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Molecular diagnosis and phylogenetic analysis of 5.8s rDNA of gene of cutaneous leishmaniasis isolated from patients in holy Karbala

A thesis

Submitted to the council of the College of Medicine/University of Karbala, for the partial fulfillment of the requirements for the degree Master of Science in Medical Microbiology

By

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Dedication

To the creative source of mercy and presence….. Allah…

To the basis of my humanity and its guide…. The prophet.

To my family

Who always stands beside me and encourage me

To all friends with love

To everyone who seeks for knowledge and learn ……

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List of Abbreviation

Abbreviation	Meaning
ACL	Anthroponotic cutaneous leishmaniasis
AVL	Anthroponotic visceral leishmaniasis
BC	Before Christ
BLAST	basic local alignment search tool
Bp	Base Pair
C3	Compelement system component 3
C3b	Clevage components of C3
CDC	Centres for Disease Control and Prevention
CL	Cutaneous Leishmaniasis
CR1	Compelement receptor1
CR3	Compelement receptor3
D.W	Distilled Water
FBS	Fetal Calf serum
DCL	Diffuse cutaneous leishmaniasis
ddH2O	[Deionized] Distilled Water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide TriPhosphates
DW	Distilled water
EMRO	Countries of Eastern Mediterranean Region
EMTM	Evan's modified Tobie's medium
Gp63	Glycoprotein of 63KD
IDT	Integrated DNA Technologies
IFN-γ	Gamma interferon
IL-12	InterLeukin 12
IL-4	InterLeukin 4
ITS	internal transcribed spacer
Kb	Kilo base pair
kDNA	kinetoplast DNA

L.d	*. Leishmania donovani*
L.m.	*Leishmania major*
L.t.	*Leishmania tropica*
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LSU	large ribosomal subunit
MCL	Mucocutaneous Leishmaniasis
MEGA	Molecular Evolutionary Genetics Analysis
MgCL2	Magnesium Chloride
mRNA	messenger Ribonucleic acid
NaCL	sodium chloride
NCBI	National Center for Biotechnology Information
Nested PCR	Nested Polymerase Chain Reaction
NK	Natural killer cell
NNN	Novy-MacNeil-Nicole medium
NO	Nitric oxide
NWCL	New World ccutaneous leishmaniasis
OWCL	Old World cutaneous leishmaniasis
PCR	Polymerase Chain Reaction
PKAD	post kala-azar dermal leishmaniasis
PKC	Protein kinase C
PMN	polymorphonuclear neutrophils
Pmol	Picomole
rDNA	Ribosomal DNA
Rpm	Round per Minutes
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal RNA
rRNA5.8	Ribosomal ribonucleic acid5.8
RT-PCR	Reverse Transcriptase-Polymerase chain reaction
SPP	Species
SSU	small subunit
Taq	Thermus aquaticus
TBE	Tris-Borate.EDTA.Buffer

Th-1	T helper cell type 1
Th-2	T helper cell type 2
TNF a	Tumor Necrosis Factor- alpha
USA	United States America
UV	Ultraviolet
VL	Visceral Leishmaniasis
Volt	Voltage
WHO	World Health Organization
WHO EMR	World Health Organization Eastern Mediterranean Region
ZCL	Zoonotic cutaneous leishmaniasi
ZVL	zoonotic visceral leishmaniasis

Summary

Leishmaniasis is a parasitic disease caused by haemoflagellate *Leishmania* . The disease is widespread and may cause serious health problems in communities throughout the Mediterranean regions and the Middle East, including Iraq. There are about 12 million cases worldwide, and there are about 1.5 million new cases of cutaneous leishmaniasis each year.

Cutaneous leishmaniasis (CL) is a major public health problem and an endemic disease in Iraqi population, so this study was conducted to find the best method for the diagnosis, by "Molecular diagnosis and phylogenetic tree analysis to detection of the cutaneous leishmaniasis species in Karbala city by Nested PCR.

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniasis that 72 males that percentage (78.3)% and 20 females that percentage (21.7)% with age ranged 10-59 years, attended to Department of Dermatology Al-Husseini Hospital in Karbala during the period between November 2017 to end January 2018.

The most infected rate of patients was between the ages of 40-49 years (40.2%), while the lowest infection rate was of patient's ages 10-19 years (6.5%).

The most infected rate of occupation patients in solder was 34 (36.9%), while the lowest infection rate in kids was 3 (3.2%), and the Most infected rate of patients was found in hands 44 (47.8) while the lowest infection rate was in Legs 11(12%).

The geographic distribution of the patients shows that 62% of the patients were from the cities of Telaefar and Dialla, while only 30% were from Karbala city. The number of lesions differs between patients. Most (65%) of the patients had a multiple lesions in comparison to the single lesions which was found in (35%) of patients.

The direct microscope examination was performed in two methods aspiration and direct microscope examination then stained with Leishman stain. Then, the positive samples were cultured on the modified Novy-MacNeil-Nicolle medium from (15 – 30) day and then active in Roswell Park Memorial Institute medium .(Only 20) sample growth.

A more specific diagnosis of the causative agent of cutaneous leishmaniasis was performed using molecular tests. Choosing 20 sample of the positively cultured samples, of the total number of pathogens that

Showed positive results were studied by Nested-PCR using two types of primers to determine the species. Because large 5.8s.r DNA gene through Nested PCR showed that all the samples were *L.major*.

The Nested PCR products of twelve isolates send to sequenced in the South Korea then compared the local species which isolated from current study with the global strains in the NCBI gene bank then recorded choose rendomly isolate K1.

It was concluded that CL is highly spread with multiple lesions more than single lesions of Cutaneous leishmaniasis diverse clinical manifestation "is prevalent in karbala city, and the molecular detection showed *L.major* was the main species causing cutaneous leishmaniasis in comparing with *L.tropica* in the present study, Nasted-PCR is a reliable method for the diagnosis and identification of *Leishmania* species because high specific and sensitive can applied in epidemiologic investigations, All *L.major* isolates in Karbala city is one isolate for sequencing 5.8s.rDNA gene and result of the (12) sequence where all similar genetically with very little change is not significant and then compare with global strain in the NCBI gene bank were similar genetically (one isolated) with Iranian isolated , finally Iraqi *L.major* isolate was genetically closest with Iranian *L.major* isolates because Iraq and iran are on the same geographic line and genetically distant for Chinese isola

Chapter One

Introduction

And

Literature Review

1.1. Introduction:

Leishmaniasis are vector-borne disease caused by protozoan parasites from the family *Leishmania* (Trypanosomatida: Trypanosomatidae) **(Akhoundi *et al.*, 2016).**

Leishmaniasis is a parasitic disease caused by haemoflagellate *Leishmania* . The disease is widespread and may cause serious health problems in communities throughout the Mediterranean regions and the Middle East, including Iraq. There are about 12 million cases worldwide, and there are about 1.5 million new cases of cutaneous leishmaniasis each year **(Ashford *et al.*, 1992).**

The disease is transmitted by female sandflies (Phlebotomus or Lutzomya) that feed on the blood of an animal or human host **(Neva and Brown, 1994)**. Most of sandfly species feed at night or dawn, usually on plant juices, they require blood meal for egg development. Species Phlebotomus is the main vector host of Leishmaniasis in Europe, Asia and Africa, while Lutzomyia is the Leishmaniasis vector in the Americas **(Cheesbrough, 1998)**.

Leishmania parasites are called "digenetic," because they need two hosts to complete their life cycle. Its life cycle included two forms: the promastigote form with in the insect vector they present an extra-cellular and amastigote form in the mammalian host as intracellular **(CDC, 2011)**.

There are three main types of leishmaniasis: cutaneous leishmaniasis (CL) caused by *Leishmania tropica*, *L. mexicana* and *L. major*. Visceral leishmaniasis (VL) caused by *L. donovani* and *L. infantum* and mucocutaneous leishmaniasis (MCL) caused by *L. braziliensis*. **(Igbineweka *et al.*, 2012)**.

The disease is becoming more important because of globalization, climate change and other circumstances which allow the parasite and its vectors to spread **(Antoniou *et al.*, 2013; Loria-Cervera, 2014).**

In Iraq, two species are present: *L tropica*, the agent of anthroponotic cutaneous leishmaniasis (ACL), and *L. major*, the agent of zoonotic cutaneous leishmaniasis (ZCL). Both ACL and ZCL were reported as causative agents of leishmaniasis in Iraq, but ACL is found mainly in suburban areas **(WHO, 2003)**.

The Cutaneous leishmaniasis lesions appeared as the sores which can change in size and appearance over time. It may start out as papules (bumps) or nodules (lumps) and may end up as ulcers (like a volcano, with a raised edge and central crater); skin ulcers may be covered by scab or crust. The sores usually are painless but can be painful. Some people have swollen glands near the sores (for example, under the arm, if the sores are on the arm or hand). The lesions of CL in normal infection appeared in the arms, legs, faces and ears, showed solid, dry like volcano area in shape and characterized by erythematous papule, with ulcerative border **(CDC, 2012)**.

The evidence confirmed infection due to a bad situation for hundreds of thousands of people who exposed to the displacing and dived in camps, in addition to the presence of the war and bad conditions, and presence of swamps near their camps which are important for reproduction sand fly **(Younis, 2018)**.

Diagnoses depend on visualizing the organisms within macrophage or collecting samples from the patient lesion by aspirate or biopsy. These collected materials can be stained by Giemsa, cultured or analyzed by PCR (Polymerase Chain reaction), which sensitively detect the parasite **(Guerin *et al.*, 2002; Alrajhi, 2003; Berman, 2005)**

In the last few years, polymerase chain reaction (PCR) has been widely used due to high sensitivity as a parasitological diagnostic test **(Singh *et al*., 2015).**

The internal transcribed spacer (ITS) refers to the non-coding spacer DNA located between the SSU and LSU rRNAs. The ITS1 region ranges from 50 to 350 bp and is located between the 18S rRNA and 5.8S rRNA genes It has sufficiently high conservation to be a *Leismania* PCR target but its polymorphism allows species typing, such as differentiating *L. aethiopica*, *L. tropica*, *L. major*, *L. turanica* and the *L. donovani* complex, *L. mexicana*, *L. amazonensis, L. guyanensis, L. braziliensis*.

The ITS2 region is of 50 to 650 bp-long and is located between the 5.8S rRNA and LSU rRNA genes. Amplification of ITS2 with generic PCR primers revealed substantial differences between the Old and New World *Leishmania* spp. But also between species complexes and species of these subgenera **(Odiwuor *at al.*, 2011)**.

1.2 Research Questions

a) what is the best method for the diagnosis of cutaneous leishmaniasis?

b) what is most common type of cutaneous leishmaniasis species in city of karbala?

1.2.1 Objectives

General Objective: Determination of species clinical infections of cutaneous leishmaniasis and methods of diagnosis with microscopy and laboratory culturing in karbala city.

Specific Objectives: To detect the Molecular diagnosis of cutaneous leishmaniasis by Nested PCR technique through Study the sequencing of

5.8.Sr DNA gene in isolated species by multiple sequence alignment analysis. And phylogenic tree analysis by MEGA 6 program to compare between locally *Leishmania* SPP. With Global species that recorded in NCBI.

1.3 Taxonomy and classification of leishmaniasis

Leishmaniasis is a parasitic disease caused by a heamoflagellate protozoan parasite of genus *Leishmania* (Schuster and Sullivan, 2002). Genus *Leishmania* belongs to Eukaryota (Domain), Kingdom Protista, Phylum: Mastigophora, Class: Zoomastigophorea, Order: Kinetoplastida (Peters and Pasvol, 2002) Family: Trypanosomatidae. Genus *Leishmania* is divided into two subgenus (Attar, 1997), further more divided into seven main complexes, which can affect human health **(Cheesbrough, 1998)** (Figure1-1).

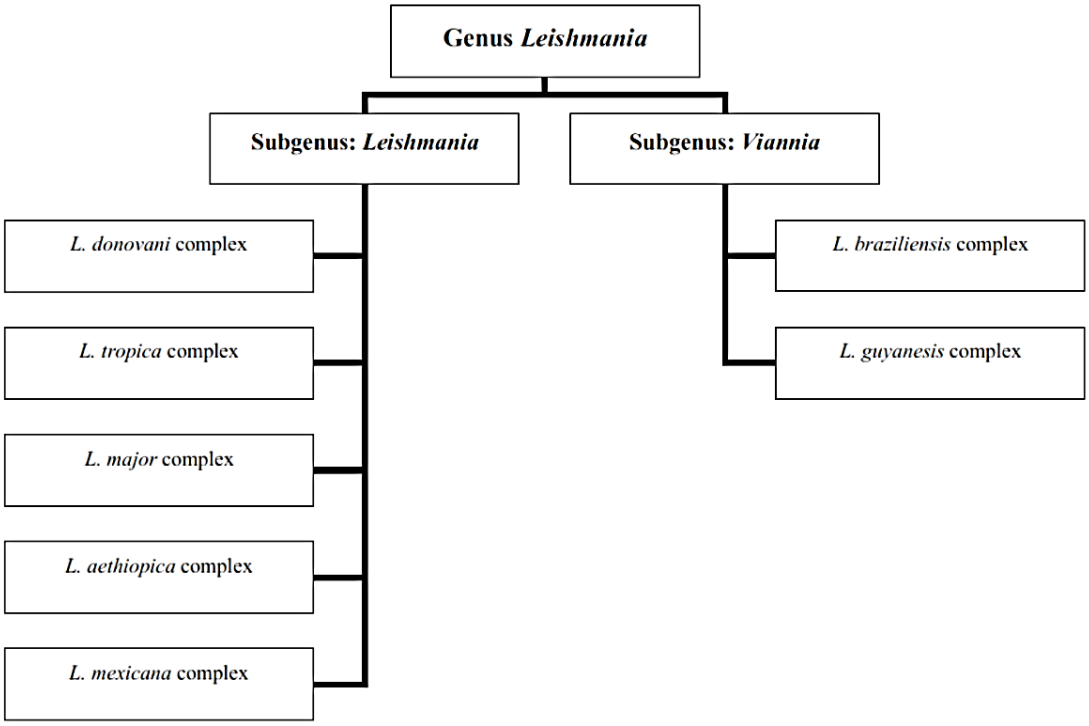

Figure (1-1) classification of Genus *Leishmania* l Adapted from **(Attar, 1997)**

1.4 History of Cutaneous Leishmaniosis:

Old World Cutaneous Leishmaniasis (an ulceration of the skin and subcutaneous tissue due to parasitic infection by *Leishmania major L. aethiopica* and *L. tropica* **(Neva and Brown, 1994)** has been described in texts dating back to 1500-2500 BC.

El-Razi described Cutaneous Leishmaniasis in Iraq in his book BKhulaset El-TagaribC (Al-Jaser, 2005) but the most important clinical description of Cutaneous Leishmaniasis was given by Alexander Russell in 1756, following examination of Turkish patient **(WHO, 2007)**.

Cutaneous leishmaniasis has been known for many hundreds of years, as one of the first clinical descriptions made in 1756 by Alexander Russell and called Aleppo boil. It is also known as (tropical sore, oriental sore, chiclero's ulcer or chiclero ulcer) **(Calvopiña *et al*., 2013)**, and it is the most common form of leishmaniasis affecting humans **(James *et al*., 2006)**. But before that, the parasite was previously observed in cutaneous lesions in India in 1885 by David D. Cuningham and then in 1889 by Peter Borovsky. The genus *Leishmania* was proposed by James Wright in1903 **(Vannier *et al*., 2002).**

Many observers tried to find out the causative agent of Cutaneous Leishmaniasis. David Cunningham of the British Indian army saw protozoan parasite first in 1885 without being able to relate it to the disease **(Cunningham, 1885)** as will as and Borovsky (a Russian military surgeon). The credit of the discovery of Old World Cutaneous Leishmaniasis is given to James Homer Wright **(Cox, 2002)**, who found the protozoan parasite and described it from a tropical ulcer of an American girl in 1903 **(Jacobson, 2003)**. New World Cutaneous Leishmaniasis was

described in the writing of Spanish missionaries in the 16th century **(Cox, 2002)**.

It was thought that the causative agent of Old World Cutaneous Leishmaniasis and New World Cutaneous Leishmaniasis was the same until Gaspar Vianna found out that they were caused by different species in 1911.

The sergeant brothers, Eduard and Etienne demonstrated the mode of *Leishmania* transmission to humans in 1921. They declared that sand flies belonging to genus Phlebotomus are responsible for the Infection. In 1922, it was found that Lutzomyia is the vector of New World Cutaneous Leishmaniasis by Ralph Lainson and his colleagues **(Cox, 2002)**.

1.5.1 Leishmaniasis distribution in the world:

Human Leishmaniasis is a worldwide infectious disease, it occurs mainly in the tropics and subtropics **(Croft and Coombs, 2003)**. Some of *Leishmania* species parasitize animals and not parasitic to human **(Neva and Brown, 1994; Attar, 1997)**. According to the World Health Organization (WHO,(Leishmaniasis affects 88 countries, 72 out of which are developing ones. There are half a million cases of Visceral Leishmaniasis (VL) and 1.5 million cases of Cutaneous Leishmaniasis (CL) occur each year. Ninety percent of CL cases occur in Afghanistan, Iran, Brazil, Kingdom of Saudi Arabia and Syria. Over 90% of VL cases occur in five countries: India, Nepal, Bangladesh, Sudan and Northeast Brazil **(WHO/TDR, 2005)**.

1.5.2 Leishmaniasis distribution in Iraq:

Sand flies from the genus phlebotomus (old word). Lutzomyia (new world) are the primary vectors responsible for disease transport **(De Lima *et al*., 2008).**

At least five species causing cutanous leishmaniasis in the world that appear in (Table1-1) the *L.major* and *L.trpica* causetive agent of the majority for cases, *L.donovani* causes post Kala-azar dermal leishmaniasis as well *L.tropica* was found to cause viceral disease among American soldiers in the Arabian peninsula **(Shiraz and Syed, 2007)**.

Table (1-1) *Leishmania* species causing cutaneous leishmaniasis in the Old World **(WHO, 2011)**.

Parasite species	Geographic distribution
L. major	Middle East, Indian subcontinent, north western China,Africa
L.tropica	Middle East, Indian subcontinent, Mediterranean littoral, western Asiatic areas
L. aethiopica	East Africa and Yemen
L. infantum	Mediterranean basin
L. donovani	Sudan, East Africa

there are two species which present in Iraq *L. tropica* and these origin of anthroponotic cutaneous leishmaniasis (ACL) and *L. major* which it's source for zoonotic cutaneous leishmaniasis (ZCL) and from the other hand both ACL and ZCL were accounted for as causative specialists of leishmaniasis in Iraq however ACL is discovered for the most part in rural territories **(WHO, 2009)**. The CL was known in the idiom Baghdad boil these term because Baghdad was think that the endemic area and the center of these diseases in Iraq **(Al–Aubaidi, 2007)**.

In Gulf war at 1990 – 1991 more than 500,000 case from Leishmaniasis recorded in the USA army and about 25,000 cases from CL were deployed in these region. While in 2003 about 150 case from CL have been reported in the USA soldiers during inter Iraq when infected with *L. major* in urban area of Iraq after a median period of deploy of 50 – 90 day **(Dedet and Ptalong, 2006)**. Cutaneous leishmaniasis has been accounted for in United States military staff, basically among those positioned in Iraq and Afghanistan likewise from August 2002 through April 2004 the department of defense reported 522 parasitological affirmed instances of cutaneous leishmaniasis acquired in Iraq and most from infections were acquired near the borders of Iraq with Syria or Iran **(Khalaf, 2010)**.

All the 176 isolates composed were *L. major*. **(Al–Aubaidi, 2007)**. Furthermore, following the resolution adopted at the 60th World Health Assembly in 2007, the global burden of Leishmaniasis and its control strategies were prioritized for countries where leishmaniasis are considered a public health problem. Two reports published by WHO in 2016 and 2017 updated the information. WHO Eastern Mediterranean Region (WHO EMR) is the highest affected region, despite the underreporting bias and the lack of epidemiological information from some countries (due in general to

political instabilities or weak monitoring system) **(Alvar *et al*., 2012)**. The table (1-2) below, based on the global health observatory 7, lists the overall number and the yearly average of CL cases reported in WHO EMR between 2006 and 2015.

Table (1-2) Yearly and total CL cases declared in high burden WHO EMR countries between 2006 and 2015.

WHO EMR CL high burden Countries*	Average annual number of CL cases	Total CL cases declared between 2006 and 2015 (Number of years declared)
Syria	44,499	444,995 (10)
Afghanistan	27,694	276,940 (10)
Iran	18,519	222,229 (12)
Pakistan	4821	43,393 (09)
Tunisia	4465	44,652 (10)
Morocco	4165	41,651 (10)
Iraq	3110	37,329 (12)
Libya	2807	19,655 (07)
Yemen	2785	27,855 (10)
Saudi Arabia	2497	24,970 (10)

1.6 Types of human Leishmaniasis:

Clinical symptoms present by the multiplication of the parasites within the human host macrophages, whose immune system failed to destroy the parasite **(Berman, 2005)**. According to the location of macrophage which harbors the parasite, four major clinical forms of Leishmaniasis are distinguished: Cutaneous Leishmaniasis (CL), Diffuse

Cutaneous Leishmaniasis (DCL), Visceral Leishmaniasis (VL) and Mucocutaneous Leishmaniasis (MCL) **(Cheesbrough, 1998)**.

1.6.1 Cutaneous Leishmaniasis (CL):

The clinical manifestations of CL have been first described in 1756 by Alexander Russell following an examination of a Turkish patient **(WHO, 2007)**. CL is characterized by Lesions on the patient skin which ulcerates later to give a disfigurement scare after healing **(Peake *et al.*, 1996; Cheesbrough, 1998)**. CL Lesions usually heals spontaneously within months. But in some patients complication may occur as the parasite metastasizes through the lymphatic to the lymph node, leading to the formation of subcutaneous nodules or enlargement of the region lymph nodes **(Bryceson, 1987)**. Ulceration of the infection may be a consequence of the host immune response **(Boakye *et al.*, 2005)**

Cutaneous Leishmaniasis is divided into two sections according to the geographical distribution and the parasite species **(Smyth, 1996)**: Old World and New World Cutaneous Leishmaniasis. However, the clinical spectrum of the disease and its response to treatment vary according to the species.

A. Old World CL: Including Southern Europe, the Middle East, parts of South-West Asia and Africa. Several species of *Leishmania* cause Cutaneous Leishmaniasis in the Old World the main of which, are *Leishmania major (L.m.)* and *Leishmania tropica (L.t.)* **(Alrajhi, 2003; Berman, 2005)**. *Leishmania* major causes a boil of 5-10 mm in diameter which changes into a large uneven ulcer or moist type lesion characterized by reddish raised edge **(Neva and Brown, 1994; Cheesbrough, 1998)**. Lesions may be multiple and differ in size **(Al-Jaser, 2005)**. *L. tropica* causes dry-type lesion 25-70mm in diameter, the ulcer is characterized by

crusted scab. Lesion formed by *L.major* infection requires 3-6 months for self-healing, while the lesion formed by *L. tropica* requires 1-2 years for self-healing **(Neva and Brown, 1994; Cheesbrough, 1998; Alrajhi, 2003)**.

The incubation period of *Leishmania major* and *Leishmania tropica* varies from 1-2 weeks to several months **(Neva and Brown, 1994)**. It used to be thought that long lasting immunity against Cutaneous Leishmaniasis is acquired after curing the infection **(Cheesbrough, 1998)**, but recent study reported that no life lasting immunity is existed and re-infection may occurs **(Al-Jaser, 2005)** (Figure 6).

B. New World CL*: Leishmania mexicana* spp. and *Leishmania vianna* spp. are the primary species which cause New World CL. The lesion caused by *Leishmania mexicana* can be self-healing, but if the ear was infected, it may last for 30 years and destroy the pina of the ear. In the case of *Leishmania vianna* spp infection, ulcers formed might be self-healing **(Cheesbrough, 1998)**.

1.6.1.1 A- Localized form of leishmaniasis:

In the localized form the parasite is confined to the skin. After an incubation period of 1- 12 weeks a papule or bump develops at the site of the insect bite. The papule grows and turns into an ulcer. A typical lesion of the localized form of CL is a painless papule or ulcer covered with an adherent crust of dried exudates. Most people with CL have one or two lesions varying in size from 0.5 to 3cm in diameter, usually occur on exposed parts of the body such as the face, arms or legs. There is, however, considerable variation: people may have as many as 200 simple skin lesions; some lesions grow but do not ulcerate (nodules), and some *Leishmania* species also infect the lymphatic system producing lesions

along the lymphatic channels (nodular lymphangitis). Secondary bacterial infection is common, causing pain and serious disability. Most lesions heal spontaneously over months or years, leaving permanent scarring with skin thinning. Scarring of leishmaniasis is typical with a de-pigmented centre and a pigmented border **(Blum *et al*., 2014)**.

1.6.1.2 B- Diffuse Cutaneous Leishmaniasis (DCL):

It occurs both in New World and Old World. Characterized by a wide, firm and smooth skin lesion which become scaly and rough later. DCL in the New world caused by *L .amazonensis* is resistant to treatment, while Old world DCL caused by *L .aethiopica* relapse after treatment **(Cheesbrough, 1998; Alrajhi, 2003)** (Figure 3-9, 3-10).

1.6.2 Visceral Leishmaniasis (VL):

It caused by *Leishmania donovani (L.d.)* and *L. infantum*. Symptoms of this form of Leishmaniasis include Fever, hepatomegaly (greatly enlarged liver), splenomegaly (greatly enlarged spleen), diarrhea **(Peake *et al*., 1996; Cheesbrough, 1998; Guerin *et al*., 2002; Peters and Pasvol, 2002)**, weight loss, anemia, skin darkening **(Smyth, 1996)** and death if patient remain untreated **(Cheesbrough, 1998; Guerin *et al*., 2002; Boakye *et al*., 2005)**.

Twenty percent of Indian patients, who cured from previous *L. donovani*, individuals suffer the symptoms of Post Kala Azar Dermal Leishmaniasis (PKDL), which is characterized by a raised erythematous patch, and hypo pigmented face, limbs, or body trunk **(Cheesbrough, 1998)** (Figure 8).

1.6.3 Mucocutaneous Leishmaniasis (MCL):

Although, it is usually caused by New World *Leishmania* species such as *L. panamensis* and *L. guyanensis* **(Peake *et al.*, 1996)** , immune compromised patients also can show MCL symptoms by other *Leishmania* species including *L. major*, *L. infantum* and *L. donovani*. MCL begins as lesions that ulcerate and become large and long-lasting that involve of human mucousal system **(Neva and Brown, 1994; Peake *et al.*, 1996)**.

The parasite attacks the nasal (nasopharynx) or the buccal cavity and slowly degenerate the cartilaginous and soft tissues to cause disfiguration and destruction of the nasal septum, lips and larynx **(Peake *et al.*, 1996; Barral-Netto *et al.*, 1998; Cheesbrough, 1998; Boakye *et al.*, 2005)** (Figure 9).

1.7 Transmission

Mammals can be infected asymptomatically for long periods, and they often remain chronically infected even after clinical cure. Subclinically infected animals can transmit *Leishmania* spp. to sand flies. These parasites have also been transmitted via blood transfusions in people and dogs, and by transplacental transmission in dogs, mice and humans **(Celeste *et al.*, 2014)**. In canine leishmaniasis caused by *L. infantum*, the parasites can sometimes be found in saliva, urine, semen and conjunctival secretions, as well as in blood **(Gradoni *et al.*, 2015)**.

Leishmania spp. is usually transmitted indirectly between hosts by sand flies of the genera Phlebotomus and Lutzomyia, which are biological vectors. Each species of *Leishmania* is adapted to transmission in certain species of sand flies.

Sandfly activity occurs when it is humid, and there is no wind or rain. These insects are usually most active at dawn, dusk and during the night, but it will bite if they are disturbed in their hiding places (animal burrows, holes in trees, caves, houses and other relatively cool, humid locations) during the day. They are attracted to light and may enter buildings at night **(De Silva *et al.*, 2015)**.

sand flies are thought to transmit the disease to people from wild mammals. The risk of direct transmission from infected dogs to humans is unknown **(Özbel *et al.*, 2011)**. In Iraq, suggested that Phlebotomus. sergenti might be the vector, while in the USA **(Tiwananthagorm *et al.*, 2017)** has suggested that both Phlebotomus. papatasi and Phlebotomus. alexandri might be the vector.

1.8 The Life Cycle:

Leishmania sp. life cycle is complex **(Guerin *et al.*, 2002)**. These parasites remain in nature by transmission between mammalian hosts through infected female Sandfly bite. Human becomes infected if he intrudes into this cycle **(Berman, 2005)**. Different species of *Leishmania* share similar morphology **(Al-Jaser, 2005)** and are characterized by having three main developmental stages; Promastigote, metacyclic promastigotes and amastigotes.

A. Procyclic Promastigotes: reproduced in the lumen of the digestive tract by longitudinal binary fission. They are characterized by delicate, long, slender form of 1.5-4 μm by 14-20 μm and a motile with anterior flagellum 15-28 μm which function in locomotion and attachment to the insect gut wall **(Neva and Brown, 1994).**

B. Metacyclic promastigote: A non-dividing infective stage found within the vector **(Neva and Brown, 1994)**. The parasite slender shape body is differentiated from procyclic promastigotes by elongated flagellum, shorter, narrower body and increased expression of some surface molecules such as Lipophosphoglycan (LPG) and Glycoprotein of 63KD (g36), which result in a form of the parasite that is adapted to infect macrophage cells **(Saraiva *et al.*, 2005.)** as in figure (1-2)

C. Amastigotes: a rounded, non-motile and unflagellated form about 2-5μm in diameter located within macrophages Phagolysosomes of mammalian hosts or any other mononuclear phagocytes **(Chang, 1990; Neva and Brown, 1994)** as in figure (1-3) .

Under light microscopy *Leishmania* has a rod shaped kinetoplast (a specialized portion of the highly extended single mitochondrion), central nucleus and sometimes a basal body that contains the centriole construction **(Chang, 1990; Neva and Brown, 1994; Smyth, 1996**) .

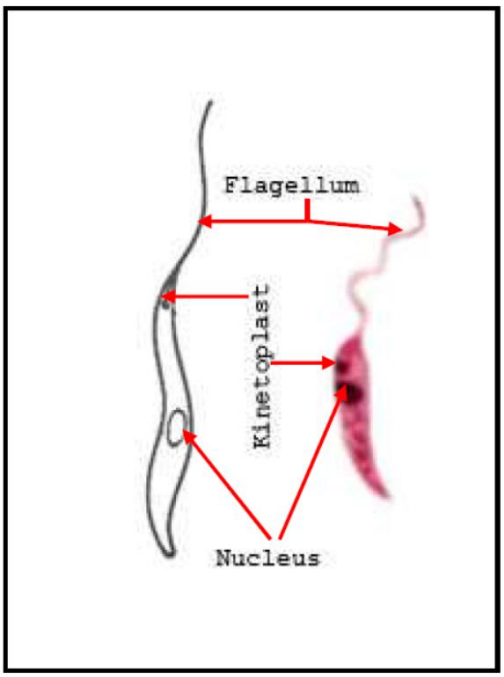

Figure (1-2) Promastigote Diagram Adapted from **(Neva and Brown, 1994)** and Image Adapted from **(Peters and Pasvol, 2002)**

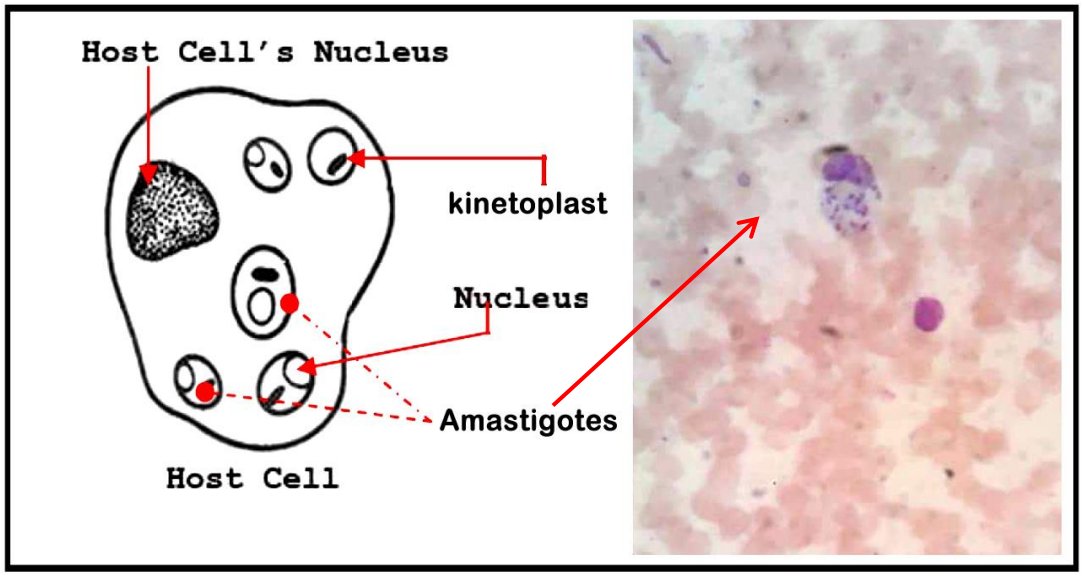

Figure (1-3) Amastigote Diagram Adapted from **(Neva and Brown, 1994)** and image by Al-Hussein hospital in Karbala city.

The parasite plasma membranes exterior consists of a coating of variant surface glycoprotein that has an important role in protecting the parasite from the host immune attack, facilitating the adhesion and entering of macrophages and manipulating the macrophage functions in its favor **(Chang, 1990; Smyth, 1996)**.

