Comparative Analysis of Microscopic and PCR Methods for Leishmaniasis Diagnosis

ABSTRACT

Leishmaniasis is a vector-borne disease caused by protozoan parasites of the genus Leishmania, transmitted primarily through the bite of infected sandflies. It manifests in various forms, including cutaneous, mucocutaneous, and visceral leishmaniasis, each presenting unique clinical challenges. Accurate diagnosis is critical for effective treatment and management. Traditional diagnostic methods include microscopy, which involves identifying Leishmania amastigotes in stained tissue samples or smears. While microscopy is cost-effective and widely accessible, it has limitations in sensitivity and specificity, particularly in cases with low parasite loads or atypical presentations. In contrast, polymerase chain reaction (PCR) offers a molecular approach that can detect even minute quantities of parasite DNA, potentially improving diagnostic accuracy.

Aims: This study aims to compare the diagnostic efficacy of microscopy and PCR in patients suspected of leishmaniasis

Study design: This study involved a comparative analysis of microscopy and PCR methods for diagnosing leishmaniasis using clinical samples from patients presenting with symptoms consistent with the disease we have compared the sensitivity of the conventional methods microscopy against PCR amplification of parasite kinetoplast DNA from these samples.

Place and Duration of Study: This was a retrospective observational study conducted between March and December 2024, study performed in NCDC *leishmania* dermatology clinical in Tripoli, the study included 116 patients who presented with skin lesions in various regions of Libya with cutaneous leishmaniasis referred to the Dermatology and Leishmaniasis clinic who had signs of cutaneous leishmaniasis

Methodology: The samples (n=116) were obtained from National Center for Diseases Control - LIBYA the patients clinically suspected of CL

Results:

The majority of lesions were found on the **lower limbs** (46%), followed by the **upper limbs** (28%), and **facial** lesions (18%). PCR result showed that 104 (89.66%) were positive for Cutaneous Leishmaniasis. 12 (10.34%) were negative for both PCR and microscopy. Preliminary results indicated that the Microscopy has high sensitivity (100%) but lower specificity (77.4%). PCR's positive predictive value (84%) and negative predictive value (100%) suggest high diagnostic accuracy.

Conclusion: The results suggest that PCR is a reliable diagnostic tool for detecting Leishmaniasis. These findings can inform future research and public health strategies aimed at controlling and preventing CL in endemic regions

Keywords: cutaneous leishmaniasis, PCR, Microscopic, specificity, accuracy, sensitivity

1. INTRODUCTION

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania*, which are transmitted to humans and other animals through the bite of infected female phlebotomine sandflies, over 20 species of the *Leishmania* parasite have been characterized and are transmitted from approximately 70 different types of phlebotomine sand flies (of the Diptera Family Psychodidae genera subdivided into Phlebotomus in the Old World and Lutzomyia in the New World (1) . There are several forms of leishmaniasis, primarily categorized into three main types; Cutaneous Leishmaniasis: It is the most common form of leishmaniasis and causes skin lesions, mainly represented by ulcers, in the visible parts of the body, which can cause permanent scars and serious disability or stigma. with approximately 95% of cases occurring in regions such as the Americas, the Mediterranean Basin, the Middle East, and Central Asia. Globally, it is estimated that there are between 600,000 and 1 million new cases of cutaneous leishmaniasis each year. However, the World Health Organization (WHO) reports notably lower figures, with only around 200,000 cases officially documented annually. (2). Mucocutaneous Leishmaniasis causes partial or total damage to the mucous membranes of the nose, mouth and throat. More than 90% of cases of cutaneous leishmaniasis are concentrated in the Plurinational State of Bolivia, Brazil, Ethiopia and Peru (3). Visceral Leishmaniasis (Kala-azar**),** this is the most severe form of leishmaniasis and affects internal organs such as the liver, spleen, and bone marrow. It can be fatal if left untreated and is characterized by symptoms such as fever, weight loss, anemia, and enlargement of the spleen and liver, it is a fatal disease if left untreated. Most of its cases are concentrated in Brazil, East Africa and India. The number of new cases around the world is estimated at between 50,000 and 90,000 cases per year, of which only 25 to 45 per cent are reported by the World Health Organization (2). It is a disease that can cause outbreaks and deaths (2). The disease affects some of the world's poorest people and is associated with malnutrition, population displacement, poor housing, a weakened immune system, and a lack of financial resources. The number of cases of this disease is estimated between 700,000 and one million cases per year (2).

