abbreviations

Abbreviate	Term
WHO	World Health Organization
L.	*Leishmania*
VL	*Visceral leishmaniasis*
CL	*Cutaneous leishmaniasis*
MCL	*Mucocutaneous leishmaniasis*
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
MLEE	Multilocus enzyme electrophoresis
ITS1	Ribosomal internal transcribed spacer 1
PGM	Phosphoglucomutase
HK	Hexokinase
LNCIDC	Centre for Infectious Diseases and Control
µl	Microliter
˚C	Celsius
s	Second
g	Gram
bp	Base pair
GelDoc	Gel documentation system
NC	Negative Control
PC	Positive Control
LD	Ladder
MW	Molecular weight

Chapter One

1. Introduction

1.1 Leishmaniasis

Leishmaniasis is considered a tropical disease transmitted by correspondent sandfly vectors and it is caused by a protozoa *Leishmania* parasite consisting of over 20 species. This Parasis is transmitted by the bite of infected female phlebotomine sandflies. In addition, more than 90 types of sandfly species are known to transmit *Leishmania*. According to the WHO report 700 000 to 1 million new cases occur annually (WHO).

1.2 *Leishmania* Classification

Leishmaniasis, a disease caused by protozoan parasites of the *Leishmania* genus, exhibits three distinct clinical forms. *Visceral Leishmaniasis (VL*), commonly known as kala-azar, is characterized by irregular episodes of fever, anemia, weight loss, and hepatosplenomegaly. It remains a significant public health concern with the potential for outbreaks and high mortality rates. *Cutaneous Leishmaniasis (CL)* represents the most prevalent form and manifests as skin lesions, predominantly ulcers, typically found on exposed body parts. The global annual incidence of new CL cases is estimated to range from 600,000 to 1 million. Lastly, *Mucocutaneous Leishmaniasis (MCL*) results in the partial or total destruction of mucous membranes in the nose, mouth, and throat, leading to severe morbidity. These variations in clinical forms highlight the diverse nature of Leishmaniasis and its considerable impact on global health(Akuffo, Costa et al. 2018).

1.3 *Leishmania* Life Cycle

Leishmaniasis, a parasitic disease, is primarily transmitted through the bite of infected female phlebotomine sand flies. During the bite, the sand flies inject infective stage metacyclic promastigotes into the skin. Once deposited at the puncture wound site, these promastigotes are phagocytized by macrophages and other types of mononuclear phagocytic cells. Within these cells, the promastigotes transform the tissue stage amastigotes. Subsequently, the amastigotes multiply through simple fission and proceed to infect other mononuclear phagocytic cells, perpetuating the infection. The cycle continues when sand flies feed on an infected host's blood, ingesting infected cells in the process. Within the sand flies, amastigotes transform back into promastigotes within the midgut and subsequently migrate to the proboscis, from where they can be transmitted to new hosts during subsequent blood meals. This intricate process of transformation and transmission between hosts and sand flies highlights the complexity of Leishmaniasis transmission and its dependence on the interactions between the parasites and the vectors involved.

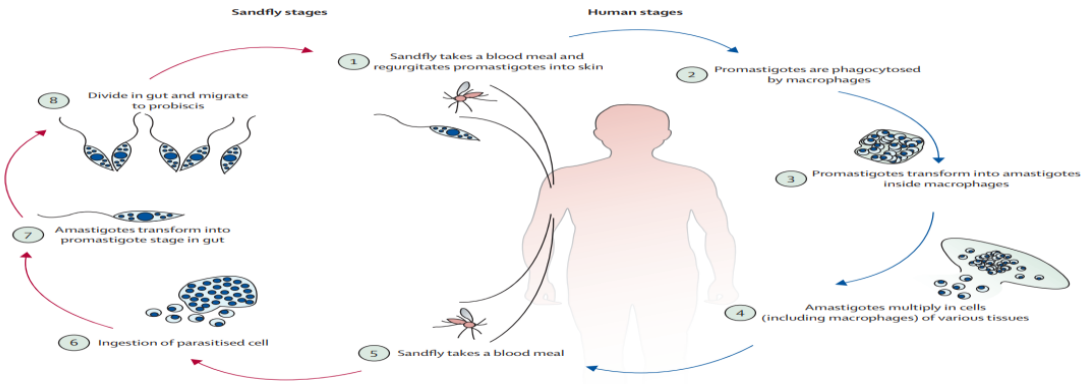


Figure 1 : *Leishmania* Life cycle

(https://www.sciencedirect.com/science/article/abs/pii/S1473309907702098)

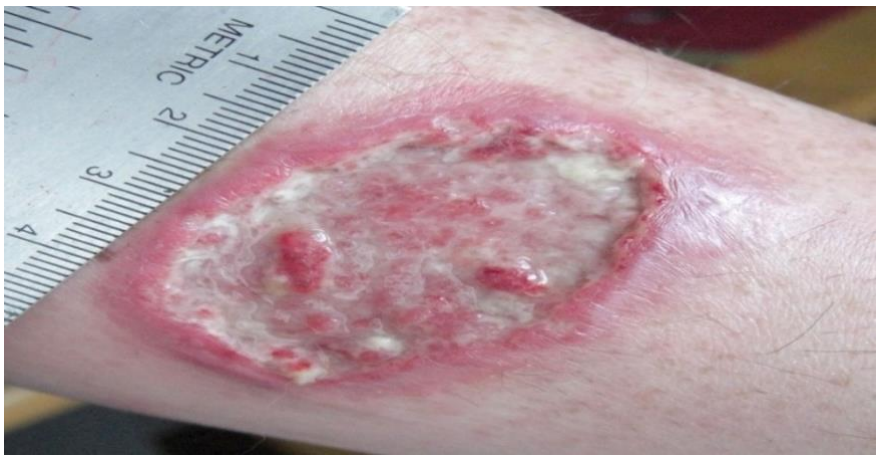
1.4 *Cutaneous leishmania* (CL)

Cutaneous leishmaniasis is endemic in more than 70 countries worldwi de.(1)CL species are divided into Old World species: *L. (L.) major*, *L. infantum*, and *L. (L.) tropica* present around the Mediterranean basin, the Middle East, the horn of Africa, and the Indian subcontinent, and New World species, such as *L. (L.) amazonensis, L. (L.) chagasi, L. mexicana L, L. (V.) naiffi, L. (V.) braziliensis, and L. (V.) guyanensis* occurs in Middle and South America(Martins, Oliveira et al. 2014).

1.5 Cutaneous *leishmaniasis* Symptoms

The first sign of an infection is typically a small erythema that develops after a variable prepatent period at the site where an infected sand fly has bitten the host. The erythema develops into a papule, then a nodule that progressively ulcerates over 2 weeks to 6 months to become the lesion that is characteristic of CL**.** (‘The Leishmaniases in Biology and Medicine, Vol. 2. Clinical Aspects and Control. Ed. W. Peters and R. Killick-Kendrick. 390 Pages plus Index. ISBN 0 12 552102 2. Academic Press, London, 1987. £55.00.’, 1988) However most infections probably remain asymptomatic and misdiagnosed(Escobar 1992) .

Figure 2 : Cutaneous leishmaniasis ulcer on a forearm. (*Leishmaniasis (Pathology)* | *Britannica*, n.d.)



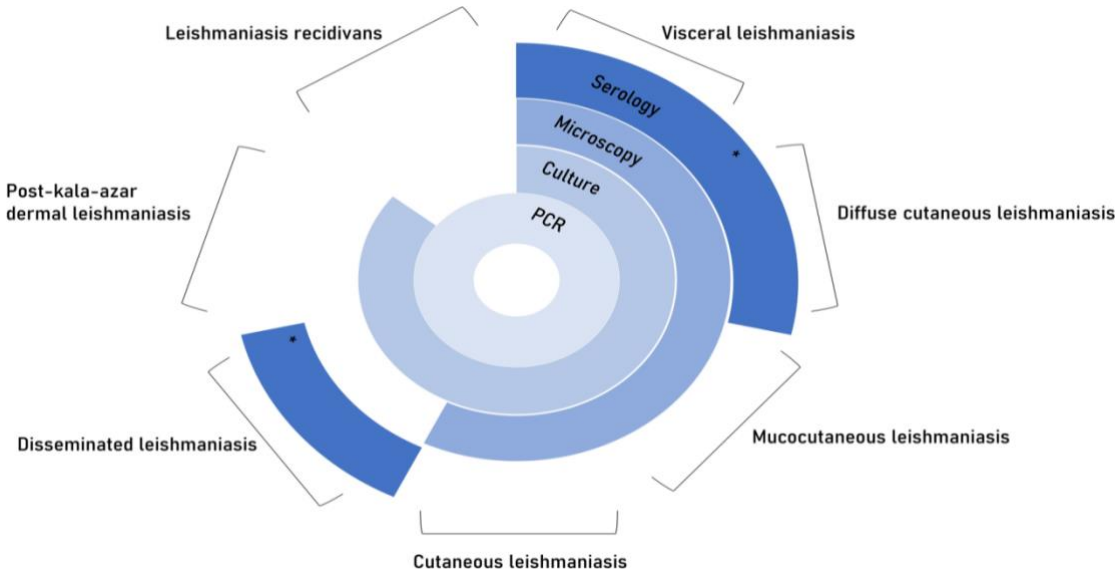
1.6 Cutaneous *Leishmania* Diagnosis Tools

1.6.1 Conventional Detection Methods

Microscopic examination, histology, culture, and other methods, are commonly used by laboratories globally, especially in endemic, resource-poor nations (Carregal, Lanza et al. 2019)**.**

Direct aspirate smears with Giemsa or Leishman staining are used for microscopic detection of *Leishmania* parasites; amastigotes appear round in shape and about 2–4 μm in diameter, and cultured promastigotes range between 15 and 25 μm in length and are ellipsoid to slender in shape(Torres-Guerrero, Quintanilla-Cedillo et al. 2017) (Gow, Johnson et al. 2022). However, the sensitivity of detection via conventional methods varies (54.0–96.4%), and specificity is as low as 46.0%, depending on the quality of the reagent used for staining, technical expertise, and the primary sample taken from patients. (Smakowska-Luzan, Mott et al. 2018, Gow, Johnson et al. 2022) (Abd El Salam, Ramadan et al. 2021) (Bensoussan, Nasereddin et al. 2006).