1.8.1 Life cycle in human:

Infected female Sandfly takes a blood meal from a healthy person and injects the metacyclic promastigotes into the host blood stream **(Peters and Pasvol, 2002)**. Macrophages will be attracted to the infection site to up take the pathogen **(Zer *et al.*, 2001)**, which enters a sac-like organelle known as parasitophorous vacuole **(Ali and Bahador, 2005)**. Multiple functions of macrophage are recognized in *Leishmania* life cycle. They serve as host cells for *Leishmania* replication and as a source of cytokines

which modulate the T cell-mediated response that may kill the parasite **(Teixeira *et al.*, 2006)**. Inside the macrophage the promastigotes stage change shapes, lose the flagellum and become round amastigote as

An adapting mechanisms to allow the parasite survival within the macrophages **(Chang, 1990; Neva and Brown, 1994)**. The mechanisms through which the parasite resists killing within the toxic environment of the phagolysosome remain incompletely defined.

The location of *Leishmania* -infected macrophage differs according to the parasite species, effectiveness of the immune system, and the host temperature. For example, *L. donovani* can live at the high body temperature within deep organs such as spleen, liver, lymph glands, bone marrow and other tissue of the reticuloendothelial system, but *L. major* remains in the external tissue macrophages **(Cheesbrough, 1998; Peters**

and Pasvol, 2002). Amastigotes start divide by asexual binary fission and accumulate in the macrophage until it ruptured to release the amastigotes. Then amastigotes will be picked up by new circulating or other local macrophages, this cycle continues and results in one of the clinical symptoms of Leishmaniasis with different species having different tropism for macrophages in particular organ in the host **(Chang, 1990)**. Finally, the infection can be transmitted among people via sandflies feeding on macrophages containing amastigotes **(Peters and Pasvol, 2002)** as in Figure (1-4)

1.8.2 Life cycle in Sand fly vector:

Intracellular and free amastigotes are ingested by female Sandfly while feeding blood from an infected individual. In the midgut, these round amastigotes change back into elongated and flagellated promastigotes forms **(Peake *et al.*, 1996; Cheesbrough, 1998)**.

These promastigotes attach themselves to either midgut or hindgut wall of the sandfly, where they multiply, mainly by asexual binary fission **(Peters and Pasvol, 2002)**. Then *Leishmania* promastigotes develops into metacyclic promastigotes by several biochemical and slight morphological changes of *Leishmania* surface **(Neva; *et al.*, 1994; Ali and Bahador, 2005)**. Finally, metacyclic promastigotes migrate forward into the pharynx and proboscis- remains there- until they will be injected into a new host when infected female sandfly is ready for another blood meal **(Neva *et al.*, 1994)**.

Although salivary glands do not become parasitized due *Leishmania* life cycle, some studies showed that female sandfly saliva may play an important role in the infection **(Cheesbrough, 1998; Zer *et al.*, 2001),** cording to Zer and his colleagues, Low dose of *Leishmania* promastigote

fails to initiate the infection of susceptible mice strain, while similar dose flourish the infection if inoculated by female sandfly. Saliva of female sandfly might explain this phenomenon via its effect on T-lymphocytes, which secrets InterLeukin-4 (IL-4) that attracts the host macrophage to the spot of the infection **(Zer *et al.*, 2001)**.

Others suggested that the saliva contains specific peptide which inhibits macrophage production of TNF-α and reduces their ability to make nitric oxide in order to destroy the parasite **(Cunningham, 2002)** as in Figure (1-4)

.

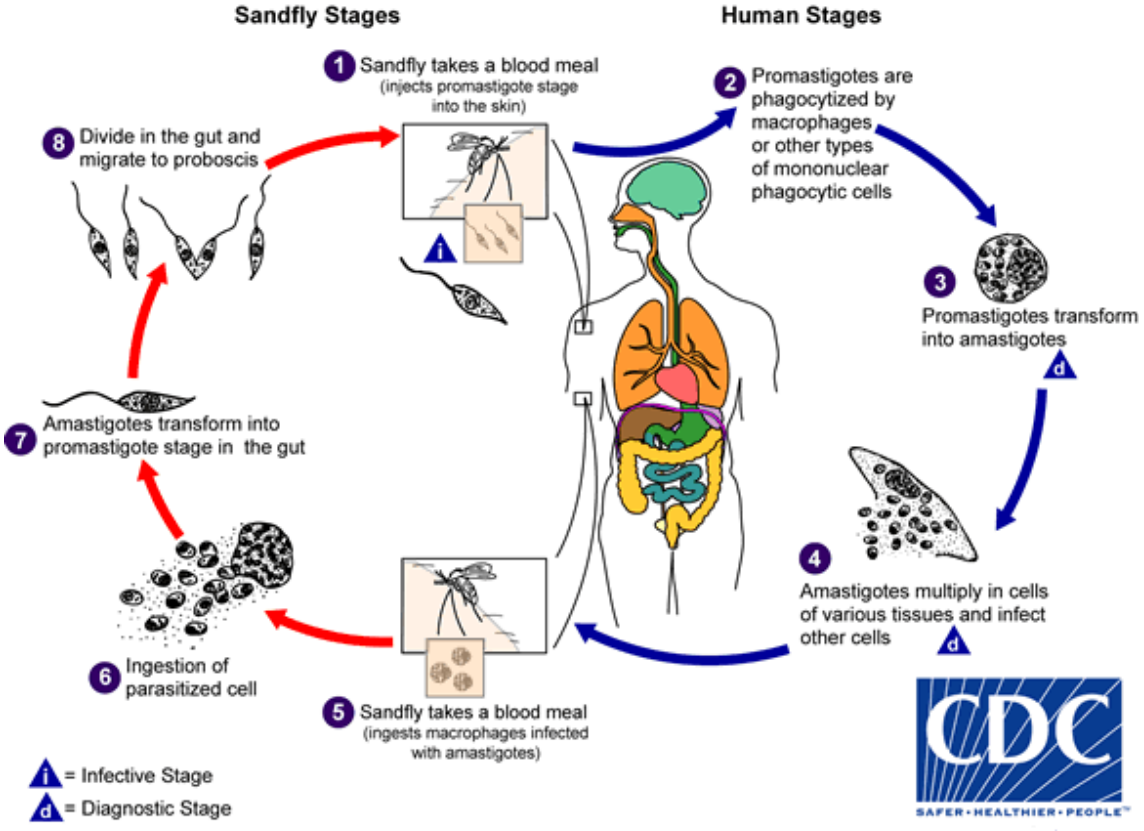

Figure (1-4) Life cycle of Leishmaniasis, **(CDC, 2011)**

1.8.3 Leishmaniasis Vector and Reservoirs:

Different species of female Sandfly are the vector host of *Leishmania* parasites **(Cheesbrough, 1998; Boakye *et al.*, 2005)**, the disease is transmitted by female sandflies (Phlebotomus or Lutzomya)

that feed on the blood of an animal or human host **(Neva and Brown, 1994)**. Most of sandfly species feed at night or dawn, usually on plant juices, they require blood meal for egg development. Species Phlebotomus is the main vector host of Leishmaniasis in Europe, Asia and Africa, while Lutzomyia is the Leishmaniasis vector in the Americas **(Cheesbrough, 1998)**.

Many vertebrate animals play a role as a reservoir of *Leishmania* parasites such as Gerbils, rodents, doges, monkeys, merinos and armadillo **(Cheesbrough, 1998; Peters and Pasvol, 2002)**.

1.9 Etiology of *Leishmania*

Leishmaniasis is caused by diphasic protozoa of the genus *Leishmania* and Viannia. *Leishmania* species are obligate intracellular parasites of mononuclear phagocytes belong to family of Trypanosomatidae **(Singh and Sivakumar, 2015)**.

There are a several species of which are pathogenic for human and animals, all species are morphologically indistinguishable and therefore *Leishmania* and Viannia subgenera are classed into complexes from species and subspecies based upon immunological, molecular, biochemical similarities.

The clinical symptoms they produce, and their ecologic characteristics **(Faulde *et al.*, 2008)**. They have been separated based on their tendency to cause visceral, cutaneous or mucocutaneous leishmaniasis **(Arfan and Rahman, 2006)**.

1.10 Immunology of leishmaniasis:

1.10.1 The interaction between *Leishmania* and human immune system:

Immunity is the body ability to resist microbes and other foreign materials or abnormal cells. Immune response may be either nonspecific (innate) or specific (adaptive) **(Sherwood, 2004)**.

Innate response includes defense in a non- specific manner by the activity of the complement system and some leukocytes, such as monocytes, resident macrophage, basophils, eosinophils, neutrophils, dendritic cells, mast cells and natural killer cells **(Sherwood, 2004)**.

Macrophages are phagocytic cells developed from migrated monocytes from the circulating blood to the tissue Macrophages engulf microbes by phagocytosis into phagosomes, Inside macrophages phagosomes binds with lysosomes to form a digestive organelles (phagolysosomes), which destroy the pathogen and represent it to the immune system **(Sherwood, 2004)**.

When the micro-organism is engulfed by macrophages an activation of numerous cellular genes occurs, several of which encode cytokines that stimulate an inflammatory response and resistance to pathogens such as Interleukin 12 (IL-12) and Tumor Necrosis Factor (TNF-α) **(Be Souza, 1995)**.

The complement system is a group of inactive plasma proteins produced by the liver and circulate the blood, when activated a sequential cascade of complement components activation reactions will form the membrane attack complex that destroys plasma membrane of foreign cells **(Abbas *et al.*, 1994; Sherwood, 2004)**.

There are additional functions of the complement system, it act as a chemotaxin, which attracts the phagocytic cells to the site of its activation and acts as an opsonin by binding on microbes to facilitate their phagocytosis **(Abbas *et al.*,1994; Sherwood, 2004)**.

Adaptive response can be cell-mediated or antibody-mediated immunity. Cell-mediated immunity is mediated by T-lymphocytes ,which secret cytokines and transform into T-cells when activated, while antibody-mediated immunity involve the production of antibodies by plasma cells the derivatives of B-lymphocytes , which identify foreign materials to the complement system or phagocytic cells **(Wilson and Waugh, 1996; Sherwood, 2004)**.

The activity of macrophage is influenced by lymphocytes and vise versa **(Wilson and Waugh, 1996; Sherwood, 2004).**

In order to develop a successful parasitic relationship with its host, the *Leishmania* must evade both the innate and adaptive immune responses **(Chang, 1990; Croft and Coombs, 2003; Oliver *et al.*, 2005)** where they are obligated to parasitize, survive and multiply inside the phagolysosomes **(Croft and Coombs, 2003)**.

Multiple tactics are used by *Leishmania* to survive the initial introduction to the blood stream. Naturally, the existence of *Leishmania* parasites in blood stream activates the complement system **(Chang, 1990**).

However, *Leishmania* avoids destruction by fixing and opsonizing C3 (the third member of the complement system) **(Mosser and Brittingham, 1997)**, C3b binds to foreign material and promotes its uptake, via C3b receptors on phagocytic cells. As expected, C3b binds to the *Leishmania* Protozoa. This results in uptake by the macrophage. The activation of the complement system by *Leishmania* parasites also results in macrophage chemotaxis to the infection site **(Wilson and Pearson, 1990)**, thereby, making the preferred host cells (macrophage) available.

1.10.2 *Leishmania* Macrophages cellular interaction:

Macrophages play a pivotal role in *Leishmania* infection. After phagocytosis, *Leishmania* promastigotes enter a parasitophorous vacuole within which the macrophage can provide a safe haven for the parasite to transform into amastigotes and proliferate in a naïve host **(Alexander and Russell, 1992)**.

However, *Leishmania* manages to escape from the destruction activity of the macrophages by inhibiting the macrophages ability to introduce the parasite antigen to other components of immune system **(Oliver *et al.*, 2005)**.

As a result, *Leishmania* manages to survive, differentiate into amastigotes, and multiply by binary fission within those cells **(Chang. 1990)**.

1.10.3 Entry of *Leishmania* in Macrophages:

Phagocytic activity of the host macrophage plays an important role of the infection. The entry of *Leishmania* into host macrophage is achieved by receptor-mediated phagocytosis **(Chang, 1990; Kane and Mosser, 2000)**. As in figure (1-5)

The parasite promastigote/amastigote must bind to the surface of the macrophage in order to be engulfed. Adhesion to macrophage receptors can be achieved through interaction between several native surface molecules of *Leishmania* **(Chang, 1990; Neva and Brown, 1994; Kane and Mosser, 2000)** or through the intervention of soluble mediators released by the host cells such as complement system **(Chang, 1990)**.

The primary method of *Leishmania* adhesion is the reaction of macrophage complement receptors and *Leishmania* surface molecule glycoprotein of 63KD (gp63), which is the most important surface molecule of this process **(Oliver *et al.*, 2005)**.

The surface protease gp63 is represented as predominant molecular surface on all pathogenic species of *Leishmania* . Research has proved that gp63 is more abundance in virulent than avirulant promastigotes **(Chang, 1990)**.

The gp63 is activated in suitable pH (4.0) and it is capable of acting on several substrates. The enzymatic activity of gp63 is important in the cleavage of complement C3 into C3b and C3bi to prevent complement mediated lyses and enhances promastigotes uptake **(Chang, 1990; Mosser and Brittingham, 1997)** as the case of *Leishmania* major **(Abbas, 1994; Cunningham, 2002)**.

C3b and C3bi present on the parasite surface assist the parasite (promastigote/amastigote)**(Mosser and Brittingham, 1997; Cunningham, 2002; Oliver *et al.*, 2005)** binding to the specific macrophages receptors CR1 and CR3 **(Abbas *et al.*, 1994; Kane and Mosser,2000)**.

Both receptors promote the phagocytosis without initiating an oxidative burst within macrophage **(Cunningham, 2002)**. Binding to CR3 has another benefit, it inhibits the induction of cell mediated immunity by prevent the production of IL-12 **(Cunningham, 2002; Oliver *et al.*, 2005)**.

In addition, gp63 is important in degrading the host antibodies as a defensive mechanism and degrading some of the host proteins for nutritional benefit of the parasite **(Kane and Mosser, 2000; Amer and Swanson, 2002)**.

Lipophosphoglycan (LPG) is another *Leishmania* major cell surface molecule of interest **(Kane and Mosser, 2000; Amer and Swanson, 2002)**, which may involves *Leishmania* promastigotes binding to macrophage as will as surviving after phagocytosis **(Chang, 1990)**.

Further binding of *Leishmania* to macrophage may occur through other receptors if the parasites opsonize some of the host molecules such as fibronectin and mannose **(Chang, 1990; Peters *et al.*, 1995)**.

The ability to use multiple routes of entry into macrophages assists the parasite protection of destruction by the outside immune components **(Cunningham, 2002)**.

1.10.4 Intracellular survival of *Leishmania* in Macrophage:

Leishmania protects itself from the harsh environment inside the phagolysosomes by several mechanisms **(Cunningham, 2002)**. It has a functional molecular surface molecules and several enzyme systems, both help to achieve its survival **(Change, 1990)** by inhibiting host phagosome-endosome fusion, hydrolytic enzymes, cytokines production, nitric oxide production and manipulating cell signaling pathways **(Cunningham, 2002)**. In another hand *Leishmania* manages to inhibit and manipulate

macrophage apoptosis in order to multiply in a hostile environment before rupturing the cell and finding another host cell **(Heussler *et al.*, 2001)**.

1.10.4.1 A. The Role of *Leishmania* Surface Molecular components:

Leishmania surface molecules - such as gp63 and LPG - protect the parasite within the phagolysosomes of the host cell by monitoring Lysosomal hydrolytic enzymes and inhibiting the oxidative burst **(Cunningham, 2002; Oliver et.al, 2005)**.

Gp63 protease destroys the host lysosomal hydrolytic enzymes. In the same time, ammonium ions released by some of *Leishmania* species might interact with lysosomal hydrolytic enzymes activity directly or by elevating the phagolysosomes pH **(Chang, 1990)**.

LPG may acts as degradation barrier which destroys the macrophage hydrolytic enzymes because of its highly anionic nature **(Cunningham, 2002; Oliver *et al.*, 2005)**.

Also, it inhibits protein kinase C (PKC), which mediates macrophage oxidative burst activation, by altering calcium mobilization to impaired responsible signal transduction for PKC activation **(Chang, 1990; Cunningham, 2002)**.

Inhibition of macrophage Nitric Oxide (NO) and IL-12 production is achieved after LPG ligation with macrophage complements receptors during the parasite invasion of the host cell **(Spath *et al.*, 2003).** The importance of LPG in preventing No production was confirmed by using *Leishmania* major mutant (specifically lacking LPG), that could not inhibit macrophage NO production **(Spath *et al.*, 2003)**.

Nevertheless LPG influences phagosome- endosome fusion by altering the membranes biophysical prosperities to influence the fusogenic property **(Cunningham, 2002; Oliver *et al.*, 2005)** such as the case of *Leishmania* donovani **(Amer and Swanson, 2002)**.

Finally, *Leishmania* surface molecules have the ability to resemble some of the lysosomal enzymes a phenomenon noun as BMolecular mimicry C such as the macrophages acid phosphatase to avoid mutual digestion **(Chang, 1990)**.

1.10.4.2 B. *Leishmania* reaction toward oxidative burst:

Oxidative burst within macrophages generates microbicidal oxygen radicals such as hydroxyl radicals, hydrogen peroxide and singlet oxygen, all of which are leishmanicidal **(Chang, 1990; Kane and Mosser, 2000)**.

Macrophage produces NO in response to extracellular signals **(Cunningham, 2002)**, *Leishmania* parasite has the ability to inhibit macrophage Nitric Oxide production by enhancing the production of IL-10 **(Oliver *et al.*, 2005)**.

If the oxidative burst was initiated by extracellular signals such as IFN-γ, *Leishmania* will dispose oxidative burst outlets by several enzyme systems **(Chang, 1990)** or by interacting with surface molecules (LPG). LPG repetitive unites scavenge the output of oxidative burst (superoxide anions and hydroxyl radicals) **(Cunningham, 2002)**.

Iron-containing superoxide dismutase and trypanothione is a representative example for *Leishmania* enzymes system **(Chang, 1990)**.

1.10.4.3 C. Inhibition of Macrophage cytokines production:

Another mechanism that assists the *Leishmania* survival is Modulating the macrophage cytokines production to bias the immune response to its benefit **(Akuffo *et al.*, 1996; Kane and Mosser, 2000 ; Cunningham, 2002)**.

Leishmania mainly modulates the secretion of macrophages Interleukin 12 (IL-12) , Interleukin 10 (IL-10) and gamma interferon (IFN-γ) for its own benefit. The proinflammatory cytokines interleukin (IL-1, IL-6 and also tumor necrosis factor TNF-alpha, were shown to affect the granulomatous response of murine and human CL. Interlukine1 participated in the initial phase of the granuloma, while IL6 and TNF-alpha were involved in the initial, maintenance and possibly also the resolution phases. Cytokine IL12 is down-regulated by *Leishmania* via ligation with macrophage receptors **(Kane and Mosser, 2000; Cunningham, 2002)**.

One of the most important initial signaling events is the release of IL-12 by the infected macrophage, leading to subsequent priming of the Th1 response and production of IFN-γ **(Teixeira *et al.*, 2006)**. In addition IL12 activates Natural killer cells (NK) and induces IFN-γ production **(Trinchieri, 1995)**, IFN-γ has a pivotal role in the activation of macrophages to kill pathogens and protect the host cell from infection **(Akuffo *et al.*, 1996; Barral-Netto *et al.*,1998)**.

Leishmania also up-regulates the production of anti-inflammatory cytokines such as IL10. IL10 is important in surprising macrophage leishmanicidal activity by opposing IFN-γ **(Akuffo *et al.*, 1996; Barral-Netto *et al.*, 1998; Belkaid *et al.*, 2001; Cunningham, 2002)**, NO, IL12 production and inhibiting PKC activity of neighboring macrophages **(Oliver *et al.*, 2005)**.

1.10.4.4 D. Inhibition of host cell signaling pathways:

Signaling pathways of host macrophages are crucial for its function. The deactivation of macrophage mediated signaling pathways relevant to *Leishmania* infection occurs once the parasite attached to macrophage by binding of complement receptors **(Spath *et al.*, 2003)**.

Leishmania tends to impair macrophage responsiveness toward Lipopolysaccharide (LPS), IFN-γ and activators of Protein kinase C (PKC) either by secreting some molecules that effect cellular transduction or by the host molecules interaction with the parasite molecular surface such as LPG. LPG impaired signal transduction by binding to soluble Ca++, thus defect PKC activation **(Kane and Mosser, 2000; Cunningham, 2002; Oliver *et al.*, 2005)**.

It has been documented that *Leishmania* defects infected macrophage response to IFN-γ by manipulating macrophage signaling pathways **(Ray *et al.*, 2000)** in order to be protected of subsequence response of IFN-γ.

1.10.5 Immune system influence on the infected Macrophage:

In an immune host, macrophages can be activated by inflammatory cytokines to produce toxic metabolites that result in intracellular killing of *Leishmania* **(Murray and Nathan, 1999)**, or their microbicidal capacity can be dampened or abrogated by suppressive cytokines, leading to disease symptoms. The immune system cells (including: Th1, Th2 and Natural killer cells) and cytokines (including: IFN-γ, IL-12, IL-10 and IL-4) effect *Leishmania* survival within the host macrophage. *Leishmania* survives within inactivated macrophage. If the macrophage becomes activated by the influence of the immune system the parasite will be destroyed by

activation of the inducible nitric oxide, which leads to oxidative burst within macrophages **(Vouldoukis *et al.*, 1995; Cunningham, 2002)**.

Immunity against *Leishmania* is cell-mediated **(Cunningham, 2002)**. If Th1 or NK cells were activated, the parasite will be destroyed by the release of principle macrophage activating factor; Interferon gamma (IFN-γ) **(Wilson & Pearson, 1990; Barral-Netto *et al.*, 1998; Stebut *et al.*,1998; Teixeira *et al.*, 2006).**

Conjugation of IFN-γ and Tumor Necrosis Factor alph (TNF-α) - released by infected macrophages- activates the inducible nitric oxide syntheses gene, resulting in the toxic nitric oxide production **(Kane and Mosser, 2000; Cunningham, 2002).**

In murine models the increased number of Th1 cells is associated with resistance to *Leishmania* major because of the fact that Th1 cells secrets IFN-γ. In non-resistance murine models the infection is associated with the increase of the number of T helper cells type2 (Th2), which secretes IL-4 and IL-10. IL-4 regulates differentiation of Nave T cells into Th2 **(Akuffo *et al.*, 1996; Barral-Netto *et al.*, 1998; Kane and Mosser, 2000; Belkaid *et al.*, 2001).**

Th2 cells inhibit macrophage IL-12 and Nitric Oxide production **(Cunningham, 2002)**. In contrast Th2 secretes IL-10, which down-regulates IFN-γ production causing the parasite survival within host macrophage **(Akuffo *et al.*, 1996; Belkaid *et al.*, 2001).**

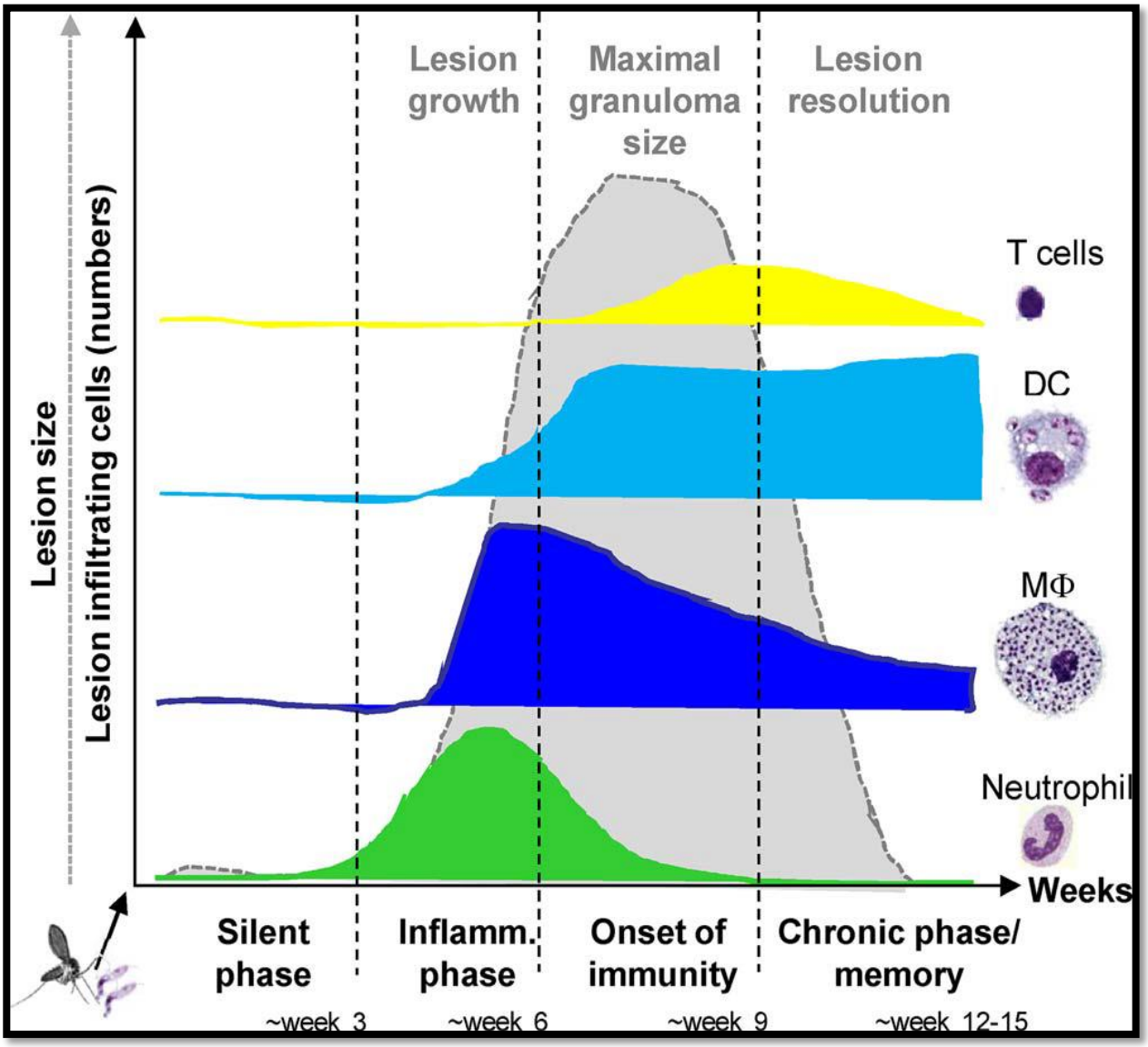

Figure (1-5): Lesion evolution in leishmaniasis **(Sacks *et al*., 2012)**

1.11 Genomic structure of leishmaniasis:

1.11.1 Chromosomal DNA:

1.11.1.1 Ribosomal DNA (rDNA):

The ribosomal RNA (rRNA) genes are located mostly on chromosome 27, usually as multiple copies of tandem head-to-tail repeats of approximately 12.5 Kb **(Yan *et al.*, 1996)**.

Among the different components of these genes, the most variable ITS regions are ideal for species-typing **(Kuhls *et al.*, 2005)**. Like most eukaryotes, large ribosomal subunit (LSU) is composed of 28S, 5.8S and 5S rRNAs, whereas the small subunit (SSU) contains 18S rRNA **(Torres *et al.*, 2010)**.

1.11.1.2 Ribosomal RNA transcription units:

The 18S rRNA is a structural RNA of the ribosomal SSU. The high conservation of this gene and its flanking regions make it suitable for reconstructing phylogenetic relationships.

The 5.8S rRNA is a non-coding component of the ribosomal LSU and is part of the 45S precursor that also contains the 18S and 28S rRNAs. The 28S rRNA acts as a ribozyme, catalysing peptide bond formation. *Leishmania* species are atypical in its composition, as they contain two large (24Sa and 24Sb) and four small rRNA molecules **(Soto *et al.*, 2004)**.

1.11.1.3 Internal transcribed spacer:

The internal transcribed spacer (ITS) refers to the non-coding spacer DNA located between the SSU and LSU rRNAs. The ITS1 region ranges from 50 to 350 bp and is located between the 18S rRNA and 5.8S rRNA genes **(Schönian *et al.*, 2003)**. As in figure (1-6)

It has sufficiently high conservation to be a *Leismania* PCR target but its polymorphism allows species typing, such as differentiating *L. aethiopica*, *L. tropica*, *L. major*, *L. turanica* and the *L. donovani* complex, *L. mexicana*, *L. amazonensis*, *L. guyanensis*, *L. braziliensis* **(Odiwuor *et al.*, 2011)**. The ITS2 region is of 50 to 650 bp-long and is located between the 5.8S rRNA and LSU rRNA genes. Amplification of ITS2 with generic PCR primers revealed substantial differences between the Old and New World *Leishmania* spp. But also between species complexes and species of these subgenera **(Schönian *et al.*, 2003)**.

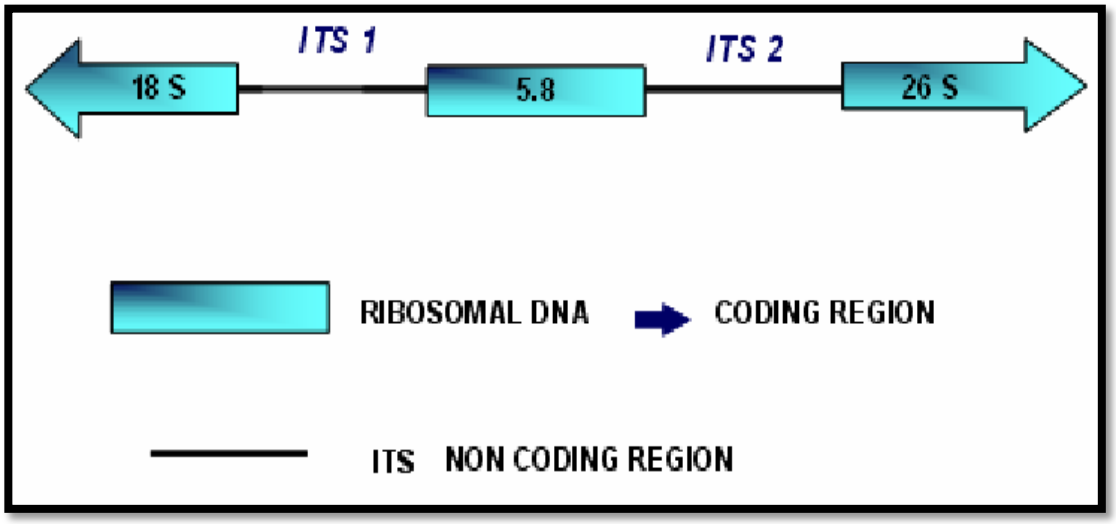

Figure (1-6): 5.8 S rDNA region (Anjana Munshi, 2012).

1.11.2 Non-chromosomal DNA:

1.11.2.1 Kinetoplast DNA (mitochondrial DNA):

All Kinetoplastid flagellates possess a single mitochondrial genome known as the kinetoplast DNA (kDNA), which consists of several thousand circular DNA molecules linked together in a concatenated network **(Luke *et al.*, 2002).**

It is a mass of circular DNA that consists of thousands of mini-circles (~1Kb each) and several dozen maxicircles (~23 Kb each) **(Haydock *et al.*, 201).**

1.11.2.1.1 kDNA maxicircle:

Usually range from 20 to 40 kb, depending on the species, and are present in a few dozen identical copies per network.

The kDNA maxicircle encode genes homologous to those present in the mitochondrial DNA of other eukaryotes **(Haydock *et al.*, 2015).**

1.11.2.1.2 kDNA minicircle:

Minicircles make up approximately 95% of kDNA and encode small RNA molecules termed guide RNAs, which provide information for RNA-editing of the maxi-circle encoded transcripts **(Stuar *et al.*, 2005)**. kDNA is traditionally the most frequently used target for detection and typing of *Leishmania* because of its multicopy nature and through high sensitivity **(Jara *et al.*, 2013).**

1.12 Diagnosis:

Differential diagnosis is important because diseases of other causes but with a similar clinical spectrum to leishmaniasis e.g. leprosy, skin cancers, tuberculosis, cutaneous mycoses, viral wart, bacterial infection and eczema are common in leishmaniasis-endemic areas **(Paniz *et al.*, 2013)**.

1.12.1 Direct examination:

Microscopic examination is probably the most common diagnostic approach used, because more sophisticated techniques are expensive and rarely available at primary, secondary, and tertiary health-care levels in

endemic areas, using Giemsa, Wright's, Leishman's or other stains **(Younis *et al.*, 2013**).

(Rahi, 2015) said that during stain the *Leishmania* amastigotes appear round to oval parasites, with a round basophilic nucleus and a small rod-like kinetoplast in macrophages or freed from ruptured cells (Peña; *et al.* 2015). The use of an Electron microscope gives the best result for identification both the size and disposition of nucleus and kinetoplast **(Marayati *et al.*, 2015).**

1.12.1.1 Parasitic isolation and identification:

Leishmania spp was isolated from skin lesion by taking aspirate from the lesion, which converted motile promastigotes after seven days by using the culture **(Habibi *et al.*, 2008).**

There are different culture media which used for culturing the *Leishmania* spp. Novy-MacNeil-Nicole medium, RPMI 1640, peptone-yeast extract, brain heart infusion and nutrient broth, Evan's modified Tobie's medium (EMTM), Grace's medium and Schneider's Drosophila medium **(David *et al.*, 2015).**

The animal inoculation into hamsters may also be valuable, especially for adaptation of the parasite **(De Vries *et al.*, 2015).**

1.12.2 Molecular tests:

Polymerase Chain Reaction is particularly sensitive and more specific also can be used to detect *Leishmania* spp. in blood, skin biopsies, lymph nodes, bone marrow and conjunctival swabs, the target sequences for characterization include either nuclear DNA such as the small subunit rRNA (SSU rRNA) gene, a repetitive genomic sequence and the miniexon

(spliced leader) gene repeats **(Nasser *et al.*, 2014)**, Also Real-Time PCR is very important techniques to detect the parasite quantitatively and qualitatively **(Fadime *et al.*, 2014)**.

1.12.2.1Nested PCR:

Nested PCR is an amendment of PCR that was designed to get better sensitivity and specificity. Nested PCR includes the use from two primer sets and two successive PCR reactions The first group of primers are designed to anneal to sequences upstream from the second set of primers and are used in an initial PCR reaction.

