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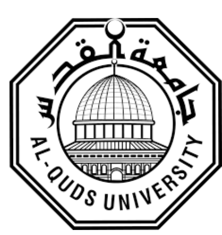

Molecular Epidemiology: Prevalence of Human Cutaneous Leishmaniasis in Palestine in the Period between 2016 and 2024 Using Next Generation Sequencing

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Molecular Epidemiology: Prevalence of Human Cutaneous Leishmaniasis in Palestine in the Period between 2016 and 2024 Using Next Generation Sequencing

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Thesis approval

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Dedication

To my family, whose unwavering encouragement has been my greatest strength, especially my husband, who has supported me greatly throughout this academic journey, my beloved sons and daughter, and my dear brothers for always being by my side.

Thank you for standing by me through the challenges and triumphs, for your love, patience, and understanding.

To my supervisors, mentors, and teachers—your guidance, expertise, and wisdom have profoundly shaped my academic journey, enriched my scientific growth, and deepened my knowledge.

With heartfelt gratitude,

Hanan Abdelmajeed Almanasra

Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except there otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signature:

Hanan Abdelmajeed Almanasra

Date: 12.01.2025

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Molecular Epidemiology: Prevalence of Human Cutaneous Leishmaniasis in Palestine in the Period between 2016 and 2024 Using Next Generation Sequencing

Abstract

Background: Leishmaniasis is a vector-borne disease caused by protozoan parasite of the genus *Leishmania*. The infection is transmitted by bite of infected female sandflies of the genus *Phlebotomus* in the old world or Lutzomyia in the new world. The diagnosis is made by stained smear microscopy, in-vitro culture, and DNA-based detection methods.

Materials and Methods: In this study that spanned from 2016 to 2024 included patients from eleven districts in the West Bank of Palestine. The diagnostic methods used in the study included microscopy of Giemsa-stained touch smears, in-vitro culture using NNN medium, and ITS1-PCR. The amplicon-based next-generation sequencing of ITS1-219 was included in the comparison part of the study. The comparison arm of the study compared two groups, CL-confirmed group in w were positive by any of the tests used in the epidemiologic part of the study and a non-CL group which were negative by all diagnostic methods. The NGS1-219-PCR was compared to ITS1-PCR, which is considered as a gold standard owing to its superiority over microscopy and *in-vitro* culture.

Results: The positivity rate during the study period was 17% (213/1262) with a prevalence of 7.0 per 100,000. The annual incidence rate 0.84 per 100,000 (25 cases per year) with approximately equal distribution between males (52%) and females (48%). The age range between 0 to 14 yrs was the most affected by CL. The CL lesions primarily affected the head (45%), followed by the upper extremities (38%) and the lower extremities (17%).

The choropleth mapping showed that Jericho is still the district with highest annual incidence rate (21 per 100,000). The study revealed that *Leishmania tropica* is the predominating species with *L. major* restricted mainly to Jericho. The year 2017 was the last year to witness a CL peak in Palestine with Jericho contributing to 40% of peak cases. More than half (52%) of the cases appeared in the months of January to March. As for the comparison arm of the study, ITS1-219-NGS was shown to have a higher sensitivity of 94% compared to the imperfect gold standard (ITS1-PCR) of 86%**.** The agreement between the two tests was fair (Kappa-0.24) only agreed on 56% of the cases only. Furthermore, the standard ITS1-PCR was unable to genotype 29 CL cases but were genotyped by ITS1-219-NGS and confirmed by BLAST search. Unlike the ITS1-PCR, imperfect gold standard, ITS1-219-NGS genotypes all its positive results correctly as confirmed by BLAST search. Moreover, 13 ITS1-PCR weak positive CL cases were found to be negative by ITS1-219-NGS and upon BLAST search shown to be contamination of human and bacterial origin.

Conclusion: Jericho area remains the main focus of CL in Palestine with *L. tropica* as the main species in the country and *L. major* restricted to Jericho area. Incidence rate has dropped compared to the two decades preceding this study as result of control measures implemented. Amplicon-based NGS is a feasible, highly sensitive, and high throughput diagnostic method with accurate species identification that can be used in clinical practice and epidemiologic survey.

List of Abbreviations

Abbreviation	Full word
BLAST	Basic Local Alignment Search Tool
CL	Cutaneous leishmaniasis
EDTA	Ethylenediamine-tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
IFAT	Indirect fluorescent antibody test
ITS1	Internal transcribed spacer 1 gene
MCL	Mucocutaneous leishmaniasis
NGS	Next generation sequencing
NNN	Novy-MacNeal-Nicolle
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
TBE	Tris borate EDTA
UV	Ultra violet
VL	Visceral leishmaniasis
WHO	World health organization

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CHAPTER ONE

1. Introduction

1.1. General background

Leishmaniasis is a vector-borne disease caused by protozoan parasite from 20 species of the genus *Leishmania*. The infection is transmitted by bite of infected female sandflies of the genus *Phlebotomus* in the old world or Lutzomyia in the new world. The disease has three main clinical forms: cutaneous leishmaniasis (CL), as the most common form characterized by skin ulcers. The second form is the mucocutaneous leishmaniasis (ML) which involves the destruction of mucus membranes of the nose, mouth or throat. Thirdly, the visceral leishmaniasis (VL); a fatal form that infects spleen and liver (WorldHealthOrganization, 2024). The life cycle of all *Leishmania* species is basically similar. The parasite exists in two morphological forms: amastigote, a non-flagellated from that exists intracellularly within macrophages of the mammalian host, and promastigote, the larger flagellated, motile via a single anterior flagellum, which lives extracellularly in the digestive system of the female sandfly vector or in *in-vitro* culture. The transformation from one form to the other occurs shortly after transfer between the mammalian host and the vector, and vice versa. Both forms of the *Leishmania* parasite reproduce asexually through binary fission. As depicted in figure 1, phlebotomine female sand fly takes a blood meal from a vertebrate host such as rodents, often called reservoir, for the maturation of eggs, the ingested blood meal may contain amastigotes if host is infected, inside the sand fly gut, amastigotes transform into, promastigotes, and attach to the gut wall. In less than a week, the promastigotes move forward again, lodging in the fly's oesophagus. When the fly feeds again, it attempts to clear out the promastigotes clogging its oesophagus and expels them into the site of blood meal incision. Promastigotes are engulfed by the host cells, often macrophages, transform into amastigotes, and multiply by binary fission until the macrophages become stuffed with amastigotes, they rupture and are destroyed. The parasites released invade new cells and begins a new cycle of infection. The result of multiplication and host immune system interaction results in tissue destruction that takes the form of ulcers and lesion in the cutaneous form and enlargement of organs such as spleen in the case of visceral form. Although the life cycle of the *Leishmania* parasite is mainly between the sandfly vector and the rodents reservoir which show

mild symptoms if at all, however, human comes into the cycle as an incidental host which is preferred by the sandfly due to its anthropophilic nature as being attracted to human blood. The dog in the visceral form plays the role of infected host showing symptoms as well as the role of the reservoir as a source of infection. Due to the involvement of animals; leishmaniasis is usually described as zoonotic. Leishmaniasis can be anthroponotic like *Leishmania tropica* where vector can transmit the infection from one human to another or even from human to animal making the human being as a host and reservoir at the same time (Ashford, 1996; Killick-Kendrick, 1999; TheCentersforDiseaseControlandPrevention(CDC), 2024).

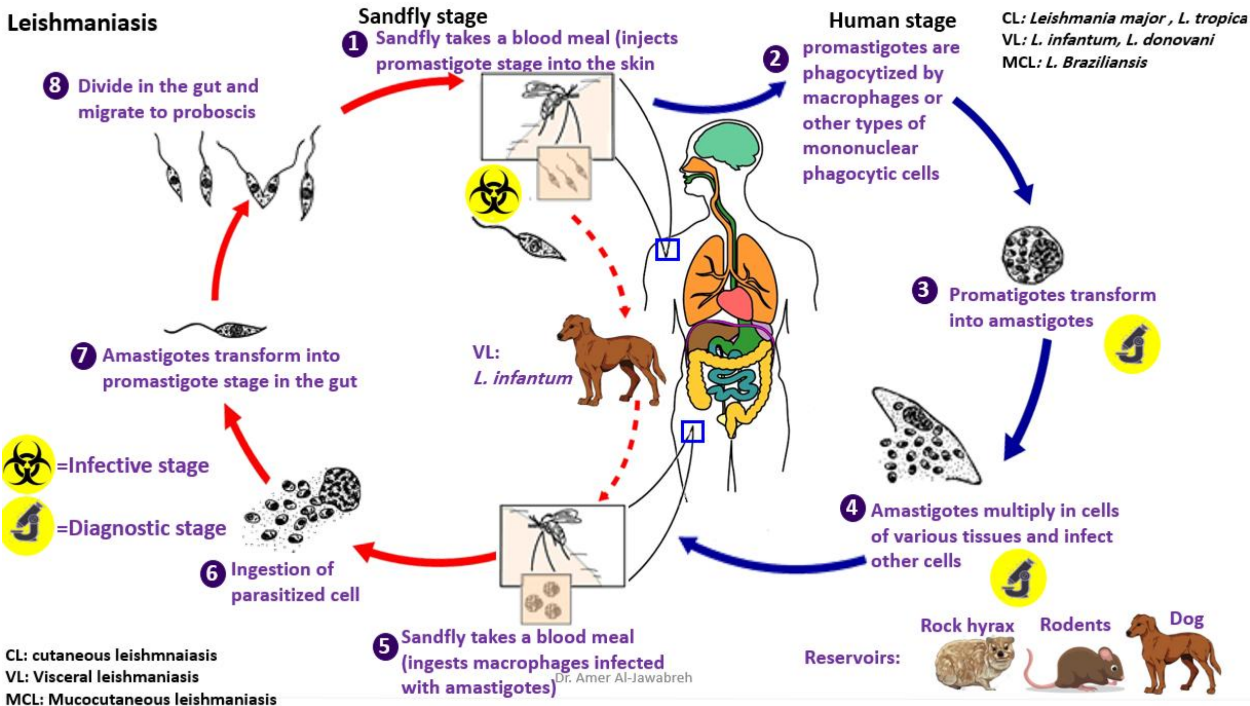


Figure 1. The life cycle of *Leishmania* parasites causing CL, MCL, and VL. Adopted from Centers for Disease Control and Prevention (CDC) with modification.

1.1. Clinical pictures

The duration of incubation period depends largely on the parasite species in with *L. major* requiring 2-3 months to show symptoms compared to 3-6 months for *L. tropica*. Factors that affect the length of the incubation period and severity of ulcers and lesions include the infective dose of promastigotes the sandfly injects into the host, age of host, and immune status of the host. The CL ulcer is typically present as painless erythematous tiny papule (1-2 mm) at the site of the sand fly bite which starts as a papule and then develops in slightly elevated nodule. With time the nodule open a shallow depression in the center and progress to ulcer with raised borders. The ulcerating lesion dry forming a crust which falls out forming a wet crater that remains from several months to a year, if left untreated. Complications such as secondary bacterial infection may occur. Atypical CL lesions such as group of papules or vesicles resembling acne, impetigo, swellings, and furuncles with varying sizes have been reported (Figure 2). The CL ulcers heal spontaneously after a period of 12 to 18 month depending species. With no or delayed treatment, a life-long shallow depressed scar will be left behind causing serious disability or stigma (WorldHealthOrganization,

2024). The shape, size, number and severity of CL ulcers depend on the host immune response, species, virulence, secondary bacterial infection, delayed therapy, frequency and duration of biting activity (Baghad et al., 2020; Franca et al., 2021; Oliveira et al., 2011; Volpedo et al., 2021).

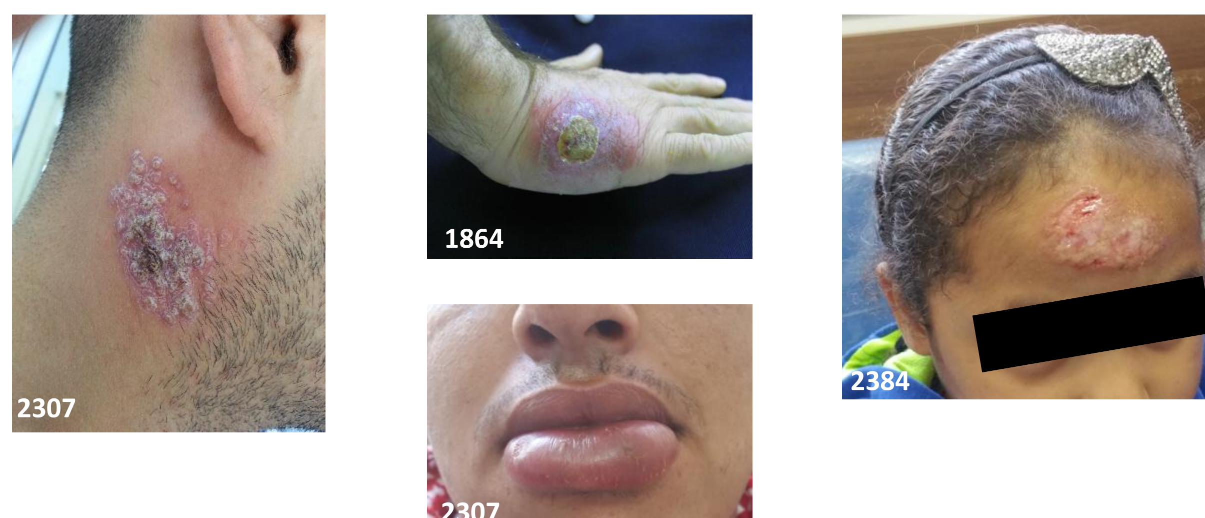


Figure 2. Confirmed CL-cases from Jericho Palestine with ulcers taking different forms.