Figure 3: Conventional detection methods used for *Leishmania* Diagnosis. (Gow, Smith et al. 2022)



1.6.2 Molecular Diagnostic tools

Various diagnostic methods employing diverse techniques and targets have been utilized to detect and characterize causative agents in *leishmaniases*. Among these, polymerase chain reaction (PCR) coupled with consecutive restriction fragment length polymorphism (PCR RFLP) has gained popularity due to its effectiveness(Azmi, Nasereddin et al. 2011). PCR-based approaches offer versatility in terms of clinical specimen types, allowing the use of skin biopsies, tissue aspirates, dermal scrapings on filter papers, stained smears, buffy coat, and even whole blood(Al-Jawabreh, Dumaidi et al. 2017). Recent advancements have introduced PCR methods that do not necessitate parasite isolation through culture and cultivation(Azmi, Nasereddin et al. 2011). Some studies have undertaken comparisons of sensitivity and specificity among different PCR-based assays (Ozerdem, Eroglu et al. 2009). On the other hand, certain methods, like Multilocus Enzyme Electrophoresis (MLEE), require parasite cultivation. MLEE serves as the standard accepted approach for identifying and distinguishing leishmanial parasites at the species level, but it is an expensive and time-consuming process that demands significant quantities of cultured leishmanial promastigotes. Therefore, the choice of diagnostic method depends on factors such as resource availability, time constraints, and specific research or clinical requirements.

1.7 *Leishmaniasis* in Libya

CL is a major public health problem in Libya. Both types of leishmaniasis are known to affect humans: zoonotic CL due to *Leishmania (L.) major* and anthroponotic CL caused by *L.*

tropica (Ghrab, Rhim et al. 2006). CL has not been fully documented in Libya. However, several studies reported the molecular prevalence of CL in Libya (El-Badry, Wetzel et al. 2016).

Diagnosis of leishmaniasis in Libya still depends on the evaluation of the clinical picture and microscopy of Giemsa-stained tissue smears (Rahi 2013). Diagnosis of leishmaniasis in Libya still depends on the evaluation of the clinical picture and microscopy of Giemsa-stained tissue smears, thus accumulating risk factors that contributed to the spread and the proliferation of CL.

1.8 Literature Review on CL in Zliten, Libya

Zliten is a geographical region in Libthat ya, that ompasses both a city and its surrounding area. The city of Zliten is located approximately 160 km (99 mi) to the east of the capital city, Tripoli. It is positioned about 35 km (22 mi) east of the ancient Roman city of Leptis Magna, while being approximately 60 km (37 mi) west of Misrata and 40 km (25 mi) east of Khoms. The city spans an approximate area of 8 km2 (3.1 sq mi). In contrast, the broader Zliten district covers a much larger area, extending over 3,000 km2 (1,200 sq mi). The district is bounded by the Mediterranean Sea to the north, Misrata to the east, Bani Walid to the south, and Khoms to the west. As for the population of Zliten, it is estimated to be around 316,000 inhabitants.

Figure 4 : Zliten Location in Libyan Map

Several studies show the epidemiology of CL in Zleiten in 2017 and a higher prevalence among males. The mean age of patients was 30.6 years, The age group most affected was between 20 and 29 years, and over 60% of patients were between 10 and 40 years old. The number of cases steadily decreased after the age of 39. In 2016, there was a significant increase in new CL cases, with 264 reported cases and an incidence rate of 0.1% (1 in 1000), compared to only 48 cases in 2015 with an incidence rate of 0.01% (1 in 10,000). Before 2015, the number of new cases registered each year was consistently less than 33. (Tabari, Youssefi et al. 2017)

Geographically, the majority of patients (41%) came from the eastern area of Zliten, while 35% came from the western area. In 2016, most CL patients were from the eastern area, whereas in previous years, the majority were from the western area. Among the patients, 3.8% had sole facial lesions, 46.2% had lesions on one extremity and 47.1% had lesions at multiple body sites (including the face, trunk, and extremities). Additionally, (Batool, Arshad et al. 2020). The male-to-female ratio in the affected population was 2.4:1. The age groups below 40 years old, with a peak prevalence between 20 and 29 years old, were the most

affected. The majority of patients originated from Majer, a rural area located in the southeast and south of the city. Around 83% of the patients had lesions on their extremities. The seasonal distribution of cases in 2017 and 2018 remained consistent with previous years, showing a peak incidence in December, similar to other areas in northwest Libya where *L. major* was identified as the causative agent of CL. Unusual clinical presentations are known to occur, which can complicate diagnosis and lead to difficulties or misdiagnosis. Nodular lymphangitis is more commonly observed in immunosuppressed individuals and the elderly. It is important to note that CL is not a life-threatening condition, and treatment approaches should prioritize the use of safe Treatments and therapies such as thermotherapy and cryotherapy, with or without intralesional Pentostam. It should be noted that CL can result in disfiguring scars on exposed areas of the skin, which can have negative social and psychological impacts on individuals affected by the disease.

In a comprehensive molecular epidemiology study conducted by Ahmad Amro in 2012, the investigation focused on Cutaneous Leishmaniasis (CL) in Libya. The study collected data from 49 areas spanning 12 districts in the north-western region of Libya, including Sirt, Misrata, Al Murqub, Tarhuna, Tripoli, Jafara, Surman, Zawia, Zuwara, Nalut, Al Jabal Al Gharbi, and Wadi Al Hayaa. Utilizing ITS1-PCR, a characteristic amplicon ranging from 300 to 350 base pairs was successfully obtained, indicating the presence of medically relevant Leishmania species. Subsequent digestion of the PCR product with endonuclease HaeIII revealed that CL in Libya is caused by at least two distinct Leishmania species. Among the analyzed samples, the majority (75.9%, 148 samples) displayed RFLP profiles with two bands at 160 and 210 base pairs, corresponding to the L. major WHO reference strain, whereas 24.1% (47 samples) exhibited RFLP profiles with two bands at 185 and 57 base pairs, similar to the L. tropica WHO reference strain. The analysis covered a period from 1995 to 2008, with both microscopic and PCR examinations conducted for species

identification and to determine positivity rates. CL cases infected with L. major were observed in all districts, while the majority of L. tropica cases were concentrated in Al Jabal Al Gharbi (46.4%), Misrata (17.8%), and Tarhuna (10.7%) districts. The age distribution at the onset of illness varied from 9 months to 87 years, with a median age of 25 years, and no significant differences were observed among different age groups. The male-to-female ratio was calculated as 1.17:1, with males constituting 54% of the total cases (Ola, Nawaz et al. 2012).

Other studies (Amro et al., 2017) showed that from 1995 to 2008, the district with the highest number of leishmaniasis cases was Jabal al Gharbi, followed by Misrata, Nuqat al Khams, and Zawiya. However, in 2011-2012, Tripoli, Murqub, and Jafara had a high number of cases, although this could be attributed to the sampling of cases in different hospitals. The male-to-female ratio in the study was 1.29:1. The average age at disease onset was 30 years, ranging from 6 months to 85 years, with a median age of 28. Among the 312 patients included in the study, 54% presented with single lesions, while 46% had multiple lesions. Among *L. tropica* cases, 77.3% had single lesions compared to 57.7% of *L. major* cases. The lesions typically appeared on exposed parts of the body, most commonly on the face and extremities (arms, legs, and feet). The lesions were usually painless and evolved.

1.9 Study Objectives

Specific and sensitive molecular diagnostic tools have not yet been implemented and information about disease distribution, parasite life cycle, and combining risk factors is confined in Libya. In this study, three PCR-based methods were compared, using PCR RFLP, targeting the ITS1, H,K, and PGM. (Azmi, Nasereddin et al. 2011) (Schönian, Nasereddin et al. 2003) .

Here, since Multilocus enzyme electrophoresis (MLEE) is not available in Libya, sequences from the genes for Hexokinase (HK) and Phosphoglucomutase (PGM) were tested (Azmi et al., 2013) as targets for methods incorporating, consecutively, a polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were carried out to identify strains of the three Old World leishmanial species *L. major, L. tropica and L. infantum* and attempt to differentiate among strains of *L. tropica* and indicate to which zymodemes they probably belong and investigate demographic characteristics of all cases.

Specific objectives:

1. To study the epidemiology of leishmaniasis disease in Zliten, Libya between 2014 and 2020.

2. To detect and identify the *Leishmania* species using the ITS1- RFLP PCR method.

3. To compare the sensitivity and specificity of different PCR-based assays, the ITS-1, Hexokinase,(HK), and Phosphoglucomutase (PGM) genes.