Amplicons resulting from the first PCR reaction are used as template for a second set of primers and a second amplification step. Sensitivity and specificity of DNA amplification may be significantly enhanced with this technique. In this study Qualitative PCR, protocol was used to detect the presence of ITS1-5.8S rDNA gene by Nested PCR **(Jeanne *et al.*, 2010).**

1.12.3 Principle of DNA sequencing

DNA sequence analysis is additional study and progress of the basis of the target gene. The term DNA sequencing discusses methods for determining the order of the nucleotides bases adenine A, guanine G, cytosine C and thymine T, in a molecule of DNA.

Academic researchers were obtained the first DNA sequence, using laboratories procedures in the early 1970 that based on 2- dimensional chromatography. By the improvement of dye based sequencing method with automated analysis, DNA sequencing has become easier and faster. DNA can be sequenced by a chemical method that breaks a terminally labeled DNA molecule partially at each recurrence of a base.

The technique will allow sequencing of at least 100 bases from the point of labeling. The dimethyl sulphate is the specific reagent of purine; and the pyrimidine specific reagent is hydrazine. In 1973, Gilbert and Maxam stated the sequence of 24 base pairs using a method known as wandering spot analysis, The chain termination method improved by Sanger and coworkers in 1975 owing to its relative easy and reliability.

The first complete DNA genome to be sequenced is that of bacteriophage ØX174, in 1975. By knowing the DNA sequence, the cause of the several diseases can be known. We can determine the sequence responsible for various disease and can be treated with the help of Gene therapy. Furthermore is useful in reviewing fundamental biological processes and diagnostic or forensic research. Sequencing can be done by different methods*:* **(Artem *et al.*, 2008; Anjana Munshi, 2012).**

1.12.3.1 Maxam – Gilbert sequencing

Allan Maxam and Walter Gilbert (1976-1977) developed a DNA sequencing method that based on a chemical degradation. This technique is based on nucleobase specific partial chemical modification of DNA and subsequent cleavage of the DNA backbone at sites adjacent to the modified nucleotides. The end labeled DNA fragments are exposed to random cleavage at adenine A, cytosine C, guanine G, or thymine T, positions by using specific chemical agents **(Artem *et al.*, 2008)**.

The chemical attack is based on three steps: base modification, the modified base that removed from its sugar and breaking the DNA strand at that sugar position. The results of these four reactions are then separated using polyacrylamide gel electrophoresis. **(Artem *et al.*, 2008)**.

1.12.3.2 Chain-termination methods (Sanger)

The chain terminator technique is more efficient and used fewer toxic chemicals and lower amount of radioactivity than the method of Maxam and Gilbert. The fundamental principle of the Sanger method 1981(enzymatic methods) was the use of dideoxy nucleotide triphosphates as DNA chain terminators. Sanger sequencing method is currently widely applied.

This technique is based on the nucleotide start at a fixed point, random in a particular base at the end, then in the back of each single base fluorescent marker, resulting in (ddATP, ddGTP, ddCTP, and ddTTP). To each reaction only one of the four dideoxy nucleotide (ddATP, ddGTP, ddCTP, and ddTTP) is added, which are the chain terminating nucleotides, lacking a 3'-OH group required for the creation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length.

Each of the relative concentrations of dNTPs and ddNTPs can be adjusted consequently that the reaction of a few to a thousand or more a head, a difference of one base a series of clips. The dNTPs and ddNTPs have a common starting point, but in a different termination nucleotide, it can be separated by high-resolution denaturing polyacrylamide-urea gel electrophoresis, fragments of different sizes, the gel can be treated via X-ray film autoradiography or non-isotopic markers detection. **(Anjana Munshi., 2012).**

1.13 Treatment:

Uncomplicated CL is usually self-limiting. Lesions resolve over months leaving a scar. Different *Leishmania* species have different self-resolution times, *L. aethiopica* and *L. tropica* being characteristically taken much longer than *L major*.

Cutaneous Leishmaniasis cases caused by *L .major* can be self-cured in 3-4 months, but most clinical Leishmaniasis doesnt cure without chemotherapy **(Berman, 2005)**. Several drugs are used for treating Leishmaniasis, such as Pentavalent antimony, Paramomycin, Liposomal amphotericin B , Funconazole and the under trial drug Miltefosine **(Guerin *et al.*,2002; Berman, 2005)**. The classic treatment is Pentavalent antimony, which if fails, can be replaced with amphotericin B as a sec line treatment **(Peake *et al.*, 1996; Berman, 2005)**. In Saudi Arabia Pentavalent antimony is the main drug used successfully against Leishmaniasis **(Al-Jaser, 2005)**. Also, cryotherapy can be used for the same purpose **(Alrajhi, 2003).**

1.14 Prevention and control:

1.14.1 Control of *Leishmania* infections relies primarily on:

Early diagnosis and treatment of infected person, Avoid sandfly bites by: using insect repellents, parathyroid impregnated bed nets and curtains, staying away of endemic areas and stopping out door activities especially at the insect active time **(Cheesbrough, 1998; Al-Tawfiq and Abukhamsin, 2004).**

Vector control by using light traps, sticky papers and insecticides **(Cheesbrough, 1998; Boakye *et al.*, 2005).**

Destruction of reservoirs such as: infected dogs, stray dogs and rodents, Setting human residents away from animal reservoir habitats, where sandfly usually breeds **(Cheesbrough, 1998).**

Because the strategies available are expensive and labour intensive, and cutaneous leishmaniasis is a nonfatal disease, prevention and control strategies have mainly focused on the treatment of the human disease, rather than on the elimination of reservoirs or reduction of human-vector contact **(Yusuf *et al.*, 2016).**

Chapter Two

SUBJECTS AND METHOD

2.1 Study Design

This Cross - sectional study conducted one hundred patients with lesions clinically suggestive to be cutaneous leishmaniasis as in Figure (2-1).

2.2 Study Site

The study was accomplished from November 2017 to end January 2018 in, at Medical Research Laboratory of Medicine Collage at Karbala University. The medical research laboratory has been equipped with work materials like (RPMI medium - Brain heart infusion etc.).

The syringe was orbited and the tissue liquids were delicately suctioned into the needle, while its withdrawal the aspirated material was inoculated in culture tubes containing twenty ml of NNN medium on data report.

All cases diagnosed by dermatologist as cutaneous leishmaniasis and their data were collected from Al-Hussein Hospital in Karbala city, and Biotechnology Research Center in Al-Nahrain Medical College in Baghdad for supplying the *Leishmania* cutaneous culture.

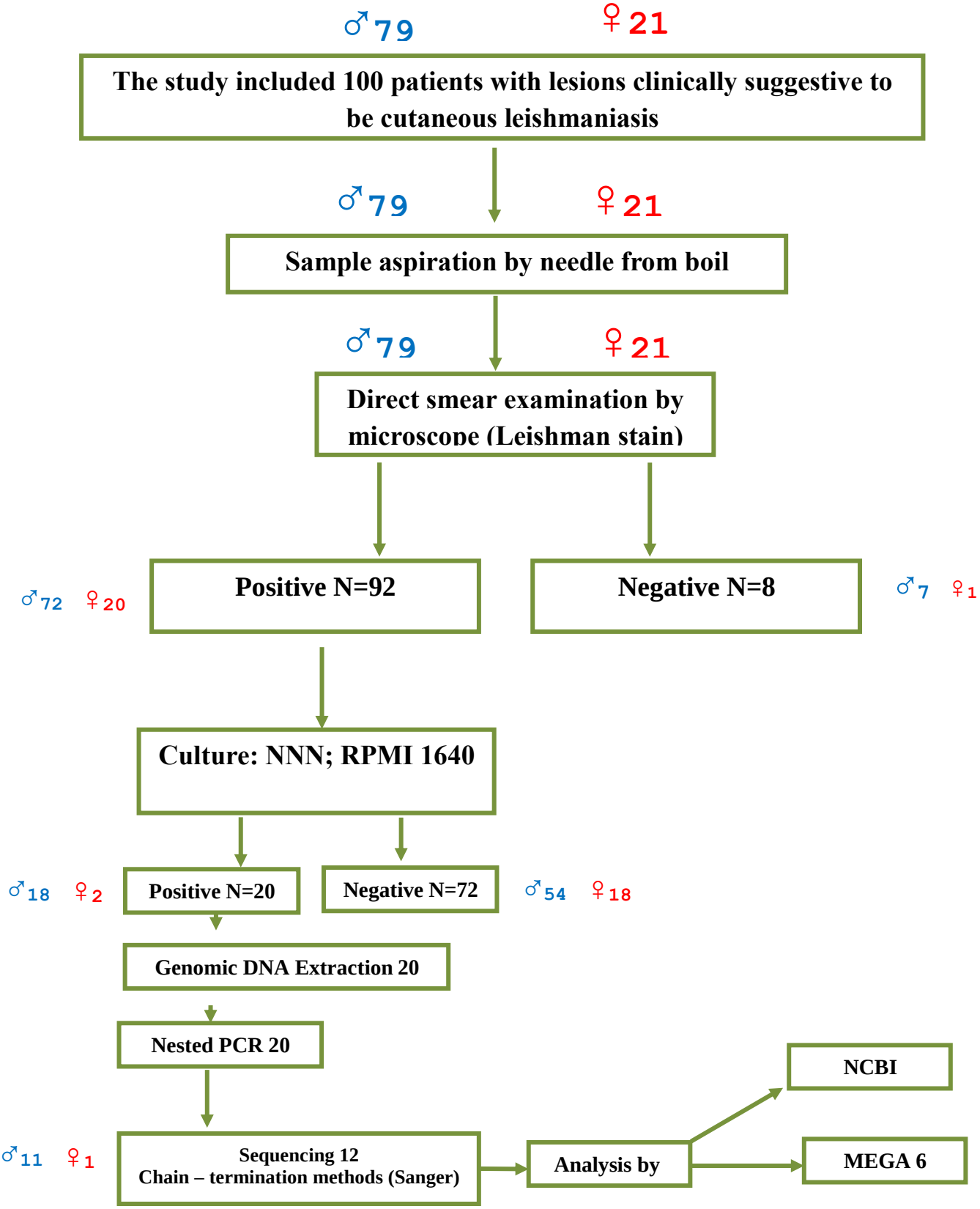

Figure (2-1) scheme diagram for study design

2.3 Instruments

Instruments used in this work are summarized in table (3-2) with their companies.

Table (2-1): Instruments used in the study

Instruments	Company
UV-visb Spectrophotometer	Cecil instruments CE7200, France
Centrifuge	H-19F Kokusan,Japan
Aura TM PCR Cabinet	Italy
Autoclave	Arneold&Sories, Germany
Tips (blue, yellow)	AFCO, Jordan
Eppendorf tube (1.5µl)	JetBiofil, U.S.A.
Multi Gene Opit Max Gradient Thermal cycler	Labnet,U.S.A
Hot plate with magnetic stirrer(L-81)	RLabinco, U.S.A.
Biopette Variable Volume 2-20ul	Biosan
Mini-power supply300v,2200v	Chain
V-1plus,personal vortex for tubes	Digsystem
Electrophoreses	CBS,Scientific,U.S.A
Minispin	Lative,Biosan
Balance	Kernpfb,Germany
Microwave	Gosonic,chain

Water distilater	Chain
Incubation	China
Hood (Fume cabinet)	USA
Refrigerator	Japan
Benzene burner	China

2.4 The following chemicals were used in the present study

Table (2-2): The following chemicals

Chemicals	Company
Agar	Difco
Bovine serum albumin	Sigma Aldrich
Agarose gel electrophoresis Kit	Conda ,U.S.A
Tris-Borate EDTA Buffer (TBE 10x)	Conda , U.S.A
Pre mix pcr	Intron, korea
Primer	IDT/Canada
G-spin DNA extraction Kit	Intron / Korea
Red safe	Intron, korea
6X Loading dye	Kapa ,U.S.A
Ladder 100bp	DisBio, korea
RPMI medium	EURO Clone
Ethanol	BDH

Penicillin/streptomycin	Sigma Aldrich
Normal saline	BDH
Ethanol	Germany
Leishman stain	Germany
Sterile cotton	China

2.5 Clinical Sample

Cutaneous leishmaniasis that 72 males that percentage (78.3)% and 20 females that percentage (21.7)% with age ranged 10-59 years, attended to Department of Dermatology Al-Husseini Hospital in Karbala during the period between November 2017 to end January 2018.

2.6 Approval of the Ethical committee

This study was approved before it's commencement by the ethical committee of the college of medicine, university of Karbala, and informed concent was obtained from all individnals.

2.7 Sample Size Determination

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniasis that 72 males that percentage (78.3)% and 20 females that percentage (21.7)% with age ranged 10-59 years, attended to Department of Dermatology Al-Husseini Hospital in Karbala during the period between November 2017 to end January 2018.

The most infected rate of patients were between the ages of 40-49 years (40.2%), while the lowest infection rate was of patient’s ages 10-19 years (6.5%).

The geographic distribution of the patients shows that 62% of the patients were from the cities of Telaefar and Dialla, while only 30% were from Karbala city.

The number of lesions differs between patients, Most (65%) of the patients had a multiple lesions in comparison to the single lesions which was found in (35%) of patients.

2.8 Sample collection

A total of 100 patients were examined by dermatologists for CL at Al-Hussain Hospital during November 2017 to end January 2018 . Their ages ranged from 10 to 50 years.

And all patients information is known by name, date of collection, gender, age, address of residence, location of injury, period of infection and type of treatment.

The patients suffering from skin lesion in an exposed portion of the body especially in the face, leg, and arm which clinically diagnosed clinical by dermatologist. The primary isolation was made from patients with cutaneous lesions.

The puncture site (lesion) was sanitizing with 70% ethanol before an aspiration. A 1 ml syringe containing 0.1-0.2 ml of sterilized saline was embedded intradermal into external fringe of the injury.

The syringe was orbited and the tissue liquids were delicately suctioned into the needle, while its withdrawal, The aspirated material was inoculated in culture tubes containing twenty ml of NNN medium. All inoculated tubes were incubated at 25Cº.

All cultures were incubated and inspected for 15-30 days before being counted negatives. Patients were positively diagnosed of CL when

actively motile promastigotes were seen in culture **(Singh and Sivakumar, 2015).** As in figure (2-2)

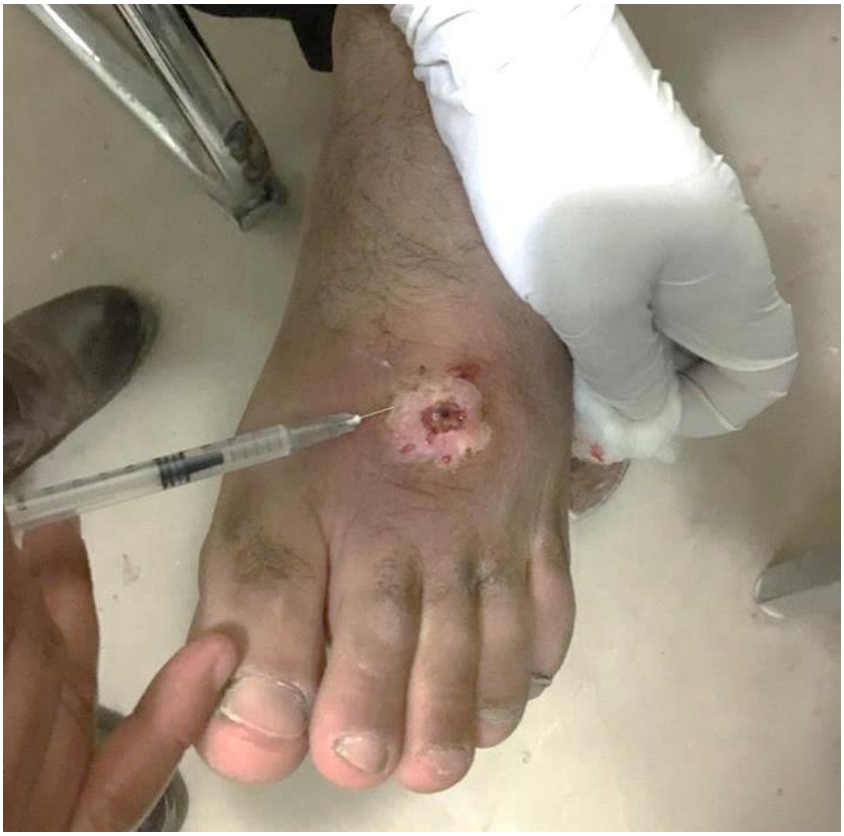

Figure (2-2) Sample aspiration by needle from boil

2.9 Microscopic Examination:

This examination conducted on each sample of aspiration prepared the smear by transferring a portion of the sample onto a clean slide. Staining with Leishman's stain solution and examined under a light microscope with a 100 objective lens, as following:

1. Smears were used as a thin as possible and air dried.
2. The smears were fully covered with Leishman's stain solution for 2 minutes.
3. The distilled water was Added to the smears as Twice the amount of Leishman's stain and mixed by swirling, incubated at least 10 min.
4. Rinsed thoroughly with distilled water.
5. The slides dried using blotting paper and air dry.

Preparation showing amastigotes is considered to be positive (+ ve) for *Leishmania* spp. and preparation with no amastigotes is considered negative (–ve) for *Leishmania* spp. All results were recorded **(Paniz *et al.*, 2013; Eksi *et al.*, 2017)**.

2.10 Growth Media

2.10.1 Biphasic Medium: Novy, MacNeil-Nicolle (NNN)

The biphasic medium was prepared and used for maintaining and cultivating *Leishmania* parasites, invitro. It is usually made up of two phases according to Kagan and Norman **(Schuster *et al.*, 2002).**

2.10.1.1 Solid phase

Solid blood agar phase consists of the following ingredients for 1-liter final volume as seen in table (3-3).

Table (2-3): Ingredients of Solid phase

Chemical	Weight/solution (g)
Brain heart infusion	33.3g
Agar	16.0 g
D-glucose	8.0g
Defibrinated human blood(-O)	40ml
Gentamicin	50µg/ml
Distilled water	900 ml

The solid phase was prepared as follows:-

1- All constituents, except the blood and the antibiotics (Gentamicin), were dissolved in distilled water. PH was adjusted to 7.4 and the solution was autoclaved at 121Cº for 20 minutes.

2- After cooling, human blood (type -O) and the antibiotics (Gentamicin), was added and mixed well.

3- Twenty ml of the medium were transferred under aseptic conditions to 20 ml volume, and were put in a slanted position to obtain a large surface area for the growth. As in Figure (2-3)

2.10.1.2 Lock's solution

Liquid phase was first described by **(Schuster *et al.*, 2002),** it consists of the followings as seen in table (2-4).

Table (2-4): Ingredients of Lock's solution.

Chemical	Weight/solution(g)
NaCl	**9.00 gm**
KCl	**0.42 gm**
$CaCl_2.2H_2O$	**0.32 gm**
$NaHCO_3$	**0.2 gm**
D-glucose	**2.00 gm**
Gentamicin	**50µg/ml**
Sulphate	**200 gm**
Distilled water	**1000 ml**

The liquid phase was prepared as follows:-

The ingredients were mixed together in a screw-capped bottle of 500ml, sterilized by autoclaving at 121 Cº for 15 minutes, left to cool before the antibiotic was added. NNN medium was used for Promastigotes were grown to stationary phase by allowing them to grow for seven days at 22Cº According to **(Ali *et al.*, 2005).**

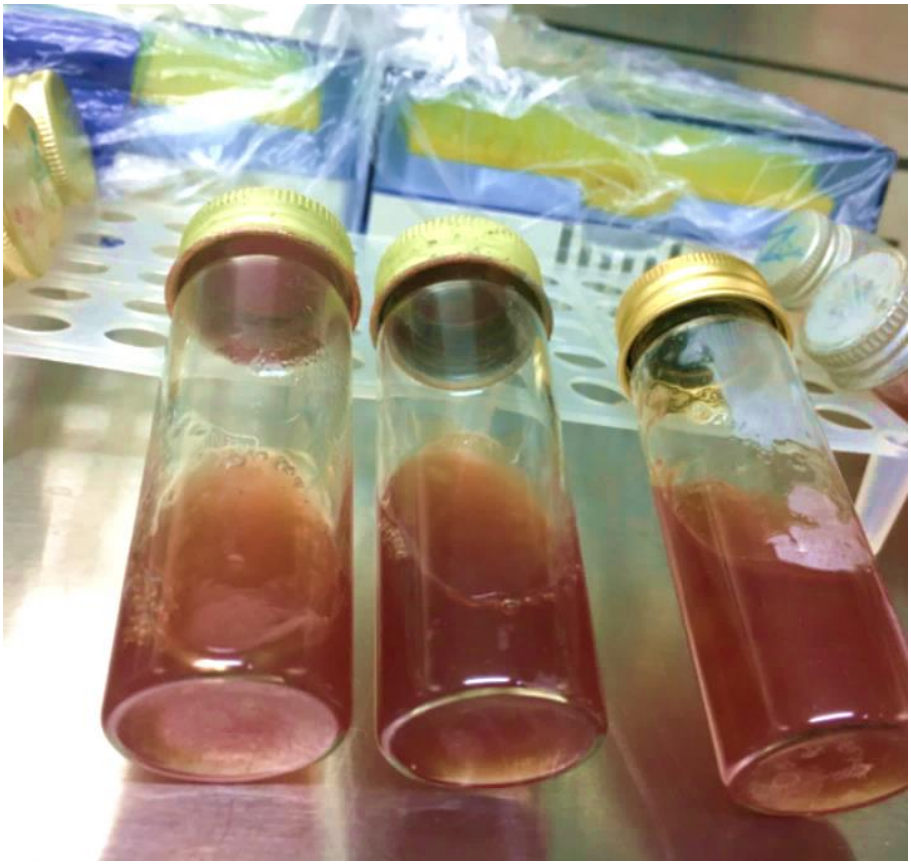

Figure (2-3) Cutaneous leishmaniasis cultured on the modified Novy-MacNeil-Nicolle medium from patients in Al-Hussein hospital in Karbala

2.10.2 Complete RPMI 1640 medium (10% FBS):

Media was prepared in sterile hood. 440 ml of RPMI1640 mixed with 50 ml of Heat-Inactivated Fetal Calf serum (FBS), and 10.000

Unites penicillin 10 µg of streptomycin/ml in media to prevent contamination with other microorganisms were filter sterilized by using disposable bottle top filter provide, Media were stored in 4 C. **(Schuster and Sulivan, 2002)**. As in Figure (2-4)

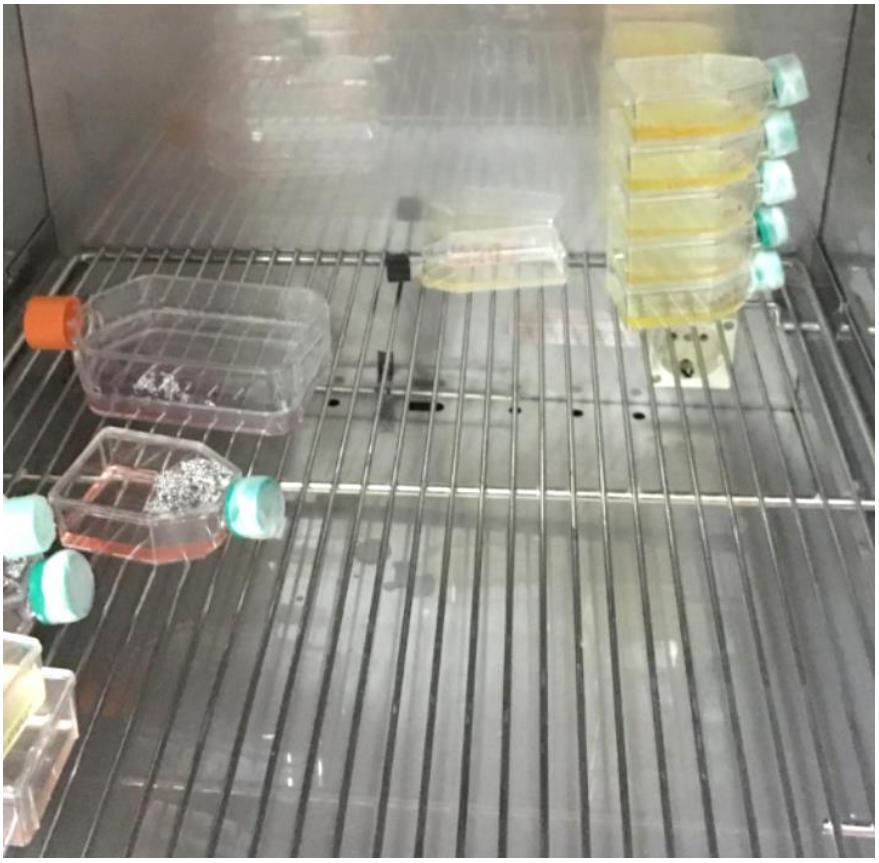

Figure (2-4) Cutaneous leishmaniasis cultured on Roswell Park Memorial Institute medium 1640 from patients in Al-Hussein hospital in Karbala

2.11 Molecular Biological Studies:

2.11.1 DNA Extraction (G-spin DNA Extraction kit)

A. Protocol

1. 200µl of *Leishmania* cultures Roswell Park Memorial Institute medium 1640 (RPMI) was add into a 1.5 ml microcentrifuge tube.

2. 20µl of Proteinase K and 5µl of RNase A Solution into sample tube and gently mix.
3. Added 200µl of Buffer BL add into into upper sample tube and mix
4. thoroughly. Incubate the lysate at 56°C for 10 min.
5. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the rid.
6. 200 µl of absolute ethanol add into into the lysate, and mix well by gently inverting 5 - 6 times or by pipetting. do not vortex. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside of the lid.
7. Carefully apply the mixture from step 6 was aplied carfully to the Spin Column (in a 2 ml Collection Tube) without wetting the rim, the cap was add, and centrifuge at 13,000 rpm for 1 min. Discard the filtrate and place the Spin Column in a 2 ml Collection Tube (reuse).
8. 700µl of Buffer WA was added to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and reuse the Collection Tube.
9. 700µl of Buffer WB was added to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and place the Column into a 2.0 ml Collection Tube (reuse), Then again centrifuge for additionally 1 min to dry the membrane. Discard the flow-through and Collection Tube altogether.
10. The Spin Column was added into a new 1.5 ml tube (not supplied), and 30 - 100µl of Buffer CE directly onto the membrane. Incubate,

for 1 min at room temperature and then centrifuge for 1 min at 13,000 rpm to elute.

2.11.2 DNA Quantitation by Gel Electrophoresis:

DNA concentration was determined by Gel Electrophoresis. This way done according to the sambrook and russel Agarose gel-electrop- horesis was used to assess the purity and mobility of purified DNA **(Miller *et al*., 1988).**

1- An agarose solution was prepared by dissolve (1) g of agarose powder in (100) ml of 1x TBE in the (100) ml flask, agarose was melted in hot block until the solution became clear.

2- The agarose solution was cooled to about (50- 55^0C), swirling the flask occasionally to cool evenly.

3- Red stain (3 µl) was added to the warm gel then sealed the ends of the casting tray with two layer of tape.

4- The combs were placed in the gel-casting tray.

5- Melted agarose solution was poured into the casting tray.

6- The agarose was allowed to solidify at room temperature, the comb pulled out carefully and the tape was removed. The gel was placed onto the electrophoresis chamber that was filled with TBE (1x) buffer.

7- DNA samples (5µl) were mixed with (3µl) DNA loading day and loaded in agarose gel wells.

8- The agarose gel electrophoresis was completed at 70V, 65Amp for 1hour. The DNA was observed by viewed under UV transilluminator.

2.11.3 Nested PCR

Nested PCR is a modification of PCR that was designed to improve sensitivity and specificity. Nested PCR involves the use of two primer sets and two successive PCR reactions.

The first set of primers are designed to anneal to sequences upstream from the second set of primers and are used in an initial PCR reaction. Amplicons resulting from the first PCR reaction are used as template for a second set of primers and a second amplification step. Sensitivity and specificity of DNA amplification may be significantly enhanced with this technique. However, the potential for carryover contamination of the reaction is typically also increased due to additional manipulation of amplicon products.

To minimize carryover, different parts of the process should be physically separated from one another, preferably in entirely separate rooms. Amplicons from nested PCR assays are detected in the same manner as in PCR above **(Jeanne *et al*., 2010).**

2.11.3.1 Preparation of Primers:

The primer used to identify and amplify the Nested PCR method. ITS1-5.8S rDNA gene was investigated by (IDT/Canada). A fragment with the size of 462bp of ITS1-5.8S rDNA gene were amplified with a specific primer. The lyophilized primers were, resolved in free DdH_2O to give a final concentration of (100 pmol/µl) as stock solution and the stock was kept at (-20) to prepare (10 pmol/µl) concentration as work primer

suspended, (10 µl) of the stock solution (90µl) of the free DdH_2O water was added to reach a final volume (100 µl).

2.11.3.2 Primers Selection:-

Materials of polymerase chain reaction for primer1 (IR (SSU rRNA) and primer 2 (ITS1-5.8S rDNA gene)(as shown in table (2-5):

Table (2-5): Primers used in Current study **(Nargis *et al.*, 2014)**.

Primers	Sequence(5'-3') direction	GC (%)	Tm (◦C)	Size of gene(bp)
IR (SSU rRNA)				
Forward	GCTGTAGGTGAACCTG CAGCAGCTGGATCATT	66	53.1	642
Reverse	GCGGGTAGTCCTGCCA AACACTCAGGTCTG	66.6	60	
ITS1 -5.8 S rDNA				
Forward	GCAGCTGGATCATTTTCC	51.4	50	462
Reverse	ATATGCAGAAGAGAGGAGGC	53.9	50	

2.11.3.3 Working principle of PCR

Polymerase chain reaction (PCR) is chain reaction which is a little piece of the DNA area, recognized which serves as the template for producing the primers that initiate the reaction. One DNA atom is used to create two duplicates, at that point four, at that point eight et cetera. This continuous doubling by particular proteins known as polymerases compounds that can string together individual DNA building blocks to

shape long atomic strands. To do their job polymerases require a supply of DNA building squares, i.e. the nucleotides comprising of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They additionally require a little part of DNA, known as the primer. PCR contained 1.5µl genomic DNA, 5µl go Pre Master Mix,1µl of Primer Forward , 1µl of Primer Reverse and 16.5µldistilled water. Thermal cycling condition for the IR (SSU rRNA), ITS1-5.8S rDNA were: initial denaturation 1 step for 3 minutes at94°C, followed by 1 cycles and {denaturation step 2 for 30 seconds at 94°C , Annealing step for 30 seconds at 58°C and extension 1 step for 90 seconds at 72 °C} followed by 39 cycle.

The final extension step 2 was performed at 72°C for 10 minutes. The essential components of polymerase chain reaction were adopted as seen in table (2-6).

Table (2-6): The mixture of working solution

PCR components	Amount
PCR Master Mix	5µl
Primer Forward(10 pmol / µl)	1µl F
Primer Reverse(10 pmol / µl)	1µl R
Distilled water (D.W)	16.5µl
DNA template	1.5µl
Final volume	25µl

2.11.3.4 PCR Program:

Table (2-7): PCR program that was applied in the Thermocycler devices.

Steps	Temperature (0C)	Time	No. of Cycles
Denaturation 1	95	3 min.	1
Denaturation 2	94	30 sec.	38
Annealing	58	30 sec.	
Extension 1	72	90 sec.	
Extension 2	72	10 min.	1

The products of PCR from amplification of IR (SSU rRNA), and ITS1-5.8S rDNA were then electrophoreses on 2% agarose gel stained with Red stain.

2.11.3.5 Preparation of Tris-Borate. EDTA. Buffer (TBE10X)(TBE buffer):

1X Tris Boric EDTA buffer prepared by dilution the stock solution (TBE10X) buffer by using 100 ml of 10X TBE to 900 ml of distilled water to prepared 1 litter.

2.11.4 Sequencing and Sequence Alignment:

The PCR products were separated on 2% agrose gel electrophoresis and visualized by exposure to ultra violate light (302nm) after red stain staining.

Sequencing of gene was performed by national instrumentation center for environment management (nicem) online at (http://nicem.snu.ac.kr/main?en_skin=index.html).

Biotechnology Lab, Homology search was conducted using basic local alignment search tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and BioEdit programs Sequence analysis was done by using software Bio Edit \MEGA6 was used to perform the multiple sequence alignment .

The sequences result compared with NCBI control strain.DNA pairrwise alignment ,single nucleotide polymorphsim was also done with the same software.

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CHAPTER THREE

RUSELTS

3. Results:

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniasis, and direct staining smear from patients lesions are positive and they showed the amastigotes in the phagocytic cell (macrophage) as neutrophil, that 72 males that percentage (78.3)% and 20 females that percentage (21.7)% with age ranged 10-59 years, attended to Department of Dermatology Al-Husseini Hospital in Karbala during the period between November 2017 to end January 2018.

The evidence confirmed infection due to a bad situation for hundreds of thousands of people who exposed to the displacing and dived in camps, in addition to the presence of the war and bad conditions, and presence of swamps near their camps which are important for reproduction sand fly **(Younis, 2018)**.

3.1 Diagnosis of Cutaneous leishmaniosis by Direct Microscopic examination:

The direct microscope examination was performed in two methods aspiration and direct microscope examination then stained with Leishman stain, direct staining smear from patients lesions are positive and they showed the amastigotes in the phagocytic cell (macrophage) as neutrophil As in Figure (3-2).