1.2 Epidemiology

1.2.1 Global, regional and local distribution

Leishmaniases is considered a neglected tropical disease. In 2023, 86 countries out of 194 countries and territories reporting to WHO are endemic for leishmaniasis with over 350 million at risk. The total number of reported cases worldwide was 271,776 CL cases. More than 83% of the cases were reported from six countries, Afghanistan, Pakistan, Syria, Peru, Algeria, and Brazil having more than 5000 CL cases (Figures 3 & 4). In 2023, there were 393 imported cases of cutaneous leishmaniasis in 22 countries. About 95% of CL cases occur in the Americas, the Mediterranean basin, the Middle East and central Asia. It is estimated that 600 000 to one million new cases occur worldwide annually but only around 270, 000 are reported to WHO (WorldHealthOrganization, 2024). There are 22 countries that did not provide data to WHO despite having CL cases. In the Mediterranean basin, 63,653 CL cases were reported in eight countries Syria; Tunisia, Morocco, Egypt, Libya, Italy, Spain, and Greece. There were nine countries that did not send any data to WHO and for unknown reasons the state of Palestine was excluded from the WHO report despite having CL cases. The Syrian Arab republic reported the majority of CL cases (89%) followed by Tunisia (6%) and Morocco (4%)(WorldHealthOrganization, 2024).

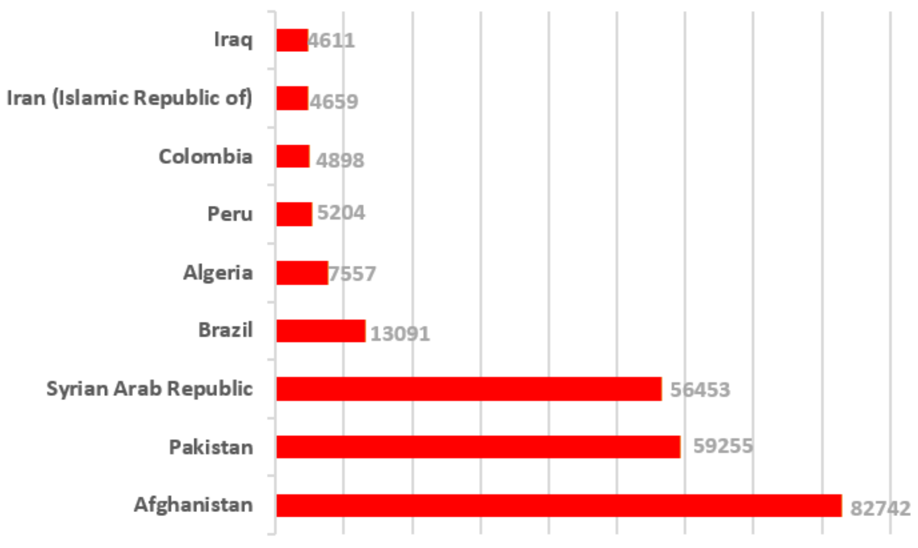


Figure 3. The countries with the highest number of reported CL cases in the world- 2023 (WorldHealthOrganization, 2024)

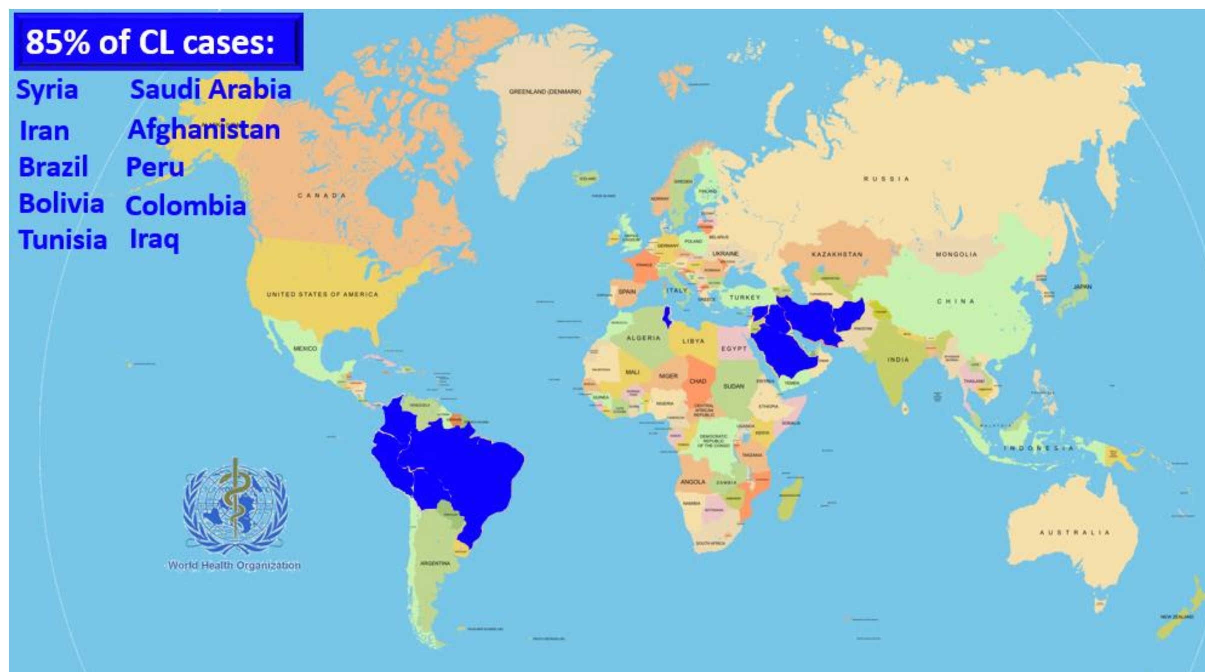


Figure 4. World map showing the countries with highest number of reported CL cases in 2023 shown in blue (WorldHealthOrganization, 2024).

In Palestine, CL cases, called 'Jericho boil' then, were reported as early as 1910 by Palestinian and foreign scientists and practitioners in which patients with skin ulcers were proven microscopically to have the parasite in their ulcers (Canaan, 1916; deBeurman, 1910; Huntemüller, 1914). A

century ago were reported from many areas extending from Haifa on the Mediterranean Sea in the west to Jericho in the Jordan valley in the east and localities in between such as Al-Quds, Beit-Jala, Beit Sahour, Ein-Karim village, A'rtuf village, Nablus, Tulkarem, al-Lidd, and AL-Khalil (Canaan, 1916; Canaan 1929; Canaan, 1945). The *Phlebotomus papatasi* sand fly was described as the vector for the disease (Canaan 1929). The CL scientific research in Palestine remained idle following the Nakba in 1948 and remained so until the six-day war in 1967, with first work published on the control of the CL in the Jordan valley and epidemiology in the West Bank as a whole (Arda & Kamal, 1983; Blum, 1978; Naggan et al., 1970). With the introduction of molecular-based methods and the rise of a new generation of Palestinian researchers, CL research gained momentum that spun off studies describing the distribution of CL in Jericho area and adjacent areas (Al-Jawabreh et al., 2003; A. Al-Jawabreh et al., 2001; Al-Jawabreh et al., 2004; Azmi et al., 2017; Azmi, Schnur, et al., 2012; Azmi, Schonian, et al., 2012; Sawalha et al., 2022; Sawalha et al., 2017; Sawalha et al., 2003).

1.3 Diagnostic Methods

Leishmaniases can be diagnosed depending on the form of disease, cutaneous, mucocutaneous, or visceral (Figure 5). Specimen of choice for cutaneous and mucocutaneous leishmaniasis is skin scrapings from the ulcer (Al-Jawabreh, 2020). While, the specimen of choice for visceral leishmaniasis is splenic puncture, bone marrow aspirate, lymph node aspirate, and buffy coat of peripheral whole blood with a sensitivity of >95%, 60-85%, 50-65%, and 20-70%, respectively (Babiker et al., 2007; Diro et al., 2017; Osman et al., 1997; Sundar & Rai, 2002). The sensitivity of methods vary depending on the diagnostic method used ranging from low sensitivity by microscopy to high sensitivity by PCR. The methods of diagnosis that have been used for the identification of *Leishmania* spp. include the following:

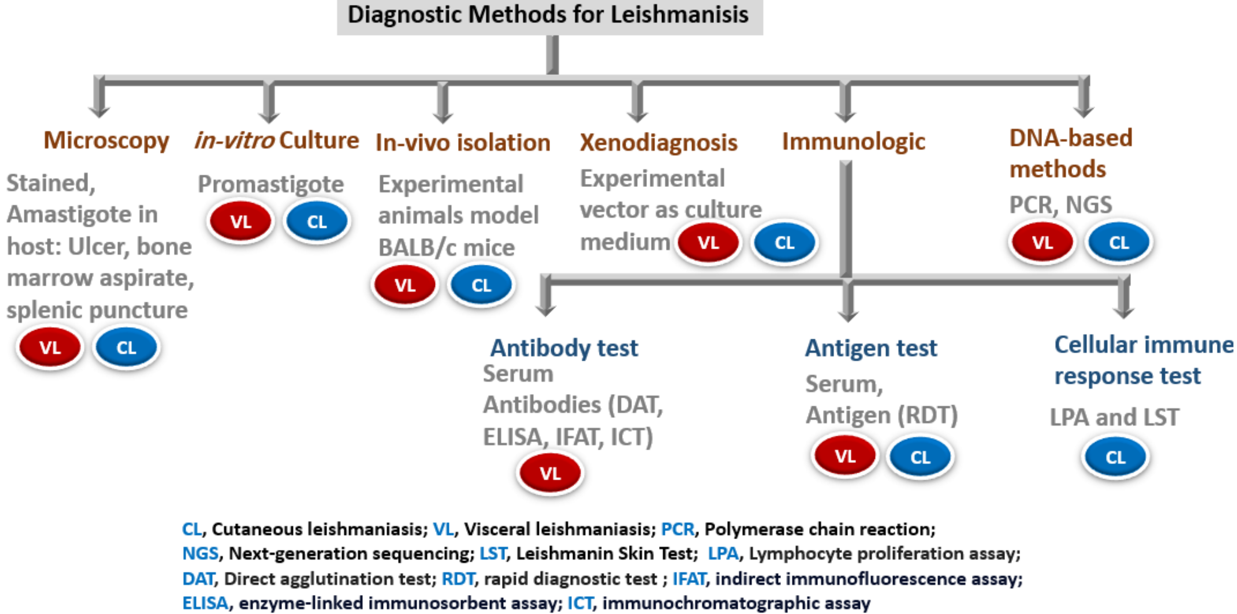


Figure 5. Diagnostic method for the identification of *Leishmania* parasite causing VL and CL. CL includes MCL. In addition to VL, antibody test includes MCL, but not CL.

1.3.1. Microscopy in which the touch smears are taken as described elsewhere (Al-Jawabreh, 2020) and stained by Giemsa stain. Under the microscope, the amastigote stage will appear as small (2 x 5µm) oval-shaped structure with large eccentric pale blue pinkish nucleus. Close to the nucleus is a small dark violet dot-like structure called kinetoplast. Amastigotes can be seen intracellularly in the macrophages or extracellularly around cells (Figure 6)(Al-Jawabreh et al., 2017; A. Al-Jawabreh et al., 2001; Al-Jawabreh et al., 2004).
Once this structure is demonstrated in the stained tissue smear, the cases is confirmed to be Leishmaniasis, but without identifying the species. The parasite load can be graded as applied elsewhere (Al-Jawabreh et al., 2006).

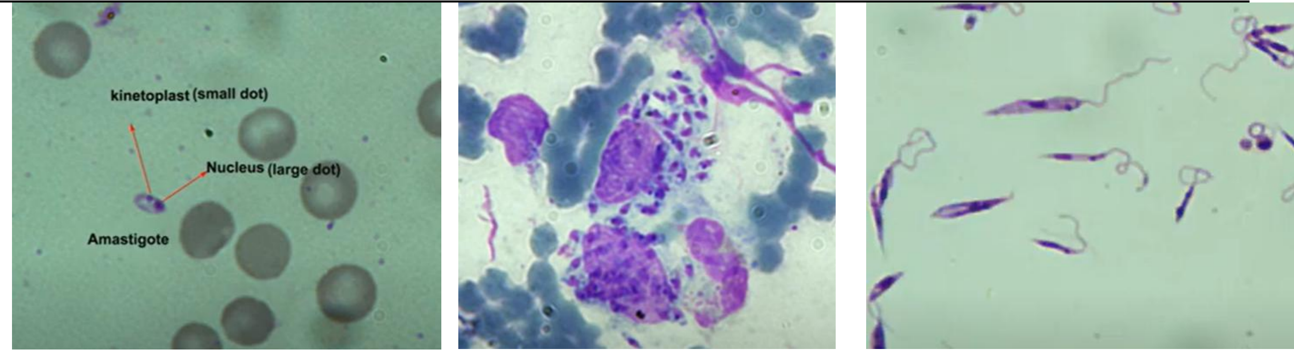


Figure 6. (a) Extracellular amastigote showing the large nucleus and small kinetoplast. (b) Intracellular amastigotes in a macrophage. (C) Promastigotes from a mass culture. Giemsa stain smears viewed under 1000x magnification.

