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Epidemiological, Immunological and Molecular Study of *Leishmania donovani*

A Thesis

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

يَرْفَعُ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا
الْعِلْمَ دَرَجَاتٍ

صدق الله العلي العظيم

سورة المجادلة / الآية 11

Dedication

To my respected **father**, whose unwavering support has driven my academic success. Your joyful applause for my achievements and cherished prayers inspires me daily. You have guided my life, providing strength and self-esteem, may God bless you.

To my beloved **mother**, the embodiment of love and generosity, whose comforting embrace and prayers are the motivator of my success. May God grant you a long and healthy life.

To my dear **brothers**, who enrich my life, and to my precious **sister**, my constant companion, may God protect you always.

Marwa

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Marwa

Summary

Visceral leishmaniasis is a widespread disease in several countries around the world, including Iraq. Infection occurs by injection of the *leishmania* parasite through a female fly belonging to the genus *Phlebotomus sp*, when it feeds on the blood /mammalian host. The distinctive feature of visceral leishmaniasis is its effect on many internal organs (usually the spleen, liver, and bone marrow).

The current study was conducted on 332 blood samples from men and women, distributed among 276 from Diwaniyah Governorate and 56 clinical samples from different hospitals in Iraq, during the period from March to December 2023.

The study dealt with two aspect, the first of which was an epidemiological study of visceral leishmaniasis in Diwaniyah Governorate, which included 276 people, where all suspected persons participating in this study were examined in the laboratories of the College of Science - Al-Qadisiyah University, where 18 people were detected and confirmed to have visceral leishmaniasis (VL) 6.52% through rapid Immunochromatography (I.C.T) screening test , which has been proven successful through its high sensitivity and specificity, in addition to clinical signs and symptoms.

The results showed that the age group (< 18) was more susceptible to infection (18.38%). that males were infected at a higher rate (9.7%) than females (4.9%). They also showed a higher number of infected people in rural areas (7.5%) compared to the city center (2.5%).

The second aspect included a study of 56 cases in which the clinical diagnosis of visceral leishmaniasis (VL) was confirmed from various hospitals in Iraq. The diagnosis was confirmed through two laboratory tests that included a rapid immunochromatography examination and an indirect

fluorescent antibody test (IFAT), in addition to signs the clinical symptoms, which were evaluated by the consulting medical staff and included in most cases: fever, weight loss, fatigue and anemia, as well as splenomegaly and hepatomegaly.

The results appeared identical in both the epidemiological study and clinical samples, as the highest infection rate was recorded in the age group (<18) and males had a higher infection rate (53.85%) than females (37.15%), and infected patients in rural areas were higher (60.7%) than in the city (39.3%), the results of clinical signs showed that the highest incidence of infection was in fever (47.37 %)The highest infection rate was in Diwaniyah Governorate (24.3%) and the lowest infection rate was in Babylon and Najaf Governorates (5.4%).

The biopsies taken from the bone marrow were examined with a light microscope, stained with Giemsa stain, and after confirming the presence of the parasite, it was cultured on NNN culture medium (Novy - MacNeal - Nicolle) and RPMI (Roswell Park Memorial Institute) medium at a temperature of 23°C.

As for the molecular examination, four samples were examined using PCR technology based on the diagnostic gene KDNA with a size of 562 bp

In this study the level of cytokines secreted by the innate immune response to visceral leishmaniasis infection, such as cytokines IL-2, IL-10, IL-12 and TGF- β , was detected using ELISA technology. The results showed a significant increase in the concentration of these cytokines in the test group (131.58 ± 15.92 , 335.68 ± 15.57 , 823.62 ± 98.75 , 341.16 ± 15.19), respectively for patients infected with visceral leishmaniasis compared to the healthy control group.

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LIST OF Abbreviations

VL	Visceral Leishmaniasis
I.C.T	Immunochromatography
IFAT	Indirect Fluorescent Antibody Test
NNN	Novy - MacNeal - Nicolle
PCR	Polymerase chain reaction
KDNA	Deoxyribonucleic acid
IL	Interlukin
IL-2	Interlukine- 2
IL-10	Interlukine- 10
IL-12	Interlukine- 12
TGF-β	Transforming growth factor- β
ELISA	Enzyme-linked immunosorbent assay
IFN-γ	Interferon gamma
TNF-α	Tumor necrosis factor alpha
NTD	Neglected Tropical Disease
WHO	World Health Organization
LPG	Lipophosphoglycan
PSG	Promastigote Secretory Gel
CL	Cutaneous Leishmaniasis
LCL	localised cutaneous leishmaniasis
PCR-ITS1	PCR-internal transcribed spacer 1
RFLP	Restriction Fragment Length Polymorphism
HIV	Human Immunodeficiency Virus
NO	Nitric oxid
CD8	Cluster of differentiation
PKDL	Post kala-azar Dermal Leishmaniasis
MCM	Micro-Culture Method
PBMC	Peripheral Blood Mononuclear Cells

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WBC	Weight Blood Cell
LFB	Lateral flow biosensors
IgG	Immunoglobulin
NK	Natural Killer Cells
DC	Dendritic Cell
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
iNOC	Inducible Nitric Oxide Synthase
Th	T helper cells
M-CSF	Macrophage Colony-Stimulating Factor
CSIF	Cytokine Synthesis Inhibiting Factor
MHCII	Major Histocompatibility Complex Class II
MP	Members Parliament
FcR	Fc Receptor
CR3	complement receptor 3
PKC	protein kinase C
MRP	multidrug resistance associated proteins
ERK1/2	signal-regulated kinases 1/ 2
JAK	Janus Kinase
SLA	Soluble Leishmania Antigen
CBA	cytometric bead array
PHA	phytohemagglutinin
mRNA	Messenger Ribonucleic Acid
SSG	sodium stibogluconate
MA	meglumine antimoniate
PM	injectable paromomycin
MF	miltefosine
AmpB	amphotericin B
LAMB	Liposomal AmpB
FDA	Food and Drug Administration
IDSA	Infectious Diseases
KMP-11	Kinetoplastid membrane protein 11
HASPB	Hydrophilic acylated surface protein B
EDTA	Ethylene diamine tetraacetic acid
RPMI	Roswell Park Memorial Institute
ROC	Receiver Operator Characteristic
PPV	positive predictive value
NPV	negative predictive value
FBS	Fetal bovine serum

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Chapter one

1.Introduction:

Leishmania is an obligate intracellular protozoan parasite that serves as the causative organism for leishmaniasis, a major cause of mortality and morbidity worldwide. The threat posed by leishmaniasis is significant particularly among economically weaker sections in tropical and subtropical regions (**Burza et al., 2018**).

Visceral leishmaniasis, one of the four types of the disease, is caused by *Leishmania donovani* and affects liver, spleen and bone marrow in humans , *Leishmania* gains access to the host cell as a consequence of the bite of the infected female sandfly (*Phlebotomus spp.*) during its blood meal from a host , In the course of its lifecycle , *Leishmania* exists in two distinct forms: (i) the flagellated extracellular promastigote form, and (ii) the amastigote form devoid of flagella that resides within host cells (**Chappuis et al ., 2007**).

The first step for *Leishmania* toward establishing infection is to cross the plasma membrane of the host cell by phagocytosis (**Kumar et al ., 2016**) . The process of internalization has been shown to involve receptors on the host plasma membrane that recognize cognate ligands on the parasite (**Ueno & Wilson , 2012**).These include the complement receptors CR1 and CR3, the fibronectin receptor.

An essential prerequisite for an intracellular pathogen to establish infection is to traverse the host plasma membrane (**Kumar et al ., 2016**). Internalization of extracellular matter into the cellular interior is a complex, multistep process involving a concerted interplay between a large number of membrane-associated cellular components. In this context, we previously explored the role of host membrane lipids such as cholesterol and the underlying actin cytoskeleton in the entry and infection of *Leishmania donovani* using multiple complementary approaches (**Kumar et al., 2016**) . The complexity involved in the process of internalization of pathogens is confounded by the multiplicity in pathways of phagocytosis exploited by the parasite. The major pathways

implicated in internalization include clathrin- and caveolin-mediated endocytosis (**Robinson , 2015**). Although there appears to be an inherent redundancy in the membrane receptors implicated in the entry of different pathogens into host cells (**Nish& Medzhitov, 2011**).

Biomarkers play a vital role in the aforementioned challenges by expanding the follow-up of patient information including immune status, determining patient response to treatment, as well as epidemiological feedback, exposure of humans and animals to vectors, and reservoir hosts model (**Ibarra-Meneses *et al.*, 2020**). Some macrophage-secreted cytokines are considered indirect biomarkers for leishmaniasis infection, including IFN- γ , TNF- α and IL-10 (**Kip *et al.*2015**). Recently, leishmaniasis investigation in humans has been associated with the detection of several biomarkers, including TNF- α , and/or other circulating proteins (**Bosch-Nicolau *et al.*2019**).

1-2.Aims of the study:

- 1-Epidemiological study of *L. donovani* in Al-Diwaniyah governorate.
- 2- prevalence of *L. donovani* in some governorates of Iraq.
- 3- Isolation the *L. donovani* in culture .
- 4- Molecular diagnosis of the *L. donovani*.
- 5- Determine the levels of some cytokines and studied as potential biomarkers of VL disease.

Chapter two

2. Literature view:

2.1. Overview of *Leishmania*:

Leishmaniasis is a neglected tropical disease NTD. More than one billion people are at risk of contracting this disease in all continents. This disease may debilitate many people and prevent them from living a decent life and preventing productive work properly (**Wamai *et al.*, 2020**). The physicians Leishman and Donovan were the first to describe the visceral leishmaniasis pathogen at 1903, it is found in stained smears from the spleen of infected patients who showed symptoms of a disease similar to malaria, and later they called it *Leishmania donovani* (**Bessat *et al.*, 2015**).

Leishmaniasis is a protozoan disease occurring in most tropical and subtropical countries worldwide and acquired through the bite of infected sand flies. The parasites causing is leishmaniasis may live and multiply in humans, domestic or stray dogs, and rodents, Multiple strains of the parasite exist, each of which may result in different disease manifestations (**Al-Kattan and Al-Hayali, 2021**).

The current epidemiological statistics indicate that there are 12-15 million cases of leishmaniasis around the world, one to two million new cases are recorded annually. Visceral leishmaniasis is estimated in 500 thousand new cases annually and that 70 thousand of these injuries lead to death (**Iddawela *et al.*, 2018**). *Leishmania* are sand fly-vector parasites that appear as intracellular amastigotes multiplies inside the phagolysosomes of mammalian and as extracellular flagellated promastigotes either in the culture or in the gut of the sand fly (**Rahi *et al.*, 2013**).

Leishmaniasis is becoming more common worldwide because of urbanization and vector distribution. Recently, some studies discussed the crucial role of travelling between endemic and non-endemic areas and the possibility of production of new *Leishmania* hybrid (**Sabzevari *et al.*, 2020**). Currently, the World Health Organization (WHO) recognizes leishmaniasis

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amongst the nine most important tropical and subtropical diseases occurring in all continents: Africa, Americas, Asia, and Europe (**Torres-Guerrero et al., 2017**). The disease transmission cycle for this group is very complex due to the many species of *Leishmania* parasite. circulating in different types of mammals by the bite of *Phlebotomies*. Humans are an incidental host and become infected when bitten by a female vector in searching for a blood (**Ovalle-Bracho et al., 2019**).

2.2. History of Leishmaniasis:

One of the earliest known epidemics of *L. donovani* infection (kala-azar as it was called in Hindi) was known in India just after the Indian Rebellion of 1857. The first medical record was however only in 1870 by British medical officers from Assam. In 1900, an English soldier stationed at Dum Dum, West Bengal, died at the Army Medical School in Netley, England cited by (**Roy, 1900**).

The autopsy was performed by William Boog Leishman. He processed the tissue sample of the enlarged spleen using a staining technique (now known as Leishman's stain) which he had just developed, and discovered the protozoan parasites using microscopy. But he mistakenly considered the parasites to be degenerate trypanosomes, already known protozoan parasites in Africa and South America (**Vincent, 2017**).

Another British medical officer Charles Donovan, who was serving in the Indian Medical Service, had found the parasites in April of which year at the Government General Hospital in Madras. After reading Leishman paper, Donovan confirmed on 17 June that the parasites (by then known as "Leishman bodies") were definitely the causative agents of Kala-azar. He wrote a commentary of his discovery in relation to that of Leishman in the same journal using the same title as that of Leishman's that appeared on 11 July 1903 (**American Society for Microbiology, 2014**).

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Soon a controversy arose as to whom such a monumental discovery should be credited. Donovan sent some of his slides to Ronald Ross, who was at the time in Liverpool, and to Alphonse Laveran at the Pasteur Institute in Paris. Laveran and his colleague Félix Mesnil identified the protozoan to be members of Piroplasmida, and gave the scientific name *Piroplasma donovani* cited by **(Roy, 1900)**.

Ross was resolved the conflict of priority in the discovery and correctly identified the species as member of the novel genus *Leishmania*. He gave the popular name "Leishman-Donovan bodies", and subsequently the valid binomial *Leishmania donovani*, thereby equally crediting the two rivals **(Dutta, 2008)**.

2.3. Classification of *leishmania*

Honigberg is considered the first to create a website to classify the parasite, which was amended in 1966. He developed a more precise classification in 1980, where modern methods of classification were identified, including serotype classification, enzyme analysis, and analysis of the magic story of DNA **(Al-Qadhi, 1995)**. The classification of the parasite depends on Sequence of the transcript of nickel-acid and isoenzyme **(Chappuis *et al.*, 2007)**.

Domain: Eukaryota

Kingdom: Protista

Subkingdom: Protozoa

Phylum: Sarcomastigophora

Subphylum: Mastigophora

Class: Zoomastigophora

Order: Kinetoplastida

Suborder: Trypanosomatina

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Family: Trypanosomatidae

Genus: *Leishmania*

The beginning of the classification of the *Leishmania* parasite was based on several criteria, including the parasite vector, clinical manifestations, antigenic characteristics, and geographical distribution (Annne-Laure 2008). Clear differences have emerged between *Leishmania spp.*, they cannot be distinguished based on their shape or appearance, as the diversity depends on several factors, including the difference in geographical distribution and behavior of the parasite in humans, the difference in the endemicity of the disease and the areas where it is found, as well as the type of animal that stores the parasite and its transmission by different types of insect (**Rassam and Al-Mudhaffar, 1997**). The genus *Leishmania* was classified into two subgenus Subgenera, one of which is *Leishmania* and the other is *Viannia*, based on evolutionary relationships (**Requena et al., 2012**).

2.4. Life cycle:

Visceral leishmaniasis (VL) is a vector-borne disease caused by replication of parasites in macrophages, mononuclear phagocytic system. The *Leishmania* has a digenetic complex life cycle with an extracellular developmental stage in the insect vector, a female *phlebotomine* sandfly (**Lievin-Le and Loiseau, 2016**) and a developmental stage in mammals, which is mostly intracellular. The parasite undergoes major morphological and physiological transformations during the different stages of its life cycle. *Phlebotomine* female sandflies are dipteran insects within the family of Psychodidae. The transmission of leishmaniasis is attributed to about 70 of around 1000 known sandfly species Those belonging to the genus *Lutzomyia* are prevalent in the New World and those belonging to the genus *Phlebotomus* are prevalent in the Old World.

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Phlebotomine sandflies are nocturnal, and in fact they become active at dusk when the temperature drops and humidity rises, and seek out dark and humid places that allow it to survive in hot and dry climates (**Murray et al., 2005**).

The *phlebotomine* sand fly acts as a vector for *Leishmania*, so it needs it not only to complete its life cycle, but also to reproduce.

There are many adaptations like secretion of phosphoglycans, which protect the parasite from digestive enzymes; production of chitinases that degrade the stomodeal valve of the sandfly, secretion of a neuropeptide that arrests midgut and hindgut peristalsis; and attaching to the midgut to avoid expulsion (**Kamhawi, 2006**). In sandflies, development of the parasite occurs in the alimentary canal with the formation of a motile, flagellated and elongated form termed as promastigote. The promastigote multiplies and matures in the insect midgut into an infective metacyclic form which migrates to its proboscis. When sandflies bite mammalian host for blood meal the metacyclic promastigotes reach the punctured wound (**Tripathi et al., 2007**). A typical inoculation contains about 100-1000 promastigotes. Once inside the host they quickly get phagocytised by leucocytes, particularly macrophages, neutrophils and dendritic cells. The parasites which are internalized by these cells through phagocytosis are trafficked to endosomes and lysosomes. The resulting phagolysosome, also called parasitophorous vacuole (PV), is an acidified and hydrolytically active compartment where the *Leishmania* is not only capable to survive but also to multiply (**Antoine et al., 1998**). Then they immediately undergo a further morphological change by shedding its flagellum and taking on an ovoid shape hence termed ‘amastigote’ and possibly a metabolic change with a switch to anaerobic metabolism under acidic conditions found chiefly in the phagolysosome compartment. The parasite’s persistence in such a hostile environment is attributed to its differentiation in amastigotes, which resist to macrophage hydrolases (**Desjardins and Descoteaux, 1997**). Phagocytic cells are ruptured by amastigotes to further infect other cells, thus

eventually damaging the whole reticuloendothelial system and to sustain the development (Sachdeva, 2016).

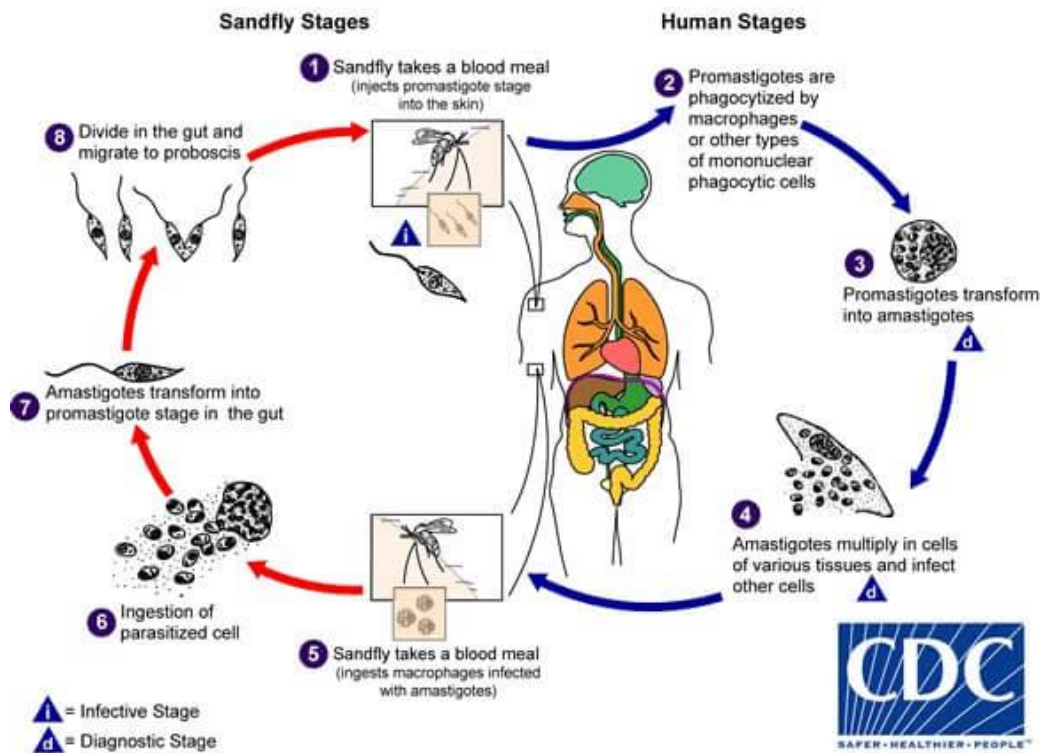


Image Source: DPDx – Laboratory Identification of Parasites of Public Health Concern

Figure (1): Schematic representation showing the life cycle of *Leishmania*

2.5. The Reservoir host:

There are two epidemiological types of leishmaniasis:

first: a zoonotic disease

The infection is transmitted from animals to humans by sand flies, and the animal is a reservoir host for disease

Second: Anthroponotic disease

The infection is transmitted from one person to another through the sand fly, and the person is a reservoir host for the disease (WHO, 1996). The reservoir host varies according to regions and their differences.