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while. As in Figure (3-1)

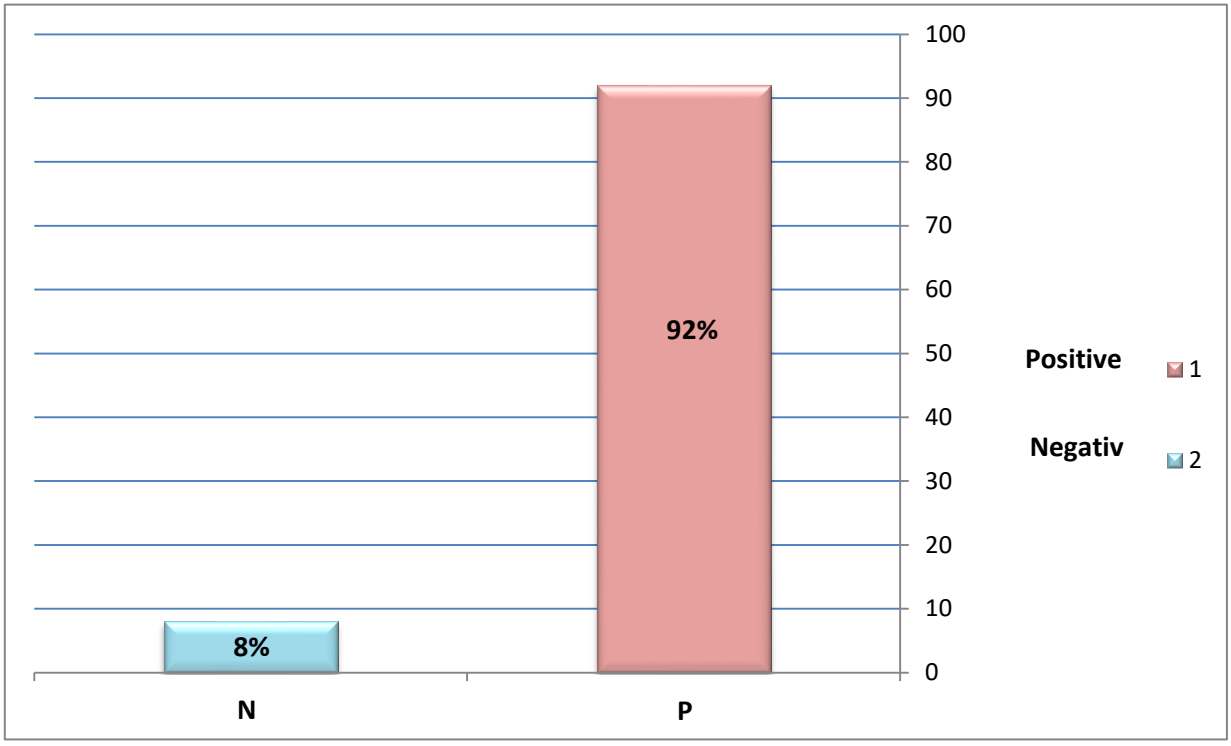

Figure (3-1) the percentage of Direct Microscopic examination

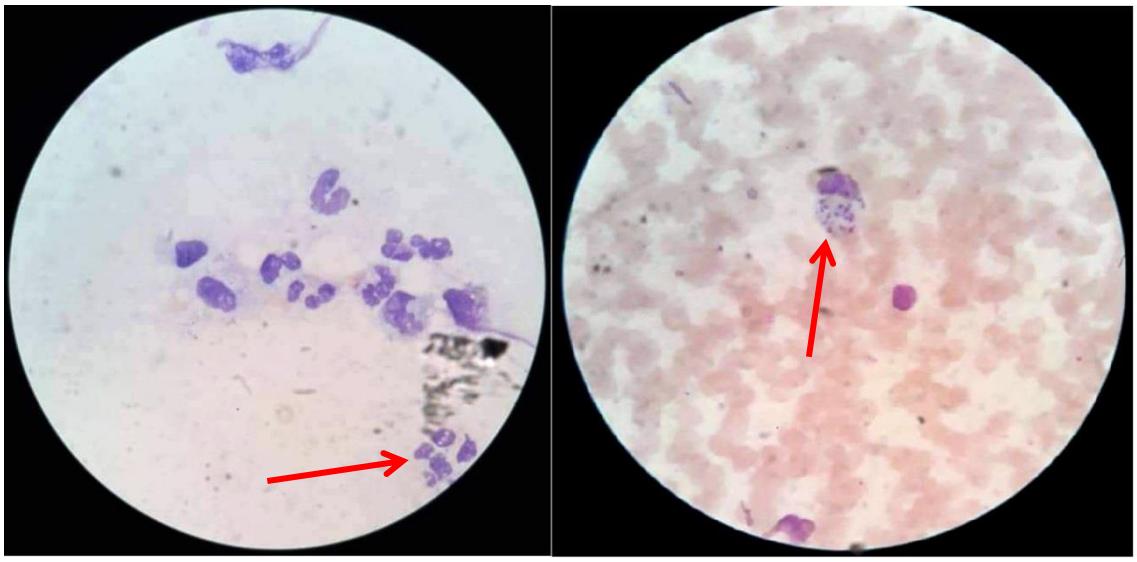

Figure (3-2): Smear from Skin Lesion Stained with Leishman stain show Amastigotes in WBC (macrophage) under 100X lens

3.2 Gender:

The distribution of cutaneous leishmaniosis infection between males and females by using microscopic examination was indicated in table (3-1), The patients were of different sex, microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniosis that 72 males that percentage (78.3)% and 20 females that percentage (21.7)%. As in Table (3-1)

Table (3-1): Frequencies and percentages of patients characteristics of gender

Socio-demographical characteristics	Group	Frequencies	Percentage (%)
Gender	**Male**	**72**	78.3
	Female	**20**	21.7

3.3 Residency:

The geographic distribution of cutaneous leishmaniosis by using microscopic examination was referred in the Figure (3-3), of the patient's shows that 62% of the patients were from the cities of Telaefar and Dialla, while only 30% were from Karbala city.

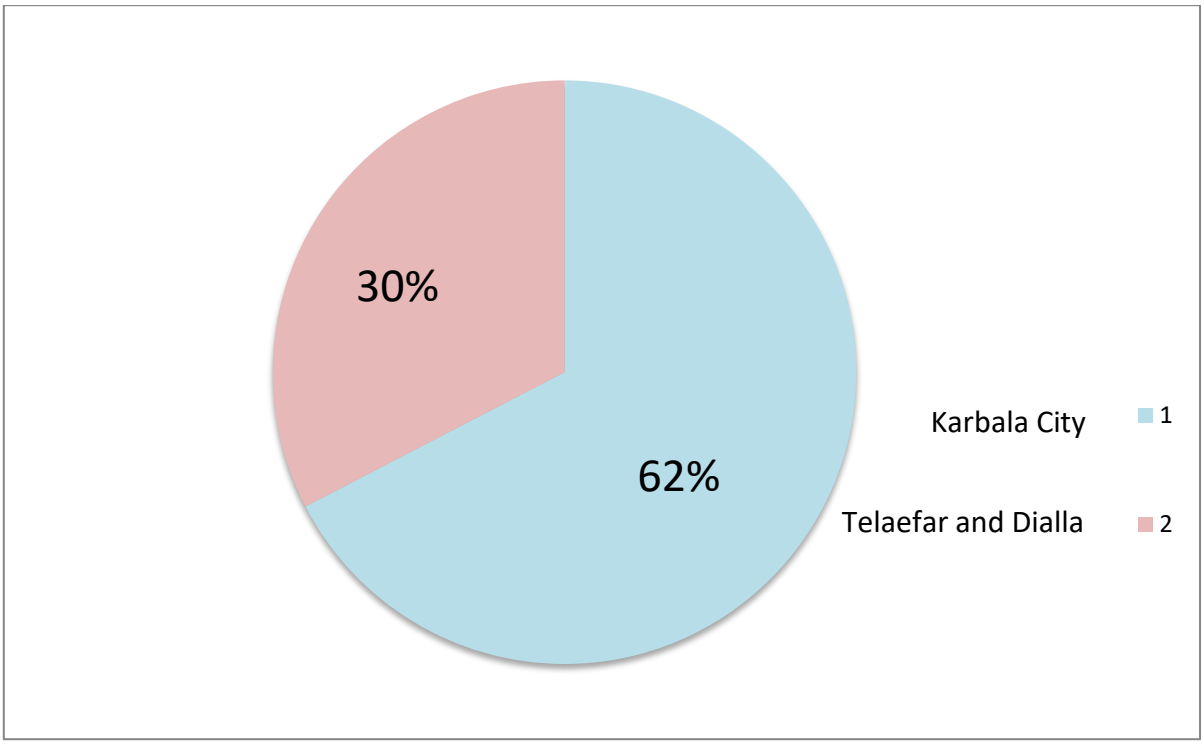

Figure (3-3) the percentage of geographic distribution of the patients

3.4 Age groups:

The distribution of infection of cutaneous leishmaniasis, table (3-2) shows infection according to the age groups was from (10-19 years) 6 (6.5%), ages ranged from (20-29 years) 11 (11.9%) and ages ranged from (30-39 years) 17 (18.5%), and the age (40-49) 37 (40.2%) and (50-59) 21 (22.8%).

So we found the higher infection with cutaneous leishmaniosis according to the age groups was in the age group (40-49) was (40.2%) while the minimum rate of infection was in their ages ranged from (10-19 years) 6 (6.5%) and the table (3-2) explains that .

Table (3-2): Frequencies and percentages of patients characteristics of age

Socio-demographical characteristics	Group	Frequencies	Percentage (%)
Age	**10 – 19**	**6**	6.5
	20 – 29	**11**	11.9
	30 - 39	**17**	18.5
	40 - 49	**37**	40.2
	50 - 59	**21**	22.8

3.5 Occupations:

The occupation in kids 3 (3.2%), student 11(11.9%), solder 34 (36.9%), worker 20 (21.7%), Employee 9 (9.8) and house wives 15(16.3%), the most infected rate of occupation patients in solder was 34 (36.9%), while the lowest infection rate in kids was 3 (3.2%), as in Table (3-3)

Table (3-3): Frequencies and percentages of patients characteristics of Occupations

Socio-demographical characteristics	Group	Frequencies	Percentage (%)
Occupations	**Kids**	**3**	3.2
	Student	**11**	11.9
	Solder	**34**	36.9
	Worker	**20**	21.7
	Employee	**9**	9.8
	Housewives	**15**	16.3

3.6 Body site of infection:

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniasis, and most of the lesions were found on the exposed areas of the body: face 25 (27.2%), hands 44(47.8%), Legs 11(12%), and ears 12(13%).

Most infected rate of patients was found in hands 44 (47.8) while the lowest infection rate was in Legs 11(12%), as in Table (3-4)

Table (3-4) Frequencies and percentages of patients characteristics of Site of lesions

Socio-demographical characteristics	Group	Frequencies	Percentage (%)
Site of lesions	**Face**	**25**	27.2
	Hands	**44**	47.8
	Legs	**11**	12
	Ears	**12**	13

3.7 The number of lesions:

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniasis, differs between patients. Most (65%) of the patients had a multiple lesions in comparison to the single lesions which was found in (35%) of patients, as in figure (3-4).

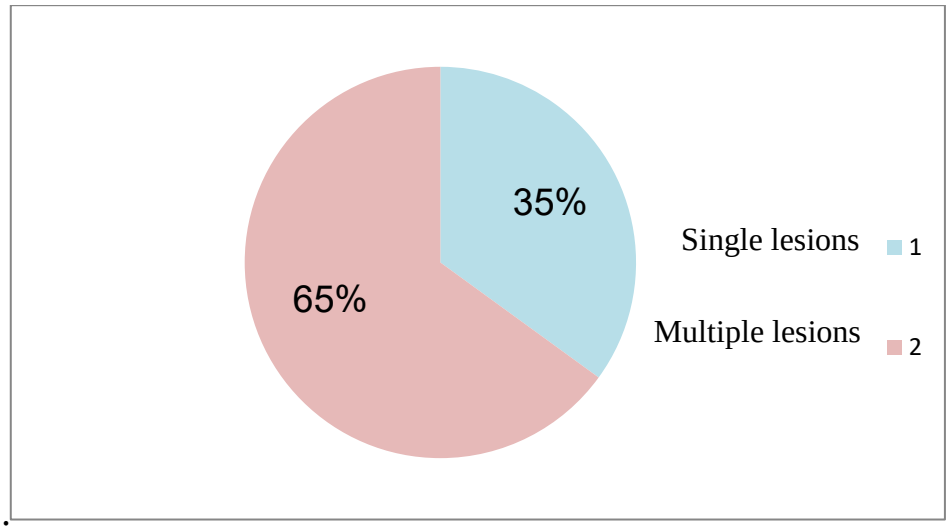

Figure (3-4) Distribution of patients percentage according to the Number of lesions

3.8 Clinical signs in infection patients

In humane the lesions, which appear in the arms, legs, faces and ears, showed solid, dry like volcano area in shape and characterized by erythematous papule, with ulcerative border, as in Figure (3-5), (3-6), (3-7), (3-8), (3-9).

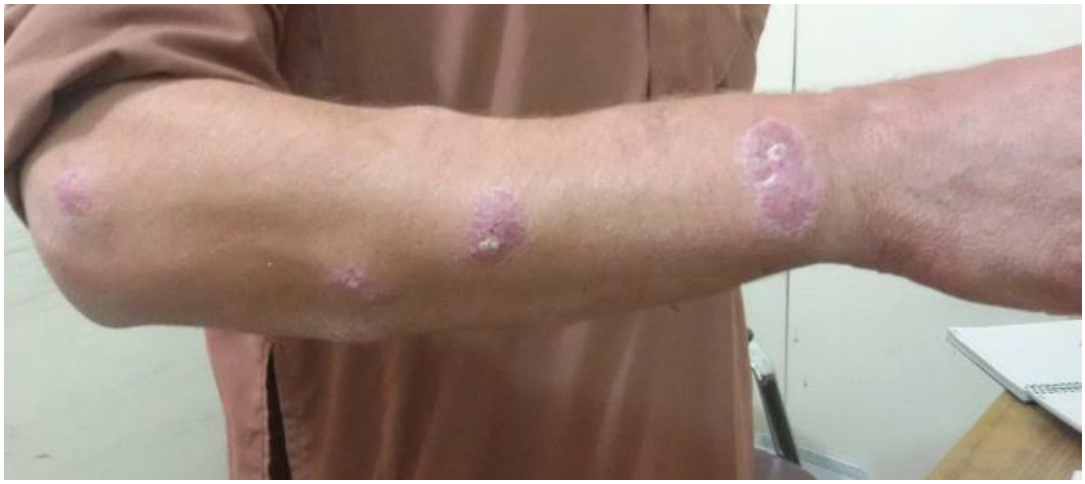

Figure (3-5) Male 30 years old with typical lesion of CL on the upper limb, an ulcerated nodule lesion on the arm, well circumscribed with a necrotic base and indurate margin.

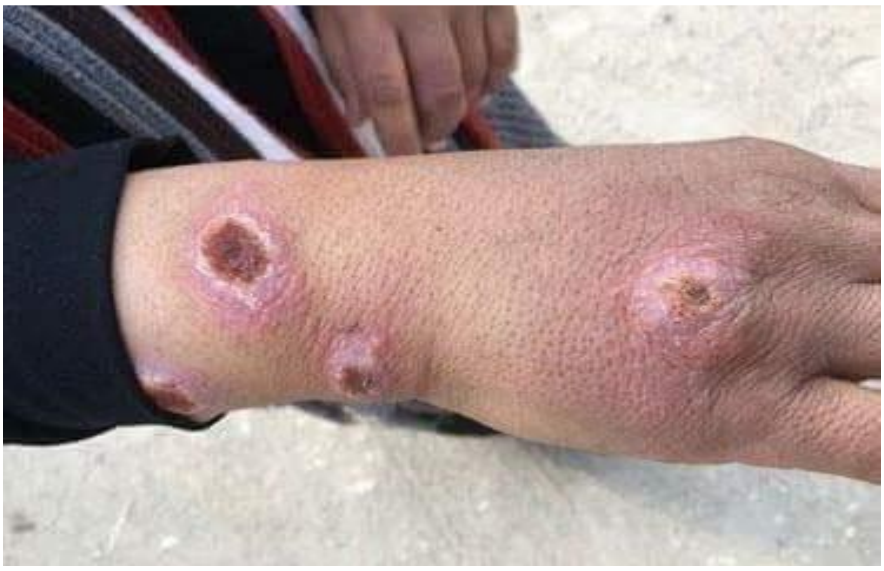

Figure (3-6):Male 23 years old with typical lesion of CL on the upper limb, an ulcerated nodule lesion on the arm, well circumscribed with a necrotic base and indurate margin.

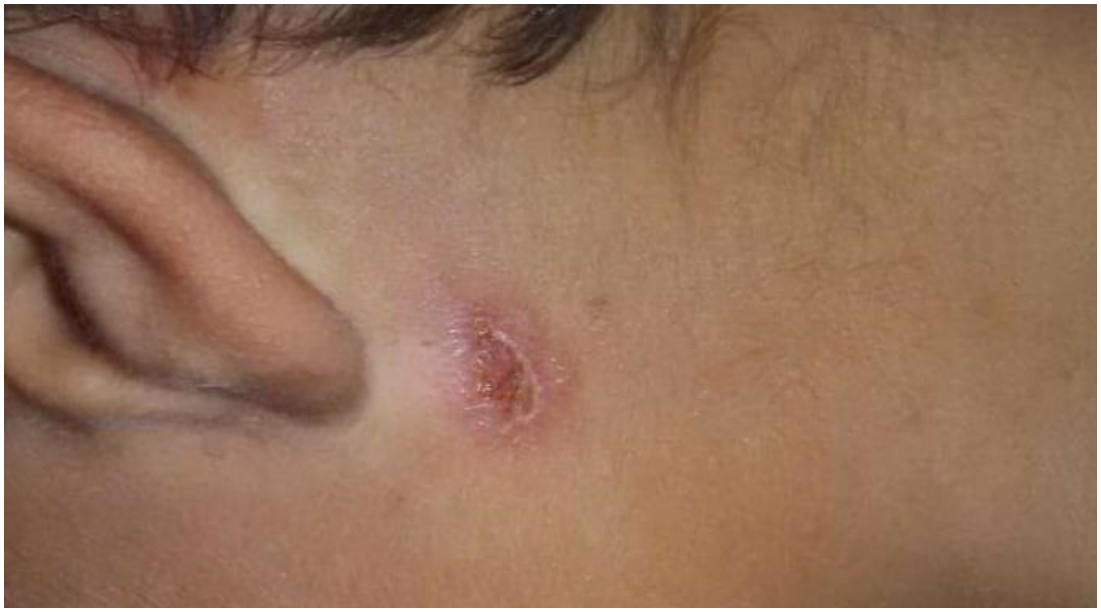

Figure (3-7): Child 10 years old with typical lesion of CL, an ulcerated nodule lesion behind the ear, well circumscribed with a necrotic base and indurate margin

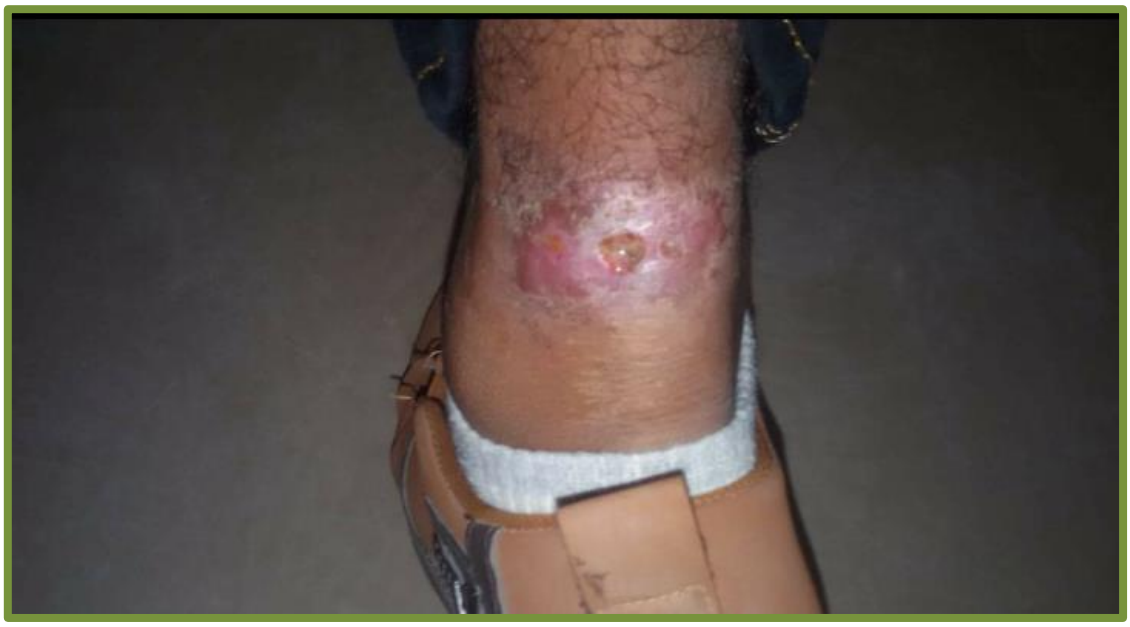

Figure (3-8):Male 20 years old with typical lesion of CL on the lower limb, an ulcerated nodule lesion on the feet, well circumscribed with a necrotic base and indurate margin

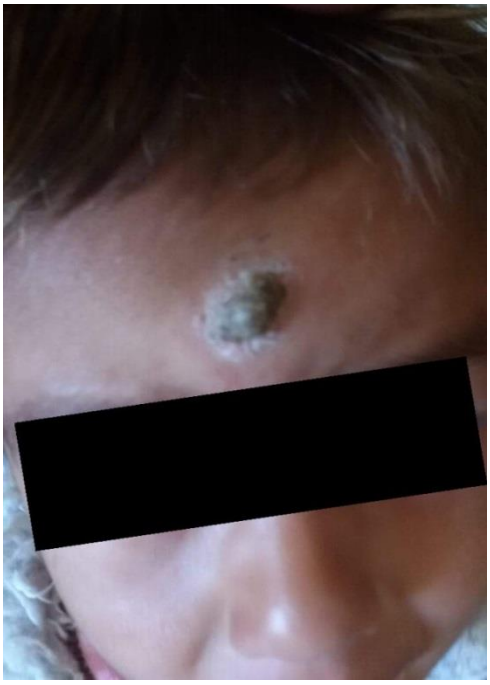

Figure (3-9): Male 10 years old with typical lesion of CL, an ulcerated nodule lesion below in the face, well circumscribed with a necrotic base and indurate margin.

3.9 Culture Examination:

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniasis.

The parasitic diagnosis depend on aspirate samples from skin lesions of patients suspect CL infection and directly culture NNN media and confirmed diagnosis of these isolation depend on activity motile promastigotes that seen in culture as in figure (3-10).

The direct microscope examination was performed in two methods aspiration and direct microscope examination then stained with Leishman stain. as in figure (3-11), Then, the positive samples were cultured on the modified Novy-MacNeil-Nicolle medium from (15 – 30) day and then active in Roswell Park Memorial Institute medium .(Only 20) sample growth as in figure (3-12).

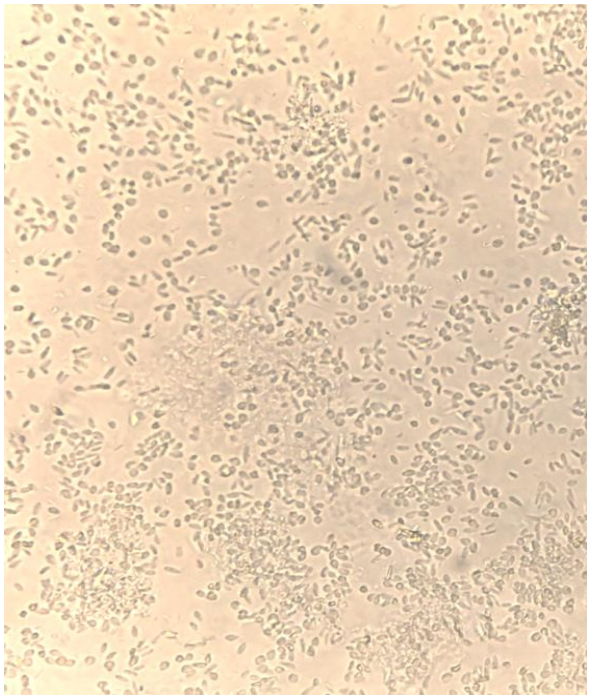

Figure (3-10): Smear from NNN Culture without Stained show promastigotes in under 40X lens

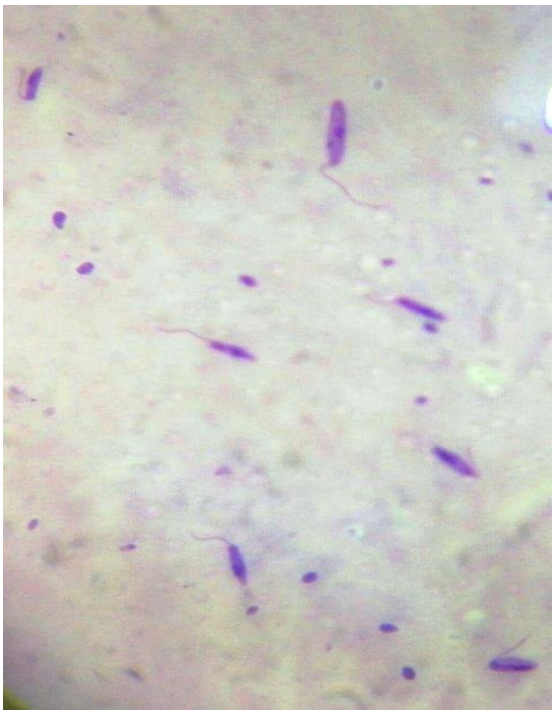

Figure (3-11): Smear from NNN Culture with Leishman Stained show promastigotes in under 100X lens

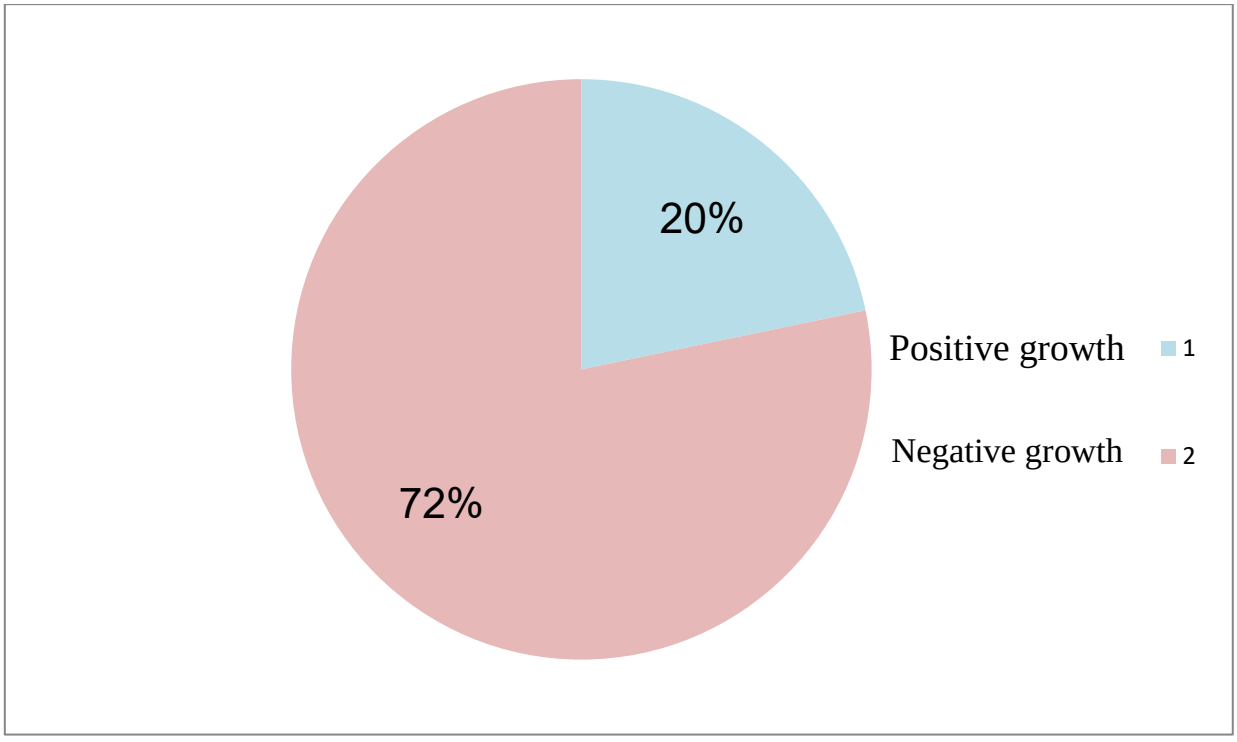

Figure (3-12) Distribution of patients percentage according to the NNN Culture.

3.10 Molecular biology studies

3.10.1 DNA Extraction:

The wizard genomic DNA purification Kit (G-spin DNA extraction kit) is used to DNA isolation from the *Leishmania* cultures in a short time. All samples showed bands, which indicated the genomic DNA on Agarose Gel Electrophoresis. As seen in figure (3-13).

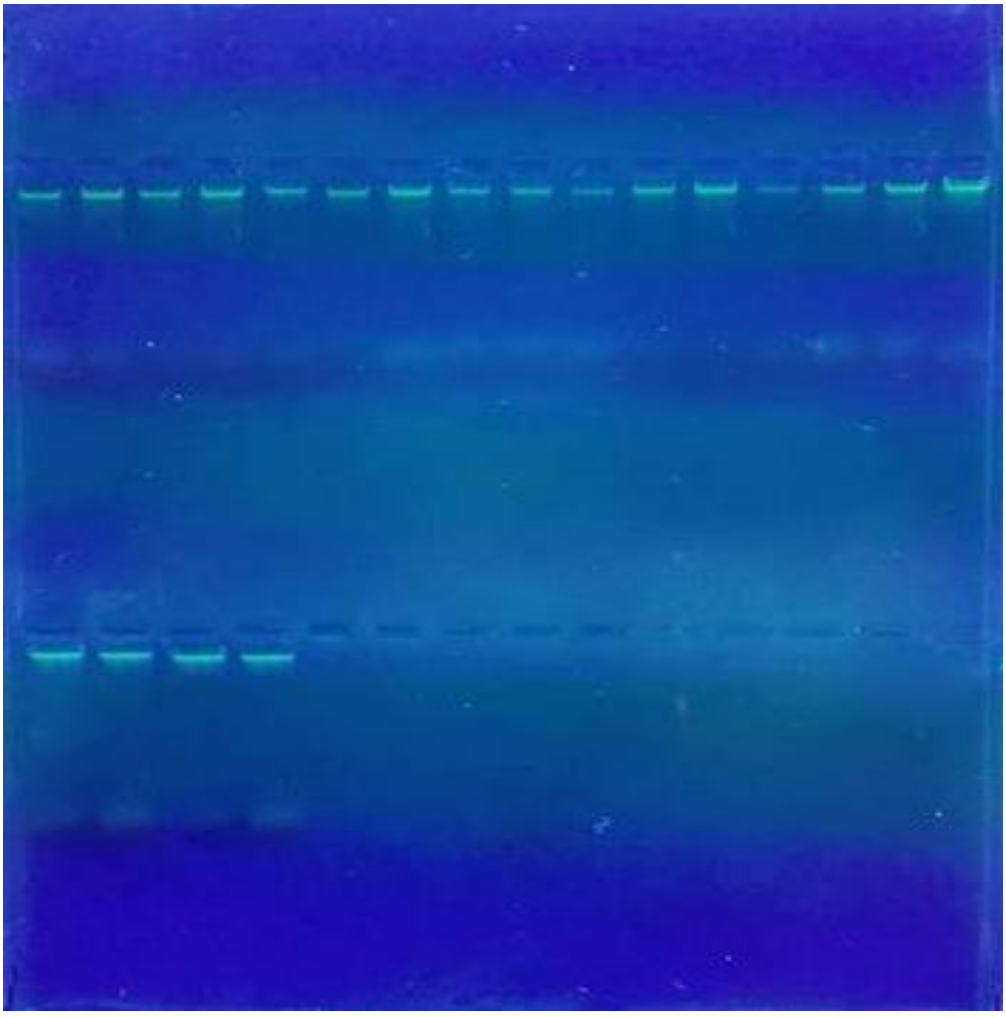

Figure (3-13): Gel Electrophoresis of genomic DNA on 1%agarose gel, (70 Volt/30 minute).

3.10.2 Molecular diagnosis by Nested PCR for 5.8sr DNA gene

Amplification of the 5.8s rDNA region by Nested PCR were electrophoresed in 2% agarose gel, for twenty sample growth from NNN media, as in figure (3-14)

The 5.8s rDNA gene through nested PCR determined by the observation of expected bands 462 bp for cutaneous leishmaniasis, showed that out, as in Figure (3-15).

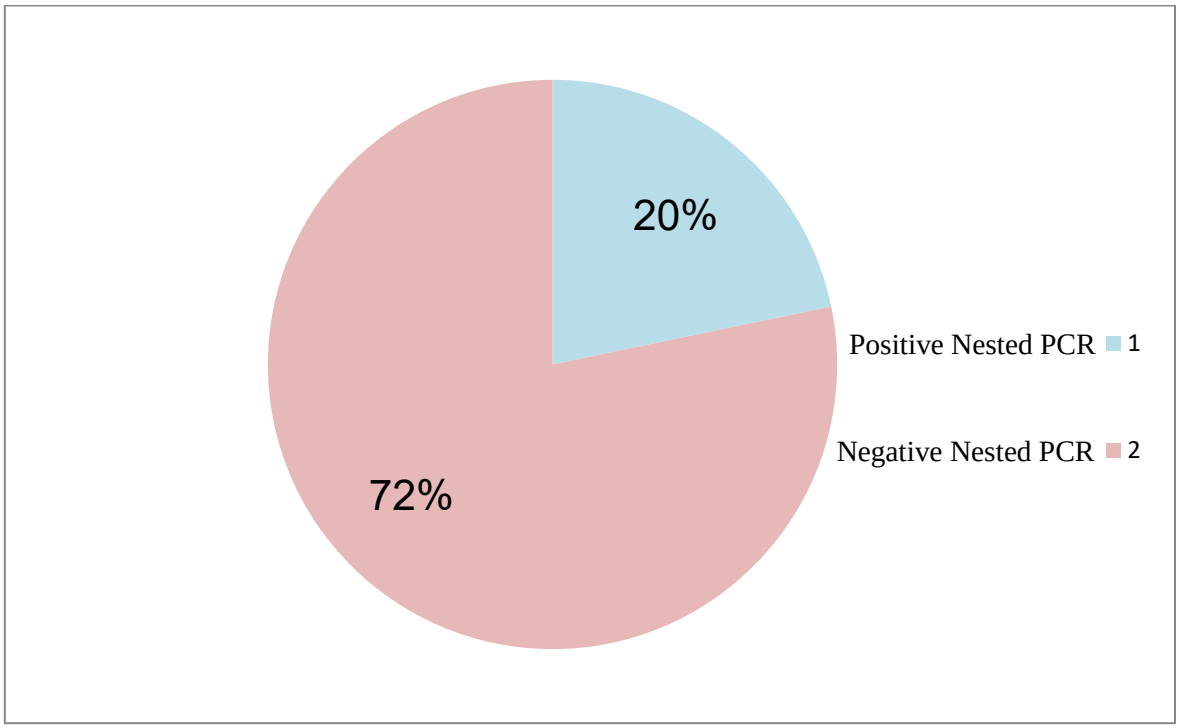

Figure (3-14) Distribution of patients percentage according to the Nested PCR.