1.3.2. *In-vitro* culture: For research purposes, the Leishmania parasite can be *in-vitro* cultured as described elsewhere (WorldHealthOrganization, 2010). *Leishmania* can be simply seeded using NNN (Novy-MacNeal-Nicolle) medium supplemented with defibrinated rabbit blood or using various enrichment media such as Schnierder's Drosophila medium, RPMI-1640, and M-199. have been used to produce mass cultures (de Oliveira Filho et al., 2024; WorldHealthOrganization, 2010). The *in-vitro* culture produces the actively-motile flagellated form of the parasite called promastigotes (Figure 6). It is a slender spindle-like fusiform shape longer than amastigotes (5 x 15µm) with large centric nucleus, small kinetoplast at the anterior part of the parasite, and a single anterior flagellum. Despite low sensitivity and inability to speciate, the demonstration of the two forms of *Leishmania*, amastigotes and promastigote, from the clinical samples is considered the gold standard and included in the WHO cases definition (WorldHealthOrganization, 2010),
1.3.3. Experimental animal model (*in-vivo* isolation)*:* Another mothed of diagnosis, though not common, is the use of experimental animal model such as BALB/c mice, Syrian golden hamster (*Mesocricetus auratus*) in which the patient sample is injected into the hind footpad and re-isolated after few days and inoculated into *in-vitro* culture medium. The presence of promastigotes indicates infection (Kamala & Nanda, 2009; Loria-Cervera & Andrade-Narvaez, 2014).
1.3.4. Xenodiagnosis: defined as exposing suspected *Leishmania* patient to a feeding uninfected sandfly female and after eight days the sandflies are dissected for the presence of promastigotes in their gut (Myskova et al., 2008; Sadlova et al., 2015; Singh et al., 2021). Xenodiagnoses and animal model are mainly restricted to research not used in clinical practice and diagnostic settings as they are time consuming and costly.
1.3.5. Immunologic methods: a commonly used field serology tests for VL and MCL in endemic areas are direct agglutination test (DAT) and rK39, a rapid immunochromatographic test (ICT), that

detect anti-leishmanial antibodies with high sensitivity of 95% and 94%, respectively. The classical IFAT and ELISA tests were less sensitive, 88% and 75%, respectively (Mniouil et al., 2018). The sensitivity of serology tests drops dramatically in VL-HIV coinfection and can remain positive for long period after cure (Boelaert et al., 2008; Lockwood & Sundar, 2006). Serology has shown a poor performance for the diagnosis of CL (Al-Jawabreh et al., 2003). Less commonly used is the detection of *Leishmania* antigen in a rapid detection test (RDT). RDT detects a membrane-based amastigote antigen (peroxidoxin) found in VL, CL, and MCL causing species such as CL Detect Rapid Test™, and for VL widely used rK39 test (REF) The sensitivity of such tests were found to be 64-73%(Azam et al., 2024; Bennis et al., 2018). Other less used tests for the diagnosis of CL are the Leishmanin skin test (LST) or Montenegro test and lymphocyte proliferation assay LPA. LPA is based on the idea that human defence against leishmania is a cell-mediated immunity type in which lymphoproliferation and the production of IFN-γ and TNF-α are capable of eliminating *Leishmania* parasite. The test assesses the the lymphoproliferative responses of peripheral blood mononuclear cells (PBMCs) of CL patients. With respect to LST, CL patient is intradermally injected with leishmanin antigen and then induration is measured after 48 to 72 hours (≥5mm for positive CL). Despite high sensitivity (97%), LST and LPA do not distinguish between active and cured CL. LST is not used for the screening of active VL as it shows negative results due to failure of immune response (anergy)l.,(Alvarado et al., 1989).

1.3.6. DNA-based detection methods: These methods depend on the amplification of a certain *Leishmania* DNA target followed by amplicon visualization. One study located some 51 DNA targets for the diagnosis of leishmaniasis (Gow et al., 2022). The selection of the DNA target depends on copy numbers, increased variation to detect species level, and stability of target (Gow et al., 2022). DNA-based methods were attractive to researchers and diagnostic service due to high analytical sensitivity, specificity, rapidity, species identifications, easiness, wide range of clinical specimen and acceptable costs (Gow et al., 2022). Real time PCR (qPCR) has been additionally used to monitor therapy by sequentially detecting parasite load and further identify asymptomatic cases (Ortalli M et al., 2020; Tsokana CN et al., 2014). DNA- based detection methods, mainly PCR, have shown high sensitivity and specificity ranging from 62-93% (Deborggraeve et al., 2008; Eberhardt et al., 2018). It all depended on the DNA target, type and source of specimen, quality of extracted DNA, storage and transportation of specimen, amplification protocols, work setup (research vs clinical practice), and method of result visualization, chemical (ethidium bromide) or fluorophore (fluorescent dye). One main disadvantage DNA- based methods is the lack of agreement on the best possible target for the detection of *Leishmania*. This disagreement may have resulted in not including these very sensitive methods in the WHO case definition of CL and just restrict it to the less sensitive methods, microscopy and *in-vitro* culture (WorldHealthOrgabization, 1999). The large number of targets caused result inconsistencies with varying levels of sensitivity and specificity, yet still higher than conventional diagnostic methods (da C Gonçalves-de-Albuquerque S et al., 2015). However, such inconsistencies can be reduced by utilizing a stringent control panel including negative and positive controls, inhibition control by spiking specimen tube with a minimum concentration of positive control to check for the presence of inhibitors if results are negative, DNA extraction control by targeting host (not *Leishmania*) housekeeping genes such as β–actin or β–globin which should be positive indicating successful DNA extraction (Al-Jawabreh et al., 2004; Al-Jawabreh et al., 2006).

Since 2020, amplicon-based next-generation sequencing (NGS) technology has begun to be applied to the etiological diagnosis and species identification of *Leishmania* in DNA samples by high-throughput sequencing and database comparison without the need to isolate pathogens or run

gel electrophoresis. Compared with traditional methods; NGS, whether amplicon-based (AmpNGS) or metagenomic (mNGS) is more advantageous for the identification and speciation of *Leishmania*. A growing number of publications, mostly case reports, have utilized NGS technology in the detection and genotyping of *Leishmania* have proved the potential of NGS in this field as described in Table 1.

Table 1. Publications showing the use of NGS in the diagnosis of *Leishmania* spp

Ref.	Type of NGS	Type of article	Leishmania spp	Country	Target
(Williams et al., 2020)	mNGS	Case report	*L. infantum*	Australia	All DNA
(Chen et al., 2020)	mNGS	Case series	*L. infantum*	China	All DNA
(Ren et al., 2021)	mNGS	Case report	*L. infantum?*	China	All DNA
(Song et al., 2021)	mNGS	Case report	*L. donovani*	China	All DNA
(Wang C et al., 2021)	mNGS	Case report	*?*	China	All DNA
(Lin et al., 2021)	mNGS	Case report	*L. infantum*	China	All DNA
(Ren et al., 2021)	mNGS	Case series	*L. donovani?*	China	All DNA
(Castillo-Castaneda et al., 2022)	Amp-NGS	Case series	Inf-don	Colombia	HSP70
(Gao et al., 2022)	mNGS	Case series	*L. donovani*	China	All DNA
(Han et al., 2022)	mNGS	Case series	*L. infnatum*	China	All DNA
(Nasereddin et al., 2022)	Amp-NGS	Full article	*L. tropica*	Palestine	ITS1, (vector)
(Liang et al., 2022)	mNGS	Case report	*L. donovani*	China	All DNA
(Chang et al., 2023)	mNGS	Case report	*L. donovani*	China	All DNA
(Al-Jawabreh et al., 2003)	Amp-NGS	Full article	*L. tropica*	Palestine	ITS1, (vector)
(Li et al., 2024)	mNGS	Case report	*L. donovani*	China	All DNA

1.3.7. WHO cutaneous leishmaniasis case definition

The definition of CL adopted by WHO concluded the following points:
a**. Clinical description**: Appearance of one or more lesions, typically on uncovered parts of the body. The face, neck, arms and legs are the commonest sites. At the site of inoculation, a nodule appears, which may enlarge to become an indolent ulcer. The sore remains in this stage for a variable time before healing and typically leaves a depressed scar. Other atypical forms may occur. In some individuals, certain strains can disseminate and cause mucosal lesions. These sequelae involve nasopharyngeal tissues and can be disfiguring.
b. **Laboratory criteria for diagnosis**: a CL case is confirmed by a positive parasitology (stained smear or culture from the lesion), however, for a mucocutaneous leishmaniasis only, positive serology (IFAT, ELISA) is included to the definition.

c. **Case classification by WHO operational definition**: a case of cutaneous leishmaniasis is a person showing clinical signs (skin or mucosal lesions) with parasitological confirmation of the diagnosis (positive smear or culture) and/or, for mucocutaneous leishmaniasis only, serological diagnosis(WorldHealthOrgabization, 1999).

1.3.8. Literature review

The epidemiological studies in Palestine have been going on in Palestine since the beginning of the 20th century from the days of Tawfik Canaan, De Beurman, and Huntemueller (Al-Jawabreh, 2023; Canaan, 1916, 1945) until recent years. It all started as simple descriptions of the sex, age, and address of CL patient to a more in-depth view of the CL behaviour and interaction between infection components, host agent and environment, until using sophisticated mathematical modelling. The rapid development came as consequence of the continuous improvement of the diagnostic methods. In Palestine, and until the early 1990s, diagnosis depended on the clinical picture with sporadic laboratory use of microscopy. In 1983, a report based on clinical picture was published(Arda & Kamal, 1983). In the mid 1990 until the 2000, positive laboratory testing became a prerequisite for treatment in the Ministry of Health clinics which gave rise to the first long-term (1994-1999) laboratory-confirm (microscopy) study (Al-Jawabreh et al., 2003; A. Al-Jawabreh et al., 2001). With the help of international research projects first Leishmania NNN culture was successfully seeded in 1998 and couple of years later genotyped. In the period between 1998 and 2004, cultured isolates and archived specimens of various types were genotyped by ITS1-PCR which showed the predominance of *L. tropica* (A. Al-Jawabreh et al., 2001; Al-Jawabreh et al., 2004). In 2017, a large-scale study that included over 2200 laboratory-confirmed (microscopy, culture, and ITS1-PCR) suspected-CL cases over two decades of CL surveillance. Other sporadic reports on the CL in Palestine were published in the last three decades (Table 2).
The diagnosis in countries with endemic countries still relies on microscopy of stained smears with a sensitivity of 24% (Al-Jawabreh et al., 2018). *In-vitro* cultivation is used as an adjunct diagnostic method, though it is mainly used for research purposes. Despite the low sensitivity, these two methods are adopted along with the clinical picture for the case definition of CL due to the absence of highly sensitive and specific diagnostic tests. Meanwhile, molecular-based methods such as polymerase chain reaction (PCR) have been widely used for the diagnosis of leishmaniasis. Despite the high sensitivity of PCR, it is still not adopted in the CL case definition. This is partly due to the many targets used with no less than 50 targets reported in the literature with each calming to be the optimal PCR (Gow et. al., 2022). Therefore, it becomes essential to evaluate the common DNA targets of PCR for the detection and speciation of *Leishmania* in a true large clinical setting. The NGS method is a recent technology that has been utilized in the field of leishmaniasis by a dozen publications over the last five years (Table 1). Method comparisons for the diagnosis of leishmaniasis have been conducted and published in attempt to improve analytical accuracy (sensitivity and specificity). The comparisons ranged between types of tests (microscopy, in-vitro culture and PCR) (Al-Jawabreh et al., 2018; Al-Jawabreh et al., 2006), types of samples (Al-Jawabreh et al., 2018), various DNA targets(Bensoussan E et al., 2006; Leon et al., 2017; Rosales-Chilama et al., 2020), and between DNA-based detection technologies (Leon et al., 2017). Nevertheless, the NGS technology is still new to the CL diagnosis with our study expected to be one of the pioneer studies.

Table 2. CL research in Palestine during the period between 1916 and 2022.

Ref.	Period	Target area	Sample size	Species	Diagnosis
(Canaan, 1916)	1914-1916	Palestine(4)	26	Lt(2)	Confirmed case (1)
(Canaan 1929)	1916	Palestine(4)	28	Lt(2)	Confirmed case(1)
(Canaan, 1945)	1924-1945	Palestine(4)	242	UD	Clinical picture
(Arda & Kamal, 1983)	1972-1980	Palestine(5)	237	UD	Clinical picture
(Arda & Kamal, 1994)	1980-1989	Palestine(5)	223	UD	Clinical picture
(A. Al-Jawabreh et al., 2001)	1994-2001	Jericho	471 (152)(6)	UD	Confirmed case(1)
(A. Al-Jawabreh et al., 2001)	1998-2001	Jericho	26	Lt, Lm	Confirmed case(3)
(Al-Jawabreh et al., 2003)	1994-2001	Jericho	471 (152) (6)	UD	Confirmed case(3)
(Al-Jawabreh et al., 2004)	1997-2002	Jericho	49	Lt, Lm	Confirmed case(3)
(Azmi, Schonian, et al., 2012)	2002-2009	Jenin	47	Lt, Lm	Confirmed case(3)
(Azmi, Schnur, et al., 2012)	2002-2008	Jenin	12	Lt.	Confirmed case(3)
(Azmi et al., 2017)	2000-2011	Ariha, Jenin, Bethlehem	31	Lt	Confirmed case(3)
(Al-Jawabreh et al., 2017)	1994-2015	Palestine(5)	2160 (895)	Lt, Lm	Confirmed case(3)
(Amro et al., 2022)	1990-2020	Palestine(5)	5855	UD	UD

(1) Giemsa-stained smear positive. (2) It was called *L. tropica* at that time. (3) Positive by smear, culture, and/or PCR. (4) Historical Palestine encompassed between the Mediterranean Sea and the River Jordan. Palestine indicates the West Bank area only. (6) Number in brackets is the confirmed CL cases, UD, undetermined.

Objectives:

The main objective of the study was to determine the burden of disease (BOD) and compare the amplicon-based NGS with conventional PCR. The specific objectives include:

1. Determine the burden of human CL in the Palestinian population by assessing the magnitude of CL cases (prevalence) and their distribution according to the PPT model (Person, Place, time) in the period between 2016 and 2023.
2. Compare the sensitivity and specificity between the conventionally used ITS1-PCR and the new NGS method.