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Humans are the reservoir host of the disease in India and Bangladesh, while wild and domestic dogs are reservoirs of the disease in Jordan, Tunisia, Algeria, Libya, Iran, and in some countries such as Yugoslavia, rodents are also the reservoir of the disease, as domestic rats play a role to maintain *Leishmania* (Anderson *et al.*, 1989) also to maintain the transmission cycle of *Leishmania* (Lemma *et al.*, 2017).

Leishmania infantum has been isolated from the blood of bats, although there are few studies conducted on flying animals to confirm that they are a reservoir host (Kassahun *et al.*, 2015; Berzunza-Cruz *et al.*, 2015) (Table 1).

(Table 1) Reservoir hosts of human leishmaniasis in some endemic countries (Alemayehu and Alemayehu, 2017).

Region	Countries	Reservoir hosts
Old world	North Africa, central and west Asia	Dog, human, rodent
	Ethiopia, Kenya	Rodents, dog, domestic animals, bats, human, rock hyrax
	Indian subcontinent, (India, Nepal, Bangladesh) and east Africa	Dog, human, rock hyrax, rodent
	Mediterranean basin, central, west Asia and west Africa	Dog, fox, rodent, human
	Europe	Dog, fox
New world	Argentina, Belize, Bolivia, Brazil, Colombia, Costa Rica, Dominican, Ecuador, El Salvador, French Guyana, Guadeloupe, Guatemala, Guyana, Honduras, Martinique, Mexico, Nicaragua, USA, Venezuela, Paraguay, Peru, Surinam, Panama	Dog, cats, rodent, marsupials, anteater, fox, monkey, coati, sloth, armadillo, porcupines, kinkajou, raccoon, red squirrel

2.6. The vectors

The sand fly is the main vector of the *Leishmania* parasite. This insect belongs to the order *Diptera*, family *Psychodidae*, subfamily *Phlebotominae*, genus *Phlebotomus* in the Old World and the genus *Lutzomyia* in the New World (**Kilic et al, 2008**). In 1942 a researcher **Bern and Chowdhury(2006)** demonstrated the transmission of visceral leishmaniasis by *Phlebotomus argentipes* to a human volunteer in South Asia.

The sand fly insect is characterized by small size, which ranges between 2-3 mm. The body and wings are covered with thick hair, and the mouth parts have blade-like incisor organs. It can't fly long distances, life cycle takes 5-9 weeks, females feed on the blood of vertebrate animals and humans, while males on plant sap (**Rutledge and Gupta 2009**). When a sand fly feeds on the blood of a vertebrate host, the insect secretes its saliva into the host's skin. This saliva contains anticoagulants and compounds that dilate blood vessels that increase blood bleeding (**Charlab et al., 1999**). 14 species of sand flies were recorded in Iraq, and only two species were found that feed on human blood, *Phlebotomus papatasi* and *P. alexandri* (**Abu-Al-Hub and Al-Hashimi, 1988**).

17 species of the genus *Phlebotomus* were also recorded, the most widespread of which is *P. paptasi*. This species is considered the most important from a medical standpoint and is the appropriate carrier of visceral leishmaniasis in Iraq (**Sukkar, 1985**)



Figure(2) shows a sand fly insect (<https://www.saudiwildlife.com>)

2.7. Epidemiology

2.7.1.of *Leishmania donovani* in the world:

The epidemiology of leishmaniasis is dynamic, and the disease is reappearing and spreading in many regions (**Dujardin and Decuypere, 2013**). There is an estimated 0.5 new cases of visceral leishmaniasis and annual deaths amount to 59,000 cases of the same disease, and it is the second disease after malaria in deaths worldwide due to infection with the parasites elementary (**Solano - Gallego et al., 2011**) . Leishmaniasis has spread widely throughout the world, spreading to approximately 82 to 88 countries (**Molyneux, 2006**). Statistics indicate that there are 12 million cases in the world includes two million new cases recorded annually and is divided into 0.5 million cases of visceral leishmaniasis and 1.5 million cases of cutaneous leishmaniasis (**Markle and Makhoul, 2004**).

Murray (2005) noted that about half of the cases of visceral leishmaniasis of the world is in India, especially in the eastern parts, while the cutaneous form

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is widespread in Western parts and the most important health problem in Sudan is the spread of black fever, especially in the central and eastern regions (**Zijlstra and El-Hassan, 2001**). Approximately 90% of black fever cases are found in India, Bangladesh, Brazil, Nepal and Sudan (**WHO, 2010**).

2.7.2. Epidemiology of *Leishmania donovani* in the Iraq:

Sukkar (1974) recorded 261 cases in the central regions of Iraq, most of them in rural areas. He explained that the disease spread widely among the poor classes, Reports at the Center for Communicable diseases and the Institute for endemic diseases in Baghdad recorded an increase in cases of black fever during the period (1971-1988). Then infections began to decline during the period (1995-1989), and the annual report stated the Ministry of Health in 2000 that the increase in infections over the years, which caused a serious health problem in the country, led to conducting a number of research and studies on this disease and finding out the best ways to control it, as well as the effect of the presence of dogs, rodents and animals and others on the spread of this disease (**Abdel-Nabi et al., 2009**).

2.8. Pathogenicity of *leishmania donovani*

The process of the parasite's effect on the host occurs after the anterior flagellar form of the *Leishmania* parasite is introduced into the vertebrate host through the bite of a female sand fly, and the flagellar form connects or comes into contact with phagocytic cells spread under the skin **Vidyashankar (2002)** hypothesized that the mechanism of flagellar association with the phagocytic cell depends on the presence of two surface molecules. Lipophosphoglycans in the parasite play a prominent role in the attachment process, and it appears that the outcome of infection with the parasite depends

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on a complex interaction between the severity of the parasite infection and the host's immune response, the incubation period of the disease takes several months, but usually may last up to ten years. **Vidyashankar (2002)** the infected macrophage cells spread the disease to all parts of the body, depending on the type of leishmaniasis and the disease it causes. These infected cells explode, releasing parasites that can reach to the liver, spleen, and bone marrow, visceral leishmaniasis occurs, which is characterized by symptoms that begin with intermittent fever and chills that last for several weeks. This disease often goes dormant or is suspected with *Malaria* and the patient also suffers from emaciation and fever Hepatosplenomegale, as well as an enlarged abdomen (**Bern and Chowdhury, 2006; Kilic et al., 2008; Orsini et al., 2012**).

The severity of the disease varies depending on the immune status of the host and the type of parasitic infection that may remain confined to the skin in the case of cutaneous leishmaniasis Known types or it infects the mucous membranes of the mouth and nose in the case of mucocutaneous leishmaniasis or it spreads to the reticular lining systems as in the case of visceral leishmaniasis (**Grevelink and Lerner, 1996**). The most important changes that occur in the blood due to visceral leishmaniasis are a decrease in the number of white blood cells. This is due to a decrease in granulocytic white blood cells (WBC) and a decrease in the number of red blood cells with a decrease in hemoglobin and an increase in the rate of erythrocyte sedimentation (ESR) and an increase in the ability to break down red blood cells (RBC), as well as a decrease in platelets (PLT). In advanced cases of infection, a defect in liver function occurs as a result of the parasite infecting most liver cells, which leads to a decrease in the production of prothrombin, thus Severe bleeding in the mucous membranes ((**Brito et al., 1975**). There are other changes from low levels of albumin in the blood to high levels of immunoglobulin such as IgG (**Al-Orainey et al., 1994**).

2.9. Visceral leishmaniosis Kala-azar (black fever):

The most dangerous form of leishmaniasis is called Kala-azar. Infection is caused by the parasite *L.infantum*, in the Old World, infecting millions of people around the world (**Bhattacharyya et al., 2014**). The main cause of infection in South America is the parasite *L. chagasi*. The reservoir hosts for this parasite are dogs (**Lainson,1997**). Infection with this type is called black fever or kala-azar, and it is called Dum Dum fever. It is one of the fatal diseases (**WHO, 2004**). Its incubation period is from (3-6) months and sometimes from months to years. It affects the internal organs, especially the spleen and liver. and bone marrow, and in this way, it differs from the cutaneous Leishmaniasis (**WHO, 2000**). (**WHO, 2004**) explained that this disease is a fatal and lethal disease, and its symptoms are irregular fever, loss of appetite and weight loss, in addition to Hepatosplenomegaly and the result of the enlargement occurring in both organs leads to Abdominal bloating. It is also accompanied by cough, diarrhea and a deficiency of white blood cells (leukopenia), in addition to the previous symptoms, as mentioned by (**Vidyashankar, 2002**), as well as hair loss and the skin in the area of the hands, feet and abdomen turning dark, which is why it is called black fever. The patient ends up dying within (6-24) months due to liver collapse. And the entire spleen (**WHO, 2002**). (**Laurenti et al., 2013**) indicated that this disease was endemic in Iraq, especially in Baghdad Governorate, in 1991, and it spread to other governorates (Dhi Qar, Maysan, and Basra).

The moderate infections usually self-heals and often if untreated is fatal, secondary bacterial infection usually causes the mortality additionally to immunosuppression, hemorrhage and severe anemia (**Khan et al., 2015**).

In some cases, Post Kala-azar Dermal Leishmaniasis (PKDL) a skin condition that occurs after treatment of visceral leishmaniasis due to *Leishmania donovani*. The disease characterized by patches of pustules or a

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nodule-like rash, especially around the mouth. PKDL generally seen in Africa and India as a late complication of visceral to *Leishmania donovani* and infrequently *Leishmania infantum* (Zijlstra, 2016).

PKDL develops after 6 months and occasionally more than 5 years next the incidence of previous visceral, about 15% of cases do not show the prior Kala-azar infection. It can diagnosis via slit-skin smears, culture or polymerase chain reaction. The clinical appearance is papular, macular or nodular lesions (Gedda *et al.*, 2020).

2.10. Diagnosis

The diagnosis of visceral leishmaniasis is complex from a clinical standpoint because it is similar to and overlaps with a group of other diseases that commonly occur, such as malaria, typhoid fever, and tuberculosis. Many of these diseases can be present along with visceral leishmaniasis, so that it is not possible to be certain that the person is infected. With black fever, these diseases also affect the liver, spleen, bone marrow, and lymph nodes, which are the same organs that are affected by black fever, thus increasing the complexity of the diagnosis process (Shyam and Rai, 2002).

The initial diagnosis of the disease is based on the clinical symptoms of the disease, which are characterized by fever and involvement of the liver and spleen, loss of appetite, cough, weight loss, anemia, and low blood cell counts white blood cells and platelets, an increase in immunoglobulin, and a decrease in albumin in the blood (Berman, 1997).

There are many different tests with different specificities and sensitivities available to diagnose visceral leishmaniasis, but later became less popular in regions the disease is endemic, and that a small number of them are not commercially available, or in general, they are expensive and require people with experience and skill, as well as expensive equipment, and require a high-tech laboratory to diagnose visceral leishmaniasis can be summarized as

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follows: (a) Viewing the parasite using an optical microscope during the histological examination process or causing experimental infection in animals or what is called inoculation animal (b) Detection of the DNA of parasites found in the infected person (c) Immunological diagnosis through the detection of antibodies in tissues, blood, or urine sample by specific antigens from the *Leishmania* parasite **(Shyam and Rai, 2002)**.

The method of diagnosing visceral leishmaniasis is usually by observing the parasites in the spleen or bone marrow, and aspirating the parasite is done by aspirating the spleen and bone marrow or by testing skin, but the usual test is always the direct agglutination test (Deoxyribonucleic acid), and these tests can only be performed in a small number of central laboratories, and people have been equipped and trained for this purpose **(Shyam and Rai, 2002)**, It has provided test development polymerase chain reaction (PCR) is an effective method for applying molecular biology techniques in diagnosing leishmaniasis in the early years, the PCR test established diagnostic methods with a high range of sensitivity and specificity that have a high ability to detect the pathogen **(Adhya et al., 1995; Andresen et al., 1997)**. Many serological techniques are based on the detection of antibodies, and the specificity of each antibody depends on the antigen represented by the presence of the parasite, and the latter is what stimulates the infection producing a wide range of antibodies that target or attack genus or species of various diseases, including visceral leishmaniasis. Therefore, allergies can It depends on the test or method of action, but the specificity depends on the antigen rather than the serological method of action used in the test. In most clinical tests, sensitivity and specificity data are compared against parasites in different tissues traditional methods for detecting antibodies also include gel diffusion and complement fixation complement fixation, indirect hemagglutination test, IFAT test, and immunoelectrophoresis test **(WHO, 1996; Albrecht, 1998)**

2.10.1. Immunochromatographic strip test rK-39

The current diagnostic test is known by several names, including the rapid diagnostic test, the rK-39 test, or the Dipstick test. This test includes detecting antibodies using the rK-39 antigen protein for the *Leishmania* parasite. The rK-39 strip test has been developed for use with serum for people who are likely to be infected but the test is routinely done with blood at the community level in affected countries because there are no facilities to extract serum from blood (**Matlashewski et al., 2013**). This test is one of the modern and rapid experiments to diagnose visceral leishmaniasis using the rK39 antigen, the latter of which consists of 10 amino acids, as these acids represent the main component of the parasite's protein, and the interaction occurs between the antibodies in the patient's serum and the antigens fixed on the rK39 test strip (**Singh et al., 1995**). In this test, a strip composed of a cellulose membrane was used, consisting of two parts or lines. The first line is known as the test line and is covered with the rK39 *leishmania* antigen. The second line is known as the control line and is covered with chicken protein A, which binds to a reagent dye. When the strip is exposed to the sample to be examined Serum, for example (a connection occurs between the components of the blood and the dye, and as a result of the capillary action, the contents of the serum will move upward until it reaches the test line containing the antigen. If the serum contains specific antibodies, they react with the rK39 antigen, giving a red line on the strip due to the reagent dye that adsorbs with Immune complexes This represents a positive result for the examination and the color appears in varying degrees depending on the concentration of antibodies in the serum. Patients' urine is also used to investigate the presence of antibodies against the parasite antigen. (**Sarkari et al., 2008**). This test has high sensitivity and specificity in South Asia, but less sensitivity in East Africa, which may be one of the underlying reasons for the difference in a specific continent due to the molecular diversity of the rK39 antigen within the

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visceral leishmaniasis *L.donovani* complex. However, the second reason may be the difference in the levels of IgG antibodies. Against *Leishmania* parasite in patients with Kala-azar who belong to different geographical areas either due to antigenic changes or immune response (**Bhattacharyya *et al.*, 2014**) .

2.10.2. Culture:

The culture is performed through the inoculation of the triturated tissue in special media. *Leishmania* is cultured in Novy–McNeal–Nicolle medium (currently called “N-N-N medium”) or in a variant of the Evans biphasic medium (**Murray *et al.*, 2005**). In the first culture, forms devoid of flagella are almost always obtained, but from the first re-seeding onwards flagellated forms appear which reach up to 50 µm long. Both techniques (microscopic exam and culture) have a sensitivity of 85% (**Ramírez *et al.*, 2000**).

2.10.3. The culture media:

Novy-MacNeal-Nicolle (NNN) medium:

It is a specialized culture medium used for the cultivation of *Leishmania* parasites. It provides an environment rich in nutrients and supportive conditions for the growth of these parasites. The medium is particularly designed to simulate the conditions found in the midgut of sandflies, which are the natural vectors for *Leishmania*. NNN medium typically contains a blood agar base. Agar is used as a solidifying agent, and the base provides a foundation for the medium. The medium includes blood, usually from a mammalian host, to provide essential nutrients for the *Leishmania* parasites. Rabbit blood is commonly used. Antibiotics are often added to the NNN medium to prevent the growth of bacteria and fungi that could compete with or contaminate the *Leishmania* culture. The medium is often overlaid with a liquid phase to create a semi-solid environment. This mimics the conditions in the midgut of sandflies, where *Leishmania* parasites naturally reside. The pH of the NNN medium is buffered to maintain a stable and suitable environment

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for the growth of *Leishmania*. The pH is typically kept around 7.2 to 7.4 (**Dos Santos *et al.*, 2018**).

2.11. Immune response to *Leishmania* parasite:

The successful elimination of *Leishmania* parasites from an infected host relies on the coordination of different key players of the host immune system. As the promastigotes enter into the blood stream following a sand fly bite, until the amastigotes reside within the macrophages, the tussle between the eradication of parasites and establishment of disease will be somewhat decided by the ability of *Leishmania* to modulate and/or evade host immune defenses. The components of host defense that are necessary for parasite clearance include elements of the innate and adaptive immune systems (**Ikeogu *et al.*, 2020**).

2.11.1 Cytokines:

The term Cytokines is of Greek origin (Cyto means cell and Kines means movement) they are soluble extracellular protein signals that work to transmit instructions to other cells they act in several ways either paracrine, autocrine, juxtacrine, endocrine, or Intacrine through its interaction with its receptors (**Balkwill, 2000**), cytokines affect cells that function in a paracrine manner or act as endocrine glands by affecting the cell itself autocrine, cytokines are considered the backbone of the immune system because they participate in cell growth and activity as they work to attract cells to the site of infection, and not all of them are considered peptides pleiotropic regulatory effect, which is secreted by nucleated cells and has an influential role on other hematopoietic cells and participates in repair processes (**Mahajan and Mehata, 2006**).

(**Kumar and Parkash, 2011**) confirmed that cytokines are a group of multiple proteins produced by several different cells in the body in response

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to injury to the target cells are exposed, it is necessary to estimate the level of cytokines to understand the body's immune reaction, know the immune status of the infected person, and understand the severity of the disease.

Cytokinesis consists of ten subgroups:

Lymphokines, interferons, colony stimulating factor, interleukins, polypeptides, tumor necrosis factor, transforming growth factor, B-chemokines- Lymphokines, interferons, colony stimulating factor, interleukins, polypeptides, tumor necrosis factor, transforming growth factor, a-chemokines-alpha, b-chemokines -beta, and stress proteins, but they are not considered hormones, growth factors, or neurotransmitters, but rather similar to them, as their work is not limited to the immune system only. They also participate in communication between cells in the stages of embryonic development (**Kumar and Parkash, 2011**).

2.11.2. Interleukins:

They are soluble mediators that control many vital processes in the immune system of immune cells (**Oppenheim & Ruscetti, 2001**). There are more than 25 types of them, as they branch from a large family of proteins that call for cytokinesis (**Coico , 2003**).

2.11.2.1. Interleukin 2 (IL-2):

Interferon-gamma is used to control visceral leishmaniasis (VL) caused by *Leishmania donovani* anti parasitic CD4+ T-cell responses in VL patients are ineffective for unknown reasons. GATA3, RORC, and FOXP3 showed low gene expression in VL patients, associated with reduced Th2, and Th17 in VL patient blood.

IL-2 is an important regulator of CD4+ T cells in VL patients, and studies have shown the possibility of using IL-2 as a treatment to improve immunity against parasites (**Chauhan et al., 2019**).