Diagnosis of CL is difficult because of the varied symptoms and the different species involved. There are many reliable laboratory diagnostic methods such as direct smear examination, culture, immunologic and molecular techniques. The routine diagnosis of CL patients depends on examination of skin lesions using smears and cultures of dermal scrapings or examination of sections obtained from a skin biopsy(4).
Culture of promastigotes from infected tissues and microscope smears for direct identification of amastigotes have long been considered the standard for diagnosis. While these techniques are highly specific for diagnosing leishmaniasis, they are not sensitive. The different species of *Leishmania* are not equally easy to culture; contamination is a constant hazard, and variations in efficacy among different growth medium formulations or even batches may be encountered. Likewise, the percent success for microscopic identification of amastigotes in stained preparations varies depending on the number of parasites present and the experience of the person examining the slide (5). Inappropriately, currently there is no single widely accepted standard procedure that can be used as a basis for evaluating new molecular diagnostic assays for leishmaniasis, though PCR methods using either genomic or kinetoplast DNA are now frequently cast in this role. Microscopic examination of skin

lesions or tissue samples and a Molecular technique like PCR to identify Leishmania DNA by making a comparison of the way each works, the sensitivity of the effectiveness and efficiency of the diagnosis and the time it takes each (6).

PCR is a powerful molecular technique and a valuable tool in the diagnosis of leishmaniasis due to its sensitivity and specificity. It plays a crucial role in confirming cases, especially in regions where leishmaniasis is endemic and in differentiating between species for appropriate clinical management. As molecular techniques continue to evolve, PCR remains an essential component in the fight against leishmaniasis and other infectious diseases (7).
In this research, we aim to compare the results of Microscopic Examination and Polymerase Chain Reaction (PCR) to emphasize the importance of early detection and diagnosis. Early diagnosis can significantly reduce the risk of complications and enable timely treatment before the condition worsens.

2. MATERIAL AND METHODS

Study Design: This was a retrospective observational study conducted between March and December 2024, study performed in NCDC *leishmania* dermatology clinical in Tripoli, The study included 116 patients who presented with skin lesions in various regions of Libya with cutaneous leishmaniasis referred to the Dermatology and Leishmaniasis clinic who had signs of cutaneous leishmaniasis were studied by taking microscopic slides from their lesions. The patients were aged between 0 and 69 years and included both males and females of Libyan and foreign (Syrian) descent.
Data Collection: The data for this study were collected from medical records and laboratory results, which included:

- Age, Gender, and Nationality of the Patient
- Location and Size of the Lesion
- PCR and Microscopic Test Results

Lesions were categorized based on anatomical location (e.g., face, upper limbs, lower limbs) and size (e.g., ≤4 cm or >4 cm). PCR testing and microscopic examination were conducted to identify pathogens such as bacteria, fungi, or viruses, providing critical diagnostic insights.
Inclusion Criteria:

- Patients with visible skin lesions.
- Both male and female patients aged between 0 and 69 years.
- Patients with PCR and microscopic results available.

Exclusion Criteria:

- Patients without a confirmed diagnosis of skin lesions.
- Patients with incomplete data on lesion characteristics or test results.

Microscopy:

For making stained smears, tissues were taken using a disposable lancet. A small incision was made in the cleaned margin of the nodules and lesions with the point of the blade. The blade turned 90 degrees and scraped along the cut edge of the incision to remove and pick up the skin tissue, which was smeared on a clean glass microscope slide. After the smears dried completely, they were fixed with 100% methanol, allowed to dry again, and stained with Geimsa stain for microscopic examination. At least two specimens were prepared for each case. One was stained and the other stored to be applied in the next appropriate time if necessary(8).

Detection of Leishmania DNA by using conventional PCR

DNA extraction:

Unstained smears were kept at 4uC until DNA extraction. To each glass slide: 180 ml ATL Materials were then scraped from slides by using filter tips and transferred into 1.5ml Eppendorf tube, step1, add 20 µl Proteinase K. Mix thoroughly by vortexing, and incubate in thermomixer at 56°C until the tissue is completely lysed. Then Vortex for 15 s. step 2, add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Step 3, Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing. After Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. step 4, Discard flow-through and collection tube. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), step 5, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), step 6, and pipet 50 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute. This step leads to increased overall DNA yield. Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate [8].