4. To differentiate strains of the *L. tropica* and indicate the zymodemes they have.

Chapter Two

2. Materials and Methods

2.1 Study subjects

Previously collected clinical specimens were taken from the archive of the Libyan National Centre for Infectious Diseases and Control (LNCIDC). These specimens and patient profiles have been archived from 2014 to 2020 for a total of 355 samples from patients who have been referred to hospitals with skin lesions typical for CL in the Zliten area . These cases were confirmed as CL patients based on clinical symptoms and microscopic examination. The patients came from different areas endemic to CL in Libya**.**

2.2 DNA Extraction

Tissue smears stained with Giemsa stain were made on glass slides and examined by microscopy. Unfortunately, these were prepared by local laboratory staff not well trained in diagnosing *leishmaniases*. The smears were not well prepared and the dermal tissues are very poor.

DNA extraction has been done using the NucleoSpin Tissue kit (Germany). All slides were scratched after adding 30µl of distilled water and 20µl of Pre-lysis buffer T1, then the content of the slide was added to a 1.5 ml sterile collection tube, 180µl of T1 buffer and 25µl of

Proteinase K solution were added to each tube and all tubes were been incubated at 56℃ overnight, the rest of the procedure has been done according to the manufacturer instruction.

2.3 Methodology and Statistical Analysis

Specimens were considered as true positives when at least two PCR assays were positive for leishmanial DNA. Similarly, samples were considered as true negatives, when two PCR assays were found negative for leishmanial DNA. This constituted the 'gold standard' applied here in the absence of adequately prepared stained skin tissue smears. (Bensoussan, Nasereddin et al. 2006), who compare the positivity of molecular biology tests with that of stained smears, have already shown the validity of this type of gold standard. They applied two PCR-based approaches used in this study using ITS1 and kDNA. To our knowledge, there were no studies that compared the sensitivity and specificity among different PCR-based assays, the ITS-1, Hexokinase (HK) and Phosphoglucomutase (PGM) genes using the tissue smears and thus gives a good value to this study.

2.4 Polymerase chain reaction and RFLP:

2.4.1 PCR amplification conditions for ITS-1 sequence.

ITS1 RNA gene is one of the powerful methods for detecting and identifying leishmanial parasites. ITS1 had an expected product size of 300-350 bp(Schönian, Nasereddin et al. 2003). ITS-1 PCR product digested with the restriction endonuclease *HaeIII* RFLP has the power to distinguish between the parasite strains that generated fragments separated by electrophoresis on 3% agarose gels and compared with those of WHO reference strains of *L.*

major (MHOM/PS/2001/ ISL659), *L. tropica* (MHOM/PS/2002/63JnF21) and *L. infantum* (MHOM/TN/1980/IPT1) .

Six (6ul) of DNA extract product of each sample has been mixed with 0.25 ul of each primer. The Primers designed for ITS1 (LITSR, L5.8S:) 2xPCRBIO HS *Taq* Mix Red were used. The PCR reaction conditions were as follows; step 1 initial denaturation: 95˚C for 15 minutes, step 2 denaturation: 95˚C for 30 seconds, step 3 annealing: 58˚C for 45 seconds, step 4 extension: 72˚C for 60 seconds (40 cycles), step 5 final extension : 72ºC for 10 seconds and finally left at 12˚C.

2.4.2 PCR amplification conditions for HK and PGM sequences

HK and PGM PCR reactions were done in a volume of 25 µl that contained 1 µl of DNA template and PCR-Ready Supreme mix (Syntezza Bioscience, Jerusalem, Israel), PCR amplification was carried out using BIO-RAD T100 thermal cycler, and the amplification conditions were the following: initial denaturation at 98 °C for 5 min, followed by 38 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 30 s and final elongation at 72 °C for 7 min. For each reaction, DNA from strains of *L. major* and *L. infantum*, and strains of *L. tropica* belonging to the various zymodemes mentioned above were used as positive controls, and distilled water was used as a negative control. Ten µl of each PCR product were run in 2.5% agarose gels. For all *Leishmania* species, the amplified products of the HK sequence were 197 bp and those of the PGM sequence were 278 bp.

2.5 Primers used in this study

Table 1: Primers used to detect the targeted genes

Primers	Forward	Reverse
ITS1	5'-TGATACCACTTATC GCACTT-3'	5'- CTGGATCATTTTCCGATG-3'
HK	5'-CCAACGCCTGCTACTTTGAG-3'	5'-CTTCTCTTGGCGCTGGTTCT-3'
PGM	5'-GAGGTGACAACGACAGCGTA-3'	5'- GGCCAGTCAGAGATTCCATC-3'

2.6 Gel electrophoreses

The gel was prepared by the addition of 2g Agarose to 100 ml TAE buffer followed by boiling for 30 seconds, and 8 µl ethidium bromide was added. Eight µl of each PCR product was loaded into each well. Samples were run at 120 volts for 60 minutes. Finally, PCR products were captured a by gel documentation system (GelDoc).

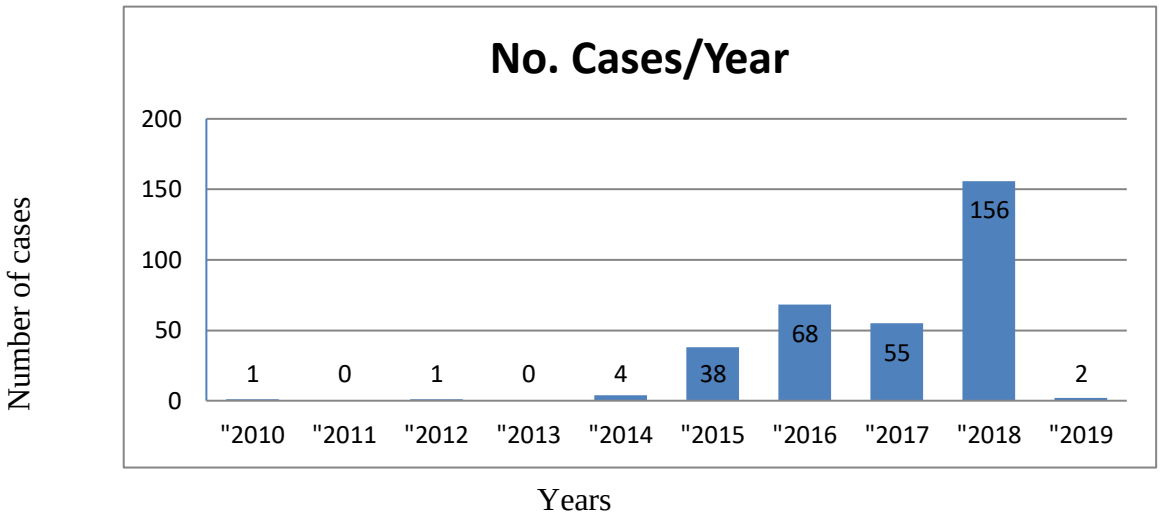
Chapter Three

3. Results

3.1Geographic distribution and demographics

A total of 347 individuals attending the Libyan National Centre for Infectious Diseases and Control (LNCIDC) were registered as human cases of CL from 2014 to 2020. This was done solely based on the development and clinical appearance of lesions and the place of habitation of the individual. Parasitological confirmation and detection of the leishmanial species were not done at that time. **Figure 5** shows the trends in the occurrence of CL during 2014–2020. Peaks of infection occurred in 2018 which has the highest peak with 48% of cases number.

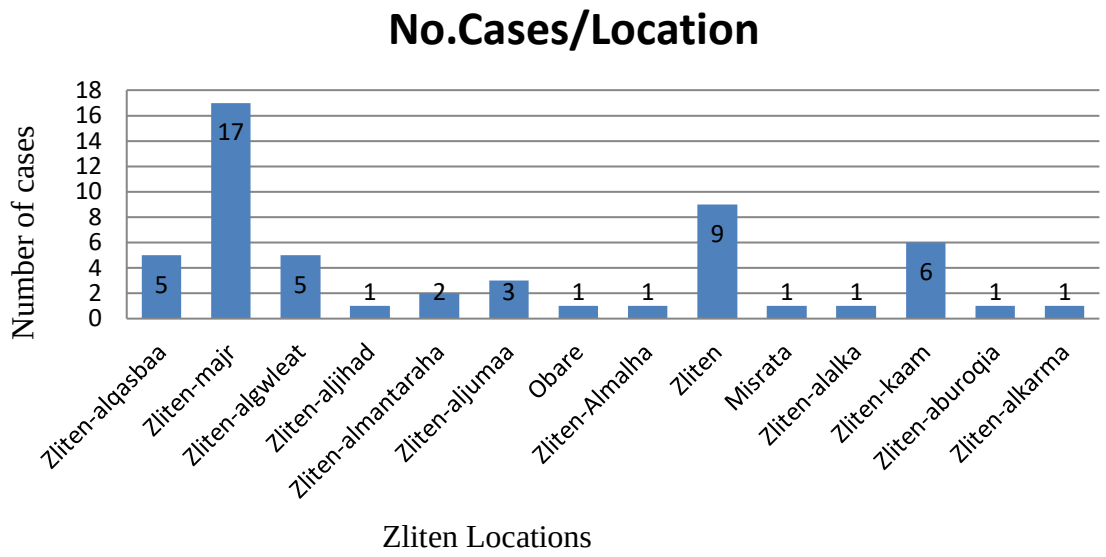
Figurer-5: Annual distribution of cases in Zliten District, Libya, during2014–2020 by main locality: Zliten City; north of Libya :Zliten-alqasbaa ,Zliten-majr ,Zliten-algwleat ,Zliten-aljihad, Zliten-almantaraha, Zliten-aljumaa ,Zliten-Almalha , Zliten-alalka, Zliten-kaam, Zliten-aburoqia, Zliten-alkarma.