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Sera from nine Sicilian patients with confirmed visceral leishmaniasis showed that IL-2 is a marker of disease activity (**Vitale et al.,1992**), since it is in high concentration at the beginning of infection and returns to the normal range following successful chemotherapy. At the same time of serum analysis for IL-2 of VL patients were stimulated with phytohaemagglutinin. IL-2 may be necessary for both the production of IL-4 and differentiation of Th2 cells, the possible contribution of IL-2 to progressive infection was examined. Four weekly injections of anti-IL-2 cured more than 80% of BALB/c mice infected with *L. maior*, as determined by diminished footpad swelling and decreased numbers of parasites in infected tissues (**Heinzel et al., 1993**). Anti-IL-2R demonstrated a similar protective effect when administered twice weekly for 4 wk. Anti-IL-2-mediated cure of cutaneous leishmaniasis was associated with increased IFN-gamma and decreased IL-4 production by regional lymph node cells compared to untreated BALB/c mice with progressive illness. IL-2, IL-4, IL-6, IL-10, IL-17, IFN- γ , TNF- α , and IL-1 β were investigated in 75 *Leishmania*-positive blood donors living in endemic areas. Significant differences were found for all the cytokines evaluated, with lower concentrations in consistently *Leishmania*-negative individuals. The exception was IFN- γ , with similar levels among all donors. No changes consistent with active disease were observed in the laboratory results for *Leishmania*-positive donors who underwent clinical evaluation, none of whom progressed to disease (**França et al., 2020**).

2.11. 2 .2. Interleukin 10 (IL-10):

Interleukin (IL-10) was first described as a cytokine synthesis inhibiting factor (CSIF); This is because it is able to suppress cellular motility produced by the main T cells (**Fiorentino et al., 1989**), which is a homodimer protein), consisting of 160 amino acids (Opal and De Palo, 2000) It has a receptor composed of two subunits, namely the α -chain (IL10-R1) and the beta chain (IL-10R2) (**Wolk and Sabat, 2006**), as it is produced by a group of diverse

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cells, including B cells (**Fillatreau et al., 2002**) (B-cell), and T cells, especially regulatory (T-reg), as well as T-cell subsets (T-subset) (**Ouyang et al., 2011**), in the beginning T helper type 2 cells (Th2). It produces interleukin (IL-10) (**Gazzinelli et al., 1992**), but it has recently been shown to be produced by T helper type 1 (Th1) cells (**O'Garra and Vieira, 2007**). It is also produced by monocytes and macrophages (**Edward et al., 2006**), and dendritic cells (DCs) (**McGuirk et al., 2002**), it is an anti-inflammatory cytokine with an immune regulatory function for various immune cells (**Saraiva and O'Garra, 2010**), effectively preventing the production of a number of cytokines' cellular activity stimulated by the activation of macrophages and monocytes, as it often has synergistic activities on pro-inflammatory pathways. In addition, it prevents the production of chemokines, which participate in inflammation. This is through the recruitment of monocytes and neutrophils, as well as T cells and dendritic cells (**Moore et al., 2001**). In addition to inhibiting the production of cytokines, interleukin (IL-10) inhibits the expression of the major histocompatibility complex class II (MHCII) and monocyte adhesion molecules, and thus it contributes to suppressing the immune response of Th1 helper cells (**deWalle Malefyt et al., 1991**).

2.11.2. 3 Interleukin 12 (IL-12):

The predominant Th1 cytokine that drives IFN- γ mediated cell activation necessary to control intracellular organisms like *Leishmania*. *Leishmania* entry into host cells is receptor mediated. These parasites are able to engage numerous cell surface receptors (1), including scavenger receptor (CD163) (**Schonlau et al., 2000**). The mannose receptor (2), Toll-like receptors 2, 3, and 4, Fc receptors (FcR) and complement receptor 3 (CR3, Mac-1, CD11b/CD18). *Leishmania* can bind CR3 directly as well as through C3bi deposition on the parasite surface. Utilization of CR3 may allow *Leishmania*

to evade innate immune responses such as production of reactive oxygen intermediates (**Balestieri et al., 2002**).

Interestingly, ligation of CR3 via antibodies in the absence of any infection has been shown to inhibit interleukin-12 (IL-12) production and suppression of T helper 1 immunity. *Leishmania* circumvent both innate and adaptive immunity through CR3 engagement. This model of receptor engagement to down-modulate host IL-12 mediated immunity may account for similar effects on MP function following infection with such diverse pathogens as viruses (**Butcher, and Denkers, 2002; Xu, et al., 2001**).

2.11.2. 4. TGF-beta (TGF-b):

The transforming growth factor plays an important role in development and chronicity of CL lesions at least in animal models of *Leishmania* infection. TGF- β is potent immunosuppressive cytokines, which act via distinct pathways to modulate excessive immune responses and immunopathology in allergy, autoimmunity, and infectious disease. TGF- β have inhibitory effects on macro- phages which are the main targets of *Leishmania*, reduce their ability to kill the parasite and their antigen presentation to effector T cells. It regulates effector T cells directly via inhibiting their proliferation and cytokine production. Secretion of TGF- β is important mechanisms of regulatory Tcell mediated immune suppression. Several studies on new world CL have revealed that TGF- β are associated with chronic forms of the disease or long-lasting atypical lesions (**Hejazi et al., 2012**).

Gamma-interferon are produced by T helper-1 cells, which dominate cell-mediated immunity, usually increases the host ability to resist disease while susceptibility and disease progression are mostly associated by the T helper-2 type of immune response. *Leishmania* and regardless of the different clinical presentations in each, resistance as well as recovery are linked to Th1 type of

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response dominance while case deterioration is linked to Th2 type of response dominance.⁵ In addition, suppression of cellular immunity (Th1 response) that results in a negative *leishmanin* skin test is a major characteristic of visceral leishmaniasis. Intracellular killing of *leishmania* parasites by interferon gamma-activated macrophages is the main mechanism for control of visceral leishmaniasis by both adaptive and innate immune responses (Elmekki *et al.*, 2016).

Chapter three

CHAPTER THREE Material Methods

3.Materials and methods

3.1. Materials

3.1.1. Equipments and Instruments

Table (3-1): The equipments and Instruments that used in this study with their companies and countries of origin:

No.	Equipment & instrument	Company
1	Autoclave	(Taiwan)
2	Camera	Samsung /China
3	Centrifuge	Hitachi (Japan)
4	Cover slides	Sail Brand (China)
5	Deep freeze Sturdy	Forma scientific
6	Disposable syringe 10 ml	Sterile EO / China
7	Distilled water	Iraq (Iraq)
8	EDTA tube	Jinsha (China)
9	Electrophoresis	Bioneer/ Korea
10	Eppendorf tubes	Bioneer/ Korea
11	Exispin vortex centrifuge	Bioneer/ Korea
12	Gel tube	Jinsha (China)
13	Glass wares(beakers)	Hysil (U.K)
14	Coplin jar	Glasswerks (U.K)
15	High Speed Cold centrifuge	Eppendorf /Germany
16	Hot plate	Stuart (England)
17	Incubator	Memmert (Germany)
18	Light microscope	Olympus (Japan)
19	Micropipettes 5-50, 0.5-10, 100-1000µl	Eppendorf/ Germany
20	Micro-plate washer	BioTeK (U.S.A.)
21	Micro-titer plate reader (450 nm filter)	BioTeK (U.S.A.)
22	Nanodrop	THERMO/ USA
23	Oven	Memmert/Germany
24	Plastic containers	Kaeaho (Russia)
25	Printer	Epson (U.S.A)

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26	Refrigerator	Concord /Lebanon
27	Sensitive Balance	Sartorius/Germany
28	Slides	Sail Brand (China)
29	Thermocycler PCR	BioRad /USA
30	Tip filter (different volumes)	Capp (U.S.A.)
31	Vortex	CYAN/ Belgium
32	Water Bath	Memmert/Germany
33	Wooden sticks	Superme (China)

3.1.2: Chemicals

Table (3-2): the chemicals with their companies and countries of origin used in this study:

No.	Chemical	Company and Origin
1	10X TBE buffer	BIO BASIC INC/ USA
2	Absolute ethanol (96%)	Himedia (India)
3	Agarose	Promega (USA)
4	DNA ladder (100bp)	Biolabs/ UK
5	Ethidium bromide 10mg/ml	BIO BASIC INC/ USA
6	Giemsa stain	Jourilabs (Switzerland)
7	Nuclease free water	Bioneer/ Korea

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3.1.1.Kits

Table (3-3): The kits used in this study with their companies and countries of origin:

No.	Kit	Company	Country
1	gSYAN DNA Extraction Kit	Geneaid	Taiwan
	GST buffer		
	GSB buffer		
	W1 buffer		
	Wash buffer		
	Elution buffer		
	GD column		
	Collection tube 2ml		
	Proteinase K 11mg		
2	Maxime PCR PreMix	iNtRON	Korea
	Taq DNA polymerase		

3.1.4.Primers

The PCR primers for confirmative detection of *Leishmania donovani* based kinetoplast DNA minicircle was designed in this study using NCBI-Genbank sequence (AF129739.1) and primer 3 plus and these primers were provided by (Macrogen company, Korea) as following table:

Primer	Sequence 5'-3'		PCR product size
KDNA <i>Leishmania donovani</i>	F	ATGAACGGGGTTTCTGCACT	562bp
	R	TGGTTGGTGATGTTGTTTGGG	

3.2.Methods

The study included (332) samples, and it dealt with two axes, the first of which was the epidemiological study in Al-Diwaniyah Governorate. Among the participants were 276 people, and 18 of them were detected infected through the rapid screening test (I.C.T). The second axis was a study of 56 cases collected from different hospitals in Iraq, who were diagnosed through two laboratory tests that included a rapid immunological examination and an indirect fluorescent antibody test (IFAT). In addition to the clinical signs and symptoms, which were evaluated by the consulting medical staff and included in most cases: the presence of long-lasting intermittent fever, large abdominal size, loss of appetite, splenomegaly, hepatomegaly, loss of weight and appetite, pale and darkening of the skin, jaundice, anemia, Cough, fatigue, vomiting). And (60) healthy people not infected with visceral leishmaniasis, aged between (9-65) years, during the time period extending from March (2023) to December (2023).

3.2.1. Blood sample collection

Three hundred thirty- two (332) blood samples were collected, including 56 samples collected from various Iraqi hospitals that were diagnosed by a specialist doctor, and (276) random blood samples from Al-Diwaniyah Governorate that were examined using an immunochromatography test (I.C.T). A venous blood sample was drawn from each patient suspected of having visceral leishmaniasis using a 5 ml medical syringe and placed in tubes containing the anticoagulant EDTA, then placed in gel tubes for the purpose of blood separation using a centrifuge (3000 rpm), where isolated. The serum was distributed into four Eppendorf tubes according to the number of immunological tests conducted in the study, and the samples were stored at (-20C°) until use.

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3.3. Reservoir Hosts

Samples of reservoir hosts were collected from domestic dogs from Al-Diwaniyah Governorate. 24 blood samples were drawn. An amount of 5 ml of blood from a vein in the sub-axillary region of the forelimbs of domestic dogs after sterilizing them with a 60% iodine solution and after anesthetizing them with (80% formalin) and under the supervision of a veterinarian, a selection was made. These dogs showed signs and symptoms of visceral disease, including swollen lymph nodes and ocular signs, as well as weight loss and loss of appetite (progressive loss of weight with decreased appetite), with bloody nosebleeds (epistaxis), kidney failure, increased urination and drinking, and an increase in urination and drinking water. . They were obtained in the backyards of homes by anesthetizing them with anesthetic releases, or through a special fishing net, or through anesthetic baits. Samples were diagnosed as follows:

3.3.1. Immunological examination of dogs:

- 1- Blood was drawn from infected dogs using a clean, sterile wine syringe with a capacity of 5 ml and placed in tubes containing EDTA.
- 2- The samples were stored in the refrigerator at 4°C until use.
- 3- The blood is then separated through a centrifuge For 5-10 minutes.
- 4- Taking serum samples

Then the Dipstick rk39 assay was used directly.

3.4. Bone marrow

A bone marrow sample of 2 ml was drawn from a person infected with visceral leishmaniasis from the pelvic bone, using a special needle for extraction by the specialist doctor. Then, bone marrow smears were prepared according to the method of **Johan and Petri (2006)** according to the following steps:

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- 1- Applying 20 microliters of bone marrow. On a clean glass slide at an angle of 45 degrees, make a thin smear, then leave the swab to dry at room temperature.
- 2-The slides were immersed in methyl alcohol for (2-3) minutes and left to dry at room temperature.
- 3- The slides were stained using Giemsa stain.
- 4- The slides were washed with water (tap water) and left to dry.
- 5- The slides were examined using an optical microscope and with an oil lens under 100X power to detect the parasite

3.4.1.Source of isolates

Visceral *Leishmania donovani* isolates were Obtained from the Yarmouk teaching Hospital in Baghdad .the media were inoculated with samples taken from bone marrow and incubated at a temperature of (25-27) °C for a period ranging from two to four weeks. Then the medium was monitored every four days, and the process was repeated for 4 weeks, where a sample of the medium was taken, stained with Gimsa staine, and examined under a microscope to confirm the presence of promastigotes. To indicate positive growth, the stages appeared in intertwined clusters in the middle. The bottles were kept in a refrigerated incubator at a temperature of (25-27) °C.

3.5. Culture Media:**3.5.1. Media for parasite culture****3.5.1.1. Biphasic medium (Nove-MacNeal-Nicolle) (NNN):**

The biphasic medium was prepared and used for maintaining and cultivating *Leishmania* parasites, *in vitro*. It is usually made up of two phases according to **Kagan and Norman (1970)**:

1. Solid Phase

Solid blood agar phase consists of the following ingredients for 1liter final volume:

Table(3-4) Ingredients per litre

Chemical	Weight/Solution
Brain heart infusion	33.3 g
Agar	16 g
D-glucose	8 g
Defibrinated rabbit blood	100 g
Penicillin / Streptomycin	50µg/ ml
Distilled water	900 ml

Solid Phase preparation

- 1.All constituents except blood and antibiotics (Penicillin / Streptomycin) were dissolved in distilled water, pH adjusted to 7.4 and the solution was autoclaved at 121C° for 20 minutes.
2. After cooling, rabbit blood and antibiotic (Penicillin / Streptomycin) were added and mixed well.
3. Twenty ml of the medium were transferred under aseptic condition to sterile universal tubes of 20 ml volume and were put in a slanted position to obtain a large surface area for growth.

2. Locke's solution

Consists of the following ingredients, pH was adjusted to 7.0:

Table 3-5: ingredients Locke's solution

Chemical	Weight / Solution
NaCl	9 g
KCl	0.42g
CaCl ₂ 2 H ₂ O	0.32 g
NaHCO ₃	0.2 g
D-glucose	2 g
Gentamicin	50µg/ ml
Sulphate	200 g
Distilled water	1000 ml

Locke's solution Preparation

The ingredients were mixed together in a screw-capped bottle of 1000 ml, sterilized by autoclaving at 121 °C for 15 minutes, left to cool before the antibiotic (Penicillin / Streptomycin) was added.

3.5.1.2.Preparation of blood added to the culture medium

10 ml of blood was drawn from the prepared rabbit's heart with a syringe, and it was transferred under sterile conditions into sterile glass bottles containing small glass balls. The bottles were shaken circularly for three minutes to separate the fibrin, then distilled water and antibiotics were added to the machine and left at a temperature of (4°C) for a period of time. 24 hours, then distributed into sterile test tubes and separated by centrifuge at 3000 rpm for 15 minutes. The sediment was discarded and the supernatant was kept at a temperature of (-20°C) until use.

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3.5.1.3. Roswell Park Memorial Institute (RPMI-1640) medium

Roswell Park Memorial Institute (RPMI) media are a series of media developed by **Minowada *et al.* (1972)** for the culture of human normal and neoplastic cells, *in vitro*. RPMI 1640 is the most commonly used medium in the series. A modification of McCoy's 5A medium, the medium was specifically designed to support the growth of human lymphoblastoid cells in suspension culture. Presently, the medium is extensively used for a wide range of anchorage dependent cell lines.

The medium needs to be supplemented with 5-20% FBS. The medium is also known to support the growth of cells in the absence of serum.

Media preparation

This media was purchased from (HiMedia Laboratories, India) and prepared according to the manufacturer's procedure:

1. A 5.2 gram powder was suspended in 450 ml tissue culture grade water and stirred constantly and gently until completely dissolved.
2. The pH was corrected to 4.0 with 1N HCL if the powder did not dissolve fully.
3. Before adding sodium bicarbonate, the pH was increased to 7.2 using 1N NaOH after it had completely dissolved. Sodium bicarbonate was added of 0.2gm for 1 liter of medium and stir until dissolved.
4. The pH rises during filtration, because the pH was reduced to 0.2-0.3 units below the target pH using 1N HCl or 1N NaOH.
5. Using pure water, the final volume was measured to be 450 ml.
6. Medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron, using positive pressure rather than vacuum to minimize the loss of carbon dioxide.
7. Fetal bovine serum of 50 ml and 100 IU/ml of penicillin and 100 µg/ ml of streptomycin were added to the filtrated mixture in step 6.
8. Liquid medium stored at 2-8°C and in dark for regular use.

3.5.1.4.culture method

The parasite-rich solution was drawn from several solid media and placed in sterile, graded bottles. The liquid medium was added to it in a volume equal to its volume. A drop of it was placed on a glass slide and examined under an optical microscope to see the parasite density and the cleanliness of the culture. In the case of a high density of the parasite, another amount of liquid medium was added to it equal to the final volume in the bottle, and so on, respectively, depending on the density of the parasite. The bottles were placed in an electric vibrator and incubated at a temperature of (26°C) and monitored daily.

3.5.1.5. Collecting parasites from the culture medium

The promastigots phase was collected from the two-phase culture media and the liquid medium and filtered, then the preflagellate phase was separated by a centrifuge at a speed of 3000 rpm for 30 minutes (the liquid culture medium was treated as the liquid phase in the process of collecting parasites from it). The flagellar front phase was washed with phosphate buffer solution, pH 7.1, and separated with a centrifuge at a speed of 3000 rpm for 30 minutes. The supernatant was discarded, the sediment was collected, and the washing process was repeated five times. The sediment was collected, after which part of it was taken for use, and the other part was stored at a temperature of 26°C until use.

All of the above-mentioned culture media were used, the best of which was liquid culture media

3.6. Immunochromatographic Strip Test (I.C.T)

The blood serum was kept at room temperature before use. Special strips were used in this test made of cellulose nitrate and saturated with the rK39 visceral *Leishmania* antigen in the test line area, as well as protein A antigen extracted from chicken in the control line area. The test was conducted at room temperature and included The method of work:

- 1-The serum was separated from the blood samples of the people subject to the test using a centrifuge at a speed of 3000 rpm for 10 minutes, and the serum was used directly after isolation.
- 2- Remove the strip from the wallet and from the end farthest from the examination pad
- 3-Put 20 microliters of serum onto the strip in the area of the pad located below the arrow.
- 4- 150 microliters of Chash buffer solution for the examination was added to flat-bottomed dishes.
- 5- Place the tape on the side of the pillow in the hole containing the Chash buffer for 10 minutes.

When the two lines (test and control) appear in red, the result is positive. The upper line represents the control line, while the test line is represented by the lower line. Whereas if only the upper line (control) appears in red, the result is negative and the test line does not appear.