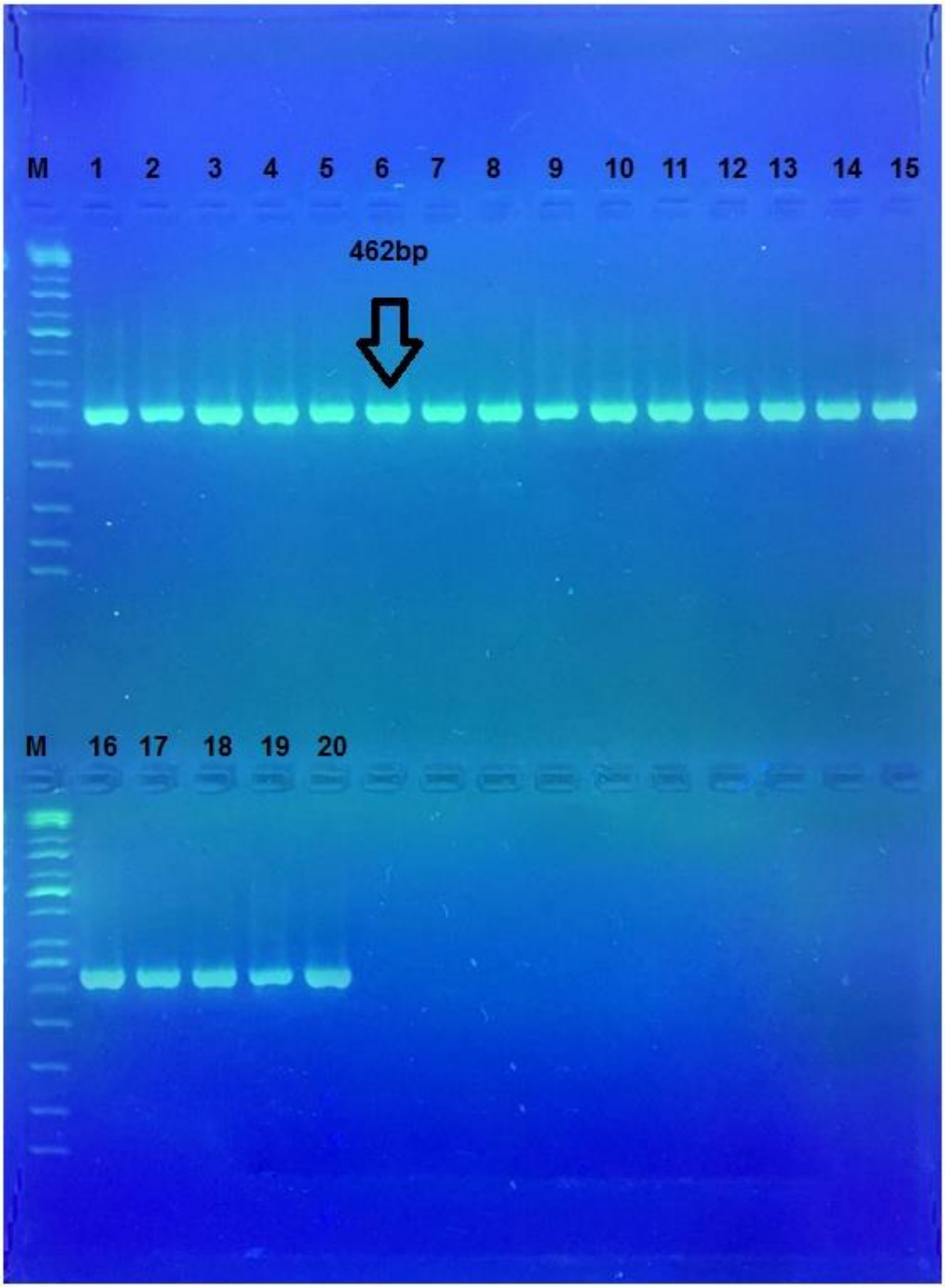

Figure (3-15): Agarose gel electrophoresis for 5.8S rDNA gene (462bp) . Bands were fractionated by electrophoresis on a 2% agarose gel (2 h., 5V/cm, 1X TBE) and visualized under U.V. light after staining with red stain. Lane: 1 (M: 100bp ladder), Lane: 1to 20 product nested PCR positive.

3.11.1 Molecular diagnosis by using genetic analyzer

The sequences for twelve isolates were arrived from Macrogen company for sequencing in South Korea after sent the PCR products of 5.8S rDNA gene and were compared the result sequences with reference sequence of 5.8S rDNA gene for *L.major* in National Center for Biotechnology Information (NCBI) gene bank, as in figure (3-16).

The results showed that the primer was successful in conducting the genetic analysis and determining the sequences between 5.8S rDNA the user in the diagnosis of isolate parasite *L.major*, all the isolates were matched with the site (NCBI) and it was found that all the isolates belong to the isolated *L.major* type from Karbala governorate.

The phylogenetic tree diagrammatic by Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 .When comparing the isolates of the current study .The phylogenic tree of the current study reflects with out significant diversity between isolates.

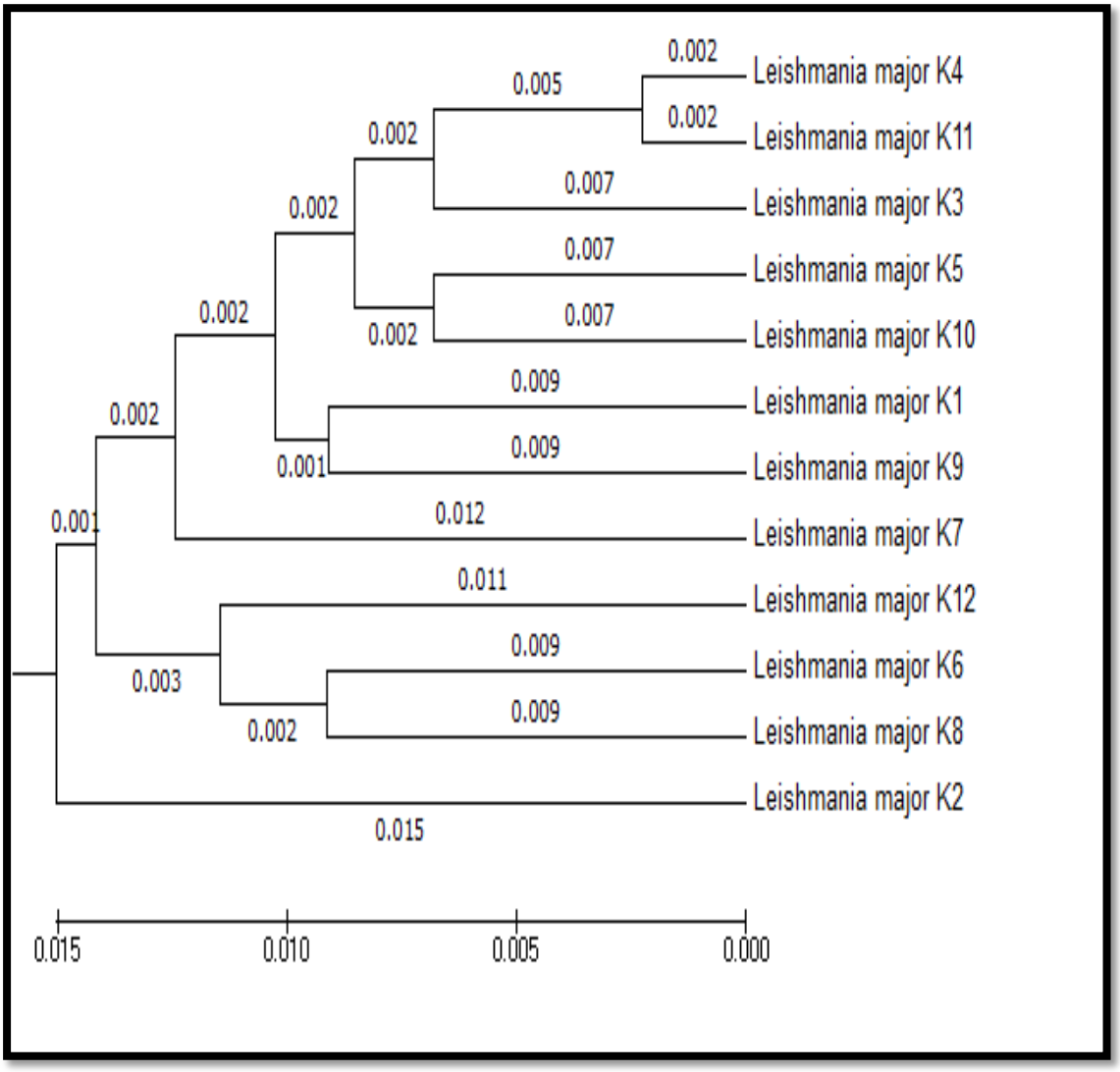

Figure (3-16): Neighbor-joining tree *Leishmania Major* of 5.8S rDNA.

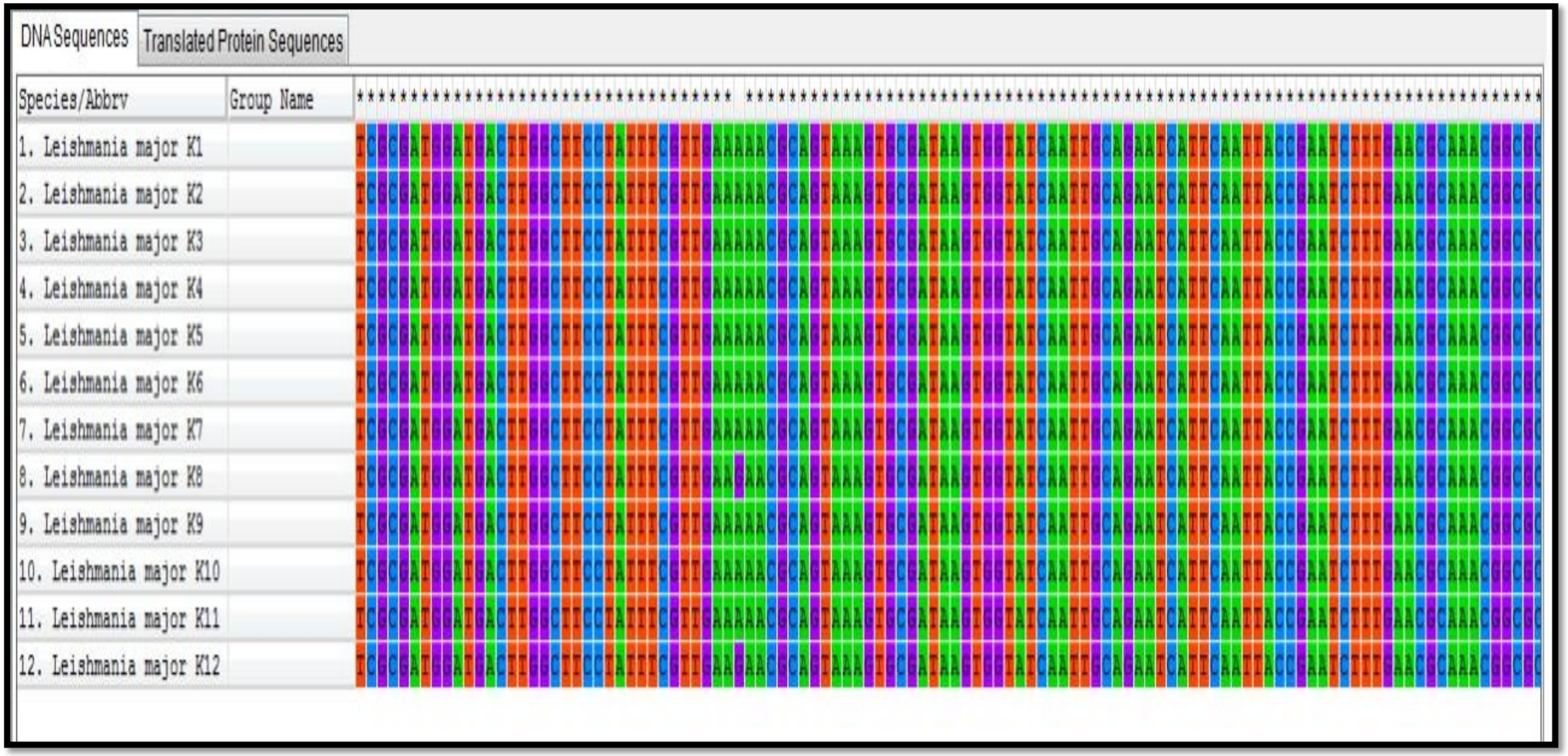

Figure (3-17): Alignment of sequence analysis of 5.8 rDNA gene of *leishmania* , karbela isolates by Mega 6

The genetic dimension between Iraq and the isolates of the world is detailed according to the Phylogenetic tree. Hierarchical cluster analysis determine the following clusters: large Cluster divided into several neck: The first root divided into two necks first neck: France, Belgium, Germany and U.S.A. Second root Sudan and Iran the genetic dimension was by 0.002, third root Iran the genetic dimension was by 0.004, fourth root Iraq-k1 isolate the genetic dimension was by 0.005 it is close to Iran the genetic dimension was by 0.004. The last cluster the genetic dimension was by 0.021. As seen in figure (3-18).

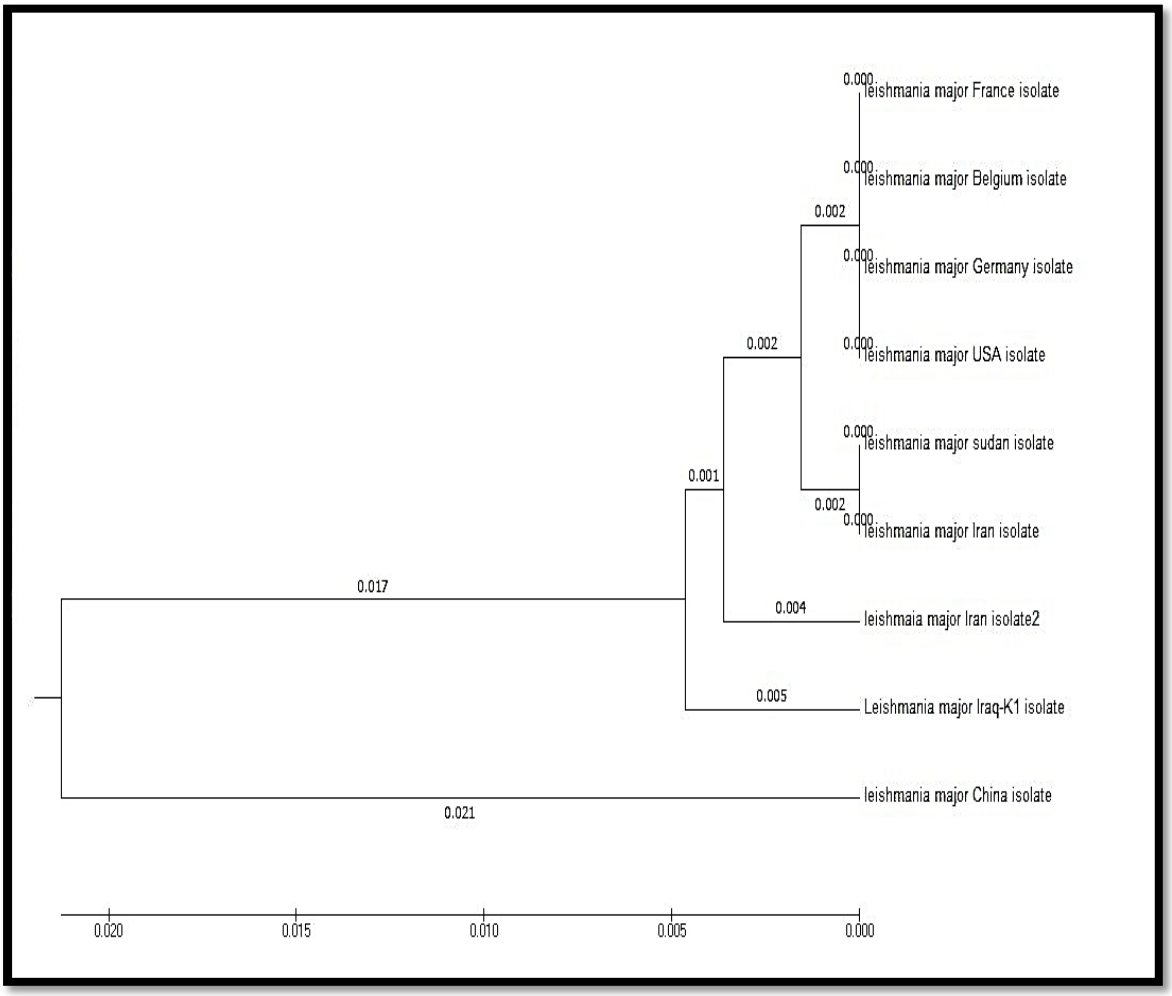

Figure (3-18): Neighbor-joining tree *Leishmania Major* of 5.8S rDNA.

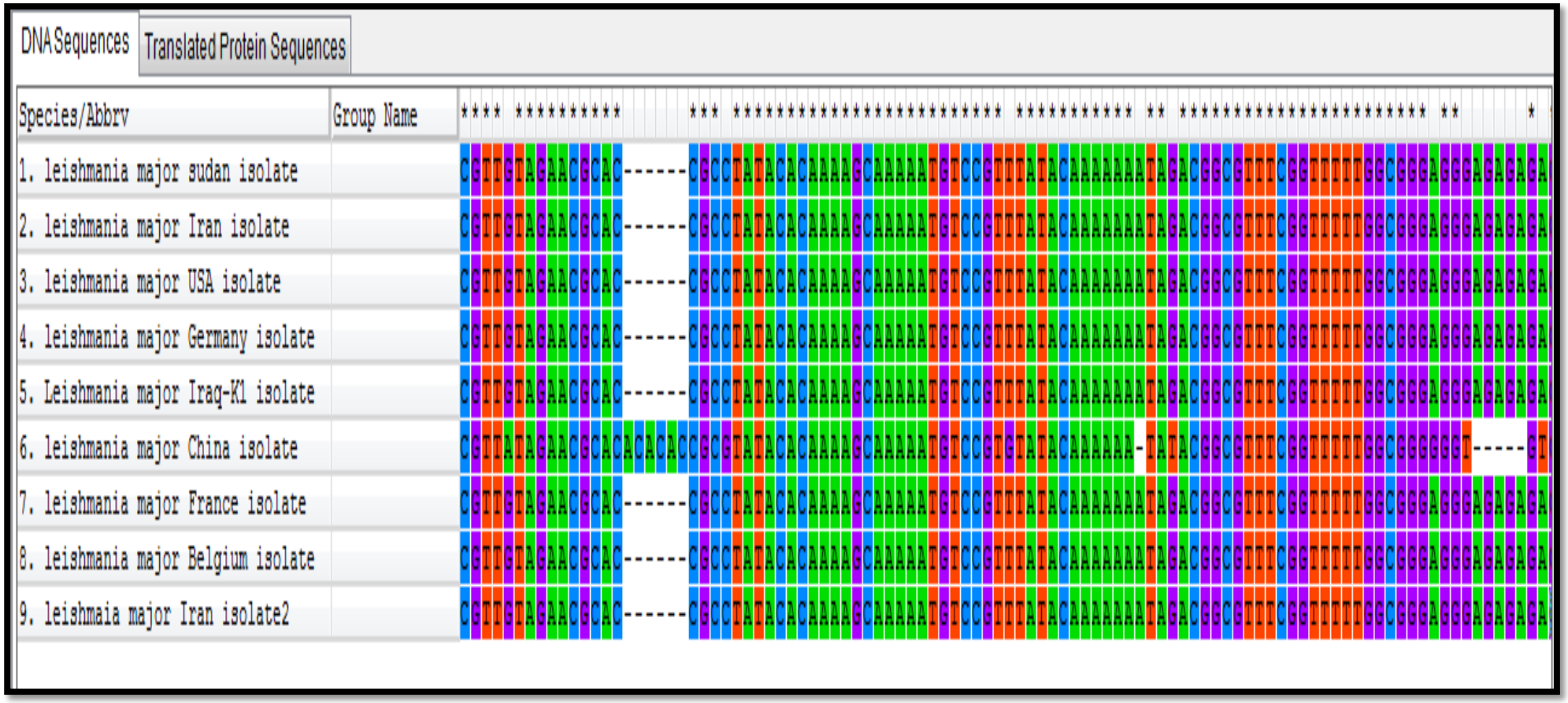

Figure (3-19): Multiple sequence analyisis of 5.8rDNA gene of *Leishmania* international isolates by Mega 6

The table (3-5) shows the degree of compatibility between Iraq isolates, and global isolates, Iranian isolates, (KU 680848) is the similar to the Iraqi isolate (MH 428844) while the China isolate (HQ 830351) genetically distant for Iraqi isolate.

Table (3-5) Homology sequence identity of local isolated to NCBI – Gene bank of *L.major* isolated.

NO.	Accession	Country	Expect	Compatibility	Max score
1	KU 680448	IRAN (2)	0	99 %	745
2	FJ 753394	USA	0	99 %	744
3	AJ 300482	GERMMANY	0	99 %	744
4	AY 260935	SUDAN	0	99 %	743
5	FM 677342	BOLGGIOM	0	99 %	616
6	KU 680846	IRAN (1)	0	99 %	743
7	KF 981807	FRANCE	0	98 %	599
8	HQ 830351	CHINA	0	94 %	599

3.11.2 Submission of local Iraq isolate in NCBI

The 5.8S rDNA gene were registered after the correspondence of the National Center for Biotechnology Information and obtained accession number and became a reference to Iraq and the Middle East and the world. Ongoing work will add to this set as more type strains are published and it is available for download at NCBI then recorded choose randomly isolate K1.

https://www.ncbi.nlm.nih.gov/nuccore/MH428844

Chapter Four

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4. Discussion

Leishmaniasis are vector-borne disease caused by protozoan parasites from the family *Leishmania* (Trypanosomatida:Trypanosomatidae **(Akhoundi *et al.*, 2016).**

Leishmaniasis is a parasitic disease caused by haemoflagellate *Leishmania* . The disease is widespread and may cause serious health problems in communities throughout the Mediterranean regions and the Middle East, including Iraq. There are about 12 million cases worldwide, and there are about 1.5 million new cases of cutaneous leishmaniasis each year **(Ashford *et al.*, 1992).**

There are three main types of leishmaniasis: cutaneous leishmaniasis (CL) caused by *Leishmania* tropica, *L. mexicana* and *L. major*. Visceral leishmaniasis (VL) caused by *L. donovani* and *L. infantum* and mucocutaneous leishmaniasis (MCL) caused by *L. braziliensis*. **(Igbineweka *et al.*, 2012)**.

The disease is becoming more important because of globalization, climate change and other circumstances which allow the parasite and its vectors to spread **(Antoniou *et al.*, 2013; Loria-Cervera, 2014).**

In Iraq, two species are present: L tropica, the agent of anthroponotic cutaneous leishmaniasis (ACL), and *L. major*, the agent of zoonotic cutaneous leishmaniasis (ZCL). Both ACL and ZCL were reported as causative agents of leishmaniasis in Iraq, but ACL is found mainly in suburban areas **(WHO, 2003)**.

Diagnoses depend on visualizing the organisms within macrophage or collecting samples from the patient lesion by aspirate or biopsy. These collected materials can be stained by Giemsa, cultured or analyzed by PCR

(Polymerase Chain reaction), which sensitively detect the parasite **(Guerin *et al.*, 2002; Alrajhi, 2003; Berman, 2005)**

In the last few years, polymerase chain reaction (PCR) has been widely used due to high sensitivity as a parasitological diagnostic test **(Singh *et al.*, 2015).**

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniasis that 72 males that percentage (78.3)% and 20 females that percentage (21.7)% with age ranged 10-59 years.

The most infected rate of patients was between the ages of 40-49 years (40.2%), while the lowest infection rate was of patient's ages 10-19 years (6.5%).

The most infected rate of occupation patients in solder was 34 (36.9%), while the lowest infection rate in kids was 3 (3.2%), and the Most infected rate of patients was found in hands 44 (47.8) while the lowest infection rate was in Legs 11(12%).

The geographic distribution of the patients shows that 62% of the patients were from the cities of Telaefar and Dialla, while only 30% were from Karbala city. The number of lesions differs between patients. Most (65%) of the patients had a multiple lesions in comparison to the single lesions which was found in (35%) of patients.

A more specific diagnosis of the causative agent of cutaneous leishmaniasis was performed using molecular tests. Choosing 20 sample of the positively cultured samples, of the total number of pathogens that showed positive results were studied by Nested-PCR using two types of primers to determine the species. Because large 5.8s.r DNA gene through Nested PCR showed that all the samples were *L.major*.

The Nested PCR products of twelve isolates send to sequenced in the South Korea then compared the local species which isolated from current study with the global strains in the NCBI gene bank then recorded choose rendomly isolate K1.

4.1 Diagnosis of Cutaneous leishmaniosis by Direct Microscopic examination:

The direct microscope examination was performed in two methods aspiration and direct microscope examination then stained with Leishman stain, direct staining smear from patients lesions are positive and they showed the amastigotes in the phagocytic cell (macrophage) as neutrophil were in agreement With these mentioned by **(Chunge *et al.*, 1989)**, This examination conducted on each sample of aspiration prepared the smear by transferring a portion of the sample onto a clean slide. Staining with Leishman's stain solution and examined under a light microscope with a 100 objective lens showing amastigotes is considered to be positive (+ ve) for *Leishmania* spp. and preparation with no amastigotes is considered negative (–ve) for *Leishmania* spp. All results were recorded **(Paniz *et al.*, 2013; Eksi *et al.*, 2017)**. As in Figure (3-2) There is another method of staining

The direct staining smear considers good in the first examination to CL, it needs a small amount of material from the edge of the lesion which stained easily by Giemsa stain **(CDC, 2011)**.

The main reason that cells are stained is to enhance visualization of the cell or the cellular components under a microscope, make them easier to see; also it can highlight **(Khademvatan *et al.*, 2012)**, Staining helps in the identification of the sample by smear colour change without getting into the

complete analysis of the sample and easy to observe the morphology, size and shape **(Khademvatan *et al.*, 2012)**.

The Giemsa stain provides a better stain intensity, and show some details that may be unclear otherwise, especially in cells, but some smears of Giemsa stain gave negative results and the parasite doesn't seen or disappeared **(Younis *et al.*, 2017)**, that attributed to many reasons, patient take treatment, mistake in time staining, smear thickness and sometimes distortion of the cell wall may occur **(Mustafa *et al.*, 2017)**. These classical methods require the presence of a relatively high number of viable or morphologically intact parasites; this may pose a problem particularly in the chronic phase of CL where parasite levels in skin lesions are very low **(Rahi, 2015)**.

4.2 Gender:

The distribution of cutaneous leishmaniosis infection between males and females by using microscopic examination was indicated in table (3-1), The patients were of different sex, microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniosis that 72 males that percentage (78.3)% and 20 females that percentage (21.7)%, as in table (3-1) Depending on genders, the present study revealed the rate of cutaneous leishmaniasis which is higher in males than in females by using microscopic examination and nested PCR assay. In Iraq **(Al-Samarai *et al.*, 2009)** also found that males were (57%) and females were (31.7%). Another study by **(Abdulwahab, 2013)** recorded that the infection in males was 65% than females (35%). **(Rahi *et al.*, 2013)** also decided that the prevalence in males was higher than females. Similar results were also established in studies by **(Al-Mousawy, 2015; Al-Obaidi *et al.*, 2016)**. In Saudi Arabia, **(Amin *et al.*, 2013)** documented that the incidence rate of CL was higher in males than in females in Al Hassa from 2000 to 2010. In Syria

(Shanehsaz *et al.*, 2013) found that Syrian males are more infected with the parasite than females, In Jordan **(Al-Athamneh *et al.*, 2014)** and in Iran **(Haddad *et al.*, 2016)** were also recorded that the prevalence rate was most frequently in males than females. The same result was in Turkey **(Mustafa *et al.*, 2017)**, this is probably happened due to the cultural habits of most areas.

Otherwise, the results of the present work appeared to disagree with the other previous Iraqi studies by **(Al-Qadhi *et al.*, 2013; Rahi *et al.*, 2014)**. Also **(Hassan *et al.*, 2017)** found in his epidemiological study of Cutaneous Leishmaniasis in Tuz found females are more infection than males, in Yemen by **(Abdul-Ghani *et al.*, 2014)** who documented that females most infected with CL than males.

These results may be attributed to the fact that males are more exposed to the insect biting more than females due to working outdoors and also due to men are less covering than women then exposed **(Al-Samarai *et al.*, 2016)**. Although it is believed that sex hormones may influence the establishment and the course of parasitic diseases, behavioural factors, making male individuals more likely to be exposed to vectors in fields and other transmission environments, are probably equally or more important **(Rahi *et al.*, 2013)**.

The males are more exposed to the environment where the sand flies present by walking near rivers or swimming beside males work in the farms, while the females mostly staying in the houses **(Kharfi *et al.*, 2004)**, **(Asmaa *et al.*, 2014)** found the highest percent of infection related to the geographical site which was near water stream flow all year and abundance of fresh water holes which provide sand flies a suitable environment to complete its life cycle and increase agriculture activities.

4.3 Residency:

The geographic distribution of cutaneous leishmaniosis by using microscopic examination was referred in the figure(3-3), of the patient's shows that 62% of the patients were from the cities of Telaefar and Dialla, while only 30% were from Karbala city, The evidence confirmed infection due to a bad situation for hundreds of thousands of people who exposed to the displacing and dived in camps, in addition to the presence of the war and bad conditions, and presence of swamps near their camps which are important for reproduction sand fly **(Younis, 2018)**.

The distribution of cutaneous leishmaniasis in a rural area was higher than the urban area. This result is agreement with other Iraqi studies as **(Rahi *et al.*, 2013; Al-Difaie, 2014; Al-Samarai *et al.*, 2016)** in Al-Qadisiya governorate, also **(Khudhur, 2018)**, in Libya **(Sabra *et al.*, 2013)**, in Syria **(Al-Nahhas *et al.*, 2016)**, in Jordan **(Al-Athamneh *et al.*, 2014)**, in Iran **(Mehdi *et al.*, 2016)**, and in Turkey **(Fahriye *et al.*, 2017)**. However a different result had been recorded by **(AL-Hucheimi, 2014; AL-Atabi, 2014)**.

Leishmaniasis is usually more common in rural than in urban areas because there are many factors that play an important role in the presence and distribution of CL in this district, including the presence of animal reservoirs such as rodents, dogs, etc.; the presence of marshes; and the use of clay to build some of the houses in villages that belong to this district area. Furthermore, as an agricultural area, attracts and harbours many kinds of insects; therefore, its population works long hours in the farms where they are more exposed to insects bites **(Al-Samarai *et al.*, 2009)**. But it is found in the outskirts of some cities **(Mustafa *et al.*, 2017)**. CDC **(CDC, 2014)** reported that the people who may have an increased risk for infection (especially with the cutaneous form) include adventure travellers, ecotourists, Peace Corps volunteers, missionaries,

soldiers, ornithologists (people who study birds), and other people who do research (or are active) outdoors at night and the transmission risk is highest from dusk to dawn because this is when sand flies are generally very active, also the duration of infection depending on Immunity of people, place of infection, number of lesions and have treatment.

4.4 Age groups:

The distribution of infection of cutaneous leishmaniasis, table (3.2) shows infection according to the age groups was from (10-19 years) 6 (6.5%), ages ranged from (20-29 years) 11 (11.9%) and ages ranged from (30-39 years) 17 (18.5%), and the age (40-49) 37 (40.2%) and (50-59) 21 (22.8%).

So we found the higher infection with cutaneous leishmaniosis according to the age groups was in the age group (40-49) was (40.2%) while the minimum rate of infection was in their ages ranged from (10-19 years) 6 (6.5%) and the table (3-2).

Disagreement whereas obtained the highest infections of CL in the age group (5-14yr), While the lowest infection of CL was observed in the age group (>1yr.) **(Al-Obaidi *et al.*, 2016**). Also **(Al-Warid *et al.*, 2017)** confirmed that majority of cases were recorded among age groups 15–45 years old. Also **(Yousif *et al.*, 2017)** who documented more frequency of the infection in the age group of (19-32) years while the lowest infection rate was noticed among age groups between (5-18) years. **(Al-Samarai *et al.*, 2016; Hassan *et al.*, 2017)** also found that the high incidence of infection occurs in older than 15 years. Another study by **(Abdulwahab, 2013)** and (**Al-Mousawy, 2015**) also established the lowest frequency among the age group older than 50 years. The reason for the low rate of elderly patients may be related to the fact that they were infected during their early ages and acquired long-term immunity during childhood, Another factor is that older people do not admit to the treatment of

CL while they know this disease and disfiguring scars are not as important for them as for youngsters **(Akçalı *et al.*, 2007)**.

Also, this difference could be due to this age playing outdoors for a long time and more exposure to the infected sand flies. Many investigators postulated that the decrease in incidence with age was due to development of immunity by previous infections **(Al-Samarai *et al.*, 2009)**.