The patients were distributed throughout Zletin, most were from Zliten-Majr .17 (31.5%) of patients came from Zliten-Majr, 9 (16.7%) from Zliten City, 6 (11.1%) Kfrom aam , 5 (9.3%) Zliten-Algwlat, 5 (9.3%) Zliten-Alqasaba ,3 (5.6%) Zliten-Aljumaa, 2 (3.7%) Zliten-Almantaraha,1 (1.6%) Zliten-AL jihad, 1 (1.6%) Zliten-Almalha , 1 (1.6%) Zliten-Alaska, 1 (1.6%) Zliten-aburoqia, 1 (1.6%) Zliten-karma, 1 (1.6%) Misrata and 1 (1.6%) came from Obare.

Figurer 6: Zliten distribution Map

Figurer 7: Geografic Distribution of cases in Zliten District, Libya, during 2014–2020 by main locality: Zliten City; north of Libya : Zliten-alqasbaa ,Zliten-majr ,Zliten-algwleat ,Zliten-aljihad, Zliten-almantaraha, Zliten-aljumaa ,Zliten-Almalha , Zliten-alalka, Zliten-kaam, Zliten-aburoqia, Zliten-alkarma, Obare and Misrata



Figures 8 and 9 show the age and gender distribution. The mean age was 36 years (median 36), with the oldest case was 105-year-old female and two youngest 0.5-year-old female and male, According to WHO age group classification in 2015; 82 (25.8%) of patients were children, 122 (38.3%) young , 67 (21.1%) Middle-aged, 34 (10.7%) elderly, 11(3.5%) senile and 2 (0.6%) patients were very old, these cases were diagnosed as CL, 185 (55.55%) were male and 148 (44.45%) were female, a ratio of 1.25:1. 18>children,,18<young<44,,45<middle<59,,60<elderly<74,,75<old seniors<89,,90<long (Bloom, Canning et al. 2015) .

Figure-8 : Age-group distribution of the cutaneous leishaniasis cases of Zliten, Libya.

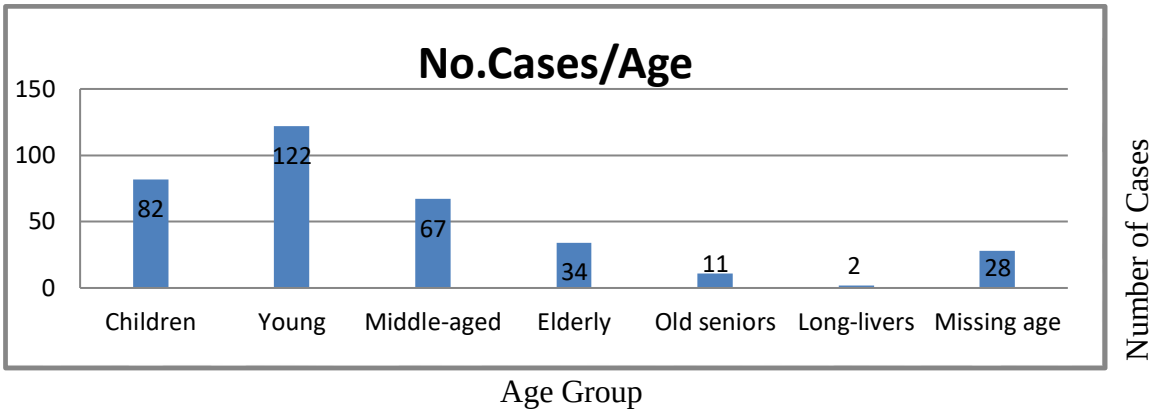
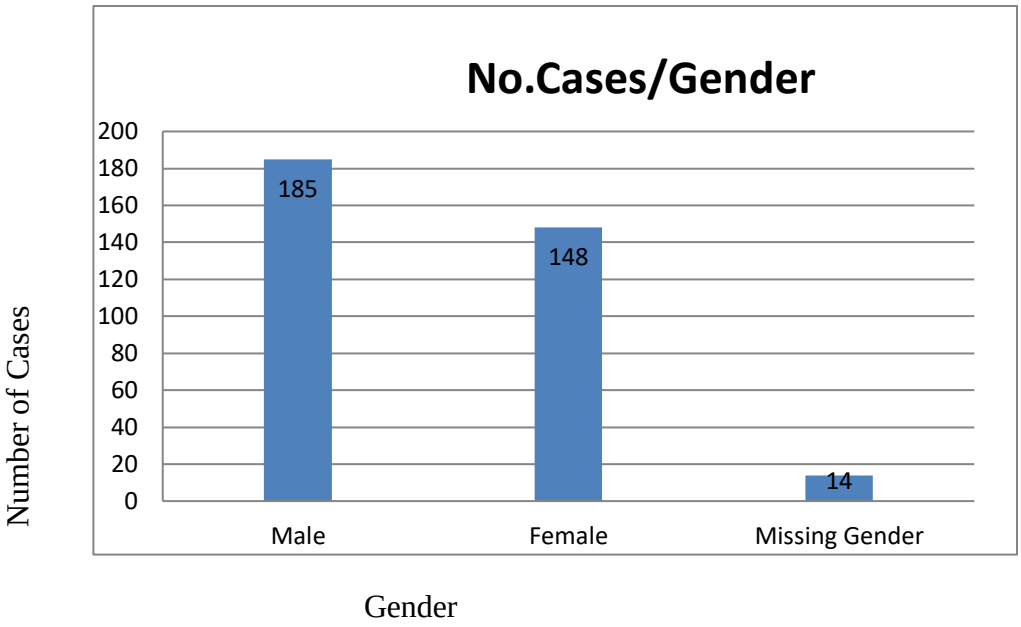


Figure 9: Gender distribution of the cutaneous leishaniasis cases of Zliten, Libya.



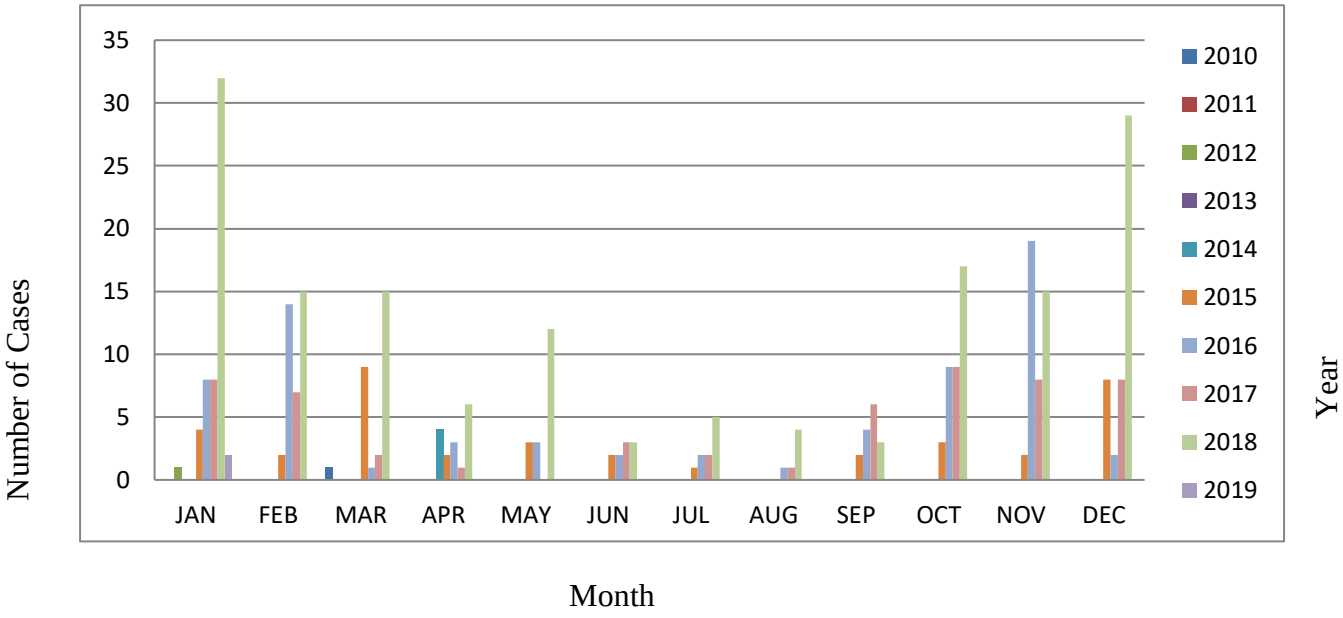
3.2 Epidemiological data

As noticed in **Figure-10** and **Table 2** the highest peak date was cited from October to March and the highest peak appears in January (16.9%) and then December (14.5%).

Table 2: Seasonal distribution per month of CL cases in Zliten Libya.