3.7.Molecular identification

3.7.1. Polymerase chain reaction(PCR)

The PCR technique was performed for detection *Leishmania donovani* based on kinetoplast DNA minicircle (kDNA). This method was carried out according to following steps:

3.7.2.Genomic DNA Extraction

Genomic DNA was extracted from skin lesion sample by using gSYAN DNA kit extracted Gene aid .Taiwan ,and done according to company instruction as following steps:

1. A 200µl of *Leishmania donovani* culture media isolates sample was transferred to sterile 1.5ml micro centrifuge tube, and then added 20µl of proteinase K and GST buffer then, mixed by vortex. And incubated at 60°Cfor 30 minutes.
2. After that, 200µl of GSB cell lysis buffer was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 60°Cfor 15 minutes, and inverted every 3 minutes through incubation periods.
3. 200µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously.
4. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tube containing the flow through were discarded and placed the column in a new 2 ml collection tube.
5. 400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow through was discarded and placed the column back in the 2 ml collection tube.

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6. 600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow through was discarded and placed the column back in the 2 ml collection tube.
7. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.
8. The dried DNA filter column was transferred to a clean 1.5 ml micro centrifuge tube and 50 µl of preheated elution buffer were added to the center of the column matrix.
9. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

3.7.3. Genomic DNA examination

The extracted genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry -wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of blood genomic DNA was added to measurement.

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3.7.3. PCR master mix preparation

PCR master mix was by using Maxime PCR PreMix and done according to company instructions as following table.

Table (3-6) PCR master mix:

PCR master mix	Volume
Genomic DNA 5-50ng	5 μ L
kDNA primers forward (10pmol)	1 μ L
kDNA primers reverse(10pmol)	1 μ L
PCR water	13 μ L
Total	20 μ L

After that, these PCR master mix reaction components that mentioned above, placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all other components needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl₂, stabilizer, and tracking dye). Then the tube placed in Exispin vortex centrifuge for 3 minutes. Then transferred in PCR thermocycler.

3.7.4. thermo cyler reaction conditions

PCR Thermo cyler conditions was designed for external primer was done as following tube.

Table(3-7) PCR thermo cyler conditions

PCR cycle	repeat	Temp.	Time
Initial denaturation	1	95C	5min
Denaturation	30	95C	30sec.
Annealing		58C	30sec
Extension		72C	1min
Final extension	1	72C	5min
Hold	-	4C	Forever

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3.7.5. Gel electrophoresis

PCR products were analyzed by loading in 1% Agarose as following steps:

- 1- 1% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.
- 2- Then 3µL of ethidium bromide stain were added into agarose gel solution.
- 3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.
- 4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 mA for 1hour.
- 5- PCR products were visualized by using ultraviolet trans illuminator.

3.8. Immunological study

The study included (74) serum samples from patients with visceral leishmaniasis and (60) serum samples from healthy people.

3.8.1. Interleukin-2-Enzyme Linked Immunosorbent Assay (IL-2- ELISA)

Human Interleukin 2 (IL-2) ELISA was used in this study for quantitative determination of IL-2 from in patient and healthy control serum samples and done according to company instruction (BT-LAB) as following:

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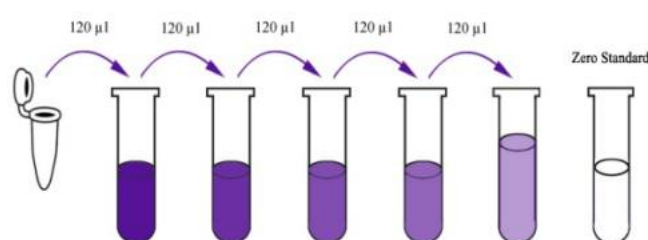
A. Table (3-8) ELISA components(IL-2)

Reagent	Quantity
Standard Solution (1200ng/L)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Bio tynylated Human IL-2 Antibody	1ml x1

B. Assay procedure:

1. All reagents, standard solutions and samples were prepared according to kit instruction and bring at room temperature before use.

1200ng/L	Standard No.5	120ul Original standard + 120ul Standard diluent
600ng/L	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
300ng/L	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
150ng/L	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
75ng/L	Standard No.1	120ul Standard No.2 + 120ul Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
2400ng/L	1200ng/L	600ng/L	300ng/L	150ng/L	75ng/L

2. A 50µl standard serial dilution was added into to standard well. Without added antibody to standard well because the standard solution contains bio tynylated antibody.

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3. A 40µl sample was added to sample wells and then add 10µl anti-IL-2 antibody to sample wells, then 50µl streptavidin-HRP added in to sample wells and standard wells (Not blank control well).
4. The ELISA mixed well and covered the plate with a sealer. Then incubated at 60 minutes at 37°C.
5. The sealer was removed and wash the plate 5 times with wash buffer. wells soaked
6. with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For
7. A 50µl substrate solution A was added to each well and then 50µl substrate solution B added to each well.
8. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.
9. A 50µl Stop Solution was added to each well, then the blue colour will change into yellow immediately.
10. The optical density (OD value) was determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

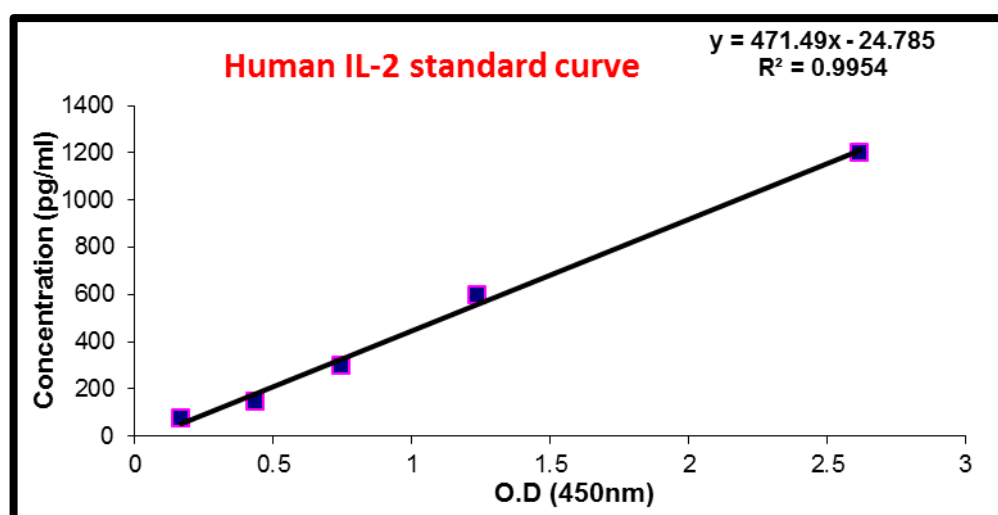


Figure (3) Standard curve for cytokinesis Il-2

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3.8.2. Interleukin-10-Enzyme Linked Immunosorbent Assay

Human Interleukin 10 (IL-10) ELISA was used in this study for quantitative determination of IL-10 from in patient and healthy control serum samples and done according to company instruction (BT-LAB) as following:

A. Table(3-9) ELISA components(IL-10):

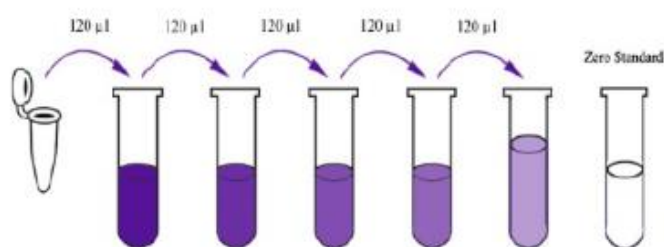
Reagent	Quantity
Standard Solution (800ng/L)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human IL-10 Antibody	1ml x1

B. Assay procedure:

1. All reagents, standard solutions and samples were prepared according to kit instruction and bring at room temperature before use.

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800pg/ml	Standard No.5	120ul Original standard + 120ul Standard diluent
400pg/ml	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
200pg/ml	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
100pg/ml	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
50pg/ml	Standard No.1	120ul Standard No.2 + 120ul Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
1600pg/ml	800pg/ml	400pg/ml	200pg/ml	100pg/ml	50pg/ml

2. A 50µl standard serial dilution was added into to standard well. without added antibody to standard well because the standard solution contains biotinylated antibody.
3. A 40µl sample was added to sample wells and then add 10µl anti-IL-10 antibody to sample wells, then 50µl streptavidin-HRP added in to sample wells and standard wells (Not blank control well).
4. The ELISA mixed well and covered the plate with a sealer. Then incubated at 60 minutes at 37°C.
5. The sealer was removed and wash the plate 5 times with wash buffer. wells soaked
6. with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For
7. A 50µ substrate solution A was added to each well and then 50µl substrate solution B added to each well.

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8. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.
9. A 50µl Stop Solution was added to each well, then the blue colour will change into yellow immediately.
10. The optical density (OD value) was determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

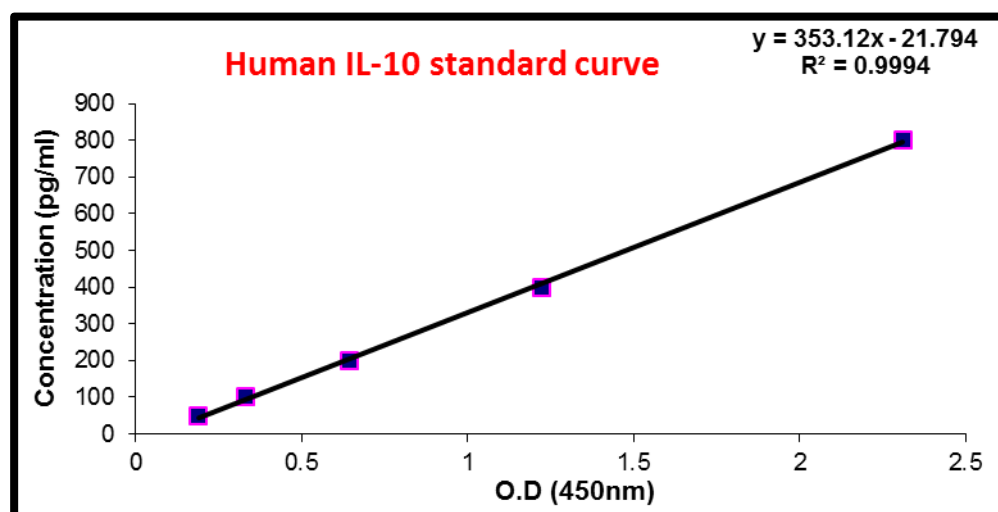


Figure (4) Standard curve for cytokinesisIL-10

3.8.3. Interleukin -12-Enzyme Linked Immunosorbent Assay (IL-12-ELISA)

Human Interleukin 12 (IL-12) ELISA was used in this study for quantitative determination of IL-12 from in patient and healthy control serum samples and done according to company instruction (BT-LAB) as following:

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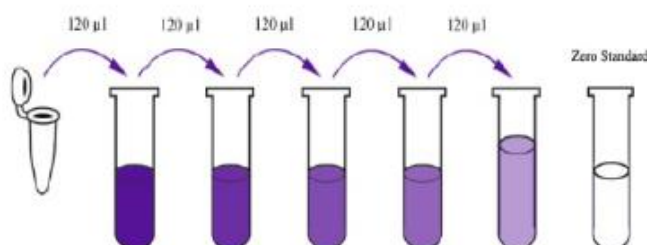
A. Table(3-10) ELISA components(IL-12):

Reagent	Quantity
Standard Solution (1200ng/L)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human IL-12 Antibody	1ml x1

B. Assay procedure:

- 1.All reagents, standard solutions and samples were prepared according to kit instruction and bring at room temperature before use.

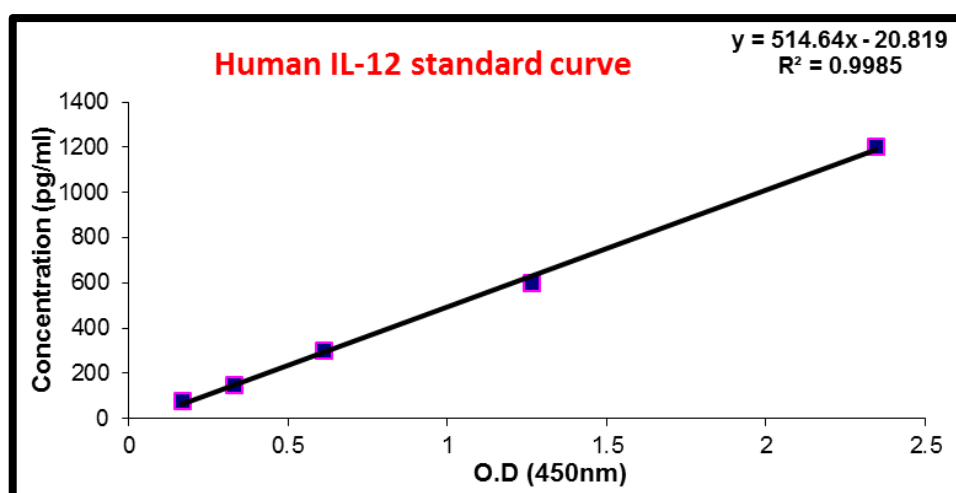
1200ng/L	Standard No.5	120ul Original standard + 120ul Standard diluent
600ng/L	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
300ng/L	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
150ng/L	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
75ng/L	Standard No.1	120ul Standard No.2 + 120ul Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
2400ng/L	1200ng/L	600ng/L	300ng/L	150ng/L	75ng/L

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- 2 A 50µl standard serial dilution was added into to standard well. Without added antibody to standard well because the standard solution contains biotinylated antibody.
- 3.A 40µl sample was added to sample wells and then add 10µl anti-IL-12 antibody to sample wells, then 50µl streptavidin- HRP added in to sample wells and standard wells (Not blank control well).
- 4.The ELISA mixed well and covered the plate with a sealer. Then incubated at 60minutes at 37°C.
- 5.The sealer was removed and wash the plate 5 times with wash buffer. wells soaked
- 6.with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash.
7. A 50µl substrate solution A was added to each well and then 50µl substrate solution B added to each well.
8. A 50µl Stop Solution was added to each well, then the blue colour will change into yellow immediately.
- 10.The optical density (OD value) was determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.



Figure(5)Standard curve for cytokinesis IL-12

3.8.4.Transforming Growth Factor ELISA(TGF- β)

Human transforming growth factor B1 (TGF- β) ELISA was used in this study for quantitative determination of TGF- β from in patient and healthy control serum samples and done according to company instruction (BT-LAB) as following:

A. Table ELISA components (TGF- β):

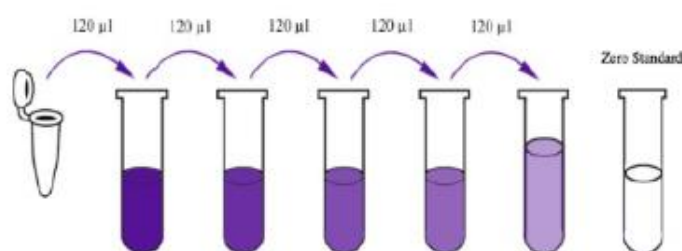
Reagent	Quantity
Standard Solution (2400ng/L)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human TGF- β Antibody	1ml x1

B. Assay procedure:

1.All reagents, standard solutions and samples were prepared according to kit instruction and bring at room temperature before use.

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2400ng/L	Standard No.5	120ul Original standard + 120ul Standard diluent
1200ng/L	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
600ng/L	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
300ng/L	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
150ng/L	Standard No.1	120ul Standard No.2 + 120ul Standard diluent

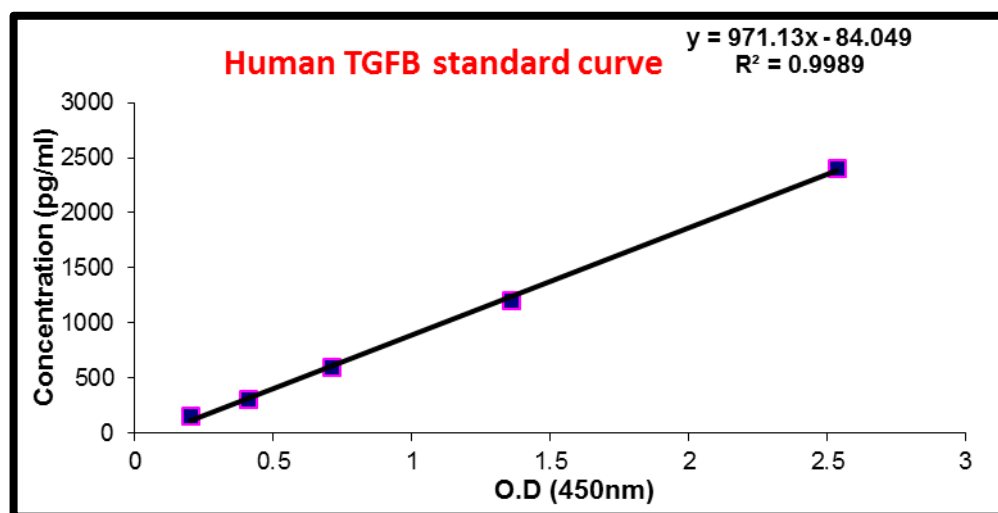


Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
4800ng/L	2400ng/L	1200ng/L	600ng/L	300ng/L	150ng/L

2. A 50µl standard serial dilution was added into to standard well. without added antibody to standard well because the standard solution contains biotinylated antibody.
- 3.A 40µl sample was added to sample wells and then add 10µl anti-TGF-β antibody to sample wells, then 50µl streptavidin- HRP added in to sample wells and standard wells (Not blank control well).
- 4.The ELISA mixed well and covered the plate with a sealer. Then incubated at 60minutes at 37°C.
- 5.The sealer was removed and wash the plate 5 times with wash buffer. wells soaked
- 6.with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash.
7. A 50µl substrate solution A was added to each well and then 50µl substrate solution B added to each well.
- 8.The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

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9. A 50 μ l Stop Solution was added to each well, then the blue colour will change into yellow immediately.
10. The optical density (OD value) was determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.



Figure(6) Standard curve for Transforming growth factor B(TGF- β)

C. Calculation of results:

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph.

3.9. Statistical analysis

The results were analyzed statistically using the statistical program SPSS version (22), where the Chi-square test and the t-test, Roce and Anova test were used to determine the significant differences between the number of samples positive for visceral leishmaniasis in the epidemiological study and to determine the significant differences between patients and healthy people for the two molecular studies. And immune serology at a probability level of $P < 0.05$ (Walk and Shostak, 2010).

Chapter Four

Results

4. Results**4.1. Epidemiology of visceral leishmaniasis in Al-Diwaniyah :**

The current study included 276 random samples from people aged between 7 and 65 years from Al-Diwaniyah Governorate and its affiliated areas, and they were examined using an Immunochromatographic test (ICT).

4.1.1. Immunochromatographic test (ICT).

The results showed that 18 (6.52%) of the samples had a positive diagnosis of visceral leishmaniasis infection

4.1.2. Characteristic of patients infected with VL .

The comparison of age group distribution according to results of (I.C.T) examination for parasite is shown in table (4-2). The present results show high rate of patients with positive VL infection (< 18) years age group, 10 (15.38%). Regarding Sex, the current results showed that the rate of infection of patients with the parasite in males is higher than in females, 9 (9.7%) versus 9 (4.9%) respectively. The comparison of residency distribution according to results of (I.C.T) examination for parasite is shown in table (4-2). The present results show high rate of patients with positive parasitic infection from rural area, 17 (7.4%).

Table (4-2): Characteristic of patients with Visceral leishmaniasis .