The result of present and other studies pointed to, these diseases can infect the individuals at any age. Also, and the **(W.H.O, 2014)** reported that people of all ages are at risk for infection if they live or travel where *Leishmania* spp is found.

4.5 Occupations:

The occupation in kids 3 (3.2%), student 11(11.9%), solder 34 (36.9%), worker 20 (21.7%), Employee 9 (9.8) and house wives 15(16.3%).

The most infected rate of occupation patients in solder was 34 (36.9%), while the lowest infection rate in kids was 3 (3.2%) as in table(3-3).

The evidence confirmed infection due to a bad situation in addition to the presence of the war and bad conditions, such as organic materials and overcrowding and presence of swamps near their camps which are important for reproduction sand fly **(Younis, 2018)**.

4.6 Body site of infection:

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniasis, and Different parts of the patient's body were observed with infection of Baghdad boil including face, arms, legs and feet, the most of the

lesions were found on the exposed areas of the body: face 25 (27.2%), hands 44 (47.8%), Legs 11(12%), and ears 12 (13%).

Most infected rate of patients was found in hands 44 (47.8) while the lowest infection rate was in Legs 11(12%) as in figure (3-4)

This result agrees with **(Al-Difaie *et al.*, 2014; Hassan *et al.*, 2017)** in Iraq who suggested that the highest rate of the lesion was on upper limbs but (**Rahi *et al.*, 2013; Al-Obaidi *et al.*, 2016**) found the face and hand the highest infection. Also, this result agrees with another study in Iran as **(Talari *et al.*, 2006; Hojat *et al.*, 2012)**.

In general, the presence and distribution of lesions depends on which parts of the body are exposed and on the susceptibility of the host **(Samarai *et al.*, 2009)**.

CDC(**CDC, 2014**) directed that, to minimize the amount of exposed (uncovered) skin, to the extent that is tolerable in the climate, wear long-sleeved shirts, long pants, and socks; and tuck your shirt into your pants.

4.7 The number of lesions:

Results of laboratory with microscope examination of one hundred patients cases showed (92) cases were infected while (8) is not in Cutaneous leishmaniasis, differs between patients. Most (65%) of the patients had a multiple lesions in comparison to the single lesions which was found in (35%) of patients.

This result is in agreement with (**Al-Difaie *et al.*, 2014**) who reported that the incidence rate of multiple lesions in CL patients was higher than of the single lesions. This can be due to the fact that some ulcers do not necessarily lead to the appearance of scars for several possible reasons, i.e. immune system

interference or early healing of the ulcers, spontaneously or due to treatment. Also which is in agreement with previous reports indicating more exposure as a result of educational and occupational situations **(Lafi *et al.*, 2007)**.

In contrast with **(Al-Mayale, 2004)** in ALQadisiya, **(Al-Hucheimi, 2005)** in Al- Kufa , **(Musa, 2011)** in Baghdad and **(Rahi *et al.*, 2013; Rahi, 2015**) in Kut city where they show the incidence rate of single lesions in CL patients which was higher (67.2%) than of the multiple lesions 32.8 %, in Iraq. And **(Khalifa *et al.*, 2004)** in Saudi Arabia.

4.8 Clinical signs in infection patients

In humane the lesions, which appear in the arms, legs, faces and ears, showed solid, dry like volcano area in shape and characterized by erythematous papule, with ulcerative border. As in figure (3-5, 3-6, 3-7, 3-8, 3-9)

The Cutaneous leishmaniasis lesions appeared as the sores which can change in size and appearance over time. It may start out as papules (bumps) or nodules (lumps) and may end up as ulcers (like a volcano, with a raised edge and central crater); skin ulcers may be covered by scab or crust. The sores usually are painless but can be painful. Some people have swollen glands near the sores (for example, under the arm, if the sores are on the arm or hand). The lesions of CL in normal infection appeared in the arms, legs, faces and ears, showed solid, dry like volcano area in shape and characterized by erythematous papule, with ulcerative border **(CDC, 2012)**.

4.9 Culture Examination:

The parasitic diagnosis depend on aspirate samples from skin lesions of patients suspect CL infection and directly culture on semi solid and NNN media and confirmed diagnosis of these isolation depend on activity motile

promastigotes that seen in culture. As in figure (3-10) only twenty sample are grow from ninety two of positive microscope examination.

This result is in the present study agreement with **(Marfurt *et al.*, 2003; Al-Heany *et al.*, 2014)** where they used DNA extraction of promastigote from culture on RPMI 1640 and NNN media, while disagreement with DNA extraction for PCR was done from direct collection sample (amastigote), therefore the result showed that all patients revealed negativism to this gene.

4.10 Molecular diagnosis by Nested PCR for 5.8sr DNA gene

There are many laboratory tests that help in the diagnosis of CL that depended mainly on a classical characteristic morphological picture. A molecular method as PCR although not available in all tests but has a crucial role in diagnosis in the level of species **(Hailu *et al.*, 2016).**As in figure (3-15)

The present study showed that the PCR was more specific and speed technique for diagnosis of cutaneous leishmaniasis, DNA analysis test was considered one of the important techniques to give the powerful criteria for the taxonomy for *Leishmania* parasite, so it has been successfully applied in order to detect the parasite DNA **(Mirahmadi *et al.*, 2018).**

PCR strategies that objective DNA can be profoundly touchy for recognizing *Leishmania* parasites on account of abundance of minicircles in each kinetoplast **(Anders *et al.*, 2002; Bensoussan *et al.*, 2006, Disch, 2006)**

In any case, high-level sequence polymorphism among minicircles is an impediment for species identification with protocols based solely on DNA PCR. Focusing on the rRNA-coding bunch presents different issues **(Van Eys *et al.*, 1992)**. Very delicate methodologies for recognizing specific *Leishmania* species, (or a species complex/subgenus), have been depicted **(Cruz *et al.*,**

2002; Parvizi *et al.*, 2008). In Iran, *Leishmania* infections are identified either biologically or molecularly, and cases of infection from rodents and sandflies are fewer **(Mohebali *et al.*, 2004).**

In the study conducted by **(Parvizi *et al.*, 2008)**, 103 out of 120 patient lived in villages and 100 out of those 103 patients had *L. major*. This is agreed with our study **(Parvizi *et al.*, 2008).**

DNA-based techniques have extended cutaneous leishmaniasis diagnosis and parasite species identification. These methods have high sensitivity and specificity **(Oliveira *et al.*, 2005)**. The present study applied the nested PCR technique. The study indicates that *L. major* was the cause of all samples collected from Karbala for cutaneous leishmaniasis.

A study found *L. major* to be the pre-dominant species for Shush **(Maraghi *et al.*, 2013)**. Cutaneous leishmaniasis in Fakeh and Musian that border the city of Mehran was caused by *L. major* **(Nadim *et al.*, 1996; Tashakori *et al.*, 2003)**. found in Dehloran, which is in the vicinity of Mehran, that *L. major* was the solitary agent for cutaneous leishmaniasis **(Kassiri *et al.*, 2012).** These studies agree with our study.

Localized conditions for leishmaniasis in this area are undoubtedly due to ecological characteristics of the disease vectors and reservoirs. Studies have documented that Tatera indica in the absence of Rhombomys opimus is the main reservoir of *L. major* (rural type) in Elam **(Kassiri *et al.*, 2012)**. This rodent has been observed in the Iran-Iraq border areas, in all cities of Khuzestan province, and in the rural areas of Mehran. The present study showed that the main vector of cutaneous leishmaniasis in the region is Phlebotomus papatasi and the definitive reservoir of the disease in the absence of R. opimus is. indica. Because the *Leishmania* species has been proven to cause the disease in this area, it is necessary to promote individual health by

educating the community and implementing appropriate control and preventive measures. It is essential to implement measures for rodent control using rodenticides and to reduce the risk of vector contact with individuals. Bed nets impregnated with insecticide should also be distributed among military personnel, especially in the border areas **(Javadian *et al.*, 1998).**

The presence of incidence of *L. major* in this study and other studies in Iraq may be due to the presence of reservoir animals in large numbers in some areas in Iraq, especially rodents and dogs. Obviously, dense populations of natural hosts of *L. major*, together with abundant vector sand flies are the key elements responsible for the high rate of human infection **(CDC, 2012)**. Also, it must know vector sand flies responsible for human infection by *L. tropica* only **(Craig *et al.*, 2013).**

The application of PCR and RFLP benefits to characterize the *Leishmania* species causing cutaneous leishmaniasis in Iraq. Two types of *Leishmania* spp., *L.major* were as mentioned previously by **(Al-Saqur *et al.*, 2013)**. and *L. tropica* was as mentioned previously by **(Sharma *et al.*, 2015)**. and that confirmed another Iraqi study **(Rahi *et al.*, 2013)** and another study in nearby countries such as Saudi Arabia **(Amin *et al.*, 2013)** and Iran **(Azizi *et ali.*, 2012)**.

The results of the molecular study were probably based on sampling location, Primer and DNA extraction. This seems to be a suitable tool for direct diagnosis and characterization of *Leishmania* species from other methods reported by **(Seray *et al.*, 2013)**

4.11 Molecular diagnosis by using genetic analyzer

The sequences for twelve isolates were arrived from Macrogen company for sequencing in South Korea after sent the PCR products of 5.8S rDNA gene

and were compared the result sequences with reference sequence of 5.8S rDNA gene for *L.major* in National Center for Biotechnology Information (NCBI) gene bank .

4.12 Neighbor-joining tree *Leishmania Major* of 5.8S rDNA:

The phylogenetic tree diagrammatic by Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 .When comparing the isolates of the current study .The phylogenic tree of the current study reflects with out significant diversity between isolates. as shown in the figure (3-16) indicates that the common diseases of the causative are from one source. In summary, most of the cases of isolation were either Displaced from the areas that included the military operations or they are members of the military forces.

4.13 Alignment of sequence analysis of 5.8 rDNA gene of *Leishmania* , karbela isolates by Mega 6:

When comparing the variation between Iraqi isolates found that variation in *Leishmania* major k8,k12 isolates, G is the different from others isolates that variation A,the result of the (12) sequence where all similar genetically with very little change is not significant. as in figure (3-17)

4.14 Neighbor-joining tree *Leishmania Major* of 5.8S rDNA:

The genetic dimension between Iraq and the isolates of the world is detailed according to the Phylogenetic tree. Hierarchical cluster analysis determine the following clusters: large Cluster divided into several neck: The first root divided into two necks first neck: France, Belgium, Germany and U.S.A. Second root Sudan and Iran the genetic dimension was by 0.002, third root Iran the genetic dimension was by 0.004, fourth root Iraq-k1 isolate the genetic dimension was by 0.005 it is close to Iran the genetic dimension was by

0.004. The last cluster the genetic dimension was by 0.021. As seen in figure (3-18).

4.15 Multiple sequence analyisis of 5.8rDNA gene of *Leishmania* international isolates by Mega 6

When comparing the variation between Iraqi isolates and the isolates of the world, we found that variation in *Leishmania major* China isolates A,A,C,A,C,A,C,G,G,T,G,T,T Also its different from others Countries. as in figure (3-19)

4.16 Homology sequence identity of local isolated to NCBI – Gene bank of *L.major* isolated:

The table (3-5) shows the degree of compatibility between Iraq isolates, and global isolates, Iranian isolates, (KU 680848) is the similar to the Iraqi isolate (MH 428844) because the same geographic line while the China isolate (HQ 830351) genetically distant for Iraqi isolate.

4.17 Conclusion

1. Cutaneous leishmaniasis with diverse clinical manifestation "is prevalent in karbala city.
2. *L.major* was the main species causing cutaneous leishmaniasis in comparing with *L.tropica* in the present study.
3. Nasted-pcr is a reliable method for the diagnosis and identification of *Leishmania* species and can applied in epidemiologic investigations.
4. All *L.major* isolates in Karbala city is one isolate for sequencing 5.8 rDNA gene.

5. Iraqi *L. major* isolate was genetically closest with Iranian *L.major* isolates and genetically distant for Chinese isolate.

4.18 Recommendations:

1. Study of disease epidemics in other cities of Iraq
2. The Real-time PCR procedure is the best methods for CL diagnosis because several advantages including more specific, faster processing time, higher sensitivity and decreased contamination risk, seem to be a suitable tool for direct diagnosis and characterization of *Leishmania* spp from other methods.
3. The samples should be collected before administering the treatment for cutaneous leishmaniasis patients because the treatment influences on the parasite .and it must use the culture method to increase the presence of parasite (promastigote) especially when detected the miniexone gene
4. Study other species of *Leishmania* and investigate causes in other cities of iraq.

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Appendix

Appendix-1

Questioner sheet for Cutaneous Leishmaniasis patients

Number of sample :-

Name:-

Date of collection sample:-

Gender:-

Age:-

Address:-

Number of lesion :-

Location of lesion :-

Duration of lesion :-

Type of treatment :-

Microscopic examination :-

DNA Extraction :-

Nested PCR of 5.8s r DNA :-

Sequencing Results :-

Phone Number

Appendix-2

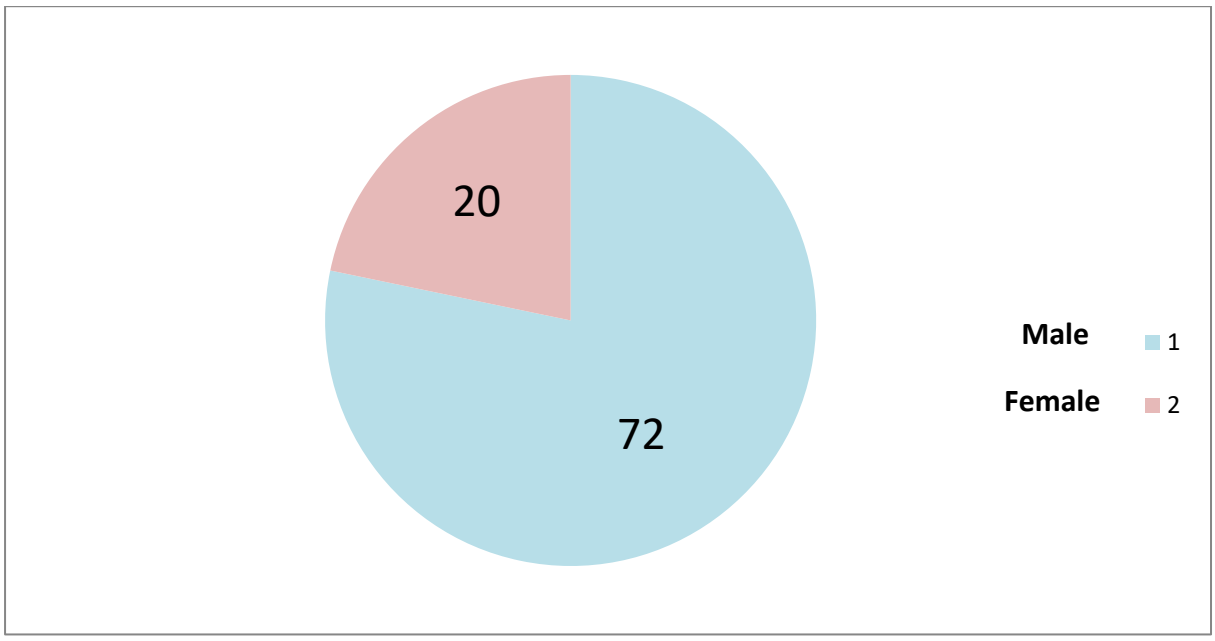


Figure: the number of gender of the patients

Appendix-3

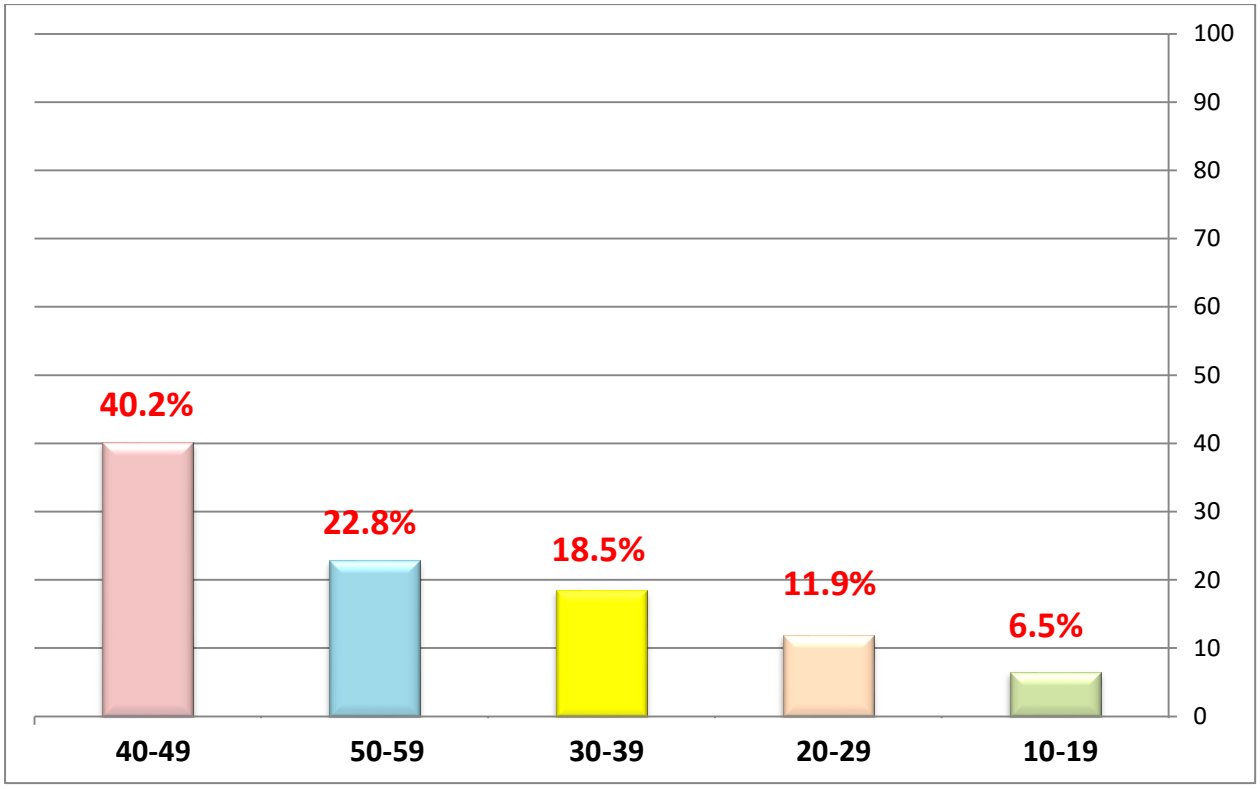


Figure: Distribution of patients percentage according to the Age.

Appendix-4

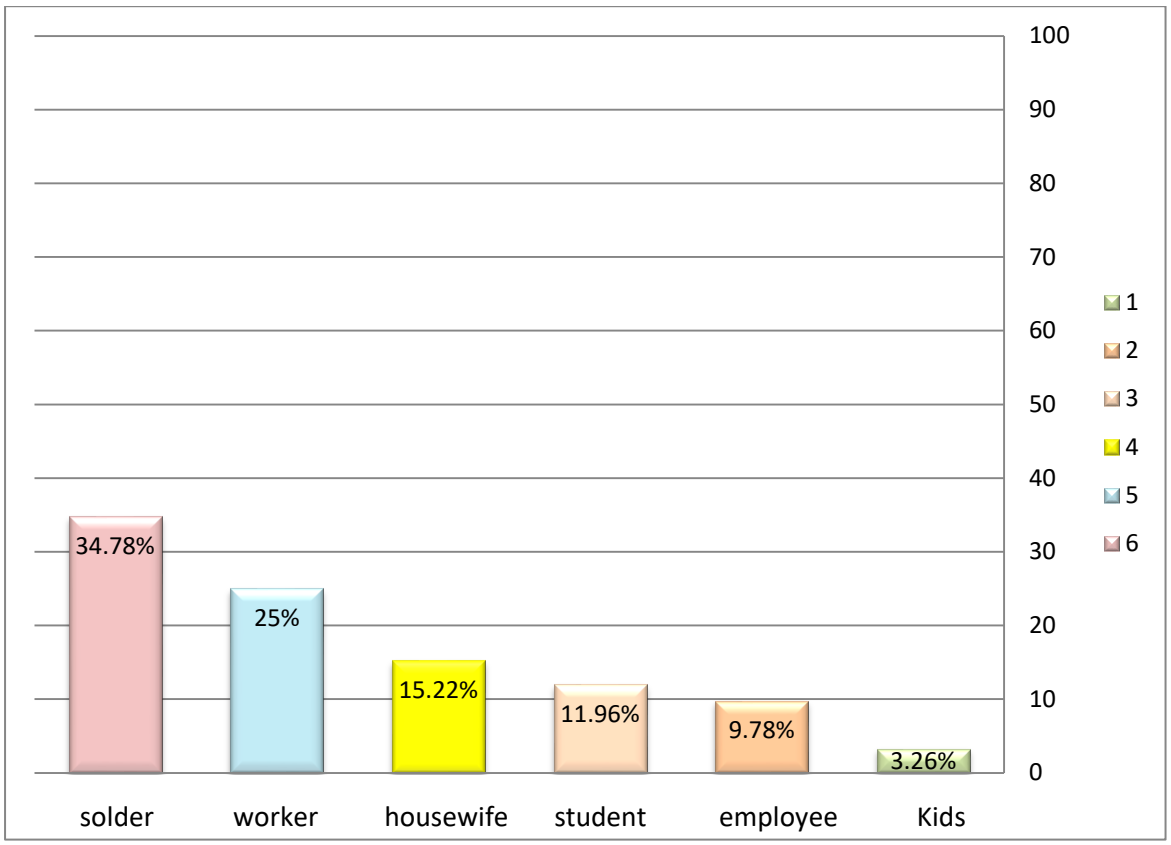


Figure: Distribution of patients percentage according to the Occupations.

Appendix-5

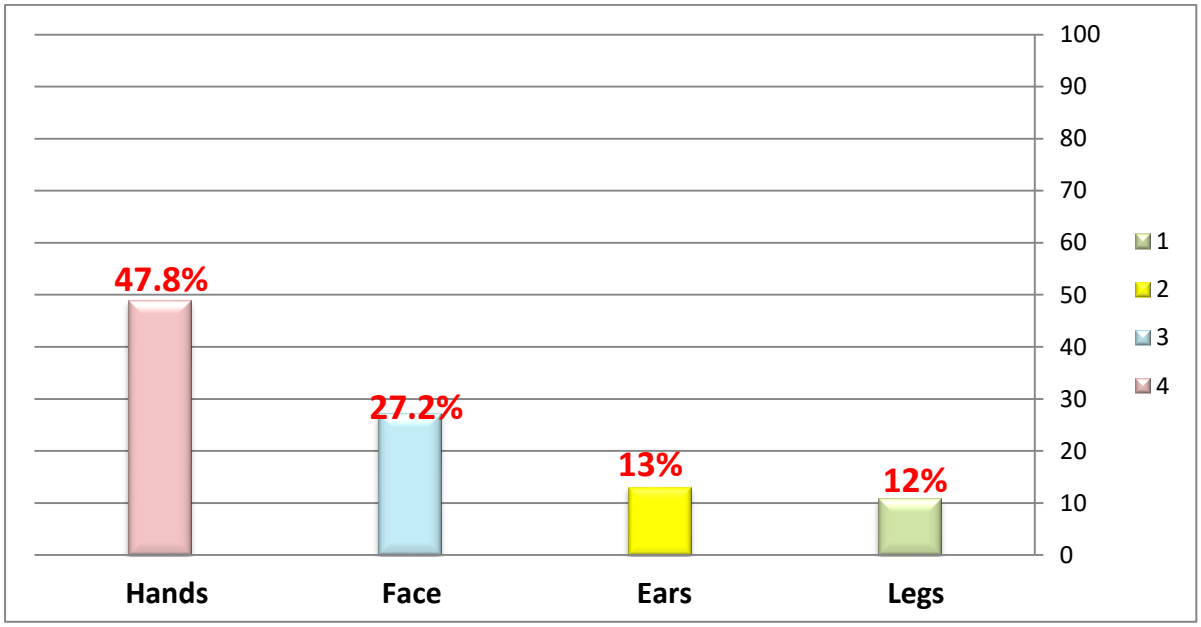
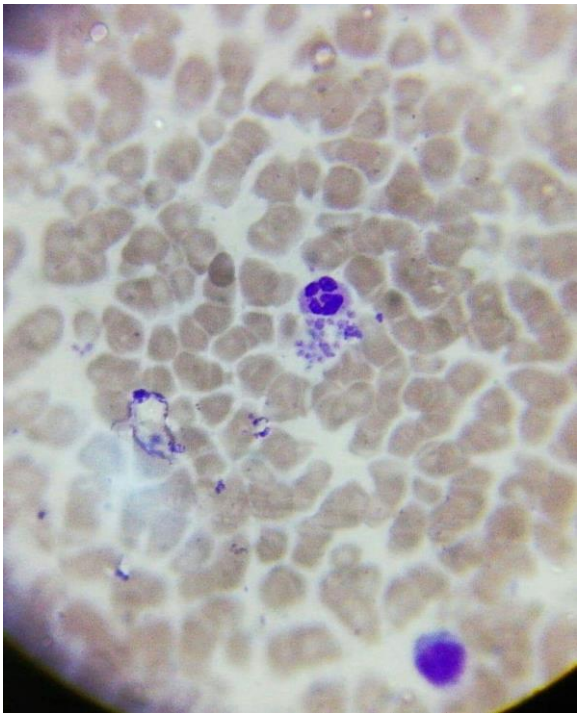


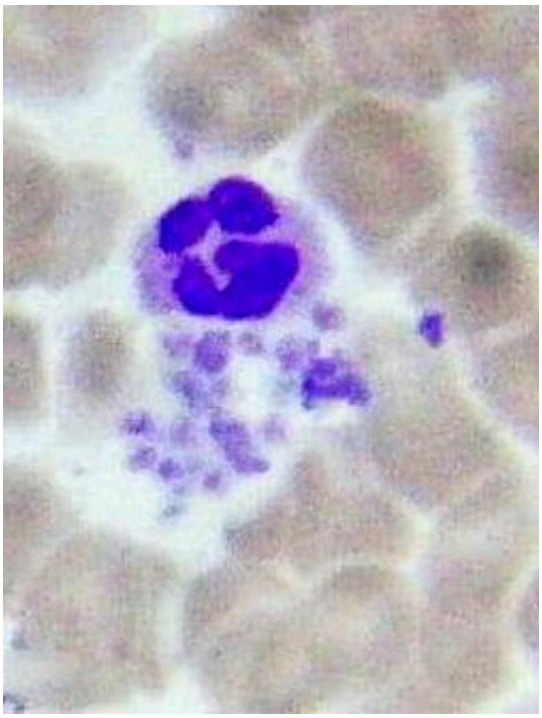
Figure: Distribution of patients percentage according to the site of lesions.

Appendix-6

Picture of working 1

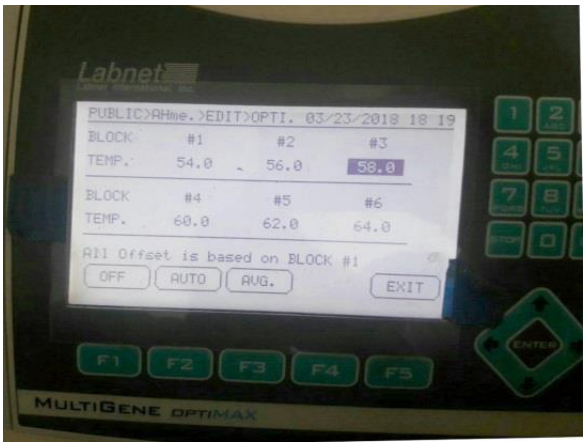


Microscope examination



Microscope examination

Roswell Park Memorial Institute medium



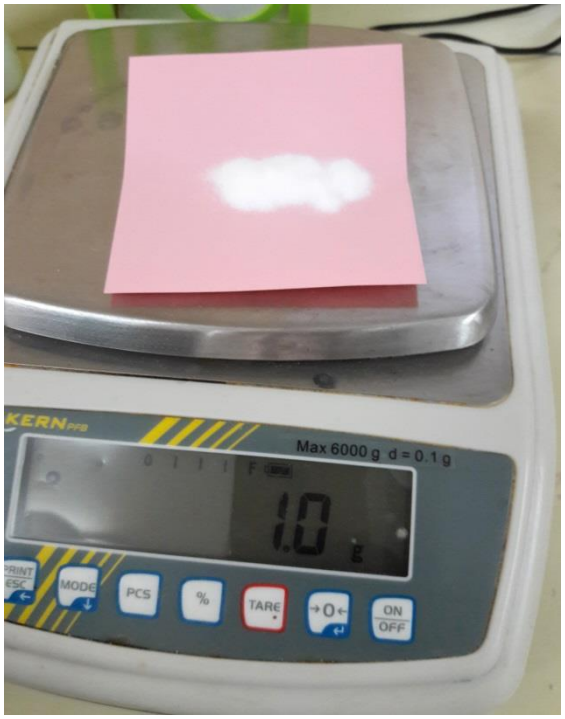
Nested PCR optimization tempter

Appendix-7

Picture of working 2

Microwave

modified Novy-MacNeil-Nicolle medium



Balance

TPE

Tris-Borate EDTA Buffer (TBE 10x)

Appendix-8

Picture of working 3

Incubation of RPMI

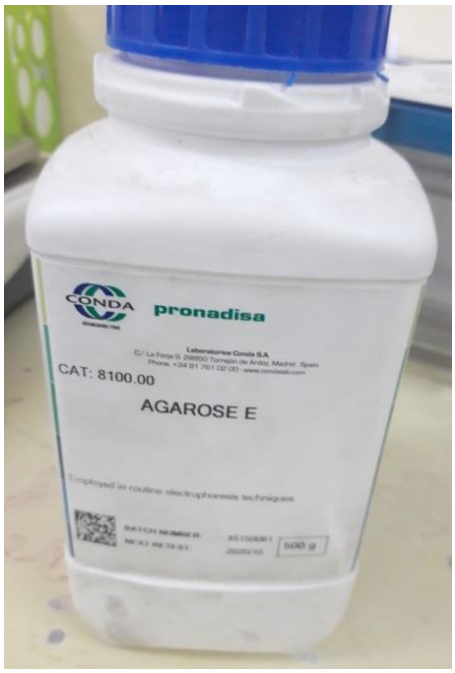
Method of sampling (DNA extraction)

Electrophoresis

Agarose gel electrophoresis

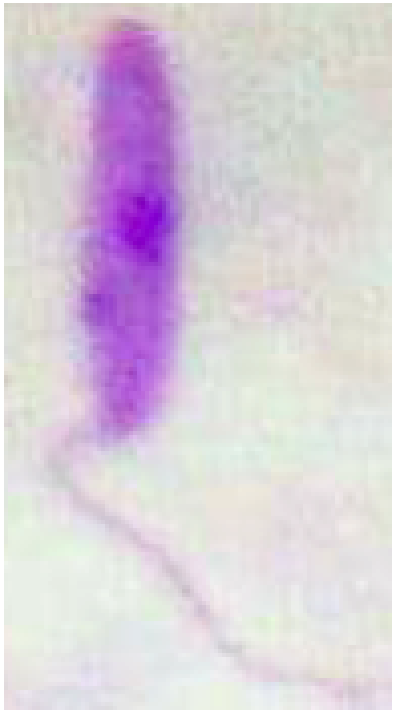
Appendix-9

Picture of working 4

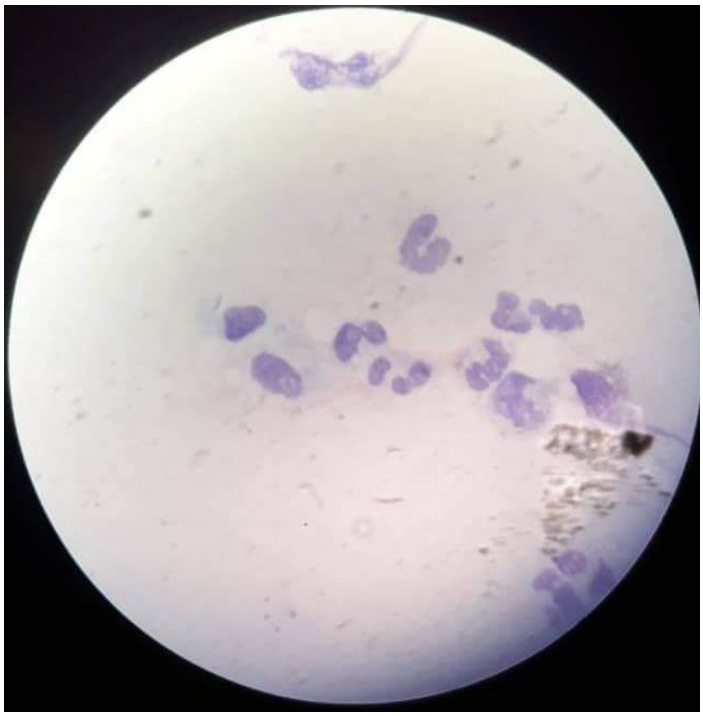


Agarose E

6X Loading dye



Microscope examination of promastigotes in NNN culture



Microscope examination of amastigotes in blood sample

Appendix-10: Procedure of G-Spin DNA extraction Kit

PROTOCOL A (for Blood, body fluids)

1. **Pipet 200 µl of whole blood or body fluids into a 1.5 ml microcentrifuge tube (not provided).**
   Note : If the volume of sample is less than 200 µl, use Buffer CL or PBS Buffer

2. **Add 20 µl of Proteinase K and 5 µl of RNase A Solution into sample tube and gently mix.**
   
   Note : It is possible to add Proteinase K to blood sample that have already been measured into 1.5 ml tube. It is important to assure proper mixing after adding the Proteinase K and RNase A solution.

3. **Add 200 µl of Buffer BL into upper sample tube and mix thoroughly.**
   
   Note : Avoid any vigorous vortexing because doing so may induce genomic DNA breakage. In order to assure efficient lysis, it is important that the blood sample and Buffer BL are mixed thoroughly to yield a lysis solution.