Year / Month	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	The sum of positives in months
JAN	0	0	1	0	0	4	8	8	32	2	55
FEB	0	0	0	0	0	2	14	7	15	0	38
MAR	1	0	0	0	0	9	1	2	15	0	28
APR	0	0	0	0	4	2	3	1	6	0	16
MAY	0	0	0	0	0	3	3	0	12	0	18
JUN	0	0	0	0	0	2	2	3	3	0	10
JUL	0	0	0	0	0	1	2	2	5	0	10
AUG	0	0	0	0	0	0	1	1	4	0	6
SEP	0	0	0	0	0	2	4	6	3	0	15
OCT	0	0	0	0	0	3	9	9	17	0	38
NOV	0	0	0	0	0	2	19	8	15	0	44
DEC	0	0	0	0	0	8	2	8	29	0	47
The sum of positive in years	1	0	1	0	4	38	68	55	156	2	325

Figure-10 : Seasonal distribution per month of CL cases in Zliten Libya.



3.3 Distribution of the study samples in Zliten

Since the civil war in Libya that affected the health, economic,c and education systems, the archived samples from the studied patients have missing information. A sum of 54 samples from patients who have been referred to Zliten Teaching Hospital with skin lesions with typical CL lesions.

3.4 Number of lesions, Treatment, and body sites involved

Out of 347 cases, only 53 (15.27%) presented the site of infection, and lesions number; 96.2% have affected the extremities, and 3.8% affected the face and extremities together. Multiple lesions involved one or more parts of the body single

lesions occurred in 17 cases (32.1%). For the number of lesions that existed; two lesions were found in 8 cases (15.1%), 20/53 cases (37.7%) found in 3-7 lesions and 8 (15.1%) cases had more than 7 lesions. In most cases 41(80.4%) were treated with Pentostam, 5 (9.8%) used Cryotherapy, 4 (7.8were %) treated with Pentostam and Cryotherapy and 1 (2%) treated with antibiotics. (figure 13)

Figure-11: Distribution of lesion number in CL cases in Zliten Libya

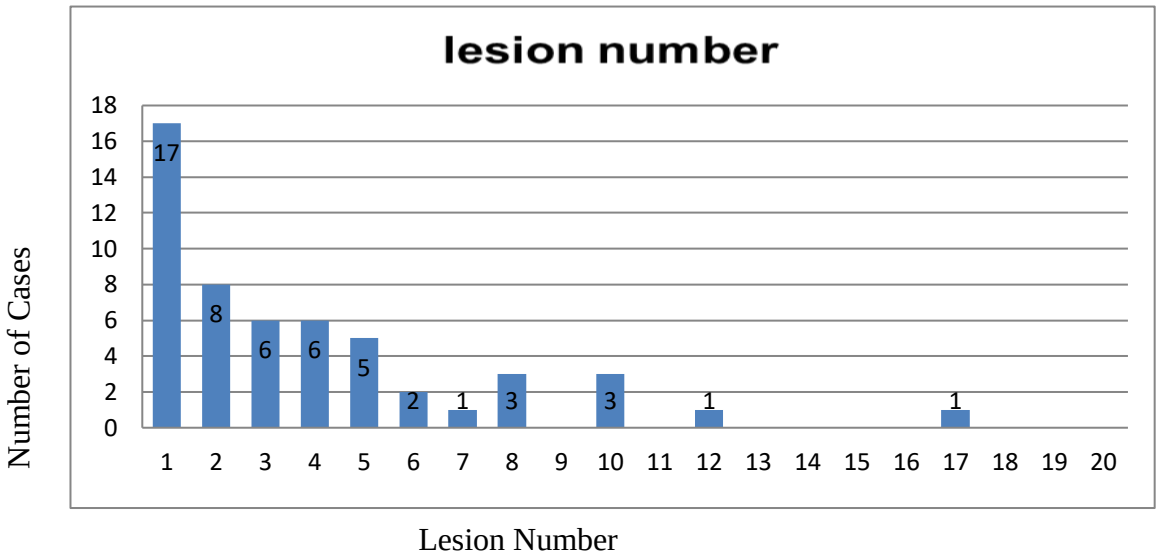


Figure-12: Distribution of infected body area in CL cases in Zliten

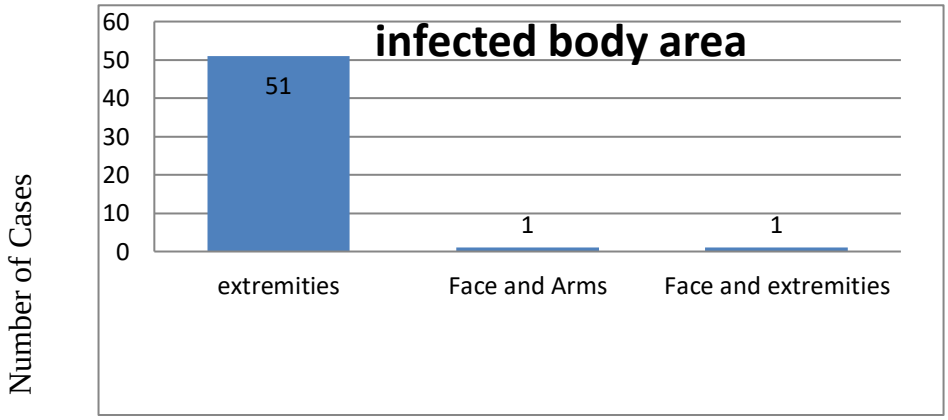
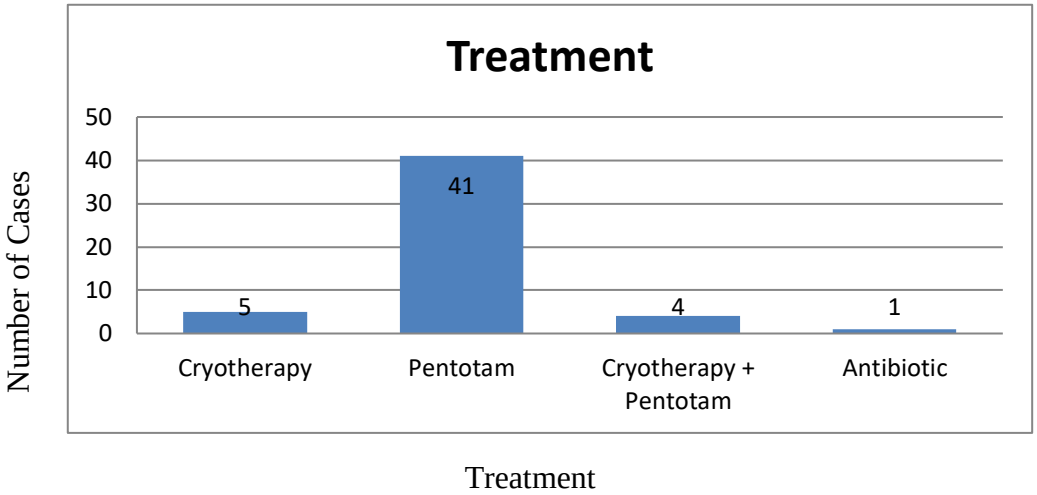


Figure-13: Distribution of Treatment used for CL patients in Zliten



3.5 Detection and identification of leishmanial parasites causing cutaneous leishmaniasis in Zletin

3.5.1 Detection of the leishmanial parasite by molecular techniques

The suspected cases that were referred to our lab at Al-Quds University, indicated that most of the skin lesions that were seen and treated were dry in appearance with granulation and papules appearing at their periphery. Skin scraping from 347 suspected cases was smeared on glass slides and stained with Giemsa stain. All slides were reported positive for amastigotes, but this result was not correct, it seems that the microscopic examination at the local laboratory in Libya was not performed by experienced staff. The microscopic examination in the laboratory failed because most slides were very poor (**Figure 14**). 65 (18.3%) of the samples were positive for ITS-1 and 81.7% were negative (Figure 15), 70 (19.8) % of samples were positive while 238 (80.2%) of samples were negative for PGM (Figure 17).

Additionally 61 (17.2%) of the samples were positive for the HK gene while 291 (82.8%) were Negative for the mentioned gene (Figure 16).

Figure 14: Representative slides in the second microscopy checking. "A" A 8 years old Male Second Microscopic examination :PCR Results : Positive ITS-1 Result: Positive PGM Positive HK "**B"** A 3 Years old Male Positive Microscopic examination the specimen has been taken at10-12-2018 PCR Results: Positive ITS-1,HK & PGM **"C"** :A 42 Years old Male Positive Microscopic examination the specimen has been taken at : 6.11.2016 from Zleten-Aljumaa the patient has 4 lesions and infected at extremities and treated with Pentostam PCR Results Negative ITS-1,HK and PGM **"D**": Age :A 24 Years old Male Negative Microscopic examination the specimen has been taken at : 30.11.2016 from Zliten-aburoqia the patient has 1 lesions and infected at extremities and treated with Pentostam PCR Results Negative ITS-1,HK and PGM.