Chracteristic	Examined number	Positive results <i>n</i> = 18
Age (years)		
< 18 years, <i>n</i> (%)	65	10 (15.38%)
18-39 years, <i>n</i> (%)	188	7 (3.72%)
≥ 40 years, <i>n</i> (%)	23	1 (4.34%)
Total number	276	18 (6.52%)
P value	0.030*	
X ²	7.000	
Sex		
Male, <i>n</i> (%)	93	9 (9.7%)
Female, <i>n</i> (%)	183	9 (4.9 %)
Total number	276	18 (6.5%)
P value	0.151	
X ²	4.331	
Residency		
Urban, <i>n</i> (%)	45	1 (2.22%)
Rural, <i>n</i> (%)	231	17 (7.35%)
Total number	276	18 (6.52%)
P value	0.001*	
X ²	14.222	

4.2.Reservoir host results

The number of dogs examined was 24, including 16 dogs (66.67%).

4.3. Clinical samples.

For patients with visceral leishmaniasis, 56 serum samples were collected from different hospitals and confirmed by two laboratory testing methods. According to the hospital laboratory protocol, cases of visceral leishmaniasis were considered positive by rapid immunochromatographic strip assay and

indirect fluorescent antibody test (IFAT). In addition to the clinical signs and symptoms that were evaluated by the consulting medical staff and included in most cases: fever, weight loss, fatigue and anemia, as well as splenomegaly and hepatomegaly.

4.3.1. Distribution of visceral leishmaniasis in clinical samples.

The mean age of patients was 33.58 ± 12.16 years and the range was 9-63 years. On the other hand, most of the patients enrolled in this study were (under 18) years old, with regard to gender males constituted (62.5%) (35 out of 56) while females constituted (37.5%) (21 out of 56). With regard to residence, the proportion of the urban population was 39.3% (22 out of 56), while the proportion of the rural population was 60.7% (34 out of 56), respectively shown in table (4-3).

Table (4-3): Characteristic of patients with Visceral leishmaniasis in clinical Samples

Age (years)	patients <i>n</i> =56	<i>P</i>
Mean $\pm$ SD	33.58 $\pm$ 12.16	0.005 † S
Range	9-63 years	
< 18 years, <i>n</i> (%)	32 (57.1%)	0.027 ¥ S
18-39 years, <i>n</i> (%)	8 (14.3%)	
$\geq$ 40 years, <i>n</i> (%)	16 (28.6%)	
Sex	patients <i>n</i> =56	<i>P</i>
Male, <i>n</i> (%)	35 (62.5 %)	0.001
Female, <i>n</i> (%)	21 (37.5%)	
Residency	patients <i>n</i> =56	<i>P</i>
Urban <i>n</i> (%)	22 (39.3 %)	0.007 ¥ S
Rural, <i>n</i> (%)	34 (60.7%)	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; ¥: Chi-square test; S: significant at $P > 0.05$

4.3.2. Clinical signs and symptoms

The clinical signs and symptoms accompanied Kala-azar parasite infection was shown in table (4.4) The present results show most signs and symptoms accompanied Kala-azar parasite infection was fever 18 (47.37%).

Table (4-4): Clinical manifestations of patients with visceral leishmaniasis

Clinical signs and symptoms	Examined number	Positive results
Fever	38	18 (47.37 %)
Splenomegaly	27	11(40.74%)
Hepatomegaly	32	13 (40.27%)
Pneumonia	13	5(38.46%)
Flatulence	37	7 (18.92%)
Anemia	31	4 (12.90%)
Weight loss	26	2(7.7%)
jaundic	17	1(5.88%)
Vomiting	20	1 (5.0%)
loss of appetite	35	1 (2.26%)

4.2.4. Distribution of visceral leishmaniasis in governorates .

The frequency distribution of samples according to governorates was shown in table (4-5) positive 18 (24.3%) of samples from AL-Diwaniyah, 13 (17.5%) of samples from Diyala, 11 (14.9%) of samples from Baghdad, 7 (9.5%) of samples from Basra, both Wasit and Dhi Qar represents 6 (8.1%) of samples, 5 (6.8%) of samples from Karbala, and only 4 (5.4 %) of samples from both Najaf and Babylon.

Table (4.5): Distribution of visceral leishmaniasis in governorates.

Governorates	No. of samples <i>n</i> =74	Percentage
AL-Diwaniyah	18	24.3
Diyala	13	17.5
Baghdad	11	14.9
Basra	7	9.5
Wasit	6	8.1
Dhi Qar	6	8.1
Karbala	5	6.8
Najaf	4	5.4
Babylon	4	5.4
P value	0.145	
Chi-square	10.875	

4.3. *In Vitro* Cultivation of *Leishmania donovani*::

After a series of follow-ups (24, 48, and 72 hours), periodic *L. donovani* promastigotes were examined in vitro in NNN culture medium. The growth result was good and the promastigotes appeared in the form of close clusters. A good parasite growth rate was observed in the biphasic culture medium when rabbit blood was used, The highest growth rate was in the liquid culture medium.

4.4. Molecular study

Species identification was another goal of the current study, and the chosen marker can identify the type of parasite based on the size of the resulting range. The size of the kDNA amplified bands in all samples, was 562 bp (Fig 4-1).

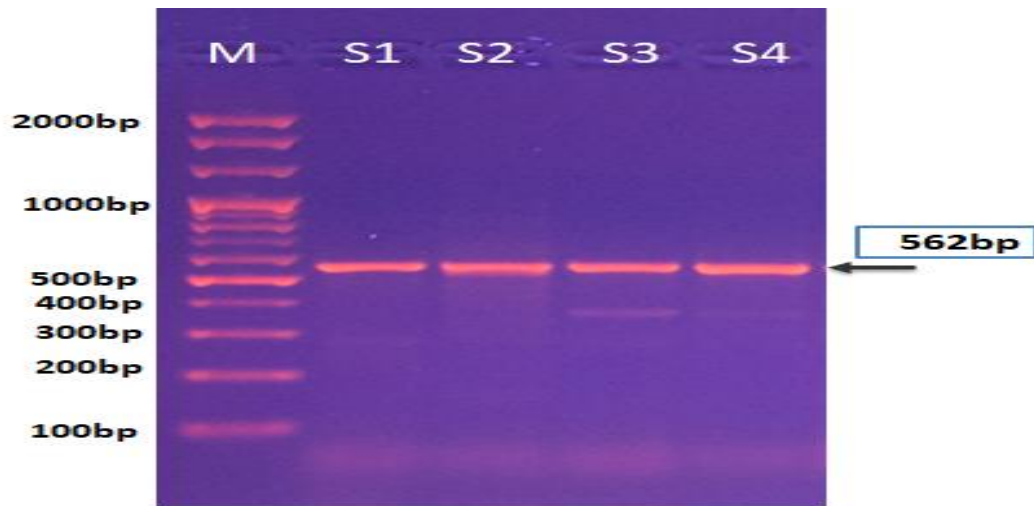


Figure 4-1: Agarose gel electrophoresis image that show the PCR product analysis of kinetoplast DNA minicircle in *Leishmania donovani* positive isolates samples. Where M: marker (2000-100bp), lane (S1-S4) showed positive *Leishmania donovani* isolates at (562bp) PCR product size.

4.5. Immunological Analysis Results:

4.5.1. Level Interleukin-2 (IL-2) in patients and healthy control.

The comparison of Interleukin-2 (IL-2) levels between patients with Kala-azar and healthy control subjects has been carried out and the results were demonstrated in table (4-6) and appendix(4-2). Mean levels of IL-2 were 131.58 ± 15.92 and 168.63 ± 21.61 , in patients with Kala-azar and healthy control respectively; the mean level was highly significant lower than in patients with Kala-azar in comparison with healthy control ($P < 0.001$).

Table (4-6): Interleukin-2 concentration in the studied groups.

	Cases –control comparison		<i>P</i>
	Patients <i>n</i> = 74	Healthy control <i>n</i> = 60	
Interleukin-2 (IL-2) levels			
Mean± SD	131.58 ± 15.92	168.63 ± 21.61	< 0.001
Range	50.29 – 197.90	104.68-258.81	† HS

n: number of cases; SD: standard deviation; †: independent samples t-test; HS: Highly significant at $P \leq 0.001$.

To evaluate the Interleukin-2 cutoff value as well as to predict Kala-azar disease as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (4-7), and figure (4-3). The Interleukin-2 cutoff value was >142.67-fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve of 81.1%, 80.0%, 83.3%, 77.4% and 0.813 (0.727- 0.900) respectively. The present results indicates IL-2 is considered as a good diagnostic marker.

Table (4-7): Sensitivity and specificity of IL-2 (> 142.67-fold) in Kala-azar disease.

IL-2 levels	Kala-azar patients <i>n</i> = 74	Healthy control <i>n</i> = 60
> 142.67	60	12
< 142.67	14	48
Sensitivity %	81.1 %	
Specificity %	80.0 %	
PPV %	83.3 %	
NPV %	77.4%	
AUC (95% CI)	0.813 (0.727- 0.900)	

CI: Confidence interval, AUC: Area under curve.

4.5.2. Level Interleukin-10 (IL-10) in patients and healthy control.

The comparison of Interleukin-10 (IL-10) levels between patients with Kala-azar and healthy control subjects has been carried out and the results were demonstrated in table (4-8) and appendix (4-4). Mean levels of IL-10 were 335.68 ± 15.57 and 260.93 ± 16.79 , in patients with Kala-azar and healthy control respectively; the mean level was highly significant greater than in patients with Kala-azar in comparison with healthy control ($P < 0.001$).

Table (4-8): Interleukin-10 concentration in the studied groups.

	Cases –control comparison		<i>P</i>
	Patients <i>n</i> = 74	Healthy control <i>n</i> = 60	
Interleukin-10 (IL-10) levels			
Mean± SD	335.68 ± 15.57	260.93 ± 16.79	< 0.00 1 † HS
Range	247.91 – 488.66	190.00-380.41	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; HS: Highly significant at $P \leq 0.001$.

To evaluate the Interleukin-10 cutoff value as well as to predict Kala-azar disease as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (4-9), and figure (4-5). The Interleukin-10 cutoff value was <271.82-fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve of 83.6%, 83.3%, 86.1%, 80.6% and 0.837 (0.751- 0.922). The present results indicates IL-10 is considered as a good diagnostic marker.

Table(4-9): Sensitivity and specificity of IL-10 (< 271.82-fold) in Kala-azar.

IL-10 levels	Kala-azar patients <i>n</i> = 74	Healthy control <i>n</i> = 60
< 271.82	62	10
> 271.82	12	50
Sensitivity %	83.8 %	
Specificity %	83.3 %	
PPV %	86.1 %	
NPV %	80.6%	
AUC (95% CI)	0.837 (0.751- 0.922)	

CI: Confidence interval, AUC: Area under curve.

4.5.3. Level Interleukin-12 (IL-12) in patients and healthy control .

The comparison of Interleukin-12 (IL-12) levels between patients with Kala-azar and healthy control subjects has been carried out and the results were demonstrated in table (4-10) and appendix (4-6). Mean levels of IL-12 were 341.16 ± 15.19 and 458.28 ± 18.14 , in patients with Kala-azar and healthy control respectively; the mean level was highly significant lower than in patients with Kala-azar in comparison with healthy control ($P < 0.001$).

Table (4-10): Interleukin-12 concentration in the studied groups.

	Cases –control comparison		P
	Patients <i>n</i> = 74	Healthy control <i>n</i> = 60	
Interleukin-12 (IL-12) levels			
Mean± SD	341.16 ± 15.19	458.28 ± 18.14	< 0.001
Range	186.07 – 457.80	317.81-674.97	† HS

n: number of cases; **SD**: standard deviation; †: independent samples t-test; HS: Highly significant at $P \leq 0.001$.

To evaluate the Interleukin-12 cutoff value as well as to predict Kala-azar disease as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (4-11), and figure (4-7). The Interleukin-12 cutoff value was > 371.62 -fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve of 83.6%, 83.3%, 86.1%, 80.6% and 0.832 (0.749- 0.916). The present results indicates IL-12 is considered as a good diagnostic marker.

Table (4-11): Sensitivity and specificity of IL-12 (> 371.62-fold) in Kala-azar disease

IL-12 levels	Kala-azar patients <i>n</i> = 74	Healthy control <i>n</i> = 60
> 371.62	62	10
< 371.62	12	50
Sensitivity %	83.8 %	
Specificity %	83.3 %	
PPV %	86.1 %	
NPV %	80.6%	
AUC (95% CI)	0.832 (0.749- 0.916)	

CI: Confidence interval, AUC: Area under curve.

4.5.4. Level Transforming growth factor- β in patients and healthy control

The comparison of Transforming growth factor- β (TGF- β) levels between patients with Kala-azar and healthy control subjects has been carried out and the results were demonstrated in table (4-12) and appendix (4-8). Mean levels of TGF- β were 823.62 ± 98.75 and 693.14 ± 68.31 , in patients with Kala-azar and healthy control respectively. The mean level was highly significant greater than in patients with Kala-azar in comparison with healthy control ($P < 0.001$).

Table (4-12): Transforming growth factor- β concentration in the studied groups.

	Cases –control comparison		<i>P</i>
	Patients <i>n</i> = 74	Healthy control <i>n</i> = 60	
Transforming growth factor-β (TGF-β) levels			
Mean± SD	823.62 ± 98.75	693.14 ± 68.31	< 0.001
Range	463.20 – 1179.39	498.0-885.14	† HS

n: number of cases; **SD**: standard deviation; †: independent samples t-test; HS: Highly significant at $P \leq 0.001$.

To evaluate the TGF- β cutoff value as well as to predict Kala-azar disease as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (4-19), and figure (4-9). The TGF- β cutoff value was < 730.24 -fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve of 70.3%, 70.0%, 74.3%, 65.6% and 0.712 (0.611- 0.814). The present results indicates TGF- β is considered as an acceptable diagnostic marker.

Table (4-13): Sensitivity and specificity of TGF- β (< 730.24 -fold) in Kala-azar disease

TGF-β levels	Kala-azar patients <i>n</i> = 74	Healthy control <i>n</i> = 60
< 730.24	52	18
> 730.24	22	42
Sensitivity %	70.3 %	
Specificity %	70.0 %	
PPV %	74.3 %	
NPV %	65.6%	
AUC (95% CI)	0.712 (0.611- 0.814)	

CI: Confidence interval, AUC: Area under curve.

4.6. Logistic regression correlations between immunological parameters.

The Logistic regression model show that the correlation of immunological parameters such as IL-2 in which have indirectly correlate with IL-10 among Kala-azar patients as in appendix (4-10), this result might be refer to that Kala-azar condition enhances production of IL-10 in contrast the expression of IL-2. Also show there was that the IL-2 indirectly correlate with TGF- β among Kala-azar patients as in figures (4-10). But this result show there was that the IL-10 directly correlate with TGF- β among Kala-azar patients as in appendix (4-14).

Chapter Five

Discussion

4.1.1. Immunochromatographic test (ICT)

Serologic diagnosis was performed by immunechromatographic rapid test (ICT), The development of rapid diagnostic tests (RDTs) is an important step for the diagnosis of VL, because they are cost-effective and rapid (**van Griensven and Diro, 2018 ; Safavi et al., 2021**). rK39 test is an immunochromatographic analysis. It qualitatively detects antibodies specific for recombinant *Leishmania* antigen rK39, a fragment of the kinesin-binding protein of *Leishmania donovani* (**Varani, 2017**).

This test is easy to perform and inexpensive and can be used for early diagnosis of VL (**Gradoni et al., 2017**). Its performance is considered high, but varies across geographical regions: a Cochrane study indicated that the sensitivity of this test is excellent in the Indian subcontinent (97%), but lower in East Africa (85%) (**Boelaert et al., 2014**)

The results of the study agreed with (**Alebie et al., 2019**) what was reported, as the rK39 strip was used, and the result was that out of 361 samples, 46 samples were infected with visceral leishmaniasis, with a percentage of (12.7%). One study in Diyala Governorate showed the highest rate of infection with visceral leishmaniasis (21.91%) from 2014 to 2017. This was during the military operations when most of Diyala's population was displaced (**Lehlewa et al., 2021**). It also agreed with the results of (**Alabaz et al., 2022**) 79.1% with rK39 dipstick test, *L. donovani* (26.7%). A study (**Varani et al., 2017**) of 643 samples recorded 94 positive samples for visceral leishmaniasis using the rK39 test strip, with a percentage of (14.82%).

Jarallah & Aabadi (2012) indicated in their study on the use of immunological test strip 39 to diagnose visceral leishmaniasis in children in Maysan Governorate that the number of positive cases of visceral leishmaniasis was 109 (69.42%) out of a total of 150 children.

The reason for the increase in the incidence of infection and its appearance in new areas may be attributed to urban and agricultural expansion and environmental changes that provide new activity for the vector insect, which is abundant throughout Iraq (**Ismail and pesson ,1992**), in addition to the increase in the number of infected people due to the increase in population and immigration, as well as activity and work of the category Young teenage girls are exposed to frequent sand fly bites due to lack of attention to public hygiene and the large amount of animal husbandry near residential areas, which constitutes a source of the spread of this disease in an epidemic form in the governorate, with the biggest problem being the decline in the health situation in the country.

4.1.2. Characteristic of patients infected with VL .

The results of the study are consistent with what was mentioned by (**Alebie et al., 2019**), as the incidence rate in females was higher than in males (17.2%) (14%), respectively, and the percentage of children under the age of five and 15 years of age was more affected. As well as a study (**Knight et al., 2023**), where the highest infection rate was in the age groups (1-4) years, at 62.4%. The results of the study also agreed with what was reported by (**Hamid Al-Azzawi et al., 2017**), as the highest infection rate was in the age groups (2-6) years. Also, in a study (**Krepis et al., 2017**), the highest infection rate was in the age group (4-6) years..

There are a variety of studies on CL and VL in Iraq In (Hawija, Kirkuk) out of 23,778 people, 107 were positive for kala azar, with an incidence rate of 45 cases per 10,000. Leishmaniasis is caused by immigration from other parts (**Obaid and Sharif, 2018**). VL is widely distributed in many parts of the world. Iraq is considered a place for the disease, especially in the central and southern parts, and most of the reasons are due to the adaptation of the vector sand fly in these areas. In the years (2010-2011) there was an increase in cases of VL disease in a number of central and southern Iraqi governorates

(Wasit, Dhi Qar , Maysan, Basra, and Muthanna) that most of the VL concentrations are located in an area of 100 km around Baghdad, mainly in (Suwayra, Yusufiyah, Mahmoudiya, Latifiyah, Salman Pak, and Al-Aziziyah, Diyala) (**Khadum, 2012**).

Also agreed with the study of **Muhammad (2007)** in Dhi Qar Governorate, as well as **Kraif (2008)** in Diwaniyah Governorate, and it also agrees with what was recorded by (**Dakel et al., 2008**), where the percentage of males was recorded at 51.79% and females was 48.21% in Dhi Qar Governorate, as well as (**Abdul Nabi et al., 2009**) in Baghdad Governorate indicated that the number of infections in males is higher than in females. The study by **Tarish (2010)** indicated that the number of infections in males (58.9%) and females (41.1%), as well as **Kadhum (2012)** where the infection rate in males was higher than in females(71.1%) and (28.8%) respectively. as well as **Abul-Doanej ,2014**) where the infection rate in males was 52.40% and in females, 47.60% .It did not agree with the study of (**Krepis et al., 2017**). The infection rate in females was higher than in males.