4. **Incubate the lysate at 56°C for 10 min.**
   Note : For complete lysis, mix 3 or 4 times during incubation by inverting tube. If it lysis perfectly, the red color of lysate becomes the dark green.

5. **Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the rid.**

6. **Add 200 µl of absolute ethanol into the lysate, and mix well by gently inverting 5 - 6 times or by pipetting. DO NOT vortex. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside of the lid.**
   Note : This step is an equilibration step for binding genomic DNA to column membrane. It is important to assure proper mixing after adding the ethanol, until not showing 2-phase which is not mixed. Also, this step conduces to pass efficiently cell lysate through a column.

7. **Carefully apply the mixture from step 6 to the Spin Column (in a 2 ml Collection Tube) without wetting the rim, close the cap, and centrifuge at 13,000 rpm for 1 min. Discard the filtrate and place the Spin Column in a 2 ml Collection Tube (reuse).**
   Note : Close each Spin Column in order to avoid aerosol formation during centrifugation. Do not transfer any solid materials.

8. **Add 700 µl of Buffer WA to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and reuse the Collection Tube.**

9. **Add 700 µl of Buffer WB to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and place the Column into a 2.0 ml Collection Tube (reuse), Then again centrifuge for additionally 1 min to dry the membrane. Discard the flow-through and Collection Tube altogether.**
   Note : It is very important to dry the membrane of the Spin Column since residual ethanol may inhibit subsequent reactions. Following the centrifugation, remove carefully the Spin Column from the Collection Tube without contacting with the flow-through, since this will result in carryover of ethanol.
   Note : Ensure that 40 (160) ml of absolute ethanol has been added to Buffer IWB.

10. **Place the Spin Column into a new 1.5 ml tube (not supplied), and 30 - 100 µl of Buffer CE directly onto the membrane. Incubate for 1 min at room temperature and then centrifuge for 1 min at 13,000 rpm to elute.**
    Note : Elution with 30 µl (instead of 50 µl) increases the final DNA concentration, but reduces overall DNA yield conventionally.
    Note : A new 1.5 ml tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, the tube can be reused for the second elution step to combine the eluates.

Appendix-11 : Reference of primer design (Nargis *et al.*, 2014)

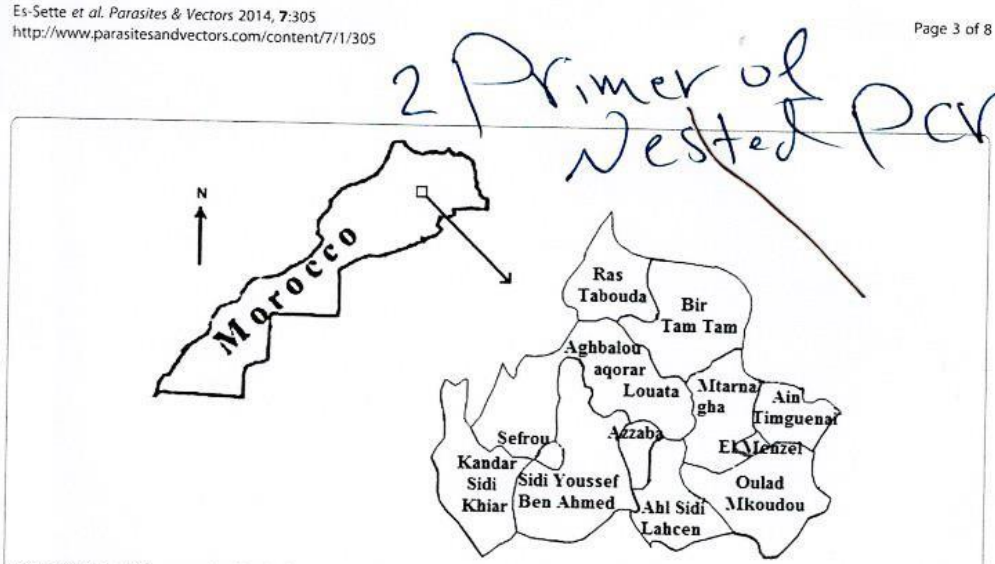


Figure 1 Map of Morocco showing Sefrou province and the Louata locality, the site of the survey.

under a cover slip in Marc-André solution for morphological identification at the species level according to morphological keys described by the Moroccan Health Ministry [20]. After dissection, the abdomen and the thorax of each female specimen were transferred to sterile 1.5 ml Eppendorf tube. They were subsequently grouped into 55 pools according to date, collection site and species, with up to 30 specimens in each pool and were stored at −20°C. The preserved pools were processed for DNA extraction. For each female, it was determined whether it was engorged or unfed.

Detection of *Leishmania* spp. in female sand flies

For each pool, DNA extraction was performed by the phenol-chloroform method [21]. DNA was purified by Qiagen kit according to the instructions of the manufacturer. Female sand flies were screened for *Leishmania* infection by nested PCR. Positive PCR were followed by direct sequencing.

Nested PCR for amplifying the ITS-5.8S rDNA gene of *Leishmania* species

Each PCR was carried out in two separate tubes [22,23]. The first-stage PCR used the forward primer IR1 (5' GC TGTAGGTGAACCTGCAGCAGCTGGATCATT 3' at the 3' end of the SSU rRNA gene) with the reverse primer IR2 (5' GCGGGTAGTCCTGCCAAACACTCAGGTCTG 3' at the 5' end of the large subunit rRNA gene).

The ITS1-5.8S rDNA gene was amplified using the nested forward primer ITS1F (5' GCAGCTGGATCAT TTTCC 3'; overlapping the 3' end of the SSU rRNA gene and ITS1) with the nested reverse primer ITS2R4 (5' ATATGCAGAAGAGAGGAGGC 3'; at the 5' end of ITS2) [23].

The first amplification reaction totaled 20 µl, containing 1xTaq polymerase buffer B (Invitrogen), 1.5 mM $MgCl_2$, 60 µM each dNTP, 1 µM primer IR1, 1 µM primer IR2, 1 unit Taq polymerase (Invitrogen). The mixture was incubated in a thermocycler involving an initial denaturation at 94°C for 3 min, followed by 37 cycles each consisting of three steps: 30 s at 94°C (denaturation), 30 s at 58°C (annealing) and 90 s at 72°C (extension). After the last cycle, the extension step was continued for a further 10 min, and then the reaction was held at 4°C.

The nested amplification was carried out in a second tube, with the reaction mix again totaling 20 µl and containing the same reagents as the first stage, except that the primers were now 1 µM primer ITS1F and 1 µM primer ITS2R4, and the target DNA was provided by adding 1 µl of the completed first-stage PCR reaction. Cross-contamination was monitored by negative controls for sample extraction and PCR solutions. The thermocycler program was as described for the first-stage.

The final PCR products of 462 bp were directly sequenced to identify *Leishmania* species infecting individual sand flies. They were purified using the Exonuclease I/Shrimp Alkaline Phosphatase (GE Healthcare, US) before sequencing by using a BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130 DNA automated sequencer (Applied Biosystems). Sequencing data were analyzed using SeqScape v.2.5 software (Applied Biosystems). Sequences were aligned using the multiple alignment program MEGA 6. A phylogenetic tree was constructed by using the Neighbor-Joining method in agreement with the

Appendix-12 : First step primer IR (SSU rRNA) design forward (Nargis *et al.*, 2014)

IDT
INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET www.idtdna.com

29-Mar-2018

(FR (SSu rRNA) forward first step

Order No. 2847894

Ref. No. 75841130

Sequence - ir1 100 nmole DNA Oligo, 32 bases

5'- GCT GTA GGT GAA CCT GCA GCA GCT GGA TCA TT -3'

Properties

Tm (50mM NaCl):* 66.0 °C
GC Content: 53.1%
Molecular Weight: 9,880.4
nmoles/OD260: 3.3
ug/OD260: 32.4
Ext. Coefficient: 305,400 L/(mole·cm)

Amount Of Oligo

26.2 = 85.7 = 0.85
OD_{260} nmoles mg

For 100 µM: add 857 µL

Shipped To

OMAR LAFI
GENETICS COMPANY FOR BIOTECHNOL
WASFI EL TAL STREET
AMMAN, 11953
JORDAN
96265536402
Customer No. 4213666 *PO No.* PO 291117 ID

Secondary Structure Calculations

Lowest folding free energy (kcal/mole): -6.11 at 25 °C
Strongest Folding Tm: 59.9 °C

Oligo Base Types	Quantity
DNA Bases	32
Modifications and Services	**Quantity**
Standard Desalting	1

Disclaimer

See on reverse page notes (I) (II) & (III) for usage, label license, and product warranties

Mfg. ID256491258

Labels - Peel here

INSTRUCTIONS

•Lyophilized contents may appear as either a translucent film or a white powder. This variance does not affect the quality of the oligo.

•Please centrifuge tubes prior to opening. Some of the product may have been dislodged during shipping.

*The Tm shown takes no account of Mg^{2+} and dNTP concentrations. Use the OligoAnalyzer® Program at www.idtdna.com/scitools to calculate accurate Tm for your reaction conditions.

M

Appendix-13 : First step primer IR (SSU rRNA) design reverse (Nargis *et al.*, 2014)

IDT
INTEGRATED DNA TECHNOLOGIES

IR (SSU rRNA)

SPECIFICATION SHEET

www.idtdna.com

29-Mar-2018

Reverse Primer First Step

Order No. **2847894**

Ref. No. **75841131**

Sequence - ir2

100 nmole DNA Oligo, 30 bases

5'- GCG GGT AGT CCT GCC AAA CAC TCA GGT CTG -3'

Properties

Tm (50mM NaCl):* 66.6 °C
GC Content: 60.0%
Molecular Weight: 9,208
nmoles/OD260: 3.5
ug/OD260: 32.7
Ext. Coefficient: 281,900 L/(mole·cm)

Amount Of Oligo

23.5 = 83.2 = 0.77
OD_{260} nmoles mg

For 100 µM: add 832 µL

Shipped To

OMAR LAFI
GENETICS COMPANY FOR BIOTECHNOL
WASFI EL TAL STREET
AMMAN, 11953
JORDAN
96265536402
Customer No. 4213666 *PO No. PO 291117 ID*

Secondary Structure Calculations

Lowest folding free energy (kcal/mole): -2.29 at 25 °C
Strongest Folding Tm: 47.0 °C

Oligo Base Types	Quantity
DNA Bases	30
Modifications and Services	Quantity
Standard Desalting	1

Disclaimer

See on reverse page notes (I) (II) & (III) for usage, label license, and product warranties

Mfg. ID 256491259

Labels - Peel here

INSTRUCTIONS

•Lyophilized contents may appear as either a translucent film or a white powder. This variance does not affect the quality of the oligo.

•Please centrifuge tubes prior to opening. Some of the product may have been dislodged during shipping.

*The Tm shown takes no account of Mg^{2+} and dNTP concentrations. Use the OligoAnalyzer® Program at www.idtdna.com/scitools to calculate accurate Tm for your reaction conditions.

M

Appendix-14 : Second step primer ITS1- 5.8 s r DNA design forward (Nargis *et al.*, 2014)

INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET www.idtdna.com

15-Feb-2018

Order No. **2826510**

Ref. No. **75504143**

Sequence - ITS1F

25 nmole DNA Oligo, 18 bases

5'- GCA GCT GGA TCA TTT TCC -3'

ITS1-5.8s r DNA] Second step
Forward primer

Properties

Tm (50mM NaCl):* 51.4 °C
GC Content: 50.0%
Molecular Weight: 5,465.6
nmoles/OD260: 6.1
ug/OD260: 33.3
Ext. Coefficient: 164,100 L/(mole·cm)

Amount Of Oligo

5.3 = 32.4 = 0.18
OD_{260} nmoles mg

For 100 µM: add 324 µL

Shipped To

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GENETICS COMPANY FOR BIOTECHNOL
EL MADINA EL MUNAWARAH STREET
AMMAN, 11953
JORDAN
96265536402
Customer No. 4213666 *PO No. PO 291117 ID*

Secondary Structure Calculations

Lowest folding free energy (kcal/mole): -0.23 at 25 °C
Strongest Folding Tm: 28.0 °C

Oligo Base Types	Quantity
DNA Bases	18
Modifications and Services	**Quantity**
Standard Desalting	1

Disclaimer

See on reverse page notes (I) (II) & (III) for usage, label license, and product warranties

Mfg. ID 235854453

Labels - Peel here

I N S T R U C T I O N S

•Lyophilized contents may appear as either a translucent film or a white powder. This variance does not affect the quality of the oligo.

•Please centrifuge tubes prior to opening. Some of the product may have been dislodged during shipping.

*The Tm shown takes no account of Mg^{2+} and dNTP concentrations. Use the OligoAnalyzer® Program at www.idtdna.com/scitoolsto calculate accurate Tm for your reaction conditions.

M

Appendix-15 : Second step primer ITS1- 5.8 s r DNA design reverse (Nargis *et al.,* 2014)

INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET www.idtdna.com

15-Feb-2018

Order No. **2826510**

Ref. No. **75504145**

Sequence - ITS2R4

100 nmole DNA Oligo, 20 bases

5'- ATA TGC AGA AGA GAG GAG GC -3'

ITS1-5.8-rDNA Reverse primer Second Step

Properties

Tm (50mM NaCl):* 53.9 °C
GC Content: 50.0%
Molecular Weight: 6,264.1
nmoles/OD260: 4.6
ug/OD260: 29.0
Ext. Coefficient: 216,200 L/(mole·cm)

Amount Of Oligo

14.0 = OD_{260} 64.9 nmoles = 0.41 mg

For 100 µM: add 649 µL

Shipped To

OMAR LAFI
GENETICS COMPANY FOR BIOTECHNOL
EL MADINA EL MUNAWARAH STREET
AMMAN, 11953
JORDAN
96265536402
Customer No. 4213666 *PO No. PO 291117 ID*

Secondary Structure Calculations

Lowest folding free energy (kcal/mole): 0.53 at 25 °C
Strongest Folding Tm: 16.3 °C

Oligo Base Types	Quantity
DNA Bases	20
Modifications and Services	**Quantity**
Standard Desalting	1

Disclaimer

See on reverse page notes (I) (II) & (III) for usage, label license, and product warranties

Mfg. ID 235854690

Labels - Peel here

INSTRUCTIONS

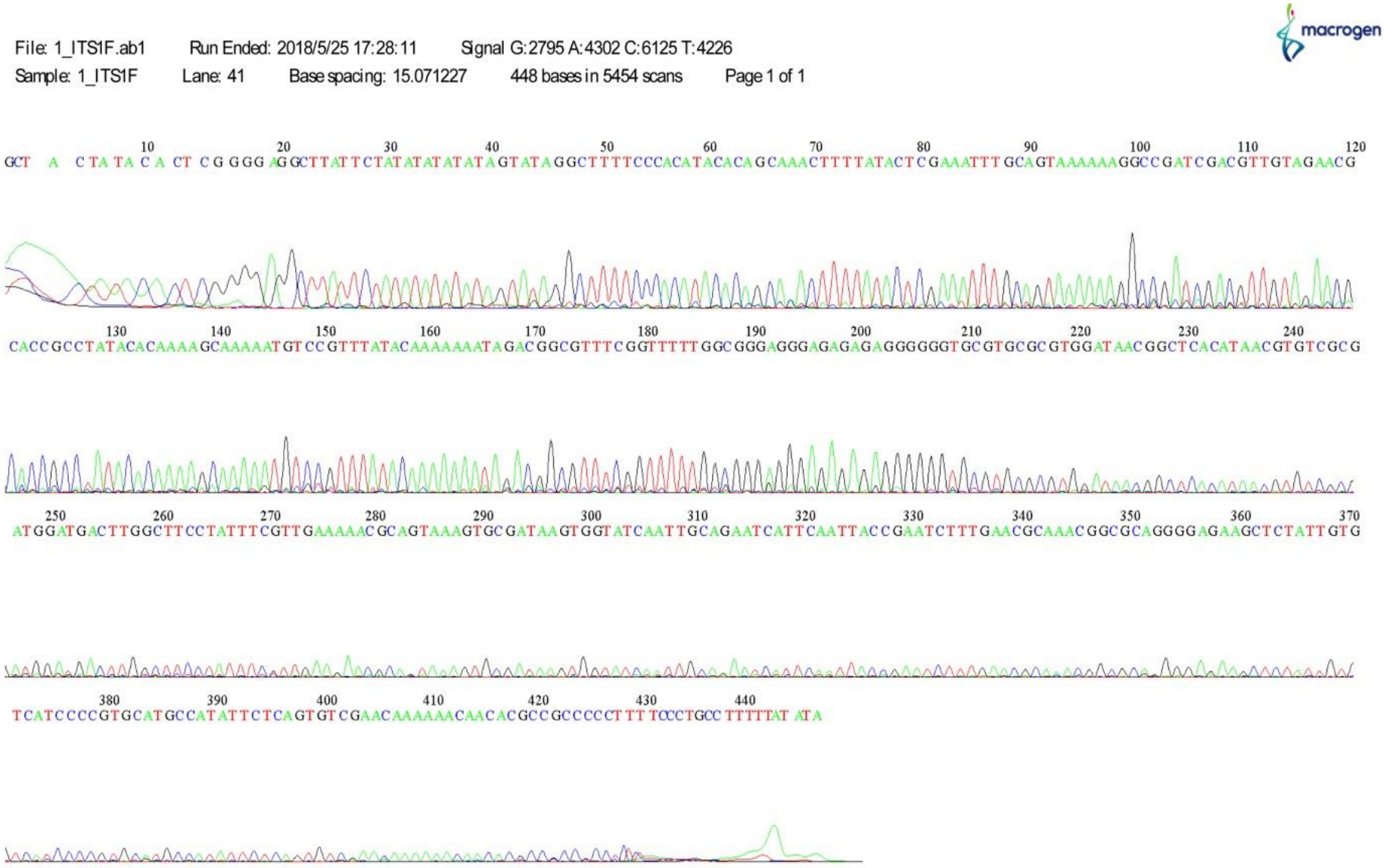
•Lyophilized contents may appear as either a translucent film or a white powder. This variance does not affect the quality of the oligo.

•Please centrifuge tubes prior to opening. Some of the product may have been dislodged during shipping.

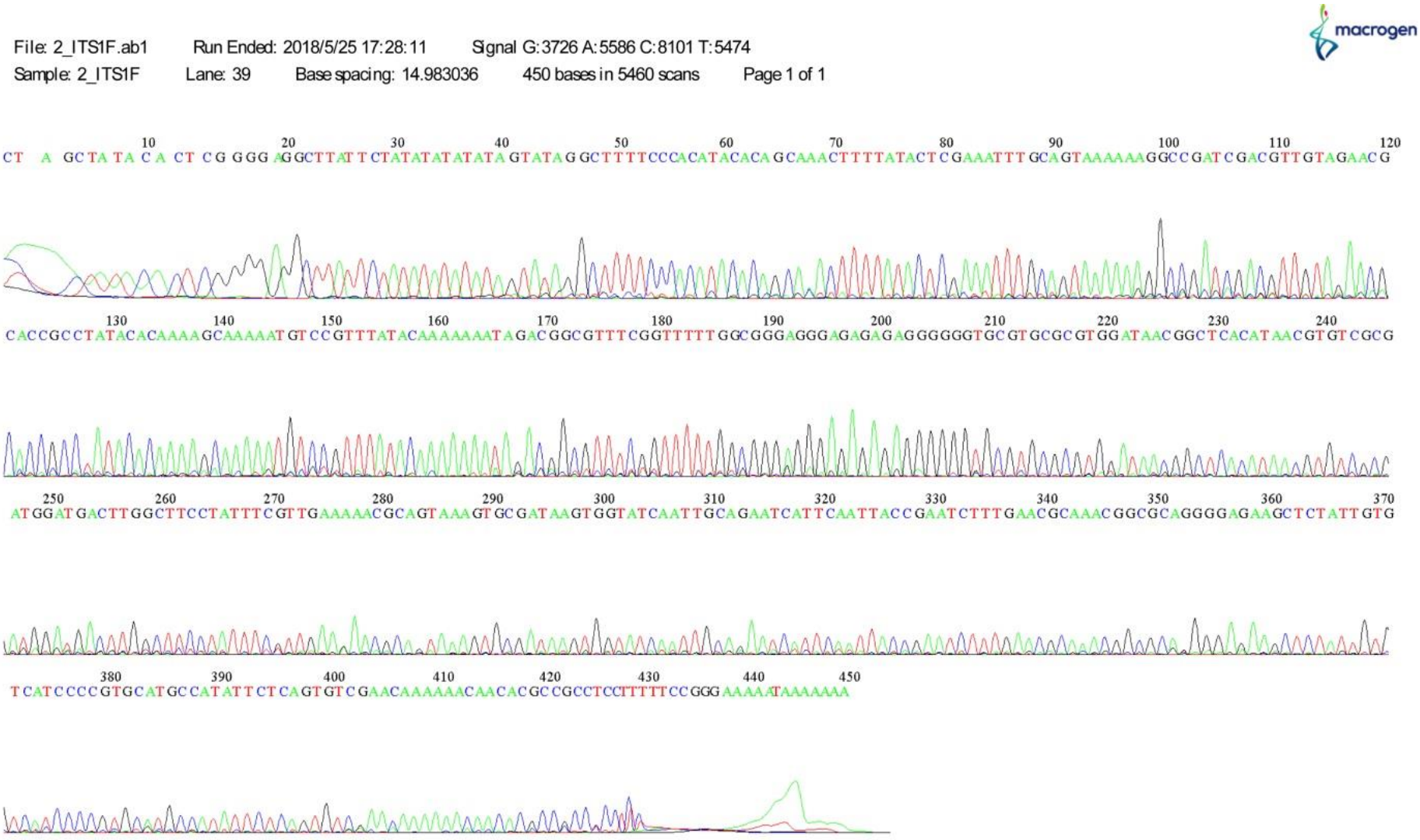
*The Tm shown takes no account of Mg^{2+} and dNTP concentrations. Use the OligoAnalyzer® Program at www.idtdna.com/scitools to calculate accurate Tm for your reaction conditions.

M

Appendix-16 Sample 1 sequences

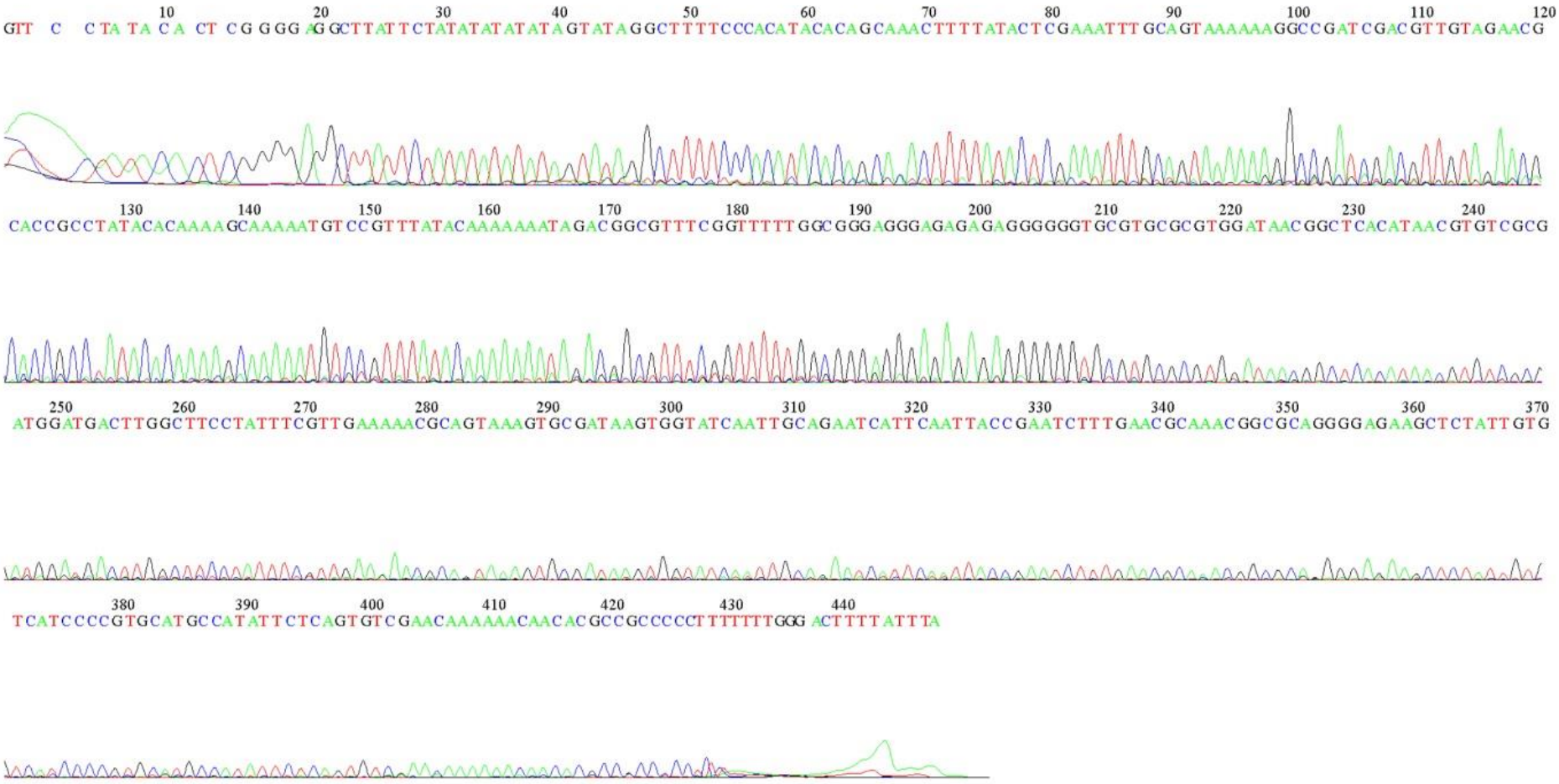


Appendix-17 sample 2 sequences



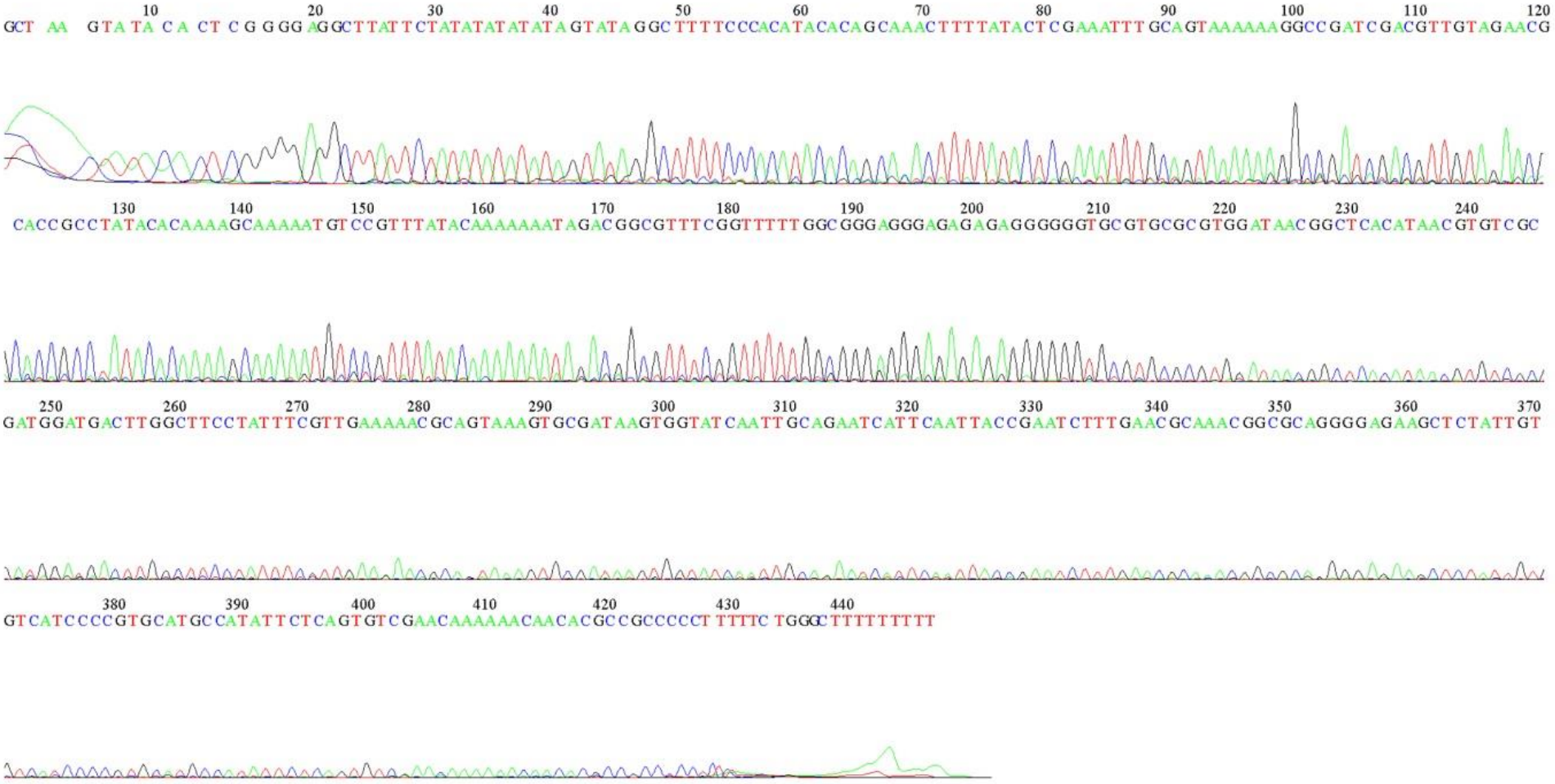
Appendix-18 sample 3 sequences

File: 3_ITS1F.ab1 Run Ended: 2018/5/25 17:28:11 Signal G:2538 A:4223 C:6163 T:4346
Sample: 3_ITS1F Lane: 37 Base spacing: 15.04211 448 bases in 5461 scans Page 1 of 1



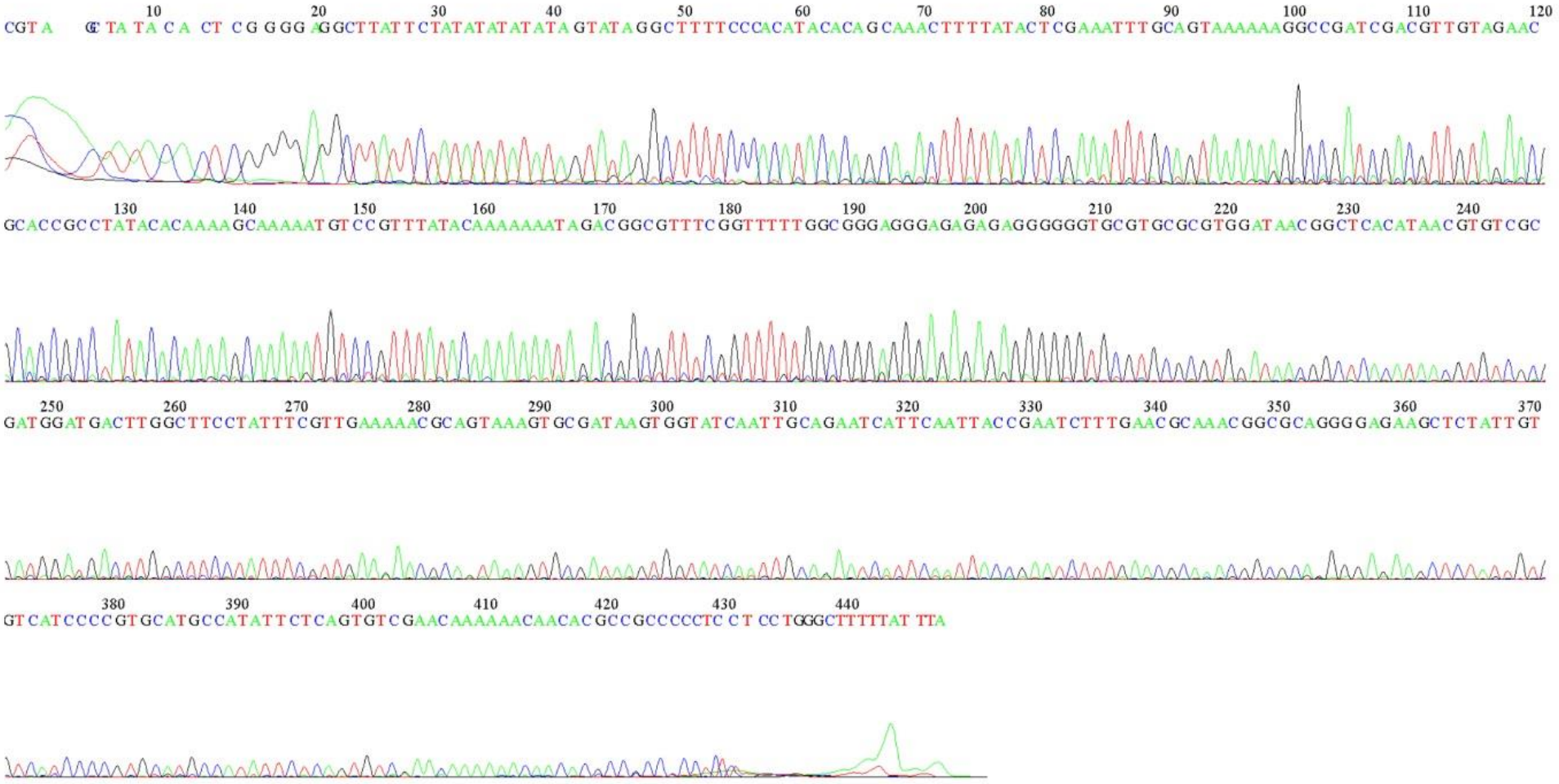
Appendix-19 sample 4 sequences

File: 4_ITS1F.ab1 Run Ended: 2018/5/25 17:28:11 Signal G:2301 A:3661 C:5160 T:3630
Sample: 4_ITS1F Lane: 35 Base spacing: 15.119242 448 bases in 5462 scans Page 1 of 1