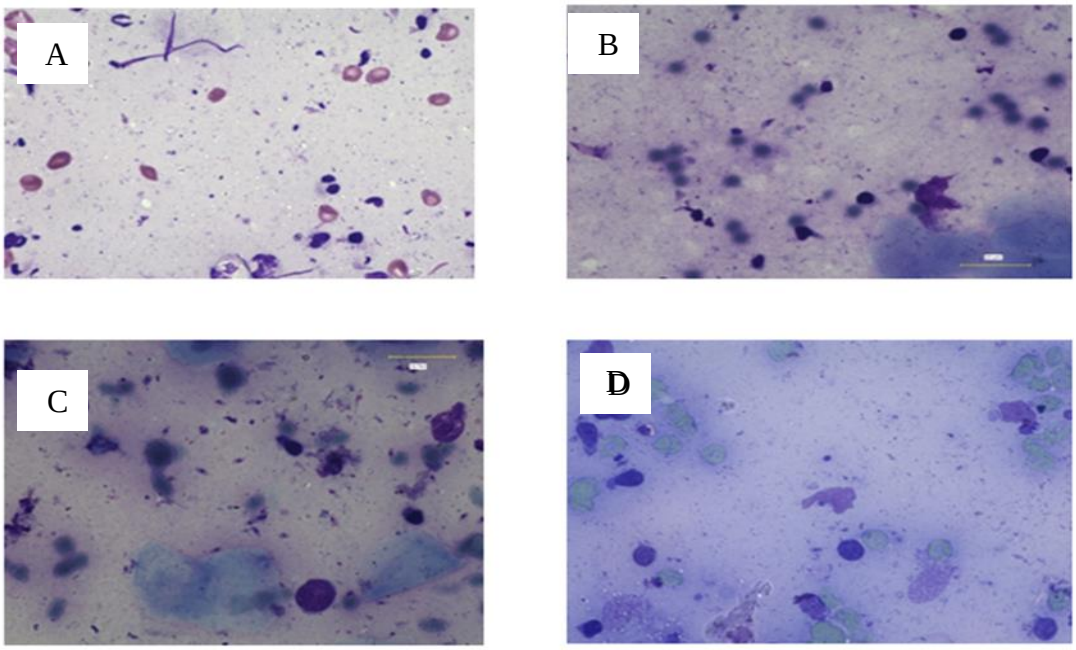


Figure 15: Distribution of ITS-1 positive samples with representative agarose gel electrophoresis**. A and B** Representative PCR Positive ITS-1 samples (product size 300-350 bp) PC1 were *Lieshmania Infantum* .PC 2,5,6 were *L.major* and PC3,4 were *L.tropica*. Positive controls lane 7,8. Negative controls (NC) were master mix with primers without DNA**. (C**) the distribution of clinical suspected cases came from Zliten; 18.3% of cases were

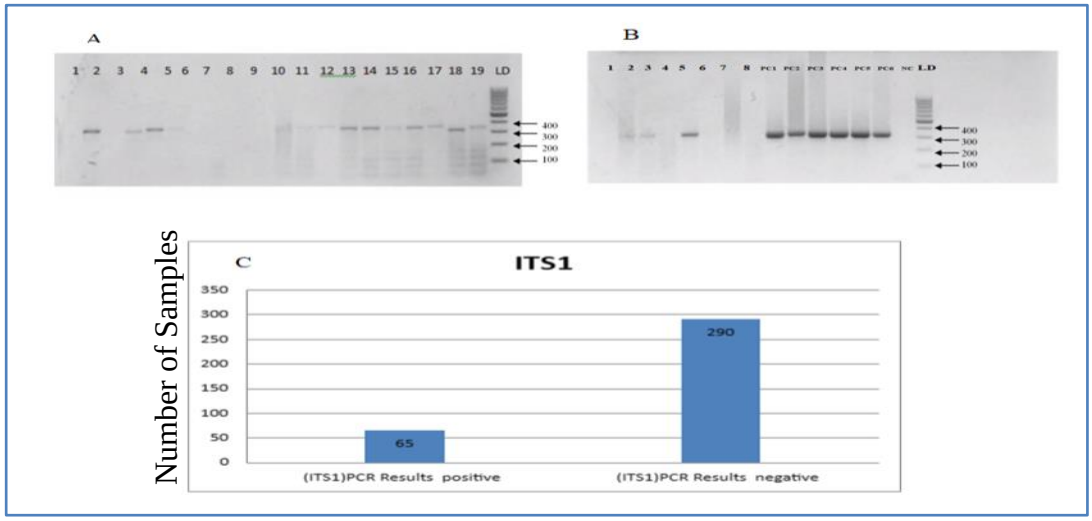


Figure 16: Distribution of HK positive samples with representative agarose gel electrophoresis: A **and B** represent PCR positive for HK (product size 197 bp), PC1 *L. infantum* . PC 2 and 5 were *L. major* and PC 3 and 4 were *L.tropica.* Positive controls were as the first well in A contains master mix with primers and known DNA of *Leishmania* species. NC used as a negative control contains mastermix and primers but without DNA**. C:** The distribution of clinical samples from Zliten shows 17.2% of cases were positive and 82.8% were Negative.

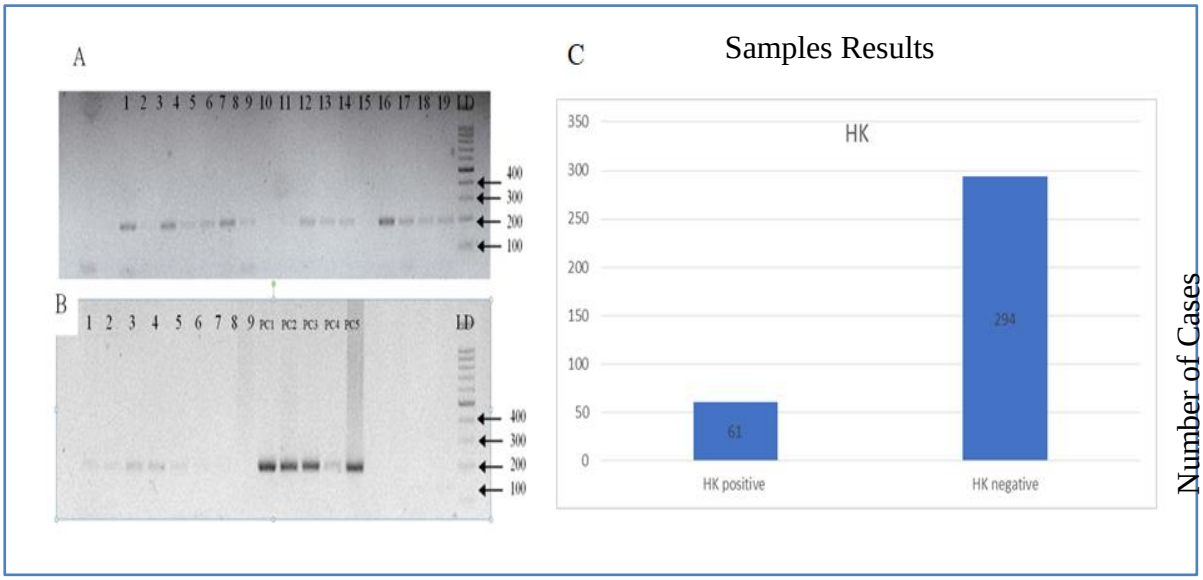
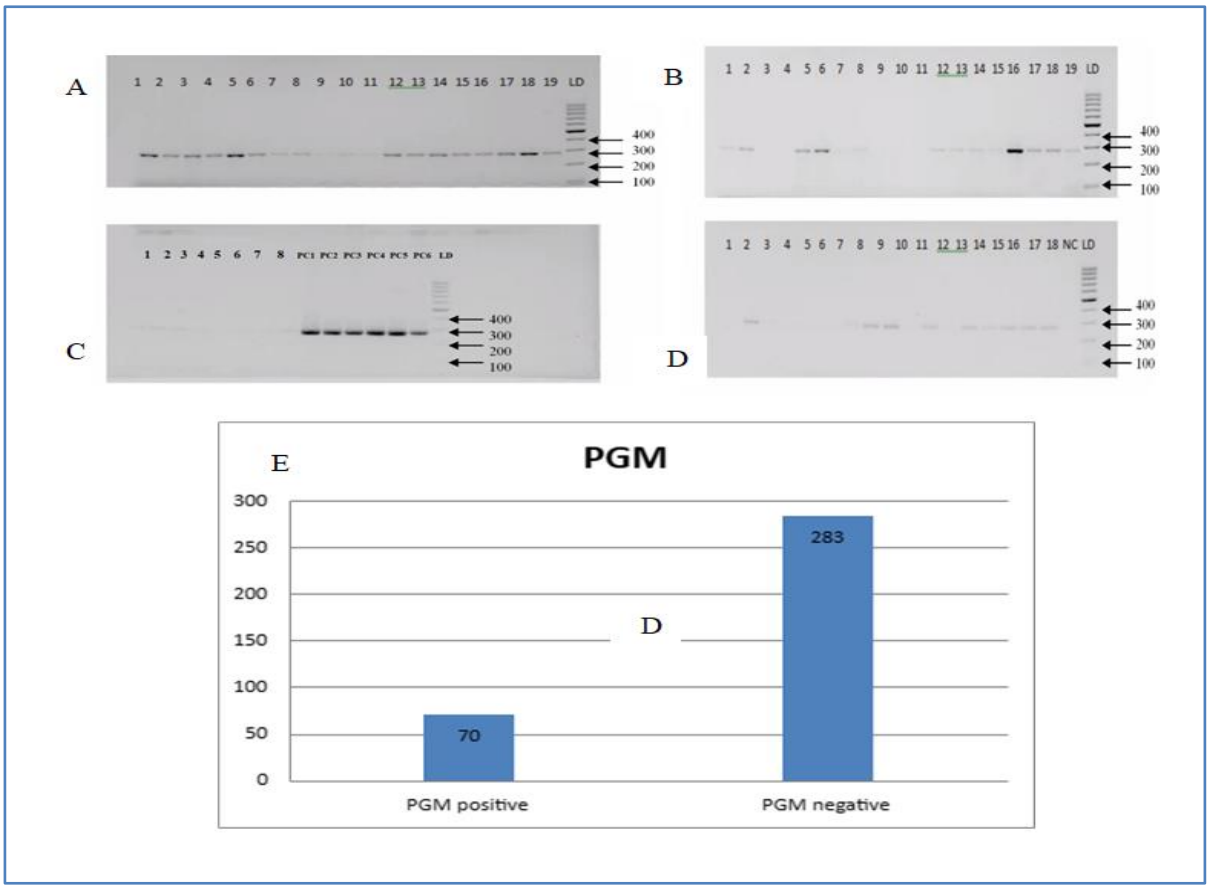