As well as the study of (**Kupani et al., 2021**) where the infection rate in males was higher than in females, and the study (**de Araujo et al., (2023)** where it was recorded the highest percentage for males (91.4%) and females (8.6%) may be due to males using the courtyard of the house to sleep in the summer, while children and women sleep inside rooms, especially in rural areas (**Uzun et al., 1999**). Also, the exposed bodies of organs such as hands and legs in males It helps in increasing the chance of infection with the parasite compared to women (**El-Deen et al., 2006**) . The reason for the high incidence of infection in the age groups (18-39) may be due, as **Al-Yasiri (2012)** pointed out, to the fact that the insect prefers ages that have more attractive factors in terms of skin vitality as well as the person's frequent contact with the external environment.

4.2.Reservoir host

(**Mukhtar *et al.*, 2000**) was mentioned in a field survey of domestic and wild animals in two villages infested with leishmaniasis in Sudan. Raising animals, including dogs, in residential areas is increases the density Rodents and an increase in the number of Poultry that do not comply with the required health conditions, including fencing and an incinerator

Faust *et al.*, (1970) indicated that there is one or more reservoir hosts for each type of Leishmania in Each region is infected with black fever, and the World Health Organization (**1986**) stated that countries differ in type The hosts that store the disease. In Jordan, Tunisia, Algeria, Libya, and Iran, dogs are the reservoirs of the disease. In some countries, such as Yugoslavia, rodents are the reservoir of the disease, and in India, Nepal and Bangladesh, humans are the reservoir.

It is one of the most important well-known reservoirs in many countries such as China, America, Algeria, Libya, Iran and Saudi Arabia. **Dangschies (2001)** also believed that the process of transporting and importing dogs from tropical or subtropical regions was the reason for the spread of some parasitic diseases, including visceral leishmaniasis.

Moritz (2001) explained the reasons for the spread of the problem in Germany is importing dogs and taking them during tourism.

In Spain, it was found that the infection rate of dogs was 10.2% when conducting the IFAT serological test. (Indirect fluorescent antibody test)

(**Fisa *et al.*, 1999**) It was also noted that an increase in the number of infections with visceral leishmaniasis in humans and dogs in the state of Minas, Brazilian in the same period of time (**Silva *et al.*, 2001**).

(Sukker *et al.*, 1981) was also able to isolate Leishmaniasis from a dog in Al-Shahimiya area in Wasit Governorate Sukker (1974) showed that the disease spreads everywhere Iraq, especially among residents of the countryside and the outskirts of cities, especially the areas surrounding Baghdad, Nouri and Al-Jubouri (1973) and Sukkar (1974) also pointed out that the disease increases in spread as they move away from central Baghdad, especially in Mahmoudiyah, Yusufiyah, Latifiyah, and Al-Mada'in, according to what everyone pointed out. In 1983, the World Health Organization office in Geneva indicated in the report of the Leishmaniasis Expert Committee that the control of leishmaniasis diseases is hampered by ignorance of the true rate of spread of these diseases and underestimation of the amount of suffering and diseases they cause to people. (Suker *et al.*, 1985) explains that the rate of infections with visceral leishmaniasis in Iraq is record Annually about 1000 cases.

4.3. Clinical samples .

4.3.1. Distribution of visceral leishmaniasis in clinical samples.

VL Mediterranean usually affects children (1–4 years of age) or immunocompromised adult (WHO, 2010) . The results of the current study showed that the highest infection rate was in category <18, with a rate of (57.1%) and the lowest infection rate was in the age group (18-93) with an infection rate of (14.3%).

The results of the study agreed with the results of the study of Kadhum (2012) who showed that the infection rate in age groups < 5 years was 50%. The results did not agree with the study (Varani *et al.*, 2017), where the highest infection rate was among adults (71%).

The results of the current study showed that the infection rate in males is higher than in females, with an infection rate of (62.5%) and (37.5%) respectively. the results of the study agreed with many studies, including the study of by (**HameedAl-Azzawi et al., 2017**), where the infection rate in males was higher than in females, at a rate of (60%) and (40%), respectively. In a study of (**Varani et al., 2017**), the infection rate in males was higher than in females (65%) and (35%) respectively. This may be due to men's increased exposure to sand flies or to immune modulation induced by androgens (**Sellau et al., 2019**). The study of **Jarallah & Aabadi (2012)** indicated that the number of positive cases in males reached 63 (57.79%) while it was in females (42.2%).

The prevalence of the disease in rural areas was higher than in urban areas, with an infection rate of (60.7%) and (39.3%) respectively. they agree With the study of **Kraif (2008)** in Al-Qadisiyah Governorate, where the number of infections in rural areas was 72 and in urban areas 45, as well as the study of (**Abdul Nabi et al., 2009**) in Baghdad city and its suburbs, where rural areas recorded (74.5%) and urban areas (25.4%), and study **Tarish (2010)** reported that the prevalence of visceral leishmaniasis in rural areas of Middle Euphrates was 62.7% and in urban areas (37.3%). Likewise, in the study of (**Tarish et al., 2009**) the percentage of infections in rural and urban areas was (68.3%) and (31.7%) respectively Likewise, **Abul-Doanej (2014)**, where the infection rate in the countryside was higher than in the city, as it was (80.96%) and (19.04%), respectively. The reason may be due to the nature of the behavior and habits of the transmitting insect and the natural conditions in the rural environment, as both pointed out. In rural areas during the year (**Hamzavi et al., 2001**), in addition to the vertebrate animals that store the disease, which increases the chance of infection with the parasite throughout the year due to the presence of continuous agriculture in summer

and winter, as well as the presence of rivers and streams that provide a good environment for the presence of sand flies (**Hamzawi et al., 2001**).

4.3.2. Clinical signs and symptoms

A study of the clinical signs accompanying infection with the visceral *Leishmania* found that those infected suffered from fever (47.37%) and hepatomegaly and splenomegaly (40.27%),(40.74%) respectively. Fever is one of the most common clinical signs among those infected, as it was noted that most of the infected suffer from high temperatures, and these results were consistent with (**Al-Orainey et al., 1994; Mehdi 2004 ; Hadi 2006**).

The results of the current study showed that patients suffer from enlarged liver and spleen at a rate of (40.74%) and (40.27%), respectively. These results were consistent with **Ghayyad (2003)** (liver enlargement 75.53% and spleen enlargement 81.41%). **Mehdi (2004)** also showed that the rate of enlargement of the liver and spleen is 76.3% and **Ghayyad (2003)** liver enlargement 85.98%and spleen enlargement 89.3%. **Hadi(2006)** explained that the rate of liver and spleen enlargement is 84.1%. While (**Al-Orainey et al., 1994**) pointed out the rate of enlargement of the liver and spleen in those infected with the visceral *Leishmania* is 100%. The reason for the enlargement of the liver and spleen is due to the accumulation of macrophages infected with the parasite inside these organs, as hyperplasia of the reticuloendothelial cells that make up the liver and spleen occurs (**WHO, 2010**). Anemia was observed in the patients, and it was similar to what was recorded by (**Mehdi,2004 ; Hadi 2006**). The cause of the anemia is due to the accumulation of macrophages cells infected with the parasite within the blood-forming tissue of the bone marrow, in addition to iron deficiency and

increased fragility of red blood cells and the ease with which they are destroyed as they progress **Varma and Naseem , (2010)**. Except a number of other symptoms accompanying the infection of the parasite, such as losing appetite, at a rate of 2.26 %, vomiting 5.0 %, pneumonia, 38.46 %, jaundice, 5.88 %, and body bloating 18.92 %, These results are similar to what was recorded by **(Hadi, 2006 ; Mehdi ,2004)**.

The results of the present study are consistent with those of **(Alabaz *et al.*, 2022)** and the most common clinical findings were fever (93.3%) and splenomegaly (90%), followed by hepatomegaly (76.6%). The most common laboratory finding was thrombocytopenia (86.6%), followed by anemia (70%).

In a study of **(HameedAl-Azzawi *et al.*, 2017)**,it indicated that the percentage of duration , Fever that was(52%), A study **(Varani *et al.*,2017)** recorded The highest incidence of clinical signs is fever, with a percentage of (78.7%), splenomegaly (55.3%), hepatomegaly (34%), and weight loss (28.7%), The results of the study agreed with what was stated by **Kadhum (2012)** that the highest incidence of infection among clinical signs is fever (54.6%), Hepatomegaly (38,8%) , splenomegaly (31.9%) ,Weight loss (19.5%) ,vomiting(4.1). Likewise, **Abul-Doanej (2014)** explained that the highest rate was in fever (100%), Anemia(71.42%), Splenomegaly(66.66%) Hepatomegaly (57.14).

4.3.3. Distribution of visceral leishmaniasis in governorates .

According to the statistics of the Ministry of Health in Iraq, in 2010, the highest infection rate was in Diyala Governorate, 501 cases, Dhi Qar 265, Wasit 207, and Diwaniyah 163. In 2011, the highest infection rate was in Maysan Governorate 189, followed by Diyala 181, Wasit 128, and Diwaniyah

128 **Abul-Doanej (2014)**. In 2012, the highest infection rate was in Dhi Qar Governorate, 212, followed by Diyala, 176, Wasit, 129, and Diwaniyah, 112. The percentages in the current study agree with the study of **Abdul Nabi et al., (2009)** in Baghdad city and its suburbs, where the number of patients was 545. As for Dhi Qar Governorate, the number of patients was 432 (**Dakel et al.,2008**) and **Kraif (2008)** mentioned in Al-Diwaniyah that the number of infections(117), **Rahi (2010)** study in Wasit Governorate recorded 100 cases, as for the Basra Governorate, the number of infections was 146, according to (**Gani et al.,2010**), and the number of infected people in the Middle Euphrates was 150 (**Tarish, 2010**). The reason is due to the low level of health in the community, such as the lack of awareness to public hygiene, as well as the raising of stray animals, especially cats and dogs, the lack of control of rodents found in and near residential areas, and the expansion of cities towards the agricultural areas and orchards.

4.4.In Vitro Cultivation of *leishmania donovani*

Isolation and culture of parasites gives better diagnostic sensitivity and can also identify organisms at the genotype and species level. Parasite species characterization and identification can be performed by combining culture and multilocus enzyme electrophoresis (**Reithinger et al. 2007**).

4.5. Molecular study

The use of molecular techniques to accurately identify *Leishmania* species, such as PCR analysis, is essential for designing appropriate control strategies (**Behniafar et al ., 2024**).

The results of the molecular examination of samples taken from the culture medium (Promastigotes) from which DNA was extracted confirmed the appearance of kDNA gene bands that were amplified using PCR with a size of pb562 base pairs. This result was close to the result of (**Al-Doori et al., 2017**) in Tikrit, which was conducted Using the kDNA gene to study the diagnosis of visceral leishmaniasis by polymerase chain reaction (PCR) and an approach to the result of (**Yaseen and Ali, 2016**) In Baghdad, they studied Using of Species-Specific Primers for Molecular Diagnosis of in Vitro Promastigotes of *Leishmania donovani*. As well as an approach to the result (**Salotra et al., 2001**) in their study Development of a Species-Specific PCR Assay for Detection of *Leishmania donovani* in Clinical Samples from Patients with Kala-Azar and Post-Kala-Azar Dermal Leishmaniasis .

Polymerase chain reaction (PCR), used to detect small-circuit parasitic DNA, has been shown to be almost 100% sensitive and specific in chronically ill individuals (**Centurion-Lara et al. 1994; Bhattacharya et al. 1996**).

A series of recent studies have focused on the use of PCR amplification of kinetoplast DNA sequences from blood, bone marrow, or spleen specimens in the diagnosis of kala azar patients (**Rodgers et al. 1990; De Bruijne and Parker 1992, 1993; Rodriguez et al. 1994; Andresen et al. 1997**).

The elucidation of some species-specific DNA fragments has made this procedure possible for the diagnosis of leishmaniasis (**Smyth et al., 1992**). PCR techniques are also more convenient than parasitological diagnostic methods (culture and microscopy) especially for samples with lower parasitic

loads (**Antinori et al., 2007**) and are therefore useful in monitoring disease progression, estimating the outcome of anti-leishmanial therapy, and describing species discrimination and resistance. (**Schönian et al., 2011**). **Moreira et al., (2007)** conducted a comparative study of different diagnostic methods and observed a sensitivity of 100%, 96%, and 95.65%, respectively, for symptomatic, minimally symptomatic, and asymptomatic groups, and a specificity of 100%. Likewise, in another study, PCR was targeted against kinetoplastid DNA (kDNA) using Uni21/Lmj4 primers, and the results were compared by parasitological methods, cultures, and smears. The results showed that kDNA PCR was the most sensitive, i.e. 100%; However, microscopy and cultures were only 71.4% and 54.3% sensitive, respectively (**Zeyrek et al., 2018**). **El-Salam et al., (2014)**, conducted an experiment in which *Leishmania tropicalis* was extracted from CL patients in Kohat and analyzed by PCR, microscopy and culture techniques. Among 113 samples, PCR, microscopy, and culture showed sensitivity of 87.61%, 53.98%, and 46.90%. Although conventional PCR shows enhanced sensitivity compared to the parasitological diagnostic method, the primary drawback of this method is its inability to differentiate between clinically active VL and asymptomatic infection (**Sudarshan and Sundar 2014**). Traditional PCR also carries a high risk of contamination, which takes longer to give results.

Mitochondrial DNA in *Leishmania* consists of about 10,000 copies of small circles of about 0.5–2 kb and about 20–40 copies of large circles of about 20–40 kb, which are called kinetochore DNA (kDNA) . These two classes of molecules contain genes that encode mitochondrial genes (in the large circles) and small RNAs, called guide RNAs (in the small circles). The high number of copies of both classes of these circular DNAs allows them to be used as a target for parasite detection directly from the clinical sample. Small circle size polymorphisms have been studied to identify and differentiate *Leishmania* species. Although some differences in the size of the

variable region of small circles occur in *Leishmania species*, some species have a similar size, which limits their use for species discrimination in both subspecies .

4.5. Immunological Analysis Results:

4.5.1. Level Interleukin-2 (IL-2) in patients and healthy control.

Proinflammatory cytokines are mainly produced to amplify the immune response -17,IL-18,IL-2,IL-12), while anti-inflammatory cytokines that counteract the effect of proinflammatory cytokines include (IL-4 ,IL-6 ,IL-10 ,TGF- β) (Andargie & Ejara, 2015).

Interleukin 2 (IL-2) is an important cytokine that influences T-cell behavior (Spolski and Leonard , 2018). It is required for the survival, proliferation, and differentiation of CD4⁺ T cells, CD8⁺ T cells, and natural killer (NK) cells , by binding to either the high-affinity trimeric IL-2 receptor (IL-2R), made up of IL-2R α (CD25), IL-2R β (CD122), and IL-2R γ (CD132), or the dimeric IL-2R (comprising β and γ chains) (Smith1988). The trimeric IL-2R receptor is highly expressed on activated CD4⁺ T cells and Foxp3⁺CD4⁺ T regulatory (Treg) cells, while memory CD8⁺ T cells and NK cells express high levels of the dimeric IL-2R(Spolski and Leonard , 2018).

(França *et al.*, 2020) studied the level of some cytokines, including IL-2, in the serum of blood donor patients infected with leishmaniasis and found that IL-2 decreases.In a study by (Tadesse *et al.*, 2021) on Antibody and cytokine levels in visceral leishmaniasis patients with varied parasitemia in patients admitted to Arba Minch General Hospital, southern Ethiopia, they found that the level of cytokinesis IL-2 decreases in the serum of patients with visceral leishmaniasis.

Leishmania donovani-infected mice receiving IL-2-blocking monoclonal antibodies (mAbs) failed to control hepatic parasite growth, associated with impaired granuloma development, whereas infected mice treated with exogenous IL-2 had reduced liver parasite burdens and increased granuloma development, relative to controls(**Murray et al.,1993**).

Similarly, intranodular injection of recombinant IL-2 in patients with disseminated cutaneous leishmaniasis reduced parasite numbers, associated with CD4⁺ T-cell infiltration(**Akuffo et al.,1990**).However, the therapeutic application of IL-2 has had limitations because of its short half-life (**Lotze et al .,1985**)and adverse side effects(**McDermott,2004**) .

A study (**Ibarra-Meneses et al., 2017**) found that IL-2 is a good indicator for diagnosing visceral leishmaniasis, as the AUC was 0.9613 (0.8767-1.000), and in studies it was confirmed that IL-2 is the most sensitive and specific biomarker in *L.infantum* (**Ibarra -Meneses et al.,2016**).

4.5.2. Level Interleukin-10 (IL-10) in patients and healthy control.

Previous studies have shown a direct role for IL-10 in the pathogenesis of human VL (**Caldas et al et al., 2005; Gautam., 2011**). Previously thought to be a Th2 cytokine, IL-10 is produced by CD4⁺ FoxP3⁺ CD25 regulatory T cells (Treg) as well as IFN- γ -producing Treg⁺ cells (Tr1 cells) in humans (**Moore et al., 2001; Spielberg and Edwards, 2001; Nylen et al., 2007; Shihara et al., 2016**). The advancement of the disease is mainly related to Th 2 type of immune response along with an increase in levels of interleukin-10, IL-5, IL-4 and transforming growth factor (**Torres-Guerrero et al., 2017**).

(**França et al., 2020**) studied the level of some cytokines, including IL-10, in the serum of blood donor patients infected with leishmaniasis and found an increase in the concentration of cytokines IL-10. (**Espir et al., 2014**) also studied the level of some cytokines, including IL-10, in the sera of patients

infected with different types of *Leishmania* and found an increase in the concentration of cytokines IL-10 compared to the control.

In a study by (**Tadesse *et al.*, 2021**) on Antibody and cytokine levels in visceral leishmaniasis patients with varied parasitemia in patients admitted to Arba Minch General Hospital, southern Ethiopia, they found elevated level of IL-10 in the serum of patients with visceral leishmaniasis. In a study by (**Kupani *et al.* , 2021**) they were found that elevated levels of IL-10 in the serum of patients infected with *Leishmania donovani* compared to control

The results agreed with a study by (**Wijesooriya *et al.*, 2023**) To evaluate the predictive value of levels of inflammatory markers to identify patients with cutaneous leishmaniasis, IL-10 showed that the (ROC) curve showed the best discrimination. AUC 1(1.00-1.00), cut off 81.13, SE(1.000), SP(1.000). Likewise, (**Ibarra-Meneses *et al.*, 2017**) IL-10 was found to be a good diagnostic marker for diagnosing Kala azar, as it was (0.9574-1.000) AUC 0.9881.

4.5.3. Level Interleukin-12 (IL-12) in patients and healthy control .

Once the initial infection starts, monocytes are recruited to infected tissue and begin to differentiate into macrophages (**Yang *et al.*, 2014**). T cells in lymphoid tissues and spleen are activated both by dendritic cells and the macrophages. The naïve T cells get activated into Th0 cells (intermediate stage) which then travel to the liver and get triggered as Th1 cells on coming in contact with dendritic cells and macrophages in IL-12 environment (**Siewe *et al.* 2016**). During this attachment, CD4⁺ T cells recognize the antigens which are bound to Major Histocompatibility Complex (MHC) (**Dhanji and Teh 2003**). CD4⁺ T cells then produce IL-12, which in turn triggers CD8⁺ T cells and enhance CD4⁺ T cell multiplication. Both CD8⁺ T cells and CD4⁺

T cells yield IFN- γ , which in turn activates the macrophages to wipe out the parasites (Siewe *et al.*, 2016).

Leishmania reduces dendritic cell-dependent immune responses by inhibiting maturation, migration, antigen presentation capabilities, and IL-12 production. (Ehrlich *et al.*, 2014), These influence on dendritic cells effects the development of effective Th1 response required to the infection clearance (Basit *et al.*, 2022). Infection with VL results in subclinical infection, leading to protective immunity or, in clinical cases, fatal outcomes if untreated. The development of protective immunity appears to depend on , host genetic factors, the infecting species (Ehrlich *et al.*, 2014).