CHAPTER 2

2. Materials and methods

2.1 Study design and study population

A retrospective population-based cross-sectional study design was used. The study included all patients attending, referred to Leishmaniases Research Unit (LRU)-Jericho for laboratory diagnosis during the period between 2016 and 2024. In addition, the study included DNA samples from the central public health laboratory (CPHL)-Ministry of Health in Ramallah-Palestine. The study covered the West Bank area of Palestine. The West Bank includes nine districts, Jericho, Jenin, Tulkarem, Qalqilyia, Salfit, Nablus, Bethlehem, Al-Quds, Al-Khalil (Map 1). Patient data including demography, clinical, epidemiology, behavior and environment, and laboratory tests were collected right before the sample collection. The data was fed into the EpiInfo™ Leishmaniasis Database.

2.3 Sample Collection

For each patient attending LRU-Jericho, data sheet was be filled, five touch smears were taken from CL-suspected lesion(s), dermal tissue aspirate with normal saline for seeding into NNN culture medium, 4 dermal tissue drops spotted on sterile filter paper as described elsewhere . (Al-Jawabreh, 2020; WorldHealthOrganization, 2010).

2.4 Laboratory diagnostic methods

2.4.1. Microscopy:

Touch smears were stained with Giemsa stain, and examined microscopically for amastigotes as described elsewhere (Al-Jawabreh, 2020; WorldHealthOrganization, 2010)

2.4.2. In-vitro culture:

Dermal tissue aspirates were cultured in rabbit enriched NNN culture media during the years 2016 and 2019, as described previously (Al-Jawabreh, 2020; WorldHealthOrganization, 2010).

2.4.3. DNA extraction and storage:

DNA was extracted from tissue drops spotted using commercial extraction kits (DNeasy Qiagen USA, Purlink, Invitrogen USA, and Nucleospin Macherey-Nagel Germany). Extracted DNA was store at -80°C until amplification**.**

2.4.4. PCR-ITS1 amplification:

The ribosomal internal transcribed spacer 1 (ITS1) region separating the genes coding for ssu rRNA and L5.8S rRNA will be amplified by a PCR, using the primers LITSR and L5.8S as described (El Tai et al., 2000; Schonian *et al*., 2003); The primer pair used for the amplification of ITS1 are LITSR / L5.8S (5´-TGATACCACTTATCGCACTT-3´)/(5`-CTGGATCATTTTCCGATG-3´). ITS1 was used as a target due to the high variability of the DNA sequence and length across *Leishmania* species, but conserved within the genus which makes it suitable for genotyping and phylogenetic studies by RFLP or DNA sequencing. ITS1-PCR is a

widely used method as indicated by the high number of citations of the original publication (>1000) (Schonian et al., 2003).

Table 3. Master Mix (MM) for PCR-Ready™ High Yield and GoTaq® mix

Component	Volume (µl)	Concentration
1. MM1: PCR-Ready™ High Yield:		
Primer-F	1.25	0.2 µM
Primer R	1.25	0.2 µM
DMSO	1.25	5%
RNase-free distilled water	16.25	
DNA template, clinical sample	4	16%
2. MM2: GoTaq® Green Master Mix:		
Primer-F	1.25	0.2 µM
Primer R	1.25	0.2 µM
DMSO	1.25	5%
GoTaq® mix	12.5	
RNase-free distilled water	3.75	
DNA template	3-5	15%

The thermocyling profile using the Thermal cyclers C1000 and CFX-96 from Biorad was run according to table 4.

Table 4: Thermal cycling profile for ITS-1 PCR

Step	^{o}C	time	X
Denaturation, initial	95	2min	1
Denature	95	30s	
Anneal	53	30s	40
Extend	72	30s	
Extension, final	72	6min	1

Moreover, the ITS1-219-NGS was developed based on the original ITS1-PCR allowing for a scientifically-sound comparison between the two methods as a major objective of this study

(Schonian et al., 2003; Talmi-Frank et al., 2010). The amplification master mixes that have been used in the study were PCR-Ready™ High Yield from Syntezza-Jerusalem and GoTaq® Green Master Mix from Promega-USA depending on the availability. The PCR reaction mix was prepared in a UV sterilized PCR box procedure for the PCR-Ready™ High Yield was conducted as shown in table 3.

2.4.5. Gel electrophoresis:
PCR products of ITS-1 were visualized by agarose gel electrophoresis and stained with ethidium bromide as described by others (Schonian et al., 2003) .

2.4.6. Control panel:
The efficiency of ITS1-PCR system was monitored by a stringent control system that included negative control with RNase free distilled water or no-sample reaction, positive control that included *Leishmania turanica* DNA (MRHO/MN/83/ MNR-6). In addition inhibition control was included in cases of suspected presence of inhibitors such as hemoglobin as described elsewhere (Al-Jawabreh et al., 2004; Al-Jawabreh et al., 2006). Inhibition control involved duplicate run of the suspected sample in which the second tube was spiked with 0.4pg/µl *L. turanica* DNA from cultured promastigotes to ensure positive result. Spiked tubes with negative results indicated the presence of PCR inhibitors. Finally, DNA extraction control was included to check the true negativity and that negative results were not due to DNA extraction failure. Two housekeeping genes were used; the β-actin gene with primer pair Aco1/Aco2(5-ACCTCATGAAGATCCTCACC-3)/ (5-CCATCTCTTGCT CGA AGT CC-3) (Al-Jawabreh et al., 2006) and the human β-globin gene with primer pair he primer pair HbG-F (5 ‘-GAA GAG CCA AGG ACAGGTAC-3 ‘)/HbG-R(5 ‘-CAACTTCATCCACGTTCACC-3 ‘) (Al-Jawabreh et al., 2004).

2.4.7. Restriction fragment length polymorphism
To identify the *Leishmania* species, each positive ITS1-PCR amplification product (15µl) was digested at 37 ^{O}C for 2 h with the restriction endonuclease enzyme *HaeIII* using the conditions recommended by the supplier (Hybaid GmbH Heidelberg, Germany). The restriction fragments were run electrophoretically in 2% agarose gel at 100 volts for one hour in 0.5x TBE (45 mM Tris-borate, 1 mM EDTA) buffer and visualized under ultraviolet light after staining with ethidium bromide (0.5 μg/ml) as described elsewhere (Al-Jawabreh et al., 2004; Schonian et al., 2003).

2.4.8. Next generation sequencing (NGS)
Two set of samples were selected for the comparison of NGS method. First, negative set, which consisted of samples that were negative by, microscopy, *in-vitro* culture, and conventional PCR (cPCR) based on ITS1. Secondly, positive set, which is the one that consisted of samples that were positive by either of the testes used in this study, microscopy culture and/or ITS1-PCR. Next generation sequencing for the samples involved in the comparison arm were carried out according to Nasereddin et al. (Nasereddin et al., 2022) using the primer pair ITS1219NGSF: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGC TGG ATC ATT TTC CGA TG and ITS1219NGSR: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GAT CG CGA CAC GTT ATG TGA G with product size of 343bp. The next-generation sequencing went through the following steps:

a. Amplicon based Library preparation, and sequencing: The aim of this step is to amplify leishmanial DNA. First ITS1-PCR run was conducted in two 96-well plates, one for CL ulcer positive and the other for the non-CL ulcers. Twenty-five microliters of PCR products were purified using AMPure XP beads (Beckman Coulter, X1) and eluted in 25µl of elution buffer. Only 7.5µl of the cleaned products underwent a secondary PCR to incorporate unique index sequences (N7XX and S5XX) for sample tagging, utilizing the Nextera XT Index Kit (Illumina, San Diego, CA, USA). Subsequently, 5µl from each tagged sample was combined, thoroughly mixed, and centrifuged. Finally, the two DNA pools (100 µl each) for the CL ulcer DNA and non-CL ulcer DNA were re-purified with X1 AMPure XP beads and quantified using the Qubit® Fluorometer (Invitrogen). A concentration of 75ng/µl for the CL ulcers and 1.25 ng/µl for non-CL ulcers were prepared from the two pools. The target number of sequencing reads per sample was least 10,000. Samples were subjected to high-throughput sequencing on the NextSeq500 system using the 150-cycle mid-output kit (Illumina, Inc., USA), generating reads from the forward strand.

b. Bioinformatics Analysis

Binary Base Call (BCL) output files from a Next Seq 500 system were transformed into FASTQ format using the BCL to FASTQ conversion tool (bcl2fastq v2.20.0.422, Copyright © 2007–2017 Illumina, Inc.). The resulting files were processed through the Galaxy platform (Galaxy Version 0.7.17.1) (https://usegalaxy.eu/). Initially, the generated sequences underwent quality assessment via Fast QC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to evaluate the integrity of the reads. The sequences were subsequently trimmed (https://usegalaxy.org/) with default settings to preserve high-quality reads, defined as those exceeding 100 base pairs in length and a minimum quality score above 20. This quality threshold corresponds to an error rate of 1 in 100, aligning with Illumina NextSeq sequencing accuracy metrics, which ensure a base call accuracy of 99%. The analysis involved creating four virtual probes for *Leishmania* spp, *L. donovani* complex, *L. major*, and *L. tropica* based on the multiple alignment of nine DNA sequences (Figure 7). The analysis of NGS data started by creating a workflow pipeline using the virtual probes to identify the *Leishmania* genus and species (Figure 8). Filtered data were matched using virtual probes specific to *Leishmania* at both the genus and species levels. Based on species-specific probes, the sequence read count for each species was quantified.

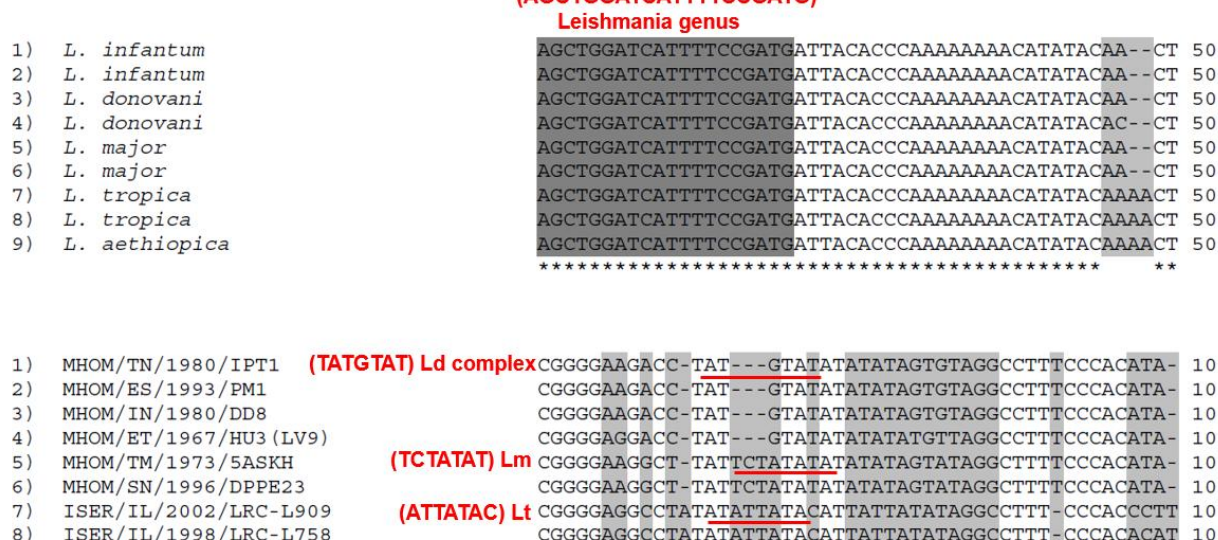


Figure 7: Multiple alignment of ITS1 of nine *Leishmania* strains including *L. infantum* (MHOM/TN/1980/IPT1, MHOM/ES/1993/PM1); *L. donovani* [MHOM/IN/1980/DD8, MHOM/ET/1967/HU3 (LV9)]; *L. major* (MHOM/TM/1973/5ASKH, MHOM/SN/1996DPPE23); *L. tropica* (ISER/IL/1998/LRC-L758 and ISER/IL/2002/LRC-909); and *L. aethiopica* (MHOM/ET/1972/L102). The dark grey areas in the 5′- and 3′-ends of the sequences represent the oligonucleotide primers used for amplification. The light gray areas represent nucleotide mismatches between the aligned sequences. The virtual probes DNA sequences (in red) for *Leishmania* genus, *L. major* (Lm), *L. tropica* (Lt) and Ld *(L. donovani)* complext probes are shown in red (Talmi-Frank et al., 2010).

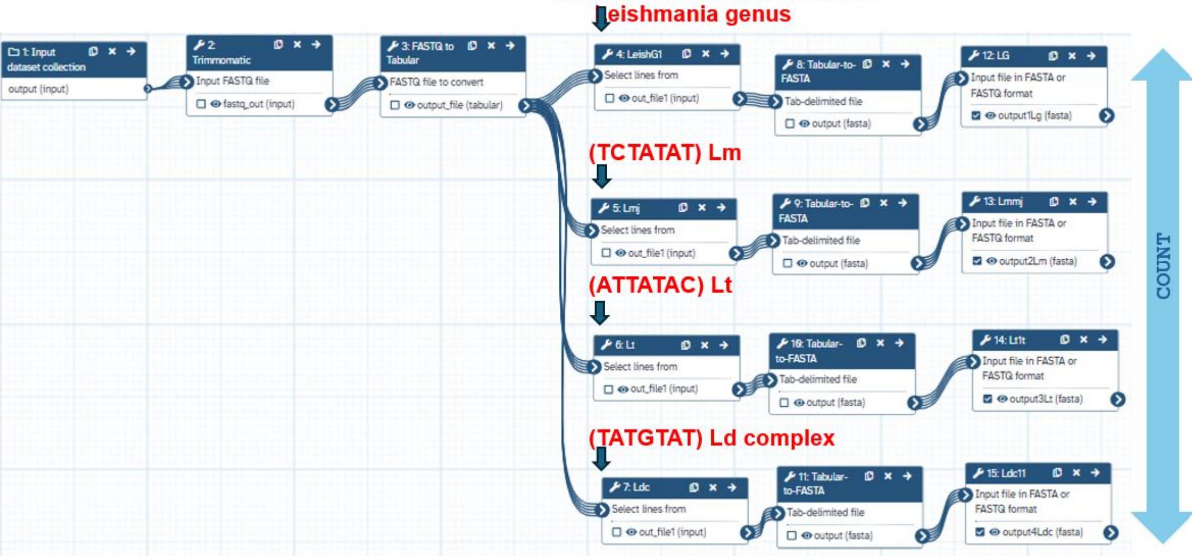


Figure 8: NGS Workflow: The sequence of the workflow pipeline tool for overall approach to analysing FastQ files as output from NGS results, the virtual probes DNA sequences (in red) for *Leishmania* genus, *L. major* (Lm), *L. tropica* (Lt) and Ld (*L. donovani*) complex probes are shown in red.