Figure 17 : Distribution of PGM positive samples with representative agarose gel electrophoresis **: A,B,C and D** Representative PCR positive PGM samples (product size 278 bp) PC1 were *L. infantum* .PC 2,5,6 were *L.major* and PC3,4 were *L.tropica*. Positive controls were master mix with primers and DNA of each *Leishmania* species known and typed before. Negative control (NC) is a mastermix with primers but without DNA**. E**: The distribution of clinical samples from Zliten shows 19.8% were positive and 80.2% were



3.5.2 Gold standard For detecting True positive samples

Since most samples were diagnosed by untrained local technicians and the conflicting PCR results. Several samples were sent to an expert to diagnose them and therefore we created our gold standard which detects the second microscopic examination and at least two positive PCR results as a true positive result. As shown in Table 3. A sum of 85 Sample were confirmed as True Positive Samples using the gold standard, 66 (77.65%) samples were ITS-1 Positive, 13 (15.29%) samples were confirmed using a second Microscopic examination done by an expert, and 6 (7.06%) of samples were confirmed by using double PCR positive test (PGM & HK) .

Table 3 : True positive samples use the gold standard (+ = Positive,- =Negative, *= Not done, +/- = Unrecognized).

+ Samples	ITS1	HK	PGM	microscopy first	microscopy second	confirmed +
34_19	+	*	+	+	*	+
36_19	+	+	+	+	*	+
38_19	+	+	-	+	*	+
53_19	+	+	-	+	*	+
75_19	+	+	+	+	*	+
78_19	+	+	+	+	*	+
80_19	+	+	+	+	*	+
82_19	+	+	+	+	*	+
84_19	+	+	+	+	*	+
97_19	+	+	-	+	*	+
98_19	+	+	-	+	*	+
107_19	+	+	+	+	*	+
108_19	+	+	+	+	*	+
110_19	+	+	+	+	*	+
111_19	+	+	+	+	*	+
117_19	+	+	+	+	*	+
118_19	+	+	+	+	*	+
120_19	+	+	+	+	*	+
121_19	+	+	+	+	*	+
128_19	+	+	-	+	*	+
129_19	+	+	-	+	*	+
130_19	+	+	+	+	*	+
131_19	+	+	+	+	*	+
132_19	+	+	+	+	*	+
134_19	+	+	+	+	*	+
139_19	+	+	+	+	*	+
152_19	+	+	+	+	*	+
154_19	-	*	-	+	+	+
158_19	+	+	+	+	+	+
161_19	-	*	-	+	+	+
159_19	+	+	+	+	-	+
162_19	+	+	+	+	*	+
168_19	-	+	+	+	-	+
173_19	-	*	-	+	+	+
174_19	+	+	+	+	*	+
175_19	-	*	+	+	*	+
176_19	-	*	+	+	-	+

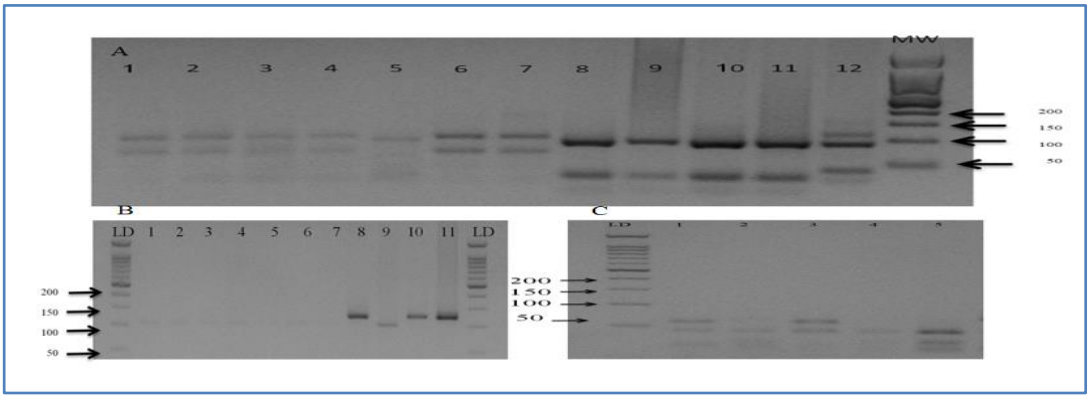
180_19	+	+	+	+	-	+
181_19	+	+	+	+	+	+
182_19	+	+	+	+	+	+
183_19	+	+	+	+	+\-	+
189_19	+	+	+	+	+\-	+
190_19	+	+	+	+	-	+
191_19	+	+	+	+	-	+
196_19	+	+	+	+	-	+
202_19	+	+	+	+	+	+
204_19	+	+	+	+	+	+
205_19	+	+	+	+	+	+
208_19	+	+	+	+	+	+
209_19	+	+	+	+	-	+
210_19	+	+	+	+	*	+
211_19	+	+	+	+	+	+
212_19	+	-	+	+	-	+
213_19	+	-	+	+	-	+
214_19	+	+	+	+	+	+
215_19	+	+	+	+	+	+
216_19	+	+	+	+	+	+
217_19	-	+	+	+	-	+
218_19	+	-	+	+	+	+
219_19	+	+	+	+	*	+
221_19	+	+	+	+	*	+
222_19	-	*	-	+	+	+
223_19	+	+	+	+	*	+
224_19	+	+	+	+	-	+
226_19	+	+	+	+	-	+
232_19	-	*	-	+	+	+
233_19	-	*	-	+	+	+
234_19	-	*	-	+	+	+
236_19	-	*	-	+	+	+
238_19	-	*	-	+	+	+
239_19	-	*	-	+	+	+
240_19	+	+	+	+	+	+
242_19	+	+	+	+	+	+
247-19	-	+	+	+	*	+
249_19	-	*	-	+	+	+
250_19	-	*	-	+	+	+
251_19	+	-	+	+	-	+
252_19	+	+	-	+	+	+
255_19	+	+	+	+	+	+
256_19	-	*	-	+	+	+
257_19	-	*	-	+	+	+
258_19	-	*	-	+	+	+
261_19	+	-	+	+	+	+

275_19	+	-	+	+	+	+
276_19	-	+	+	+	*	+

3.5.3 Identification of leishmania parasites using molecular techniques

Restriction fragment length polymorphism (RFLP) analysis of the amplified internal transcribed spacer 1 region (ITS1) digested with restriction enzyme *HaeIII* and analyzed by electrophoresis on 3% agarose gels. RFLP analysis of the amplified PGM digested with restriction enzyme *MobI* samples, and analysis of the amplified HK digested with restriction enzyme *MobI & HaeIII* respectively. The ITS1 PCR RFLP assay identified *Leishmania* species for 19 samples (29.2%), whereas HK and PGM PCR RFLP identified 12 samples (17.1%), respectively. The RFLP pattern of the ITS1 amplicon for other samples (70.8%) could not be identified owing to weak PCR amplification. *L. tropica* was identified as the causative agent in 3/36 samples (8.3%), and *L. major* in 33/36 (91.6 %) (Figure 18).

Figure 18: Molecular identification of causative CL species. **A**: Restriction fragment length polymorphism (RFLP) analysis of the amplified internal transcribed spacer 1 region (ITS1) digested with restriction enzyme *HaeIII* and analyzed by electrophoresis on 3% agarose gels. Samples 1-4 and 6 identified as *L. major*/ 5; *L. tropica*, 7; *L. major;* 1,3, and 7 positive controls ,8-11; *L. tropica* positive controls and 12; *L. infantum* positive control. **B:** RFLP analysis of the amplified PGM digested with restriction enzyme *MobI* samples 1-7; *L.major*, 8, 10 and 11 were *L.major* positive controls and 9 *L. tropica* positive control. **C:** RFLP analysis of the amplified HK digested with restriction enzyme *MobI* & *HaeIII* samples 1and 3 were



Chapter Four

4. Discussion

In this study, *Leishmania* parasites causing CL in Zliten Libya have been detected in clinical specimens and identified at the species level using ITS-1, PGM, and HK PCR-RFLP approaches, and the geographical and demographic distribution of cases and the disease dynamics were investigated.