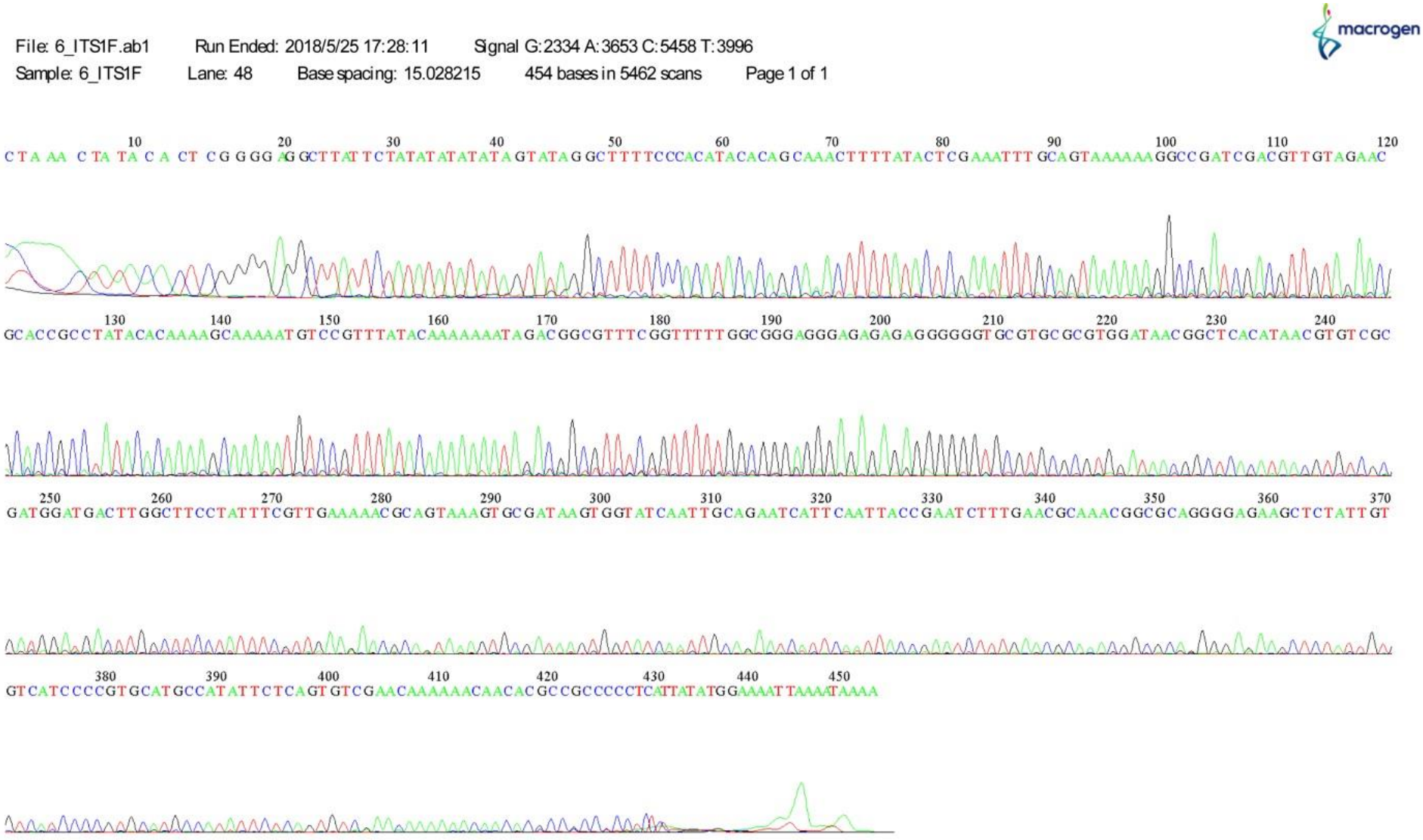


Appendix-20 sample 5 sequences

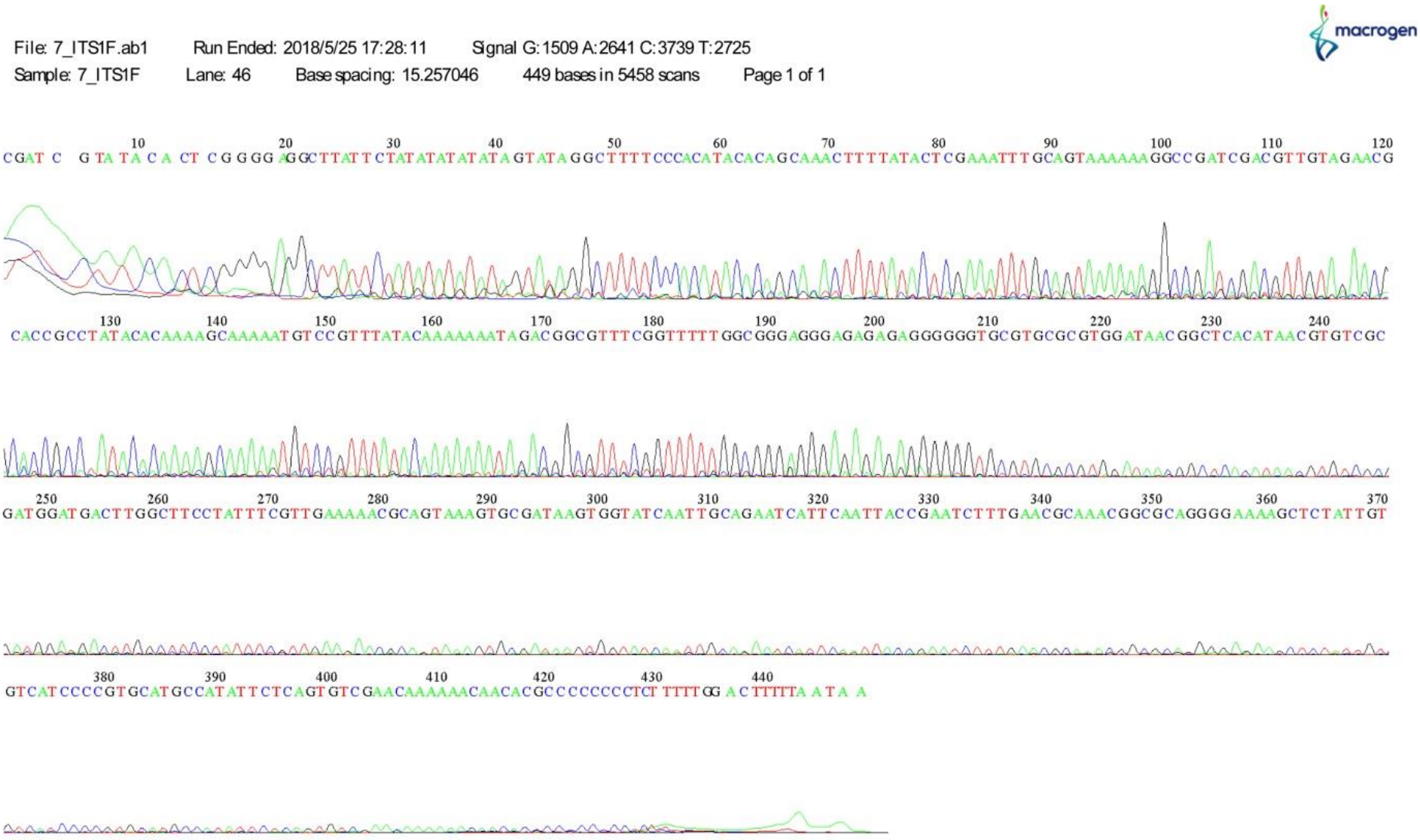
File: 5_ITS1F.ab1 Run Ended: 2018/5/25 17:28:11 Signal G:2134 A:3438 C:5157 T:3578
Sample: 5_ITS1F Lane: 33 Base spacing: 15.187694 449 bases in 5456 scans Page 1 of 1



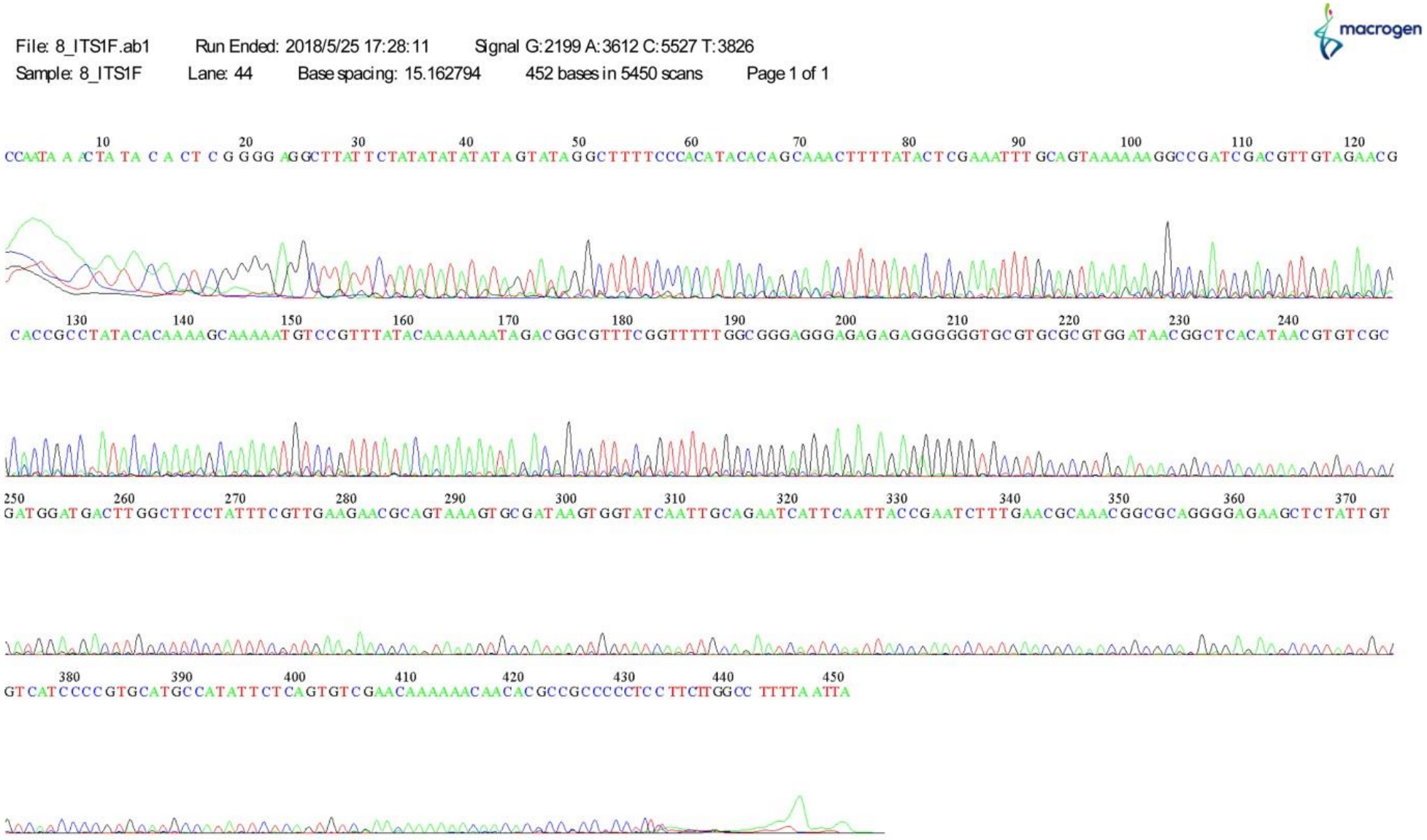
Appendix-21 sample 6 sequences



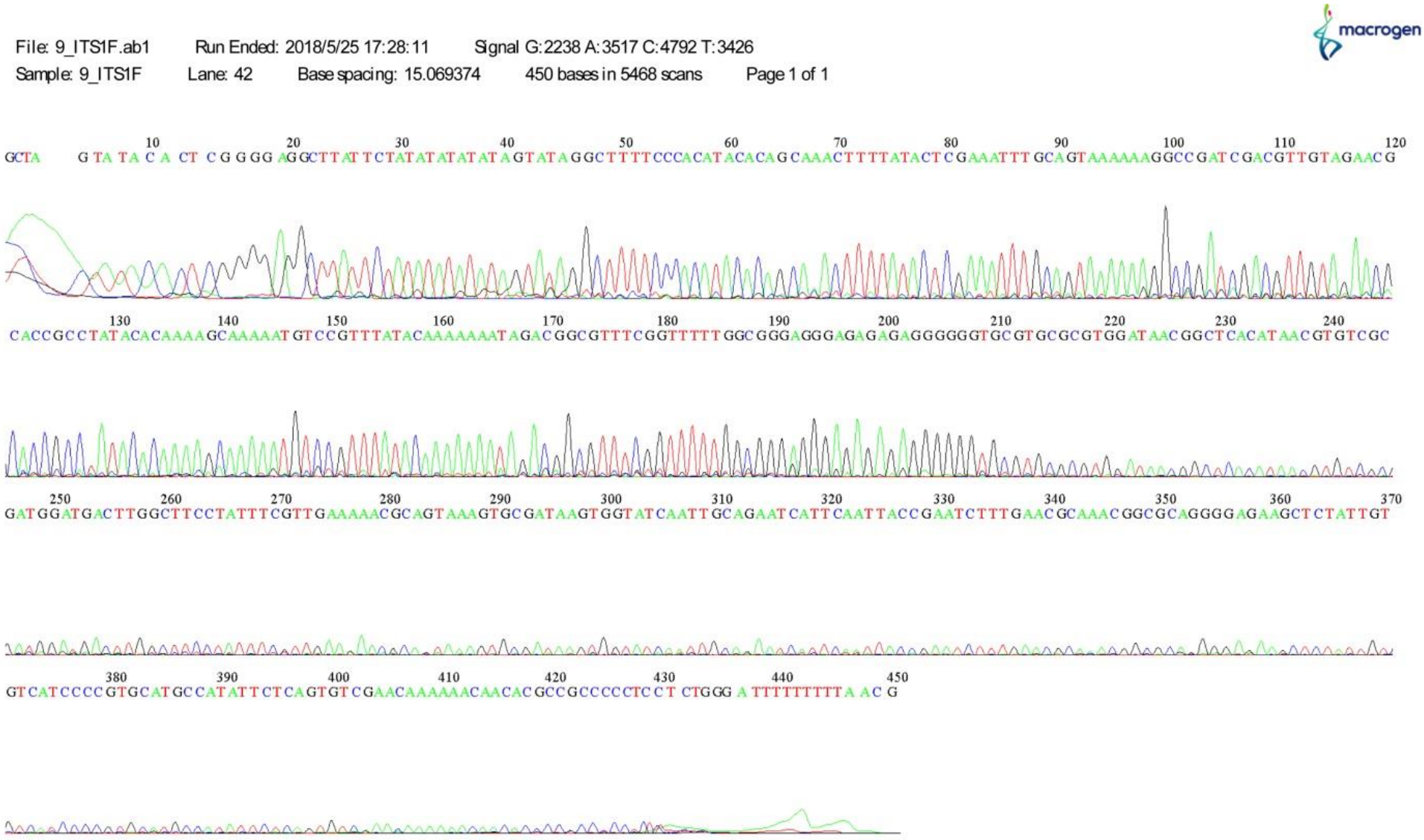
Appendix-22 sample 7 sequences



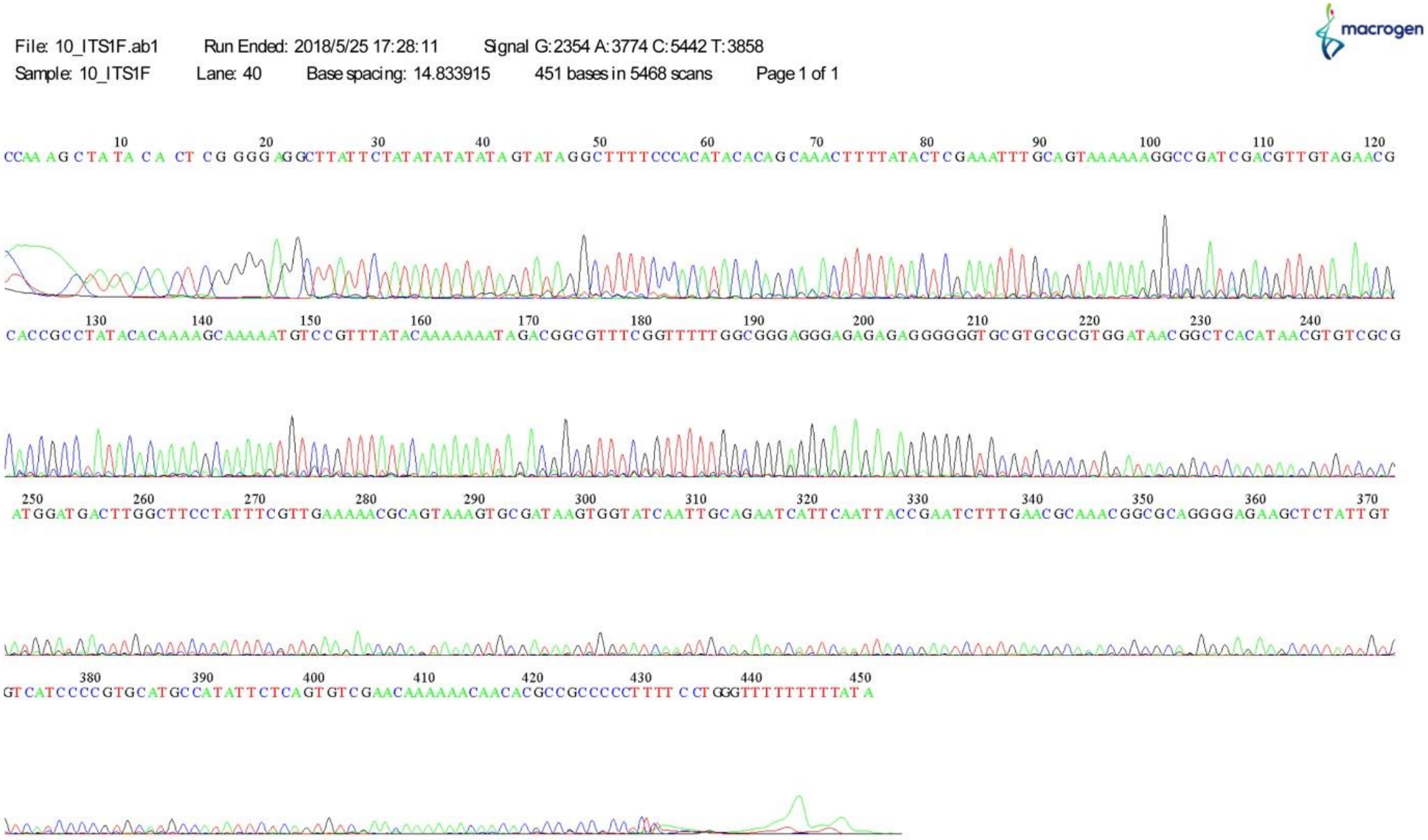
Appendix-23 sample 8 sequences



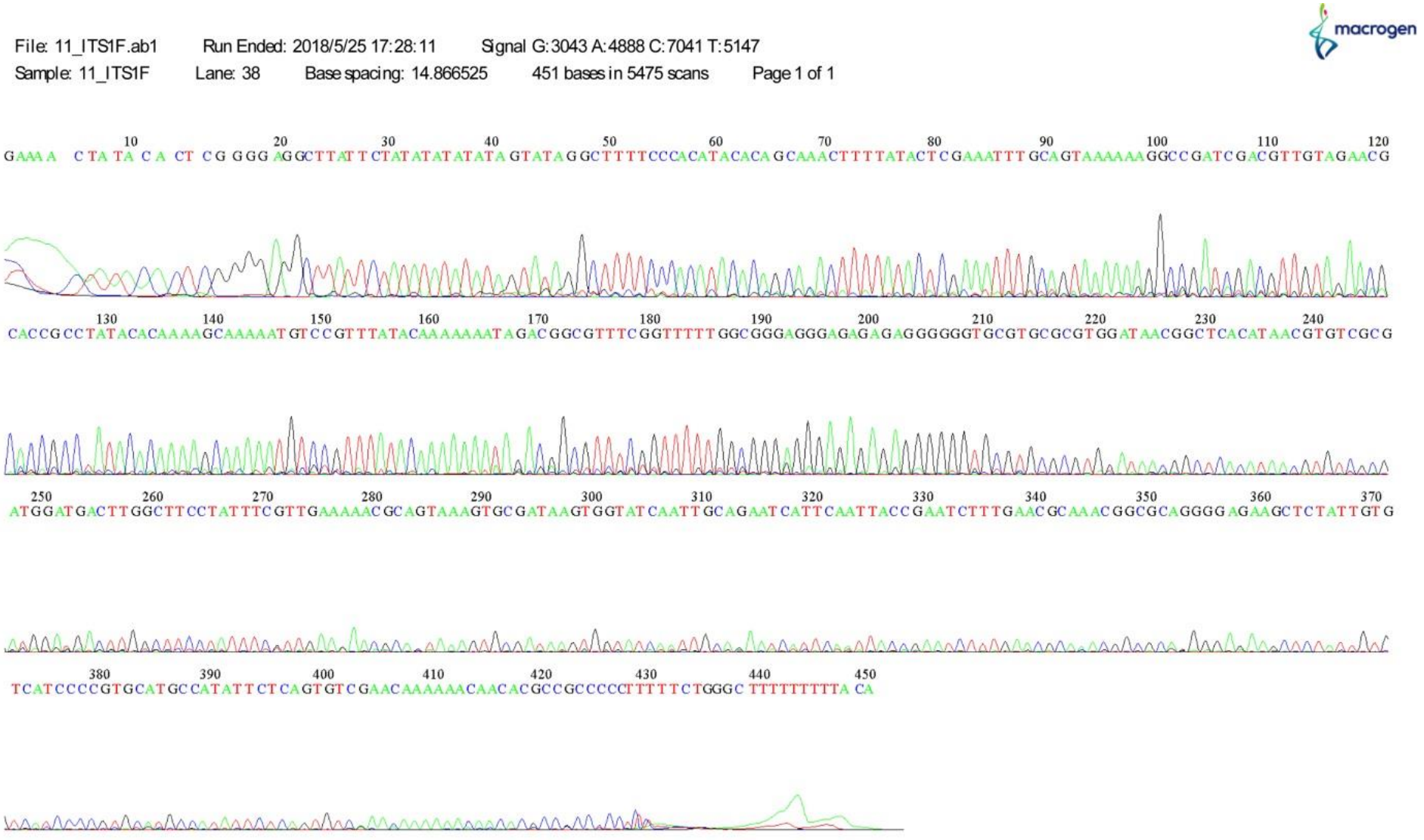
Appendix-24 sample 9 sequences



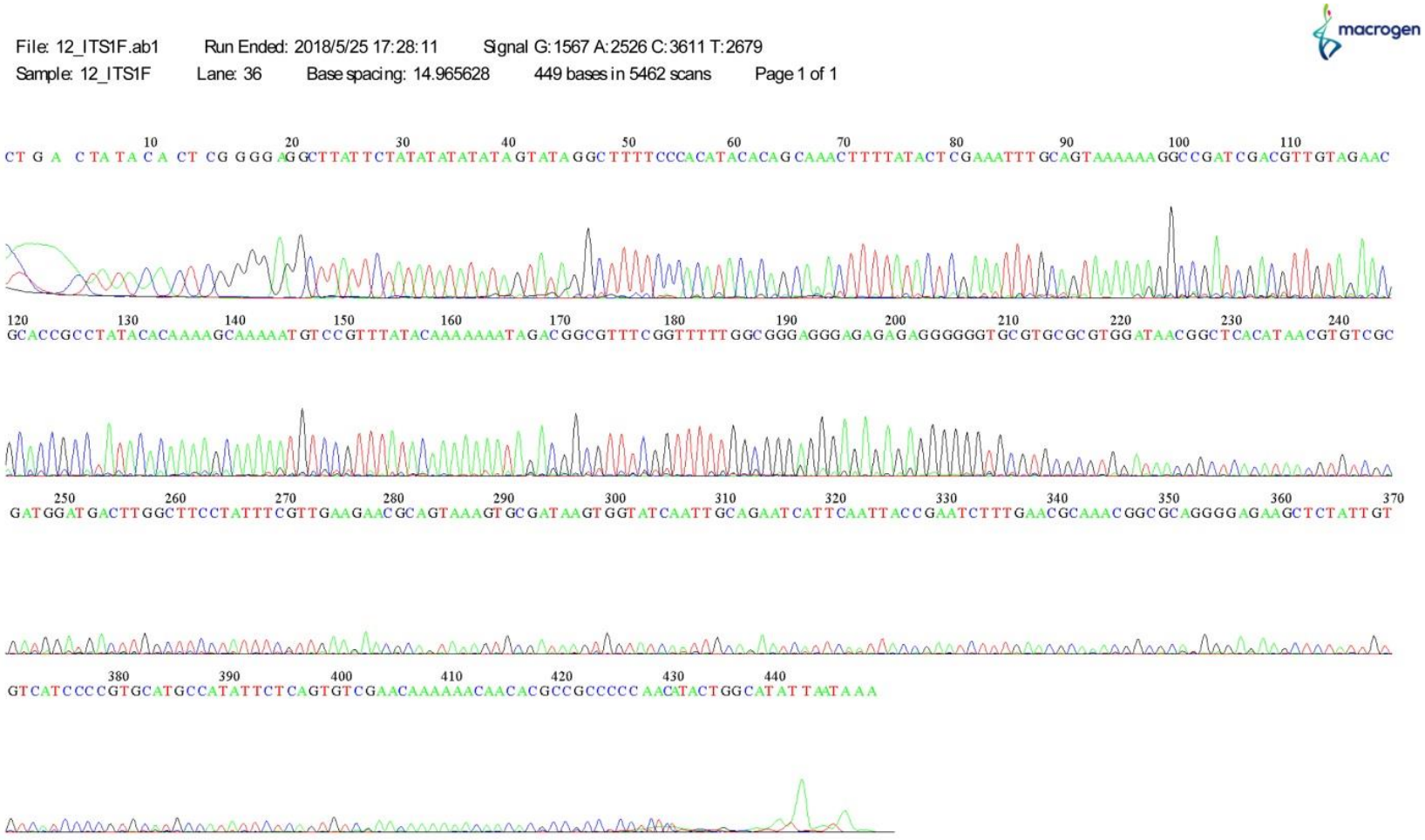
Appendix-25 sample 10 sequences



Appendix-26 sample 11 sequences



Appendix-27 sample 12 sequences



Appendix-28 Choose randomly isolate k1 then recorded in NCBI gene bank

Leishmania major internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: MH428844.1

FASTA Graphics

Go to:

```
LOCUS       MH428844                448 bp    DNA     linear   INV 27-AUG-2018
DEFINITION  Leishmania major internal transcribed spacer 1, partial sequence;
            5.8S ribosomal RNA gene, complete sequence; and internal
            transcribed spacer 2, partial sequence.
ACCESSION   MH428844
VERSION     MH428844.1
KEYWORDS    .
SOURCE      Leishmania major
  ORGANISM  Leishmania major
            Eukaryota; Euglenozoa; Kinetoplastida; Trypanosomatidae;
            Leishmaniinae; Leishmania.
REFERENCE   1  (bases 1 to 448)
  AUTHORS   Obyes,L.H., Zghair,F.S. and Hamza,D.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-JUN-2018) Biology, Al-Furat Al-Awsat Technical
            University, Kerbala University, Kerbala 00964, Iraq
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /organism="Leishmania major"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:5664"
                     /note="biotype: 2120185"
     misc_RNA        <1..>448
                     /note="contains internal transcribed spacer 1, 5.8S
                     ribosomal RNA, and internal transcribed spacer 2"
ORIGIN
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       61 cacagcaaac ttttatactc gaaatttgca gtaaaaaagg ccgatcgacg ttgtagaacg
      121 caccgcctat acacaaaagc aaaaatgtcc gtttatacaa aaaaatagac ggcgtttcgg
      181 tttttggcgg gagggagaga gagggggtg cgtgcgcgtg gataacggct cacataacgt
      241 gtcgcgatgg atgacttggc ttcctatttc gttgaaaaac gcagtaaagt gcgataagtg
      301 gtatcaattg cagaatcatt caattaccga atctttgaac gcaaacggcg caggggagaa
      361 gctctattgt gtcatccccg tgcatgccat attctcagtg tcgaacaaaa aacaacacgc
      421 cgccccctttt tccctgcctt tttatata
//
```

الخلاصة

داء الليشمانيات مرض طفيلي يسببه داء الليشمانيات السوطيات دموية. هذا المرض واسع الانتشار وقد يسبب مشاكل صحية خطيرة في المجتمعات في جميع أنحاء منطقة البحر الأبيض المتوسط والشرق الأوسط ، بما في ذلك العراق. هناك حوالي 12 مليون حالة في جميع أنحاء العالم ، وهناك حوالي 1.5 مليون حالة جديدة من داء الليشمانيات الجلدي كل عام.

داء الليشمانيات الجلدي هو مشكلة صحية عامة رئيسية ومرض مستوطن في السكان العراقيين ، لذلك أجريت هذه الدراسة لإيجاد أفضل طريقة للتشخيص ، من خلال "التشخيص الجزيئي وتحليل شجرة التطور الوراثي للكشف عن أنواع داء الليشمانيات الجلدي في مدينة كربلاء بوساطة Nested PCR.

أظهرت نتائج الفحوصات المخبرية بالفحص المجهري لمائة حالة إصابة (92) حالة إصابة ، بينما (8) غير مصابة بداء الليشمانيات الجلدي حيث بلغ عدد الذكور (72) ,ونسبتهم(78.3) % و 20 إناث ونسبتهم(21.7) % تتراوح أعمارهم بين (10) و (59) عامًا ، حضر إلى قسم الأمراض الجلدية بمستشفى الحسيني في كربلاء خلال الفترة من نوفمبر 2017 إلى نهاية يناير 2018.

كان معدل الإصابة الأكثر بين سن 40-49 سنة ونسبتهم (40.2 ٪) ، في حين كان معدل الإصابة الأدنى بين سن (10-19) سنة ونسبتهم (6.5 ٪).

كان أكثر المرضى المصابين من الجنود 34 (36.9٪) ، بينما كان أقل معدل للإصابة عند الأطفال 3 (3.2٪) ، ووجد أكثر المصابين في منطقة الايدي 44 (47.8%) بينما كان أقل معدل للإصابة كان في الساقين 11 (12 ٪).

يوضح التوزيع الجغرافي للمرضى أن 62٪ من المرضى كانوا من مدينتي تلعفر وديالى ، بينما كان 30٪ فقط من مدينة كربلاء. يختلف عدد الآفات بين المرضى. كان لدى معظم المرضى نسبه (65٪) آفات متعددة مقارنةً بالآفات المفردة التي وجدت بنسبه (35٪) من المرضى.

تـم إجـراء الفحـص المجهـري المباشـر بطـريقتين: سـحب خزعـه سـائل والفحـص المجهري المباشر ثم تصبيغها بصبغة اللشمان بعد ذلك ، تم زراعة العينات الإيجابية في وسط Novy-MacNeil-Nicolle من (15 - 30) يومًا ومن ثم تنشط في (Roswell Park Memorial Institute medium).نمت 20 عينه فقط.

تم إجراء تشخيص أكثر تحديداً للعامل المسبب لداء الليشمانيات الجلدي باستخدام الاختبارات الجزيئية. تم اختيار 20 عينة من العينات الايجابيه، من إجمالي عدد مسببات المرض .

أظهرت نتائج إيجابية تمت دراستها بواسطة Nested-PCR باستخدام نوعين من Primer لتحديد النوع. لأن حجم الجين كان كبير (5.8s.rDNA) من خلال Nested-PCR ا أظهرت أن جميع العينات كانت *L.major*.

ارسلت نواتج Nested PCR المكونة من اثني عشر عزلة متسلسلة الى كوريا الجنوبية ، ثم قارنت الأنواع المحلية التي عزلت من الدراسة الحالية مع السلالات العالمية في بنك الجينات NCBI ثم اختيرت عزلة K1 بشكل عشوائي للتسجيل.

النتائج كانت تتضمن أن الليشمانية الجلدية منتشر بشكل كبير مع آفات متعددة أكثر من الآفات المفردة لداء الليشمانيات الجلدي "المظاهر السريرية المتنوعة" منتشرة في مدينة كربلاء ، وأظهر الاكتشاف الجزيئي أن *L.major* كان النوع الرئيسي الذي تسبب داء الليشمانيات الجلدي في المقارنة مع *L.tropica* في الدراسة الحالية ، Nasted-pcr هي طريقة موثوقة لتشخيص وتحديد أنواع داء الليشمانيات لأن درجة عالية من الحساسية والخصوصية يمكن تطبيقها في التحقيقات الوبائية ، جميع العزلات *L.major* في مدينة كربلاء هي عزلة واحدة لتسلسل الجينات 5.8s.rDNA والنتيجة من التسلسل (12) حيث تكون كل العوامل المتشابهة وراثيا مع تغير طفيف غير مهمة ومن ثم مقارنة مع االعزلات العالميه المسجله في بنك الجينات NCBI. كانت متشابهة وراثيا (عباره عن عزلة واحدة) مع العزلات الإيرانية ، وأخيرا العينة *L.major* العراقية المعزولة كانت وراثياً الأقرب الى العزلة الإيرانية *L.major* المعزولة لأن العراق وإيران على نفس الخط الجغرافي وبعيدة وراثيا للعزلة الصينية.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء - كلية الطب
فرع الاحياء المجهرية الطبية

التشخيص الجزيئي والتحليل الوراثي لـ 5.8s rDNA جين لداء الليشمانيات الجلدي المعزول من مرضى في كربلاء المقدسة

رسالة مقدمة

مجلس كلية الطب/ فرع الاحياء المجهرية/ جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في الاحياء المجهرية الطبية

من قبل

ليث حسن عبيس

بكالوريوس تقنيات تحليلات مرضية/ كلية المستقبل الجامعة /2016

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1440 هـ

2019 م