In cases where non-*Leishmania* were identified, the predominant sequence was analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify species identity. A threshold of 200 reads per sample was established to confirm *Leishmania* positivity, following previously described criteria (Early et al., 2019).

2.6 Statistical analysis:

Epi Info 7™ statistical package (CDC free-software) was used for data analysis including distribution and frequency tables and graphs, spot maps and choropleth maps. Contingency 2×2 tables for infections of CL and risk factors were tested using the two tailed Fisher's exact test at a P-value <0.05. Fisher's exact test at a P-value <0.05 was used to compare the two unmatched groups, the CL ulcer group and the non-CL ulcer group. The cases definition of CL depends on demonstration of amastigotes in a stained smear taken from the ulcer or the promastigotes following in-vitro culture. Both tests suffer from low sensitivity as shown previously (Al-Jawabreh et al., 2018; Al-Jawabreh et al., 2006) making them imperfect gold standard. In order to avoid evaluating a newly advanced methods using an imperfect gold standard, the following equations were used assuming ITS1-PCR as the imperfect gold standard (Al-Jawabreh et al., 2006; Gart & Buck, 1966) as shown in table 5.

Table 5. A 2x2 table related to Equations 1 and 2

ITS1-219-NGS (New)	ITS1-PCR (Imperfect gold std)		Total
	Positive	Negative	
Positive	a	b	a+b
Negative	c	d	c+d
	a+c	b+d	n

$$Se_N = \frac{(a+b) * Sp_G - b}{n * (Sp_G - 1) + (a+c)} \quad \text{........Equation 1}$$

$$Sp_N = \frac{(c+d) * Se_G - c}{n * Se_G - (a+c)} \quad \text{..............Equation 2}$$

Se_N: Sensitivity, New (NGS)

Sp_N: Specificity, New (NGS)

Se_G: Sensitivity, imperfect gold std

Sp_G: Specificity, imperfect gold std

N: Total number of samples tested

The kappa statistic was used to measure the degree of agreement between the two methods, ITS1-PCR and ITS1-219-NGS in terms of results and genotyping. McNemar's test was used to detect any statistical difference between the two tests with dichotomous results (positive or negative) in a matched pairwise approach utilizing online GraphPad calculator (graphpad.com). As for the *Leishmania* genotyping with polytomous outcomes (*L. tropica*, *L. major* and undetermined); Bhapkar marginal homogeneity test, an extension of McNemar's test, was utilized to detect the difference between the two diagnostic tests. The level of significance was considered as P-value <0.05.

3. RESULTS

3.4 Distribution of CL: Person, Place, time model

3.4.1. Person:

The positivity rate (No. CL/tested persons) was 17% (213/1262). The prevalence of CL in the West Bank of Palestine during the eight-year study period was 7.0 per 100,000 person (CL=202), regardless of cure or healing of cases. The average annual incidence rate (IR) of CL cases in the West Bank of Palestine during the study period (2016-2023) was shown to be 0.84 per 100,000 with a total of 199 CL cases (25 cases per year) (Figure 9). The CL cases were distributed equally between males (52%, 99/190) and females (48%, 91/190). The age of the infected individuals range from 6.0 months to 71 years. The mean age of the CL cases was 19.2 years. The distribution of CL cases by age showed that the age range of 0-14 years is the most exposed to CL infection representing (49%, 63/128) (Figure 10). Approximately half of the reported CL infections sites were in the neck/head area (45%, 46/102) followed by the upper extremities (38%, 39/102), while the rest were in lower extremities. The nose and the forehead, equally, were the most infected sites in the head (29%, 7/24).

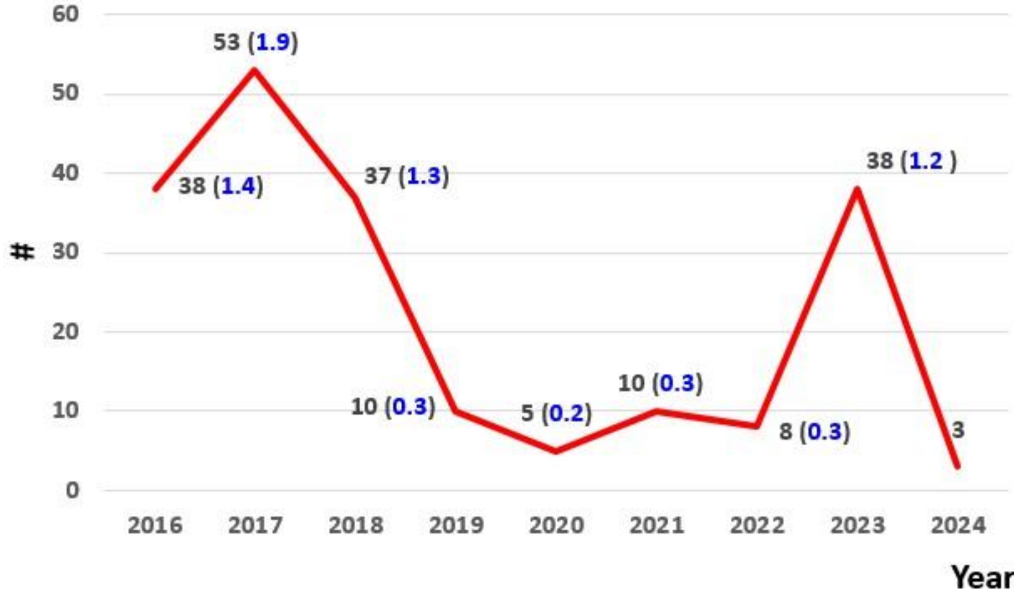


Figure 9. A line chart showing the distribution of CL cases by year during the study period 2016-2023. The numbers indicate that total number of CL cases per year while the numbers in brackets represent the incidence rate (IR) per 100,000 persons.

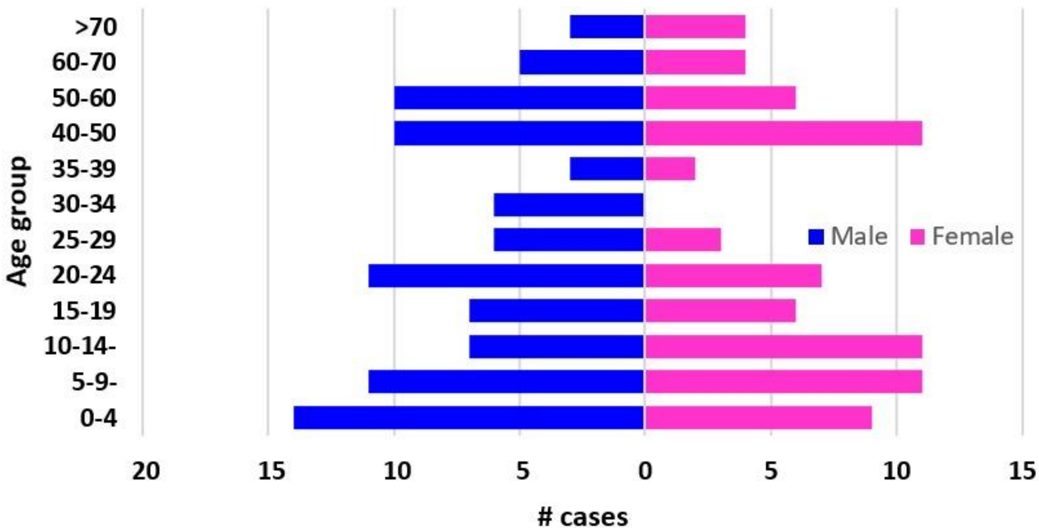


Figure 10. A pyramid graph showing the distribution of CL cases by age group and sex during the study period 2016-2023.

3.4.2. Place

Figure 11 shows the choropleths mapping of the CL cases from 2016 until 2023. The mapping showed that the Jericho District is the most prevalent district with a crude number of 79 CL cases (annual IR 21 per 100,000) followed by Tubas with 23 cases (IR 4 per 100,000). The predominating *Leishmania* spp. in Palestine is *L. tropica* which represents 75% (88/118) of the cases and spreading in all the Palestinian districts. The remaining percentage (25%, 30/118) is *L. major* which is mainly focused in the Jericho District (66%, 19/29) while the rest is reported in other districts. *L. tropica* and *L. major* are equally distributed in the district of Jericho. This study showed that the vast majority of the cases are reported from villages (69%, 92/134), followed by the cities or towns (35%, 35/134), while the rest were from refugee camps and Bedouin encampments.

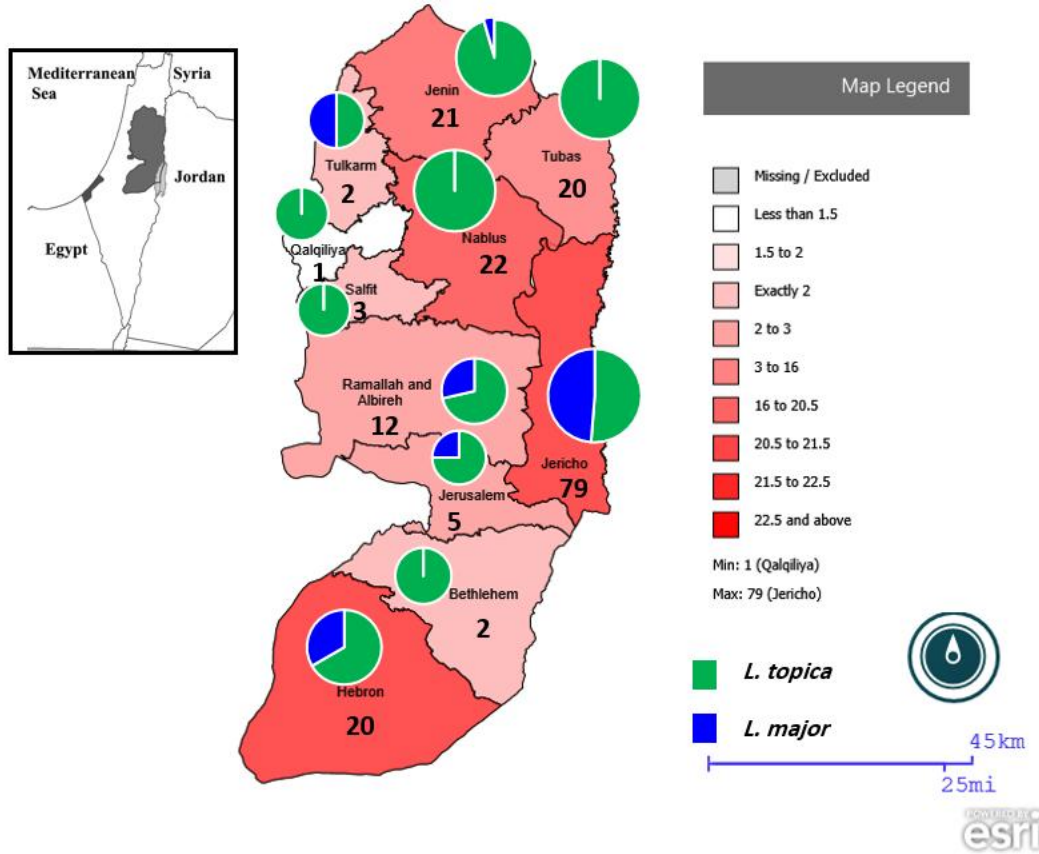


Figure 11. Choropleth mapping of confirmed CL cases in the West Bank, Palestine during the study period (2016-2023). The intensity of the red reflects the number of CL cases. The pie chart represents *Leishmania* spp., *L. tropica* and *L. major*. The size of the chart reflects the number of species. Map created by EpiInfo maps.

3.4.3. Time

The distribution-by-year revealed a peak in 2017 with number of CL cases reaching 53 representing more than half (26%, 53/202) of the cases reported during the study period. The district of Jericho has contributed with approximately 40% (18/53) of the bulk of cases. The monthly distribution of the CL cases disclosed the chronological pattern to be in the months from January to March (winter and early spring) forming approximately 52% of the depicted cases (Figure 12).