The results of the study did not agree with what was reported (Khoshdel *et al.*, 2009), as the level of cytokinetic IL-12 in patients infected with visceral leishmaniasis was high compared to control, as well as In a study in southwestern Ethiopia, serum cytokines (IFN- γ , IL-12, and IL- 15) in 70 VL patients were significantly higher than 12 healthy controls in the same endemic area (Hailu *et al.*, 2004).

A study (Ibarra-Meneses *et al.*, 2017) found that IL-2 is a good indicator for diagnosing visceral leishmaniasis, as the AUC was 0.9613 (0.8767-1.000), and in studies it was confirmed that IL-2 is the most sensitive and specific biomarker in *L. infantum* (Ibarra -Meneses *et al.*, 2016).

4.5.4. Level Transforming growth factor- β in patients and healthy control.

TGF- β is a potent regulatory cytokine that suppresses expression of inducible NO synthase and IFN- γ , and suppresses Th1 and Th2 cell development, plays an important role in the progression of leishmaniasis in rodents (Li, J *et al.*, 1999). In the study conducted by (Gantt ,2003) on the

role of TGF- β which aimed to examine whether functionally active TGF- β may be present in the local environment surrounding *Leishmania* promastigote invasion in murine models, high levels of total TGF- β were found. - β in tissues and cultured immune cells from mice and hamsters infected with *L.donovani*.

In study by (**Kumar et al., 2017**) found higher levels of TGF- β for patients with visceral leishmaniasis compared to controls. In a study by (**Kupani et al ., 2021**) they were found that elevated levels of TGF- β in the serum of patients infected with *Leishmania donovani* compared to control.

In other studies, it was found that TGF- β promotes progression or prevents the treatment of leishmaniasis in mouse models(**Gorelik, et al.,2002**). Active TGF- β is augmented during infection of inflammatory mouse peritoneal macrophage cultures with several different *Leishmania sp.* *in vitro* (**Gomes et al., 2000**).

In a study of(**Omer et al .,2000**) it was found that TGF- β alters the phenotype of innate macrophages by inhibiting TNF- α , IFN- γ , inducible NO synthesis (iNOS), and enhancing arginase expression. Conversion of arginine from iNOS to arginase results in decreased NO. and enhancing polyamines, which in turn can promote parasite growth (**Iniesta et al.,2001**).

The results agreed with a study by (**Wijesooriya et al., 2023**) To evaluate the predictive value of levels of inflammatory markers for identifying patients with cutaneous leishmaniasis based on TGF-B, the (ROC) curve showed that the best discrimination of TGF-B was at 72 hours, and the AUC ratio was 0.957 (0.901-1.00) and cut off (3834.55),SE(0.95)

Conclusions & recommendation

Conclusions:

This study has come out with the following conclusions:

- 1 . Visceral leishmaniasis is still a serious endemic parasitic disease in Iraq. It spreads and affects both sexes at different ages.
2. Inflammatory cytokines such as interleukin 10, 2 and Transforming growth factor- β , are increased in patients with visceral forms.
3. It was found that the Immunochromatographic test (I.C.T) is the easiest and cheapest in diagnosing visceral leishmaniasis and has the ability to detect the disease early and within minutes at room temperature.
4. The present results indicates IL-2, IL-12 and IL-10 can be used as markers for leishmaniasis infection, especially in endemic areas of patients at different stages of the disease and TGF- β is considered as an acceptable diagnostic marker.

Recommendations:

1. Leishmaniasis in Iraq needs greater attention from the Ministry of Health to conduct more epidemiological and survey research in different regions of the country.
2. More studies are needed to examine other immune molecules as biomarkers during the different stages of leishmaniasis.
3. Educating and raising health awareness among citizens about the seriousness of the disease and its transmission methods.
4. Emphasizing the disposal of waste and organic materials that are suitable places for the growth and reproduction of the vector medium (sand flies) and combating them during their spread season .
5. Combating reservoirs of leishmaniasis, such as dogs and rodents, in cooperation between citizens and relevant authorities, and emphasizing the raising of pets according to health conditions.

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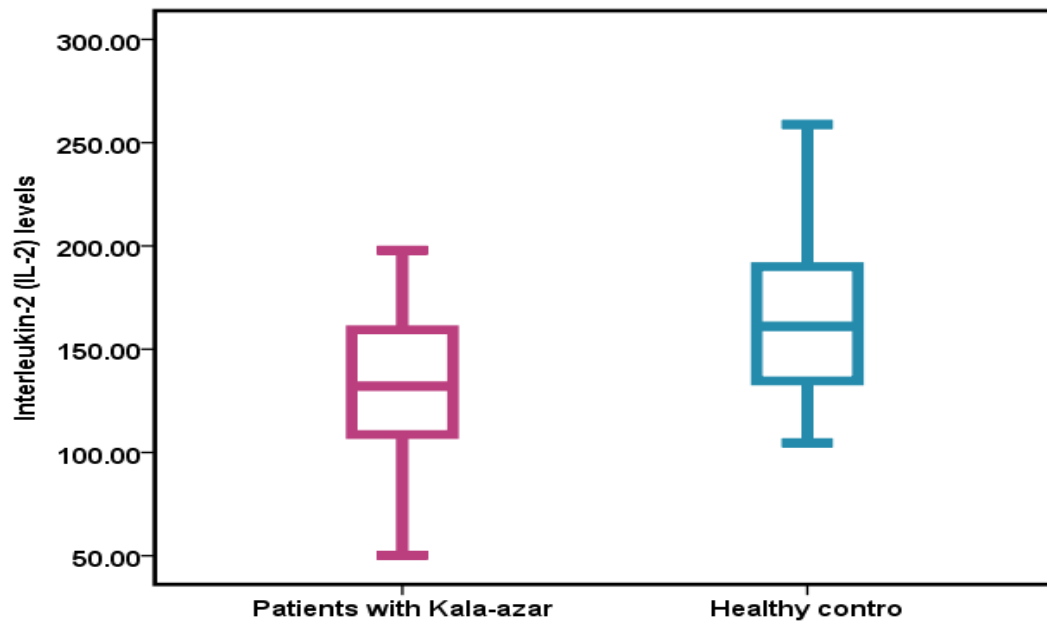
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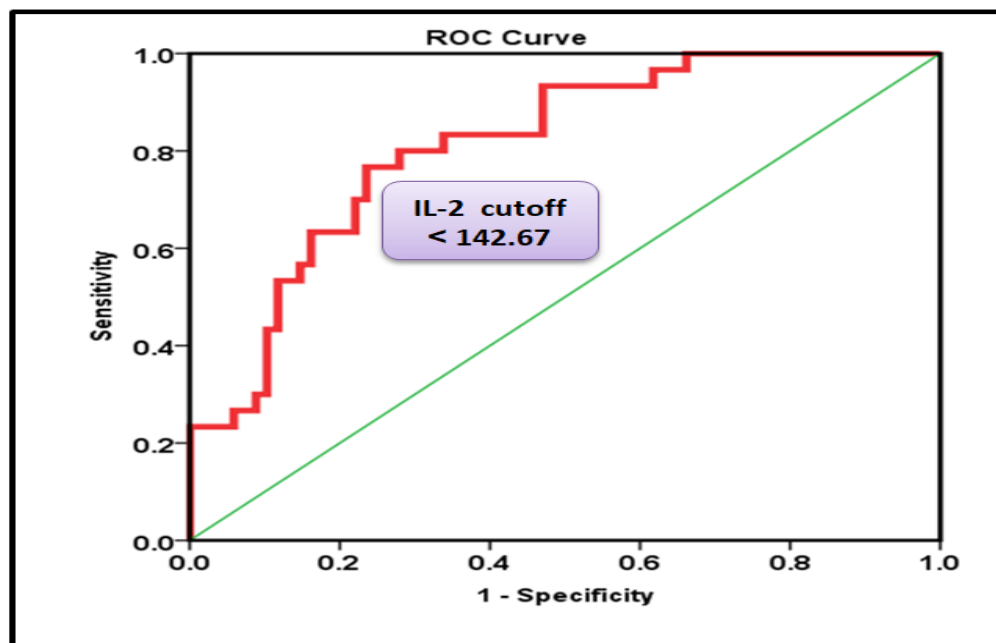
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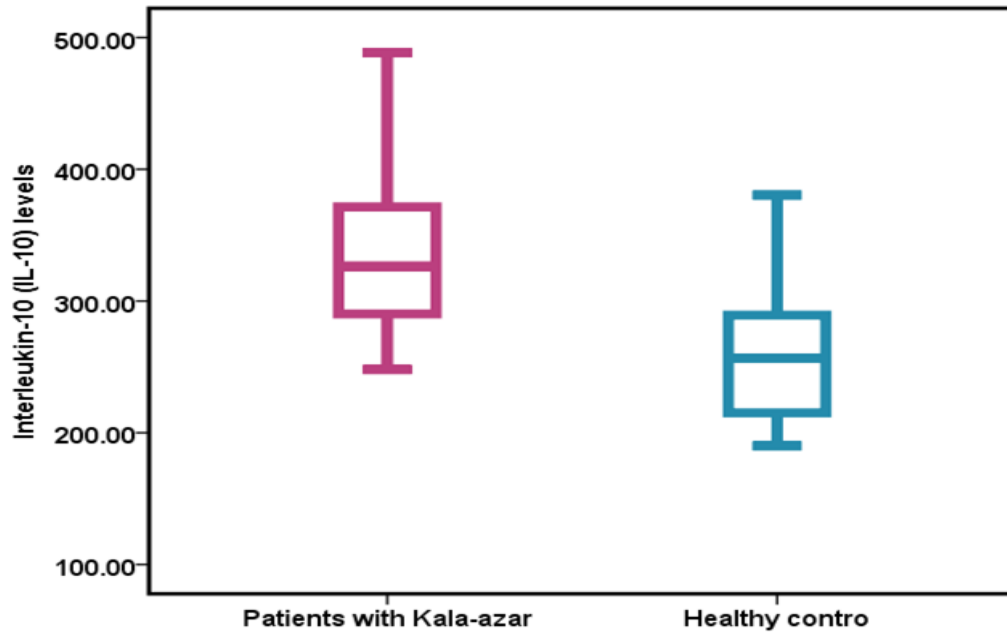
Appendices



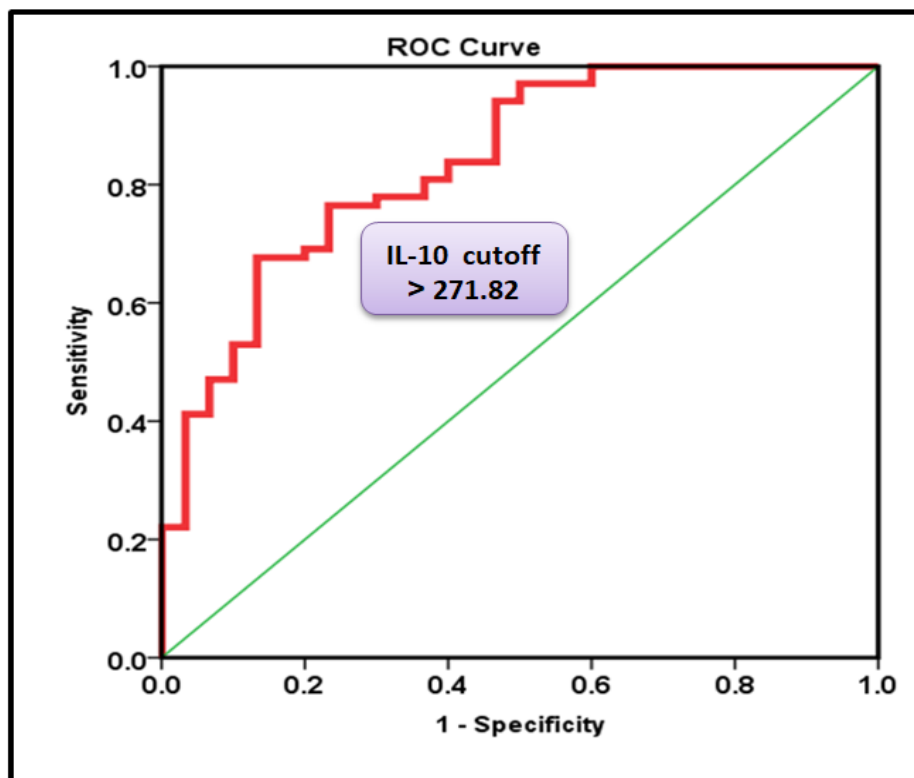
Appendix (4-2): Mean Interleukin-2 (IL-2) levels of patients and controls



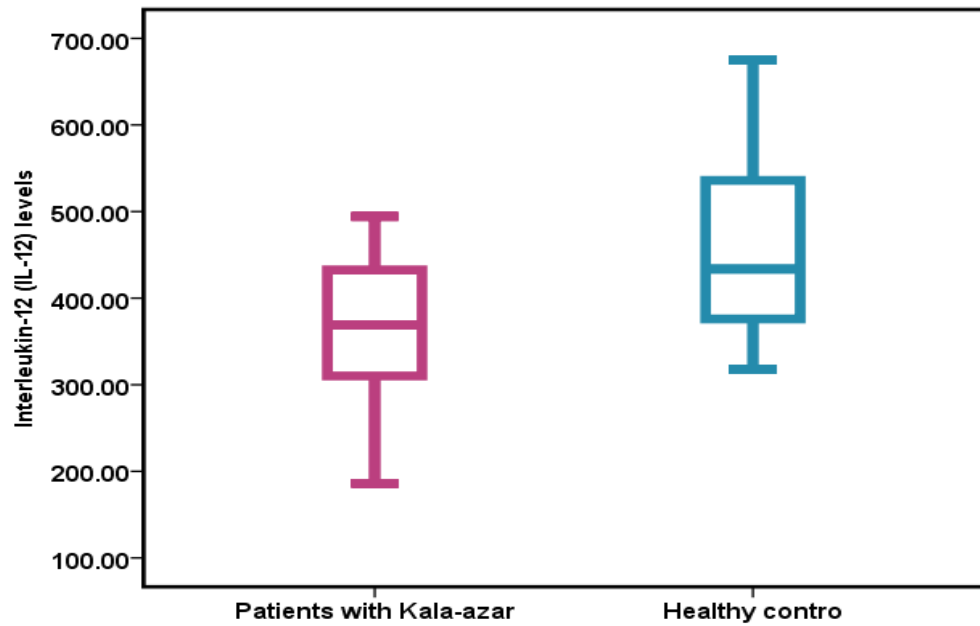
Appendix (4-3): Receiver operator characteristic curve analysis of IL-2 for the calculation of possible diagnostic cutoff value.



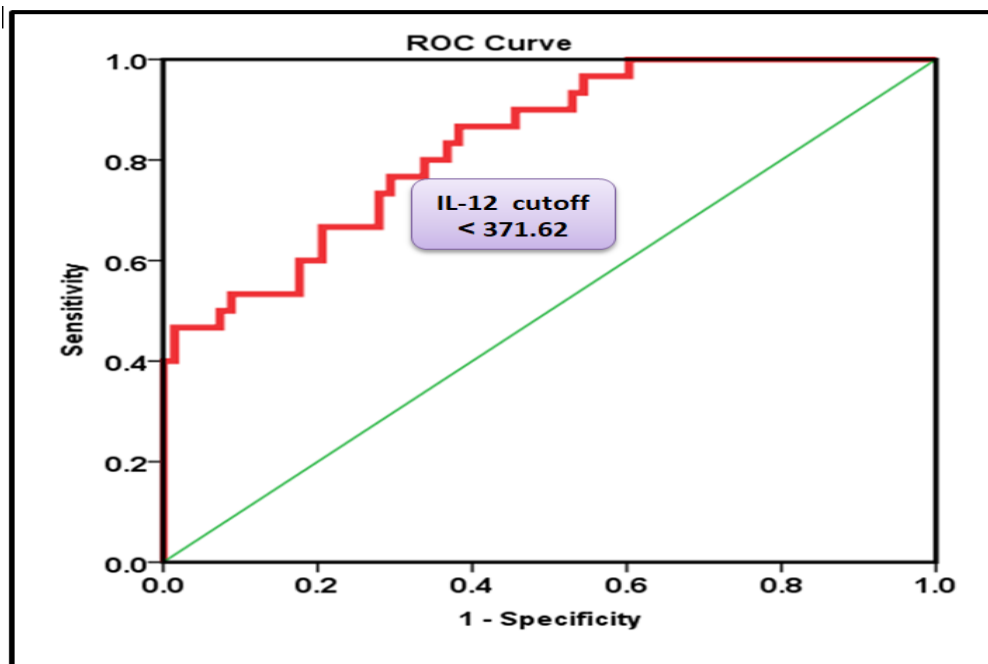
Appendix (4-4): Mean Interleukin-10 (IL-10) levels of patients and controls.



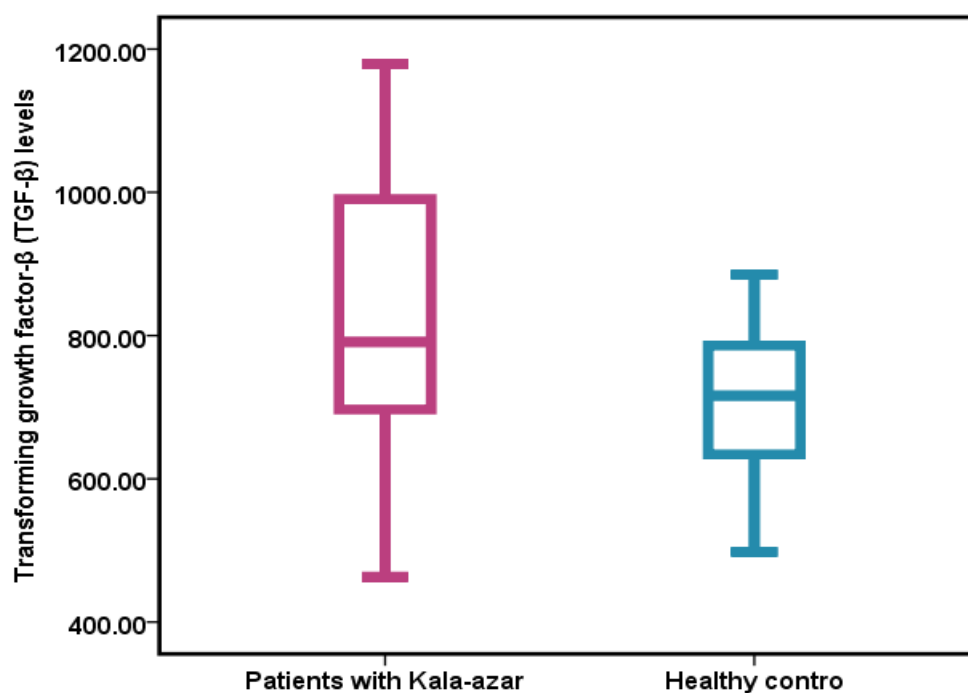
Appendix (4-5): Receiver operator characteristic curve analysis of IL-10 for the calculation of possible diagnostic cutoff value.