Out of 355 Giemsa-stained smears, only 36 were successfully amplified and characterized by PCR-RFLP. Despite the implementation of national control and surveillance programs, various North African countries, including Libya, have been unable to effectively curtail the transmission of the sandfly vector and mitigate the incidence of CL infections. The ongoing proliferation of the disease remains a significant concern. Additionally, the civilian war in Libya affected the health system badly. The samples sent to analyze from Libya diagnosed all as *Leishmania* positive Giemsa-stained tissue smears however, conflicted results were noticed when diagnosed with PCR using ITS-1, PGM, and HK genes. During DNA Extraction and PCR analysis in the early stages of the analyses, we noticed very weak bands in gel electrophoreses. So, we decided to use half the amount of the Elution Buffer to concentrate the DNA. Several samples have been confirmed by experts and it was found that not all samples received were positive as microscopic examination indication. Therefore, we applied our gold standard for diagnosis based on whether the sample is true positive or true negative. The gold standard we used was the microscopic examination which we defined as the second microscopic examination as a conformation for positive samples or used at least two PCR positives to define the sample as a true positive sample. It's been noticed during DNA extraction from Giemsa-stained tissue smears that the smears barely have a tissue content the quality is very poor and there is a possibility that the PCR for some specimens

was inhibited due to the presence of impurities in the DNA extracts, which were not sufficiently eliminated during DNA purification process, or to degraded DNA.

The application of the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique facilitated the identification of the simultaneous existence of two distinct species, namely *L. major* and *L. tropica*, responsible for the occurrence of CL in Libya. The observation of these species coexisting in the region aligns with previous reports, as they are widely recognized as the predominant causative agents of CL in the Mediterranean Basin. Notably, *L. infantum*, which has been identified as a contributing factor to CL in other countries of North Africa (Rhajaoui, Nasereddin et al. 2007) (Haouas, Garrab et al. 2010) was absent in the specimens examined in this study.

The clinical manifestations associated with CL encompass a wide range of symptoms, lacking specificity, and often resembling other types of skin infections such as staphylococcal, streptococcal, mycobacterial ulcer, and fungal infections. Microscopic examination, while utilized for diagnosis, fails to provide differentiation among *Leishmania* species and exhibits limited sensitivity. Additionally, it is important to note that CL cases attributed to *L. tropica* tend to have prolonged durations and pose greater challenges in terms of treatment compared to those caused by *L. major*. Consequently, it becomes imperative to implement sensitive and species-specific diagnostic methods at the primary healthcare level, to enhance the accurate identification and management of CL cases (Ola, Nawaz et al. 2012).

CL exhibits a wide-ranging impact across all age groups in Libya. Analysis of the male-to-female ratio revealed a slightly higher infection rate among males, with a ratio of 1.25:1. This disparity can be attributed to the male lifestyle, who often engage in outdoor sleeping practices during hot nights, rendering them more susceptible to sand fly bites and subsequent infection compared to women, who tend to spend less time outside their homes. These

findings align with the outcomes of a prior study conducted by (El Buni, Jabeal et al. 2000) (Amro, Gashout et al. 2012). The primary treatment for CL in Libya is Sodium stibogluconate (Pentecostal), which serves as the first-line therapy. While a few cases of resistance have been reported, the documentation of such instances by physicians has not been systematic. Establishing an improved reporting system is crucial to monitor resistant cases effectively and undertake investigations to comprehend the underlying factors contributing to resistance.

In Libya, all cases of CL have been traced back exclusively to the northwestern districts of the country. These specific districts exhibit a typical Mediterranean coastal climate in the upper northern regions such as Tripoli, while the climate shifts towards semiarid and arid conditions in Al Jabal Al Gharbi and Wadi Al Hayaa to the south. Similar to numerous countries in the Mediterranean region, the climatic and environmental factors, along with the development of agricultural activities, create favorable conditions for the transmission of *Leishmania* parasites (Bousslimi, Aoun et al. 2010) (Ben-Ahmed, Aoun et al. 2009) . It is noteworthy that the prevalence of *L. tropica* infection appears to be relatively hypo endemic in comparison to *L. major*, which was found in all endemic districts. This variation in infection patterns within these districts can be attributed to the distinct life cycles of these two species, which necessitate further investigation for comprehensive understanding. Interestingly our findings in Zliten show that 91.6 % of cases were *major*. In comparison to the previous study conducted by (Amro et al.2011), the analysis of RFLP profiles for the samples revealed that 75.9% of them exhibited identical profiles to that of *L. major*, while 24.1% of the samples corresponded to *L. tropica*. This comparison suggests that the prevalence of *L. major* is higher than that of *L. tropica* in the studied region, indicating that *L. major* is the main causative agent of CL in Zliten. However We aimed To differentiate strains

of L. tropica and indicate the zymodemes they belong, but unfortunately, our samples appear to have a very low number of L.tropica

The incidence of CL is closely linked to the patterns of rainfall and minimum temperature. In the context of North Africa, anticipated climatic changes are predicted to involve an overall rise in annual minimum and maximum temperatures, with a greater emphasis on the increase in minimum temperature. Conversely, precipitation levels have exhibited a significant decrease in recent decades, particularly during the winter and early spring seasons. Future projections suggest a continuation of reduced rainfall, leading to a more pronounced seasonality of dryness and warming in the region (Amro, Gashout et al. 2012), In contrast, our results show that the peaks started from late Autumn to middle Spring season which is an expected result. In our study, the highest peaks were detected from October to March, and the highest peak appeared in January (16.9%) and then December (14.5%). The highest Peak in 2018 with 48% of cases if compared with previous studies (Amro, Al-Dwibe et al. 2017) . which shows an overall increase in the number of cases per year, with the highest number of cases observed from 2008 until 2012.However missing Data appear in years 2014 and 2020 which included in the study time zone maybe this missing data occurs because of the civilian war in Libya It is noteworthy that the high number of cases in 2011 and 2012 was collected only in a single hospital in January. Analysis of the entire dataset collected from 1995 to 2012 showed that 71.6% of cases were recorded between November and March, with the highest number of cases (21.1%) in January. The seasonal distribution of the causative species revealed a peak of *L. major* cases from November to January (69.4%), while *L. Tropica* cases peaked in January and February (41%), with almost no cases (except for one exception in April) between April and August. In the period 1995-2008, the majority of cases were recorded in February, but there were continuous occurrences of several cases during the summer months (Arshah et al., 2019). The incidence of CL is strongly influenced by rainfall

and minimum temperature patterns. In North Africa, anticipated climatic changes are projected to result in an overall increase in annual minimum and maximum temperatures, with a particular emphasis on rising minimum temperatures. Conversely, precipitation levels have shown significant decreases in recent decades, particularly during winter and early spring. Future projections indicate a continued reduction in rainfall, leading to heightened seasonality of dryness and warming in the region (Amro, Gashout et al. 2012). However, our study yields contrasting results, where the peak occurrence of CL cases is observed from late autumn to the middle of the spring season, aligning with expectations. In our investigation, the highest peaks were detected between October and March, with the highest peak occurring in January (16.9%) followed by December (14.5%). These findings differ from previous studies (Amro, Al-Dwibe et al. 2017), which reported an overall increase in the number of cases per year, with the highest number of cases observed from 2008 to 2012. It is worth noting that the high number of cases in 2011 and 2012 was collected from a single hospital in January. Analyzing the complete dataset spanning from 1995 to 2012, we found that 71.6% of cases were recorded between November and March, with the highest number of cases (21.1%) occurring in January. Regarding the seasonal distribution of the causative species, *L. major* cases peaked from November to January (69.4%), while *L. Tropica* cases reached their peak in January and February (41%), with few cases recorded between April and August, except for one exception in April. From 1995 to 2008, the majority of cases were recorded in February, but several cases continued to occur during the summer months (Arshah et al., 2019).

As expected the extremities were the most infected place of the patients with about 96.2 % compared to the same author in 2019 (Veysi, Mahmoudi et al. 2020)where 83% of the patients in Zliten were infected in the extremities. We aim To differentiate strains of L.

tropica and indicate the zymodemes they belong, but unfortunately, our samples appear to have a very low number of L.tropica

Given that various *Leishmania* parasite species are responsible for causing CL in Libya, it becomes crucial to identify and study the distinct reservoir hosts and insect vectors involved in the transmission of CL. This understanding is essential before implementing control measures. A comprehensive comprehension of the life cycles of different parasite species and their interactions with vectors and reservoir hosts is pivotal for the successful application of prevention strategies, control measures, and the development of surveillance protocols or guidelines. Such protocols and guidelines are essential for monitoring the burden of CL in Libya and for evaluating the effectiveness of implemented control measures. By considering these factors, effective interventions can be designed to combat the transmission and impact of CL in the region.

Chapter Five

5. Conclusion

The prevalence of CL in Zliten, a prominent city in the northwestern region of Libya, continues to pose a significant and escalating health concern. The impact of the disease is particularly pronounced among children and female patients, with an increasing number affected each year. Given this scenario, it becomes imperative to employ molecular identification techniques to accurately determine the specific *Leishmania* parasite species involved. This molecular identification is crucial to follow up the epidemiology of leishmaniasis infection in the region and for designing and implementing the most appropriate control measures and therapeutic regimens, tailored to address the unique characteristics of the identified parasite species.

Technicians in Libya need more training to diagnose the *Leishmania* parasites using Microscopic Giemsa-stained tissue smears as well as molecular-based techniques.

Chapter six

6. Strengths and Limitations

To our knowledge, our Study is the first that compares the genetic diagnosis targets of *Leishmania* parasites (ITS-1, PGM,HK) Additionally this study provides the situation of the diagnosis system of the *Leishmania* parasites in Libya.

This study has several limitations such as the bad documentation for the received samples, poor Giemsa staining of the tissue smears, and bad sample handling and storage.

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