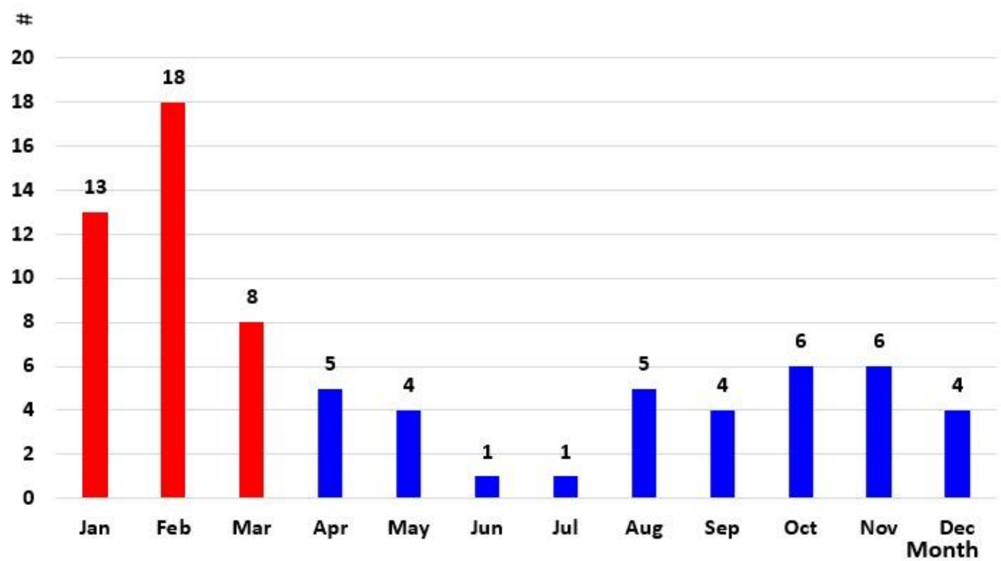


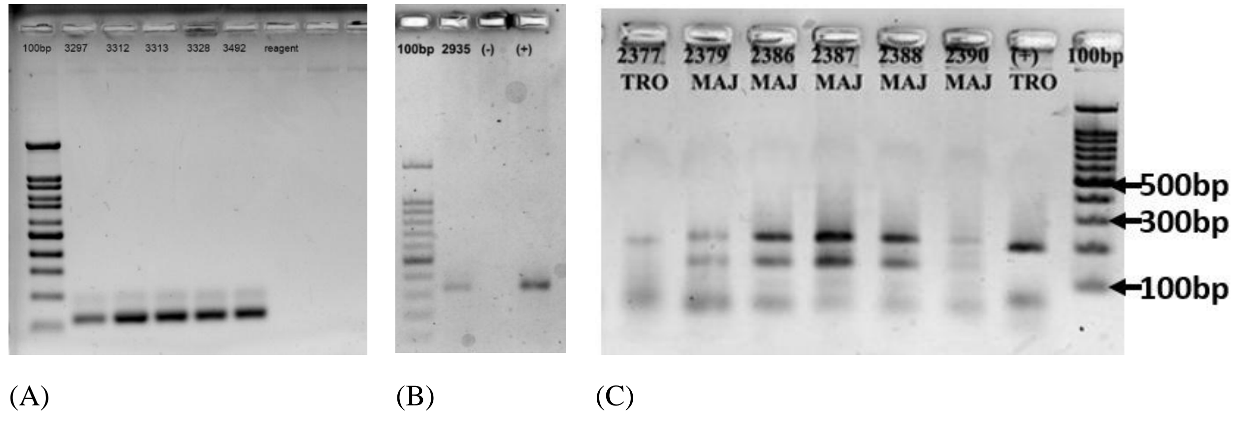
Figure 12. The monthly distribution of CL cases in Palestine during the period between 2016 and 2024. The bars show the total cases in the given months throughout the study period.

3.1 ITS1-219-NGS

From a diagnostic setup, two sets of samples tested with conventional ITS1-PCR and subsequently genotyped by RFLP as described in Annex 1 were randomly selected for ITS1-219-next generation sequencing (NGS) test (Figure 13). One of the sets consisted of 94 DNA samples negative for ITS1-PCR (plate DNA bulk concentration=1.24ng by Qubit™ fluorometer), while the other set of 94 was positive (plate DNA bulk concentration=75.2ng by Qubit™ fluorometer). In the ITS1-PCR negative set of samples, four DNA samples (4%) were shown to be positive by the new method (ITS1-219-NGS) and further confirmed by BLAST search of the four DNA sequences which completely matched Leishmanial DNA in the GenBank (Figure 14). While, 13 weakly positive DNA samples (14%) from the positive set were shown to be negative by the, ITS1-219-NGS (Table 5, 6, Annex 1). The study showed that there was an almost perfect agreement between the two test methods (kappa statistic=0.82, 95% CI= 0.73-0.90). The difference between the two diagnostic methods is a border line statistically significant (McNemar's test, P-value= 0.049-0.052) with a difference of 4.8% (95%CI=0.5-9.0) between the two tests.

Table 6. A 2x2 contingency table of the ITS1-219- NGS test assuming the ITS1-PCR as the imperfect gold standard

ITS1-219-NGS (New)	ITS1-PCR (Imperfect gold std)		Total
	Positive	Negative	
Positive	81	4	85
Negative	13	90	103
Total	94	94	188



(A) (B) (C)

Figure 13. Gel electrophoresis (1.4%) run of host β-actin amplicons for selected specimen showing a band at 120bp (A). Gel electrophoresis (1.4%) run of ITS-1amplicon for specimen 2935 showing a band comparable to Leismania spp (340bp) (A). *Leishmania* restriction pattern for the RFLP run made from ITS1-amplicon digested by *HaeIII* enzyme. *L. major* (MAJ) showed two bands at 203 and 132bp, while *L. tropica* (TRO) shows one band at 185bp while other three (57, 53, 24bp) are not seen due to small size. All came from skin scraping from skin lesion (B).

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Leishmania tropica isolate 56 clone 3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	Leishmania tropica	276	276	98%	6e-70	100.00%	966	FJ948450.1
☑	Leishmania tropica isolate 56 clone 1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	Leishmania tropica	276	276	98%	6e-70	100.00%	966	FJ948449.1

Figure 14. The BLAST search for the four ITS1-PCR negative, but positive for ITS1-219-NGS showed *L. tropica* species.

3.2. Comparative analysis between species

Table 7 Showed that the two diagnostic methods, ITS1-PCR and ITS1-219-NGS, had fair agreement (Kappa statistic=0.24, 93%CI=0.12-0.37) and that they agreed on 45 (36+9/81, 56%) observations only, while disagreeing on 36 results. The imperfect gold standard (ITS1-PCR) was unable to genotype 29 positive results, while they were accurately genotyped by ITS1-219-NGS. All 29 DNA sequences were BLAST-searched in the GenBank and found to completely (100% similarity index) match their corresponding DNA sequences in the GenBank, 28 *Leishmania tropica* and one *L. major* (Annex 1). However, nine out of the 13 DNA samples that were ITS1-PCR positive but negative by the new ITS-219-NGS had their genotype incorrectly determined as *L. tropica* (7), *L. major* (2) (Annex 1). Upon BLAST search of the NGS major reads for the ITS1-PCR positive, but ITS1-219-NGS negative; contaminations of human and bacterial origin were found predominate (Table 8). The ITS1-219-NGS was able to genotype all its positive results. The differences between the two diagnostic tests was statistically significant (Bhapkar test; P-value<0.001).

Table 7. A 3x3 contingency table of species identification by ITS1-219 NGS and ITS1-PCR, as the imperfect gold standard

ITS1-219-NGS (New)	ITS1-PCR (Imperfect gold std)			Total
	TRO	MAJ	UD	
TRO	36	7	28	71
MAJ	0	9	1	10
UD	0	0	0	0
Total	36	16	29	81

TRO, *Leishmania tropica*; MAJ, *L. major*; UD, undetermined

Table 8. The results of BLAST search for the most common NGS read of the specimen with discrepant results (ITS1-PCR negative, ITS1-219-NGS positive)

Rec	ITS1-PCR	Genotype	ITS1-219-NGS	BLAST
2070	Positive	Lt	Negative	*Ralstonia* spp.
2077	Positive	Lt	Negative	*Ralstonia* spp.
2076	Positive	Lt	Negative	*Ralstonia* spp.
2085	Positive	Lt	Negative	*Homo sapiens*
2100	Positive	Lt	Negative	*Enterococcus faecium*
2275	Positive	Lt	Negative	*SARS-CoV-2*
2727	Positive	Lt	Negative	*Escherichia coli*
2295	Positive	Lt	Negative	Phage genome
3679	Positive	Lt	Negative	*Ralstonia* spp.
3862	Positive	Lt	Negative	*Ralstonia* spp.

SARS-CoV2, Severe acute respiratory syndrome coronavirus 2

3.3 Validation of NGS test using imperfect gold standard.

As the gold standard (100% sensitive and 100% specific) for the diagnosis of cutaneous leishmaniasis (CL) is not established so far; the most sensitive test currently available was adopted as the gold standard which is described as ‘imperfect gold standard’. To overcome this dilemma, the study used Gart and Buck equations (Gart and Buck, 1966) (Table 5). Based on this, equations 1 and 2 were used to calculate the sensitivity and specificity for ITS1-219-NGS test which produced higher sensitivity of 94% compared to the 87% for the imperfect gold standard (ITS1-PCR) and a specificity of 100%. Without using Gart and Buck equations, the sensitivity of the ITS1-219-NGS test would have been be 86% (81/94) and a specificity of 96% (90/94) which are lower than the imperfect gold standard method (ITS1-PCR) (Table 6).

4. DISUSSION

Form an epidemiological point of view, the study revealed that the prevalence of CL in Palestine has reduced dramatically compared to that of the previous two decades (1994-2015). According the last wide-scale study, the average incidence rate (IR) in the last five years that preceded this study (2012-2015) was 10.9 per 100,000 while the average in this study became decreased to 0.85 per 100,000 (Al-Jawabreh et al., 2017). The plausible explanation for this reduction is the extensive pesticide spraying campaigns conducted by the ministry of health that covered the entire country twice a year (May and September) (Sawalha et al., 2022). Secondly, the COVID-19 pandemic during which the lockdown was imposed in 2020 and 2021 that restricted the movement of people and their encounter with their reservoir and vector. Thirdly, the health policies that ceased to use the clinical manifestations as the sole method for confirming a CL case and additionally imposed laboratory testing to be accepted as a CL case. Finally, the advanced diagnostic methods including PCR has significantly played part in genotyping and confirming a CL case. It is worth mentioning that MOH does no implement microscopy and *in-vitro* culture in the diagnostic protocol and rather restricted to PCR without RFLP analysis or sequencing for species identification which could have given either false positive or false positive results. Our study is in agreement with previous studies in Palestine and the region in which CL is slightly higher in males which has been explain by the dress code and behaviour (Al-Jawabreh et al., 2003; Al-Jawabreh et al., 2017; Arda & Kamal, 1983). Contradictory finding has been reported in which females were more prone to CL (Aksoy et al., 2016). In addition, in both males and females, CL infection is predominating in the age range from zero to 14 yrs The typical explanation given in this situation is the naive inexperienced immune system in which the immune response type I that activates the macrophage by increasing Interferon-γ(IFN-γ), Tumor necrosis factor (TNF), nitric oxide (NO), and reactive oxygen species (ROS) is still underdeveloped (Aksoy et al., 2016; Al-Jawabreh et al., 2003; Al-Jawabreh et al., 2017; Arda & Kamal, 1983; Rodrigues et al., 2016) (Figure 10).

The CL cases were reported in all eleven Palestinian districts, however, with Jericho (Jordan Valley) as the main focus of CL in Palestine. This has been the center of debate since the days of Canaan and Huntemuller in 1914 and the publications thereafter (Al-Jawabreh et al., 2003; Al-Jawabreh et al., 2017; Arda & Kamal, 1983, 1994; Canaan, 1916; Canaan 1929; Canaan, 1945; Dostrowsky, 1926; Huntemüller, 1914). The Jordan valley is known to be a semi-arid tropical area with low annual fall rate considered to be the lowest (-300m below sea level) and hottest spot in Palestine, This topography and climate were considered the optimal for the sand fly vectors (*Phlebotomus* spp) and reservoir animals such as the fat sand rate (*Pseumomys obesus*) and rock hyrax (*Procavia capensis*) which play a decisive role in the CL infection (Schlein et al., 1982; Schlein et al., 1984). Other districts such as Tubas, Jenin and the eastern hills of Nablus show CL cases on a regular annual basis (Al-Jawabreh et al., 2017). Older CL foci reported in Palestine which no longer exist were Haifa, Bethlehem, Artuf (a Palestinian village destroyed during the

1948 Nakba) (Canaan 1929; Canaan, 1945; Dostrowsky, 1926; Sternfeld, 1944). The study showed that *L. tropica* is most widely distributed species in Palestine which is supported by previous studies in Palestine which were related to the presence and spread of the rock hyrax (*Procavia capensis*), the probable reservoir for *L. tropica* (Al-Jawabreh et al., 2017; A. Al-Jawabreh et al., 2001; Azmi, Schnur, et al., 2012). *L. major* is mainly focused in Jericho with sporadic cases in other districts, however, the importing of *L. major* cases form Jericho could not be ruled out as a result of work in Jericho, or seasonal movement of shepherds. It is worth mentioning that the *Psamommys obesus* incriminated as the reservoir in the early 1980s is no longer existing in the Jordan Valley (Jericho) which points out the presence of other reservoir or the anthroponocity of transmission, a dilemma that remains to be solved (Schlein et al., 1984). The study affirmed that CL remains to be a rural disease (69% of case) where the infection components; vector, reservoir, and environment are favourable for the parasite. Even towns like Jericho and Tubas that are considered urban areas have agricultural and urbanization activities that make contracting CL on the outskirts highly possible. The study divulged the slight change in seasonality of CL infections which shifted from late summer to autumn in the 1940s to late autumn until early winter (October to January) in the 1990s to autumn until spring (March) in the 2000s(Al-Jawabreh et al., 2003; Al-Jawabreh et al., 2017; Canaan, 1945). This is explained by the variation in the biting season of the sandfly vector which usually occurs between may to late September which could be affected by climatic factors such as rainfall described elsewhere (Al-Jawabreh et al., 2003; Al-Jawabreh et al., 2017). With an incubation period duration of two to six months, any CL lesions in winter indicates bite from an infected sandfly in the previous summer. It is noteworthy that CL cases are seen through the year with minimum numbers occurring during summer indicating a low sandfly biting activity in winter.