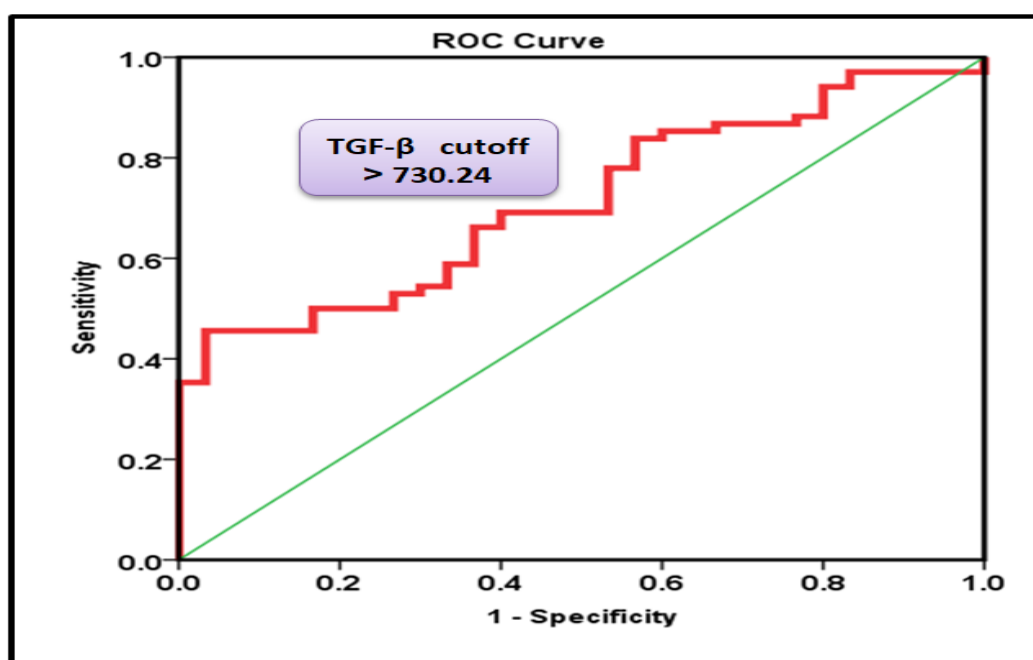
Appendix (4-6): Mean Interleukin-12 (IL-12) levels of patients and controls.



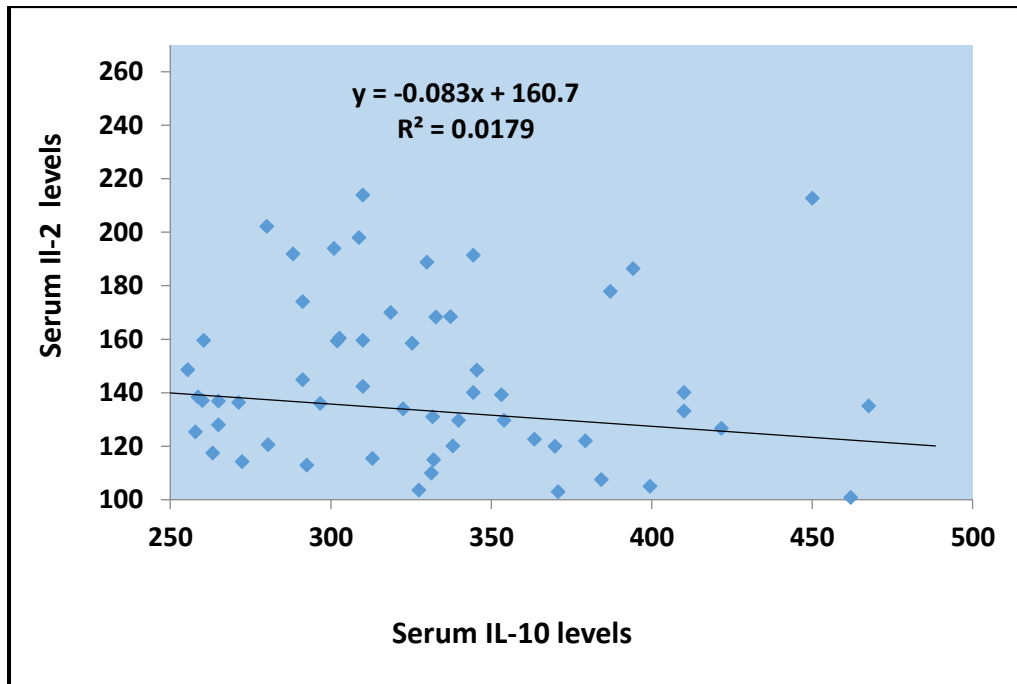
Appendix (4-7): Receiver operator characteristic curve analysis of IL-12 for the calculation of possible diagnostic cutoff value.



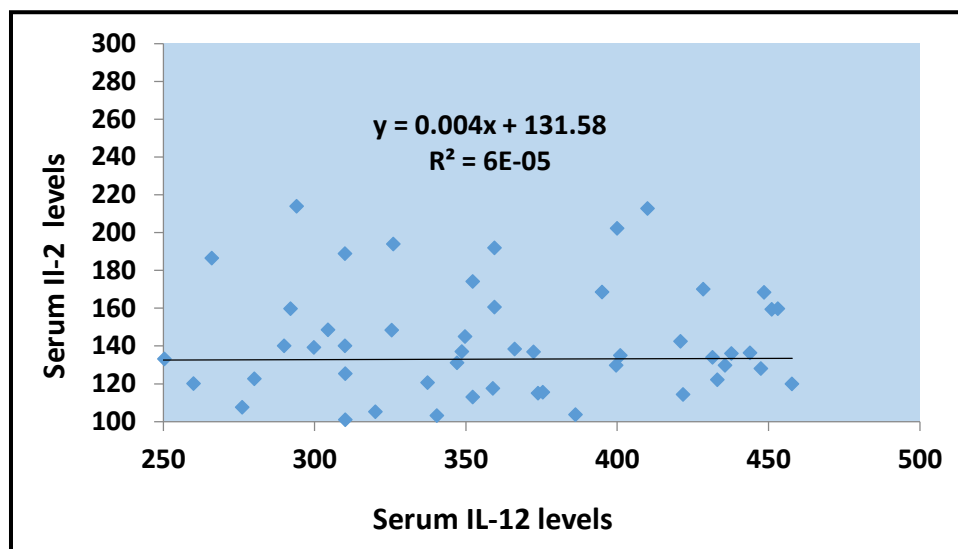
Appendix (4-8): Mean TGF-β levels of patients and healthy controls



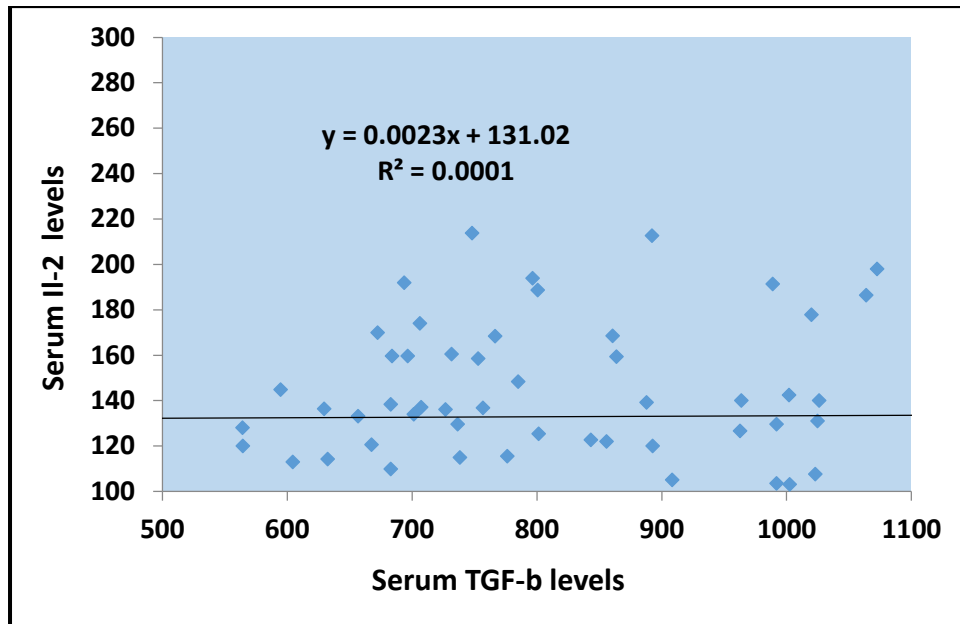
Appendix (4-9): Receiver operator characteristic curve analysis of TGF-β for the calculation of possible diagnostic cutoff value.



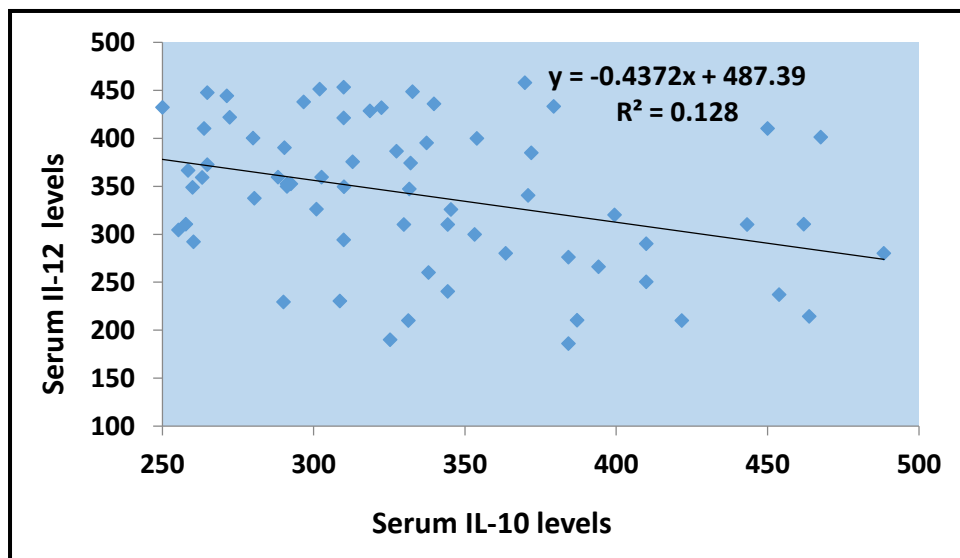
Appendix (4-10): The Logistic scatter blot of IL-2 and IL-10 among patients.



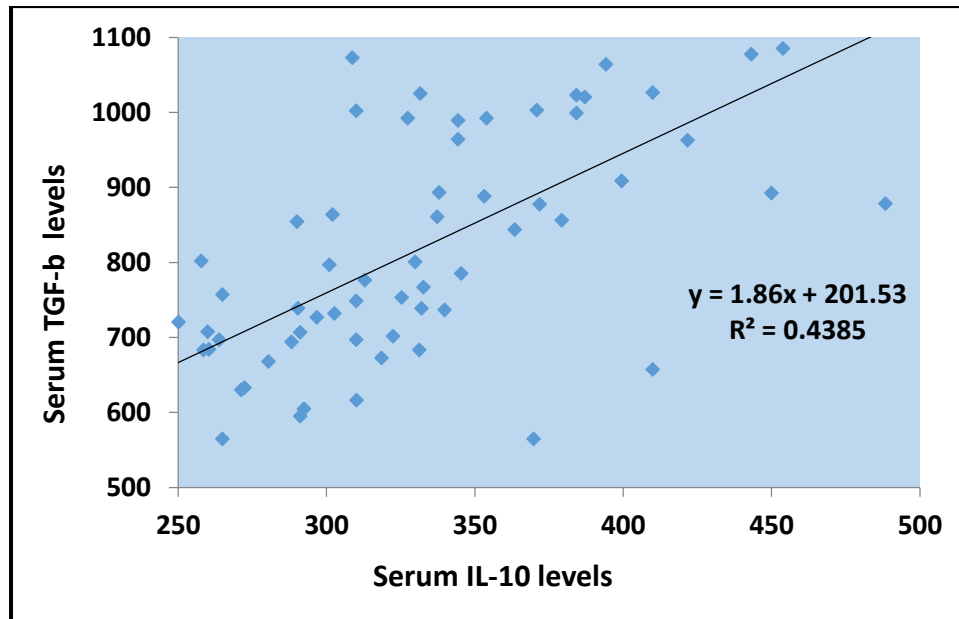
Appendix (4-11): The Logistic scatter blot of IL-2 and IL-12 among patients.



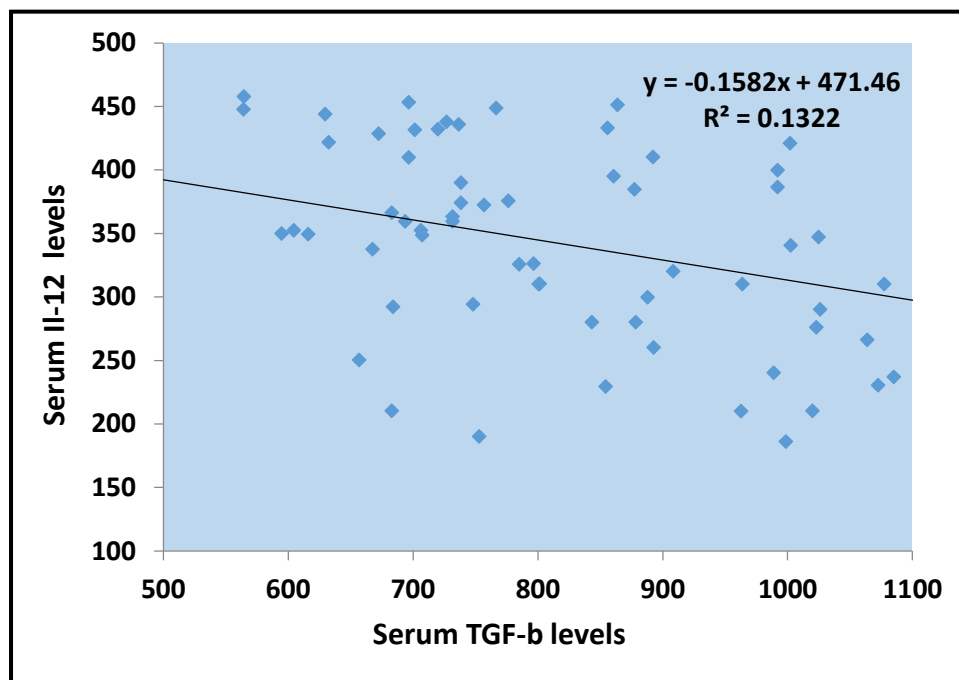
Appendix (4-12): The Logistic scatter blot of IL-2 and TGF- β among patients.



Appendix (4-13): The Logistic scatter blot of IL-10 and IL-12 among patients.



Appendix (4-14): The Logistic scatter blot of IL-10 and TGF-β among patients.



Appendix (4-15): The Logistic scatter blot of IL-12 and TGF-β among patients

الخلاصة

داء الليشمانيات الحشوي هو مرض منتشر على نطاق واسع في العديد من البلدان حول العالم، بما في ذلك العراق . تحدث العدوى عن طريق حقن طفيلي الليشمانيا من خلال ذبابة انثى تنتمي الى جنس *Phlebotomus sp.* عند تغذيتها على دم الثدييات (المضيف)، السمة المميزة لداء الليشمانيات الحشوي هي تأثيره على العديد من الأعضاء الداخلية (عادةً الطحال والكبد ونخاع العظام).

أجريت الدراسة الحالية على 332 شخصا من كلا الجنسين، توزعت على 276 ومن محافظة الديوانية و56 عينة سريرية من مستشفيات مختلفة في العراق ، خلال الفترة من اذار إلى كانون الاول 2023.

تناولت الدراسة محورين اولهما الدراسة الوبائية لداء الليشمانيات الحشوي في محافظة الديوانية والتي شملت 276 شخصا حيث تم فحص جميع الاشخاص المشتبه بهم المشاركين في هذه الدراسة في مختبرات كلية العلوم – جامعة القادسية ، حيث تم الكشف والتأكد من إصابة 18 شخصاً بداء الليشمانيات الحشوي (VL) بنسبة 6.52% من خلال اختبار الفحص المناعي السريع (I.C.T) والذي أثبت نجاحه من خلال حساسيته ونوعيته العاليتين بالإضافة إلى العلامات والأعراض السريرية. وأظهرت النتائج أن الفئة العمرية (أقل من 18) أكثر عرضة للإصابة وبنسبة (18.38%)، وأن الذكور يصابون بنسبة أعلى (9.7%) مقارنة بالإناث (4.9%). كما أظهرت ارتفاع عدد المصابين في المناطق الريفية (7.5%) مقارنة بوسط المدينة (2.5%).

وشمل المحور الثاني دراسة 56 حالة تم الكشف والتأكد من التشخيص السريري لديهم داء الليشمانيات الحشوي (VL) من مستشفيات مختلفة في العراق، وتم تأكيد التشخيص من خلال اختبارين معملين تضمننا فحصا سريعا للكروماتوغرافيا المناعية واختبار الأجسام المضادة الفلورية غير المباشرة (IFAT)، بالإضافة إلى العلامات والأعراض السريرية، والتي تم تقييمها من قبل الطاقم الطبي الاستشاري وشملت في معظم الحالات: الحمى وفقدان الوزن والتعب وفقر الدم، وكذلك تضخم الكبد والطحال.

وظهرت النتائج متطابقة في كل من الدراسة الوبائية والعينات السريرية، حيث سجلت أعلى نسبة إصابة في الفئة العمرية (أقل من 18 عاما) وكانت نسبة الإصابة لدى الذكور أعلى (53.85%) عن الإناث (37.15%)، والمصابين في المناطق الريفية كانت أعلى (60.7%) عنها في المدينة

(39.3%)، وأظهرت نتائج العلامات السريرية أن أعلى نسبة إصابة كانت في الحمى (47.37%) وكانت أعلى نسبة إصابة في محافظة الديوانية (24.3%) وأعلى نسبة إصابة في محافظة الديوانية (24.3%). أدنى نسبة إصابة كانت في محافظتي بابل والنجف (5.4%).

تم فحص الخزع المأخوذة من النخاع العظمي بالمجهر الضوئي، بعد صبغها بصبغة كيمزا، وبعد التأكد من وجود الطفيلي، تم زراعتها في وسط استزرع (NNN (Novy - MacNeal - Nicolle و RPMI (Roswell Park Memorial Institute). عند درجة حرارة 23 درجة مئوية.

أما بالنسبة للفحص الجزيئي فقد تم فحص أربع عينات باستخدام تقنية PCR اعتماداً على الجين التشخيصي KDNA بحجم 562 زوج قاعدي.

في هذه الدراسة تم الكشف عن مستوى السيتوكينات التي تفرز عن طريق الاستجابة المناعية الفطرية لعدوى اللشمانيا الاحشائية، مثل الحركيات الخلوية $TGF-\beta$, IL-12, IL-10, IL-2 باستعمال تقنية ELISA. أظهرت النتائج عن زيادة معنوية في تركيز هذه السيتوكينات في مجموعة الاختبار (15.92 ± 131.58، 15.57 ± 335.68، 98.75 ± 823.62، 15.19 ± 341.16) على التوالي للمرضى المصابين باللشمانيا الاحشائية مقارنة بمجموعة السيطرة الاصحاء.



جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة القادسية – كلية العلوم
قسم علوم الحياة

Leishmania* دراسة وبائية ومناعية وجزيئية لطفيلي *donovani

إطروحة مقدمة إلى
مجلس كلية العلوم جامعة القادسية
كجزء من متطلبات نيل درجة دكتوراه فلسفة في
علوم الحياة / علم الحيوان
من قبل

مروة حسين دخیل

بكالوريوس علوم ، علوم الحياة 2014 ، جامعة القادسية
ماجستير علوم الحياة ، كلية التربية للبنات 2019 ، جامعة الكوفة

بإشراف

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