This is the first study that compared the next-generation sequencing (NGS) technology in a real diagnostic condition for the diagnosis of cutaneous leishmaniasis (CL) from suspected human patients. To date, the utilization of NGS was limited to few case report of human visceral leishmaniasis (VL) (Castillo-Castaneda et al., 2022; Chang et al., 2023; Chen et al., 2020; Gao et al., 2022; Han et al., 2022; Li et al., 2024; Liang et al., 2022; Lin et al., 2021; Ren et al., 2021; Song et al., 2021; Wang C et al., 2021; Williams et al., 2020). Our group used the NGS method in the species identification and genetic diversity study of *Leishmania tropica* in the sand fly vector (*Phlebotomus* Spp.)(Al-Jawabreh et al., 2023; Nasereddin et al., 2022). The two basic tests that depend on the demonstration of the two parasite stages, amastigote in the host and promastigotes in the *In-Vitro* culture cannot be opted for investigating a diagnostic test that is expected to be supersensitive. These two methods (Microscopy and *In-Vitro* culture) adopted by the WHO case definition were shown to have extremely low sensitivity (Microscopy: 37-57%, *In-vitro* culture: 64%) with high false negative rate result, requiring experienced and qualified personnel, and inability to identify *Leishmania* species by parasite morphology (Al-Jawabreh et al., 2004; Azmi et al., 2017). Therefore, as a gold standard the study adopted a similar test to new one under investigation in that it is molecular-based test that targets the same DNA sequence (ITS1) with established sensitivity and specificity in the literature that is higher than the conventional methods (microscopy and *In-vitro* culture), and used in similar diagnostic setting (Al-Jawabreh et al., 2017; Al-Jawabreh et al., 2006; Schonian et al., 2003). The ITS1-PCR gold standard used in the study has a sensitivity of 87% and 100% specificity as established earlier (Al-Jawabreh et al., 2018; Al-Jawabreh et al., 2006). However, the ITS1-PCR was considered an imperfect gold standard as it depended largely on agarose gel electrophoresis for the visualization of the ITS1-PCR and its subsequent RFLP products which in considerable proportion of cases with low DNA concentration

was difficult to read results and interpret restriction patterns. Even in the rare cases where Sanger sequencing was used, such low DNA-concentration samples gave erroneous DNA sequences. The study showed that the sensitivity of the ITS1-219-NGS has increased to 94% compared to 87%, the sensitivity of ITS1-PCR (imperfect gold standard), while the specificity remained 100%. Despite the perfect agreement between ITS1-PCR and ITS1-219-NGS (kappa statistic=0.82), the 6% increase in sensitivity was marginally significant (McNemar's test, P=0.049) which justifies the use of the new assay. The four ITS1-PCR negative, but positive by ITS1-219-NGS were confirmed by BLSAT-search. These four discrepant results were due to low DNA concentration that yielded no bands on the agarose gel of the ITS1-PCR thus judged as negative, however, yielded enough reads (5016-190978) to confirm the presence of *Leishmania* DNA thus confirmed as positive. As the peripheral blood is considered the least-sensitive specimen of choice compared to the most sensitive but invasive splenic puncture or bone marrow aspirate; the high sensitivity of amplicon-based NGS assay was supported by other studies which proved that NGS detected *Leishmania* DNA in peripheral blood but was negative by conventional PCR (Chang et al., 2023; Li et al., 2024; Liang et al., 2022). Peripheral blood is considered the least-sensitive specimen of choice compared to the most sensitive but invasive splenic puncture or bone marrow aspirate in VL cases. On the other hand, the 13 ITS1-PCR samples with faint bands on the agarose gel which were considered positive; yet, they yielded negligible or no reads by the ITS1-219-PCR, thus considered negative. The ITS1-PCR depends mainly on the observer's visual reading of results compared to fully-automated detection of number of DNA sequence reads. Faint traces of DNA bands on a gel agarose electrophoresis can be rated differently depending the observer's perception and experience. Such errors are subsequently inherited in equivocal RFLP pattern.

The species identification of the *Leishmania* was shown to be significantly different between the studied diagnostic methods, ITS1-PCR and ITS1-219-NGS (Bhapkar test; P-value<0.001) with high disagreement between the tests (Kappa statistic=0.24) as ITS1-PCR was unable to determine the genotype of 29 positive *Leishmania* cases. This can be explained by the low concentration of DNA which fails to or does not show clear restriction pattern especially when the band molecular size of the restricted pattern is small as in the case of *L. tropica* (24, 53, and 57bp). The faint pattern does not provide a suitable platform for a correct genotyping as it varies depending on the observer. Sanger sequencing does not resolve the problem as noisy uninformative DNA sequence will result with sufficient amount of the purified PCR (5-50ng) is required. Conversely, the ITS1-219-NGS was able to correctly genotype all the positive cases including those missed by the ITS-PCR (imperfect gold standard). The advantages of the new index test (ITS1-219-NGS) over the ITS1-PCR is the high specificity as it produces millions of DNA sequence reads, the multiple DNA sequences sequenced from the original DNA, and then giving a consensus sequence representing all for a given *Leishmania* sample which can then be confirmed by BLAST-search ruling out any probability for non-specific sequences to be interpreted as *Leishmania*. Unlike ITS1-219-NGS, ITS1-PCR Sanger sequencing which depends on producing one sequence from the original DNA fragment leaving little chance for correct genotyping in case of partial or sequencing failure. An evident example of the supreme specificity of amplicon-based NGS is the large number of reads shown by the negative control sample which upon BLAST-search were shown to be human and bacterial DNA contaminants. In this study, maximum read length used for sequencing was 151bp which was enough to make the correct identification and differentiation between *Leishmania* species. In this case, the NGS has an average error rate of less than 1/1000 leading to high accuracy (Q30), however, fragments longer than 500bp tend to yield low quality sequences with higher error

rates (Tan et al., 2019). The low error rate and the millions of reads produced increases sensitivity and specificity of the method and thus accuracy.

Another advantage of using NGS technology is its flexibility to be utilized in targeting various genes, spacers, exome sequencing and even whole genome sequences (WGS) from different clinical specimens and from different hosts (Nasereddin et al., 2022; Patino et al., 2021). Other studies have proved that amplicon-based NGS detected co-infection with multiple *Leishmania* spp. compared to Sanger sequencing that detected one species per sample (Castillo-Castaneda et al., 2022). Additionally, the assay can detect different *Leishmania* species coinfection mainly in opportunistic diseases in immunosuppressed patients (Badirzadeh et al., 2018). Finally, the timeliness of the amplicon-based NGS method which takes 48 hours starting from DNA extraction, amplification, sequencing and ending with bioinformatic analysis is considered short taking into account the high throughput sequencing power.

The limitations of the study is the possibility of human genome contamination, host or technician, and bacterial DNA contamination from the environment which may impede the analytical sensitivity which can be mitigated by BLAST search in the GenBank and multidisciplinary interpretation of the results by microbiologists, clinicians and medical laboratory technologists. Another limitation usually raised is the high cost-per-test which could be true for a case-by-case test, but not for a bulk of samples such as in the case of epidemiologic surveys or cases in endemic areas. The main limitation for the epidemiological part of the study is the incomplete data for the patients from the Palestinian Ministry of Health.

In conclusion**,** our study revealed that Jericho, along with Tubas, Jenin and the eastern hills of Nablus remain the main foci of CL caused by *L. tropica* and *L. major,* which remains restricted to Jericho area. Moreover, the current study showed the feasibility, high sensitivity, and accurate species identification (genotyping) of amplicon-based NGS. This puts forward the need to incorporate it as a routine diagnostic method in clinical practice and epidemiologic surveys for cutaneous leishmaniasis.

The following recommendation will be submitted to the Ministry of Health:

1. Continue the surveillance of leishmaniasis in Palestine
2. Include genotyping by RFLP or sequencing or both as part of the routine testing.
3. Fill in the patient data sheet completely and accurately to able to get a clear picture of the CL behaviour.
4. Include microscopy and *in-vitro* culture to the panel of testing.
5. Open channels of cooperation with researchers and clinicians.

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ANNEX 1

Annex 1			ITS1-Microsocpy-Culture			ITS1-219-NGS		No. of reads		
	REC		Result-1	Spp.		Result-2	Spp.	Leishmania spp.	L. tropica	L. major
nonCL	**3297**		Negative			Negative		0	0	0
nonCL	**3312**		Negative			Negative		0	0	0
nonCL	**3313**		Negative			Negative		0	0	0
nonCL	**3328**		Negative			Negative		0	0	0
nonCL	**3333**		Negative			Negative		0	0	0
nonCL	**3337**		Negative			Negative		0	0	0
nonCL	**3338**		Negative			Negative		0	0	0
nonCL	**3344**		Negative			Negative		0	0	0
nonCL	**3345**		Negative			Negative		0	0	0
nonCL	**3348**		Negative			Negative		0	0	0
nonCL	**3351**		Negative			Negative		0	0	0
nonCL	**3354**		Negative			Negative		0	0	0
nonCL	**3360**		Negative			**Positive**	**TRO**	0	0	0
nonCL	**3377**		Negative			Negative		0	0	0
nonCL	**3393**		Negative			Negative		0	0	0
nonCL	**3394**		Negative			Negative		0	0	0
nonCL	**3395**		Negative			Negative		0	0	0
nonCL	**3400**		Negative			Negative		0	0	0
nonCL	**3406**		Negative			Negative		0	0	0
nonCL	**3411**		Negative			Negative		0	0	0
nonCL	**3412**		Negative			Negative		0	0	0
nonCL	**3414**		Negative			Negative		0	0	0
nonCL	**3415**		Negative			Negative		0	0	0
nonCL	**3416**		Negative			Negative		0	0	0
nonCL	**3424**		Negative			Negative		0	0	0
nonCL	**3426**		Negative			Negative		0	0	0
nonCL	**3430**		Negative			Negative		0	0	0
nonCL	**3436**		Negative			Negative		0	0	0
nonCL	**3437**		Negative			Negative		0	0	0
nonCL	**3439**		Negative			Negative		0	0	0
nonCL	**3442**		Negative			Negative		0	0	0
nonCL	**3453**		Negative			Negative		0	0	0

nonCL	**3454**		Negative			Negative		0	0	0
nonCL	**3455**		Negative			Negative		0	0	0
nonCL	**3456**		Negative			Negative		0	0	0
nonCL	**3457**		Negative			Negative		0	0	0
nonCL	**3458**		Negative			**Positive**	**TRO**	0	0	0
nonCL	**3462**		Negative			Negative		0	0	0
nonCL	**3463**		Negative			Negative		0	0	0
nonCL	**3464**		Negative			Negative		0	0	0
nonCL	**3466**		Negative			Negative		0	0	0
nonCL	**3469**		Negative			Negative		0	0	0
nonCL	**3471**		Negative			Negative		0	0	0
nonCL	**3474**		Negative			Negative		0	0	0
nonCL	**3475**		Negative			Negative		0	0	0
nonCL	**3476**		Negative			Negative		0	0	0
nonCL	**3486**		Negative			**Positive**	**TRO**	0	0	0
nonCL	**3487**		Negative			Negative		0	0	0
nonCL	**3491**		Negative			Negative		0	0	0
nonCL	**3492**		Negative			Negative		0	0	0
nonCL	**3493**		Negative			Negative		0	0	0
nonCL	**3496**		Negative			Negative		0	0	0
nonCL	**3497**		Negative			Negative		0	0	0
nonCL	**3504**		Negative			Negative		0	0	0
nonCL	**3505**		Negative			**Positive**	**TRO**	0	0	0
nonCL	**3506**		Negative			Negative		0	0	0
nonCL	**3509**		Negative			Negative		0	0	0
nonCL	**3511**		Negative			Negative		0	0	0
nonCL	**3512**		Negative			Negative		0	0	0
nonCL	**3515**		Negative			Negative		0	0	0
nonCL	**3519**		Negative			Negative		0	0	0
nonCL	**3521**		Negative			Negative		0	0	0
nonCL	**3522**		Negative			Negative		0	0	0
nonCL	**3527**		Negative			Negative		0	0	0
nonCL	**3539**		Negative			Negative		0	0	0
nonCL	**3557**		Negative			Negative		0	0	0
nonCL	**3560**		Negative			Negative		0	0	0
nonCL	**3644**		Negative			Negative		0	0	0
nonCL	**3645**		Negative			Negative		0	0	0
nonCL	**3646**		Negative			Negative		0	0	0
nonCL	**3656**		Negative			Negative		0	0	0
nonCL	**3660**		Negative			Negative		0	0	0
nonCL	**3662**		Negative			Negative		0	0	0
nonCL	**3665**		Negative			Negative		0	0	0
nonCL	**3667**		Negative			Negative		0	0	0

nonCL	**3803**		Negative			Negative		0	0	0
nonCL	**3806**		Negative			Negative		0	0	0
nonCL	**3823**		Negative			Negative		0	0	0
nonCL	**3828**		Negative			Negative		0	0	0
nonCL	**3835**		Negative			Negative		0	0	0
nonCL	**3849**		Negative			Negative		0	0	0
nonCL	**3850**		Negative			Negative		0	0	0
nonCL	**3852**		Negative			Negative		0	0	0
nonCL	**3855**		Negative			Negative		0	0	0
nonCL	**3856**		Negative			Negative		0	0	0
nonCL	**3858**		Negative			Negative		0	0	0
nonCL	**3860**		Negative			Negative		0	0	0
nonCL	**3867**		Negative			Negative		0	0	0
nonCL	**3880**		Negative			Negative		0	0	0
nonCL	**3883**		Negative			Negative		0	0	0
nonCL	**3885**		Negative			Negative		0	0	0
nonCL	**3886**		Negative			Negative		0	0	0
nonCL	**3887**		Negative			Negative		0	0	0
nonCL	**3893**		Negative			Negative		0	0	0
nonCL	**(-)CONT**		Negative			Negative		0	0	0
CL	**(+)CONT**		Positive			Positive		0		0
CL	**2076**		Positive	**TRO**		**Negative**		**36910**	**548**	**9**
CL	**2077**		Positive	**TRO**		**Negative**		**13215**	**167**	**1**
CL	**2078**		Positive	**TRO**		**Negative**				
CL	**2274**		Positive	**MAJ**		Positive	**TRO**	**30078**	**781**	**226**
CL	**2275**		Positive	**UD**		**Negative**		**24115**	**338**	**178**
CL	**2276**		Positive	**MAJ**		Positive	**MAJ**	**31488**	**202**	**8246**
CL	**2290**		Positive	**UD**		Positive	**MAJ**	**116115**	**246**	**90024**
CL	**2293**		Positive	**MAJ**		Positive	**MAJ**	**89990**	**253**	**61001**
CL	**2085**		Positive	**TRO**		**Negative**		**14498**	**41**	**15**
CL	**2295**		Positive	**MAJ**		**Negative**		**40574**	**550**	**9**
CL	**2297**		Positive	**TRO**		Positive	**TRO**	**85384**	**59356**	**105**
CL	**2299**		Positive	**TRO**		Positive	**TRO**	**130439**	**109354**	**207**
CL	**2070**		Positive	**TRO**		**Negative**		**42954**	**3**	**1**
CL	**2302**		Positive	**TRO**		Positive	**TRO**	**136355**	**104445**	**251**
CL	**2303**		Positive	**TRO**		Positive	**TRO**	**134698**	**119767**	**98**
CL	**2304**		Positive	**MAJ**		Positive	**TRO**	**112045**	**101437**	**82**
CL	**2305**		Positive	**MAJ**		Positive	**TRO**	**72387**	**20329**	**71**
CL	**2306**		Positive	**UD**		Positive	**TRO**	**56178**	**19120**	**467**
CL	**2307**		Positive	**TRO**		Positive	**TRO**	**163816**	**154859**	**115**
CL	**2069**		Positive	**TRO**		Positive	**TRO**	**33270**	**4183**	**4**
CL	**2311**		Positive	**UD**		Positive	**TRO**	**158946**	**149538**	**116**
CL	**2312**		Positive	**TRO**		Positive	**TRO**	**89453**	**712266**	**70**

CL	**2313**		Positive	**MAJ**		Positive	**TRO**	**153545**	**126835**	**81**
CL	**2314**		Positive	**MAJ**		Positive	**MAJ**	**104738**	**373**	**68240**
CL	**2317**		Positive	**UD**		Positive	**TRO**	**64001**	**47348**	**60**
CL	**2321**		Positive	**MAJ**		Positive	**TRO**	**39102**	**798**	**10631**
CL	**2322**		Positive	**MAJ**		Positive	**TRO**	**66931**	**599**	**28326**
CL	**2323**		Positive	**UD**		Positive	**TRO**	**64790**	**936**	**20937**
CL	**2325**		Positive	**TRO**		Positive	**TRO**	**50642**	**489**	**3644**
CL	**2086**		Positive	**TRO**		Positive	**TRO**	**67493**	**19575**	**411**
CL	**2327**		Positive	**MAJ**		Positive	**MAJ**	**119370**	**3097**	**25189**
CL	**2330**		Positive	**UD**		Positive	**TRO**	**40115**	**11235**	**159**
CL	**2332**		Positive	**UD**		Positive	**TRO**	**26461**	**5625**	**4766**
CL	**2079**		Positive	**TRO**		Positive	**TRO**	**28229**	**1598**	**6**
CL	**2335**		Positive	**UD**		Positive	**TRO**	**110479**	**1438**	**78293**
CL	**2095**		Positive	**TRO**		Positive	**TRO**	**42443**	**7014**	**13**
CL	**2102**		Positive	**MAJ**		Positive	**MAJ**	**107103**	**822**	**42407**
CL	**2097**		Positive	**TRO**		Positive	**TRO**	**101316**	**41293**	**437**
CL	**2344**		Positive	**MAJ**		Positive	**MAJ**	**140407**	**254**	**95796**
CL	**2093**		Positive	**MAJ**		Positive	**MAJ**	**52513**	**114**	**14818**
CL	**2345**		Positive	**MAJ**		Positive	**MAJ**	**24497**	**284**	**15607**
CL	**2347**		Positive	**MAJ**		Positive	**TRO**	**53195**	**13686**	**1543**
CL	**2350**		Positive	**MAJ**		Positive	**MAJ**	**20382**	**1527**	**3119**
CL	**2351**		Positive	**UD**		Positive	**TRO**	**87891**	**64162**	**68**
CL	**2100**		Positive	**MAJ**		**Negative**		**41019**	**180**	**424**
CL	**2724**		Positive	**UD**		Positive	**TRO**	**52933**	**8849**	**25**
CL	**2727**		Positive	**UD**		**Negative**		**486112**	**256**	**1**
CL	**2744**		Positive	**UD**		Positive	**TRO**	**112707**	**56581**	**24**
CL	**2918**		Positive	**UD**		Positive	**TRO**	**123360**	**98593**	**67**
CL	**3342**		Positive	**UD**		Positive	**TRO**	**24722**	**7669**	**13**
CL	**3353**		Positive	**UD**		Positive	**TRO**	**42525**	**19932**	**48**
CL	**3356**		Positive	**UD**		Positive	**TRO**	**120008**	**96828**	**75**
CL	**3398**		Positive	**UD**		Positive	**TRO**	**82521**	**26423**	**86**
CL	**3425**		Positive	**UD**		Positive	**TRO**	**85902**	**35221**	**38**
CL	**3364**		Positive	**UD**		Positive	**TRO**	**73915**	**17100**	**200**
CL	**3498**		Positive	**UD**		Positive	**TRO**	**168183**	**141465**	**106**
CL	**3535**		Positive	**UD**		Positive	**TRO**	**121209**	**86913**	**52**
CL	**3537**		Positive	**UD**		Positive	**TRO**	**112786**	**85743**	**85**
CL	**3553**		Positive	**UD**		Positive	**TRO**	**122745**	**81405**	**758**
CL	**3568**		Positive	**UD**		Positive	**TRO**	**130333**	**101489**	**354**
CL	**3576**		Positive	**UD**		Positive	**TRO**	**149396**	**115588**	**104**
CL	**3579**		Positive	**UD**		Positive	**TRO**	**101302**	**63093**	**665**
CL	**3580**		Positive	**UD**		Positive	**TRO**	**86766**	**41375**	**464**
CL	**3589**		Positive	**UD**		Positive	**TRO**	**195263**	**179253**	**132**
CL	**3595**		Positive	**TRO**		Positive	**TRO**	**4177**	**1836**	

CL	**3621**		Positive	**TRO**		Positive	**TRO**	**6624**	**1813**	**3**
CL	**3650**		Positive	**TRO**		Positive	**TRO**	**5061**	**2144**	**42**
CL	**3651**		Positive	**TRO**		Positive	**TRO**	**10810**	**6886**	**35**
CL	**3666**		Positive	**UD**		Positive	**TRO**	**21428**	**6657**	**141**
CL	**3672**		Positive	**TRO**		Positive	**TRO**	**24088**	**17566**	**12**
CL	**3679**		Positive	**TRO**		**Negative**		**113**	**3**	**0**
CL	**3684**		Positive	**TRO**		Positive	**TRO**	**1194**	**450**	**0**
CL	**3685**		Positive	**TRO**		Positive	**TRO**	**4037**	**1592**	**5**
CL	**3691**		Positive	**TRO**		Positive	**TRO**	**9061**	**1806**	**3**
CL	**3694**		Positive	**TRO**		Positive	**TRO**	**25053**	**17466**	**16**
CL	**3695**		Positive	**TRO**		Positive	**TRO**	**52150**	**37850**	**41**
CL	**3699**		Positive	**TRO**		Positive	**TRO**	**12635**	**5150**	**95**
CL	**3700**		Positive	**TRO**		Positive	**TRO**	**39105**	**28259**	**26**
CL	**3710**		Positive	**UD**		**Negative**		**128**	**0**	**0**
CL	**3710**		Positive	**UD**		**Negative**		**680**	**74**	**1**
CL	**3714**		Positive	**TRO**		Positive	**TRO**	**86878**	**26047**	**37**
CL	**3725**		Positive	**TRO**		Positive	**TRO**	**138512**	**78469**	**98**
CL	**3738**		Positive	**UD**		Positive	**TRO**	**115566**	**27871**	**29**
CL	**3754**		Positive	**TRO**		Positive	**TRO**	**72319**	**22926**	**206**
CL	**3766**		Positive	**TRO**		Positive	**TRO**	**137898**	**94779**	**500**
CL	**3790**		Positive	**TRO**		Positive	**TRO**	**25022**	**39**	**2**
CL	**3816**		Positive	**TRO**		Positive	**TRO**	**53104**	**7646**	**10**
CL	**3842**		Positive	**TRO**		Positive	**TRO**	**54993**	**21**	**1054**
CL	**3862**		Positive	**TRO**		**Negative**		**3357**	**30**	**0**
CL	**3865**		Positive	**TRO**		Positive	**TRO**	**17170**	**2719**	**19**
CL	**3876**		Positive	**TRO**		Positive	**TRO**	**31755**	**7263**	**8**
CL	**3894**		Positive	**TRO**		Positive	**TRO**	**49359**	**191**	**100**
CL	**3905**		Positive	**TRO**		Positive	**TRO**	**167104**	**155762**	**177**
CL	**3897**		Positive	**TRO**		Positive	**TRO**	**91208**	**48360**	**72**
nonCL	**(-)CONT**		Negative	**(-)CONT**		Negative		**7804**	**0**	**0**
CL	**(+)CONT**		Positive	**(+)CONT**		Positive	**TRO**	**3402**	**3017**	**7**

علم الأوبئة الجزيئي :انتشار داء الليشمانيا البشري في فلسطين في الفترة ما بين 2016-2024 باستخدام تسلسل الجين الوراثي

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الملخص

داء الليشمأنيا هو مرض يتناقل عن طريق الذباب ويسببه طفيلي اولي يسمى الليشمانيا، تنتقل العدوى عن طريق لدغة أنثى ذبابة الرمل المصابة، يتم التشخيص عن طريق الفحص المجهري لشريحة الدم المأخوذة من المنطقة المصابة، وعن طريق الزراعة في المختبر، وطريقة الكشف القائمة على الحمض النووي.

المواد والطرق: في هذه الدراسة التي امتدت من عام 2016 الى عام 2024 شملت مرضى من احدى عشر منطقة في الضفة الغربية الفلسطينية، تضمنت طرق التشخيص المستخدمة في الدراسة الفحص المجهري للعينة المصبوغة بصبغة جيمسا ، وفحص الزراعة باستخدام الوسط الغذائي NNN ، كذلك تم استخدام تقنية فك الشيفره الوراثيه الحديثة بالجيل التالي (NGS) القائم على مضاعفة جزءمعين من الجين ITS1-219 -NGS في جزء المقارنة من الدراسة ، حيث تم مقارنة في مجموعتين ، مجموعة ايجابية أي إصابة مؤكدة بواسطة أي من الاختبارات المستخدمة ، ومجموعة سلبية بجميع طرق التشخيص، تمت مقارنة نتائج ITS1-PCR مع نتائج NGS1-219-PCR والذي يعتبر معيار ذهبي نظرا لتفوقه على الفحص المجهري و فحص الزراعة.

النتائج: كان معدل الإصابات خلال فترة الدراسة 17% (1262/213) بمعدل أنتشار 7 لكل 100,000. حددت الدراسة أن معدل الإصابة السنوي 0.84 لكل 100,000، (25 حالة في السنة) مع توزيع متساو تقريبا بين الذكور (52%) والأناث (48%). كانت الفئة العمرية بين 0-14 عاما هي الأكثر تأثرا بهذا المرض، كانت أغلب الإصابات (45%) في الرأس، و(38%) في المناطق العلوية من الجسم والأطراف السفلية (17%). أظهرت خريطة توزيع الحالات أن أريحا لا تزال منطقة ذات أعلى معدل إصابة سنوي (21لكل 100,000). كشفت الدراسة أن الليشمأنيا المدارية هي النوع السائد مع اقتصار الليشمانيا الكبرى بشكل أساسي على أريحا. كان عام 2017 هو العام الأخير الذي شهد ذروة الإصابات بهذا المرض في فلسطين حيث ساهمت أريحا بنسبة 40% من الحالات. ظهرت أكثر من نصف الحالات (52%) في شهري كانون ثاني إلى آذار. أما بالنسبة للمقارنة طريقتي الفحص فقد تبين أن ITS1-219-NGS يتمتع بحساسية اعلى بنسبة 94% مقارنة بنتائج ITS1-PCR (85%).

وكان معامل التوافق k=0.24 فقط 56% من الحالات وكذلك فلم يتمكن فحص ITS1-PCR من تحديد النمط الجيني ل 29 عينة ولكن تم تحديد النمط الجيني لها بواسطة ITS-219-NGS وتم تأكيده من خلال بحث BLAST على عكس ITS1-PCR. تمكن ITS1-219-NGS من تحديد نوع الليشمانيا لجميع نتائجه الإيجابية بشكل صحيح كما أكده بحث BLAST في بنك الجينات، علاوة على ذلك وجد أن 13 حالة إيجابية على ITS1- PCR كانت سلبية بواسطة ITS1-PCR-NGS وعند بحث BLAST تبين أنها تلوث من أصل بشري أوبكتيري .

الاستنتاج: تظل أريحا هي البؤرة الرئيسية لمرض الليشمانيا في فلسطين حيث تعتبر الليشمانيا الاستوائية (*L. tropica*) هي النوع الرئيسي والليشمانيا الكبرى (*L. major*) محصورة في أريحا. إنخفض معدل الإصابة مقارنة بالعقدين السابقين في هذه الدراسة نتيجة لتدابير المكافحة التي تم تنفيذها. الفحص التشخيصي القائم على الامبليكون من NGS هو طريقة تشخيصية مجدية وحساسة للغاية وعالية الأنتاجية مع تحديد دقيق للأصناف ويمكن استخدامها في التشخيص وفي المسح الوبائي.