PCR amplification of Cutaneious leishmania DNA

A PCR targeting the ribosomal internal transcribed spacer 1 (ITS1) of Leishmania was performed using the primer pair ITS1F (5`-GCAGCTGGATCATTTTCC-3´) and ITS2R4 (5´-ATATGCAGAAGAGAGGAGGC-3´) (8). The first amplification reaction totaled 20 µl, containing 4µl Hot firepol blend master mix ready to load (5x), 0.2 µl forward primer, 0.2 µl reverse primer, 1 µl DNA template, 19 µl water. The mixture was incubated in a PCR machine involving an initial denaturation at 94°C for 3 min, followed by 40 cycles each consisting of three steps: 30 s at 94°C (denaturation), 30 s at 58°C (annealing) and 90 s at 72°C (extension). After the last cycle, the extension step was continued for a further 10 min, and then the reaction was held at 4°C. Amplified DNA products were confirmed with 1% agarose gel and visualized under ultraviolet (UV) light after staining with 1 µl Syber glod nucleic acid gel stain.

Statistical Analysis

Data were analyzed using descriptive statistics to determine the frequency of different types of lesions, the correlation between lesion size and site, and the prevalence of positive results from PCR and microscopy tests. Chi-square tests were used to analyze the association between lesion characteristics and the presence of pathogens.

3. RESULTS AND DISCUSSION

DEMOGRAPHICS:

A total of 116 patients were included in the study, With the following key findings:

Gender Distribution: The data indicates a predominance of male patients in the study of cutaneous leishmaniasis, with males comprising 72% of the total cases, while females constituted 28% (Figure 1).

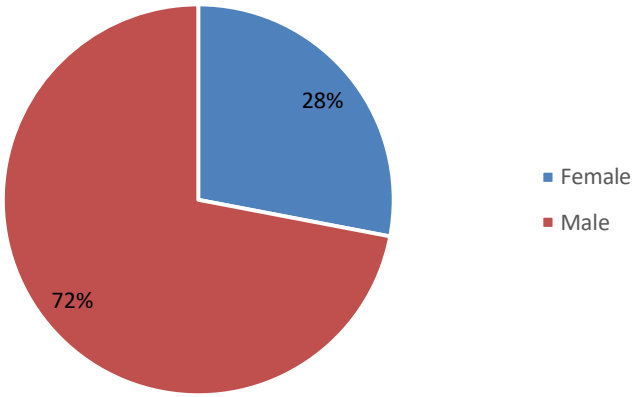

Figure 1. Cutaneous leishmaniasis cases by gender

Age Distribution: The patients' age ranged from 4 to 69 years, with the majority of cases involving adults aged 20-50 years (Figure 2).

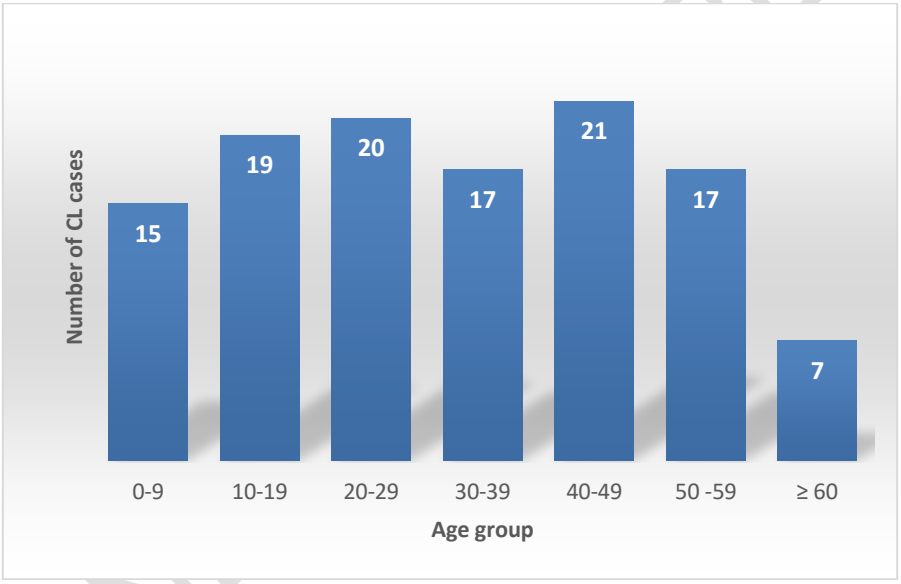

Figure2: Cutaneous leishmaniasis cases by Age groups

Lesion Distribution: The majority of lesions were found on the **lower limbs** (46%), followed by the **upper limbs** (28%), and **facial** lesions (18%) (Figure 3).

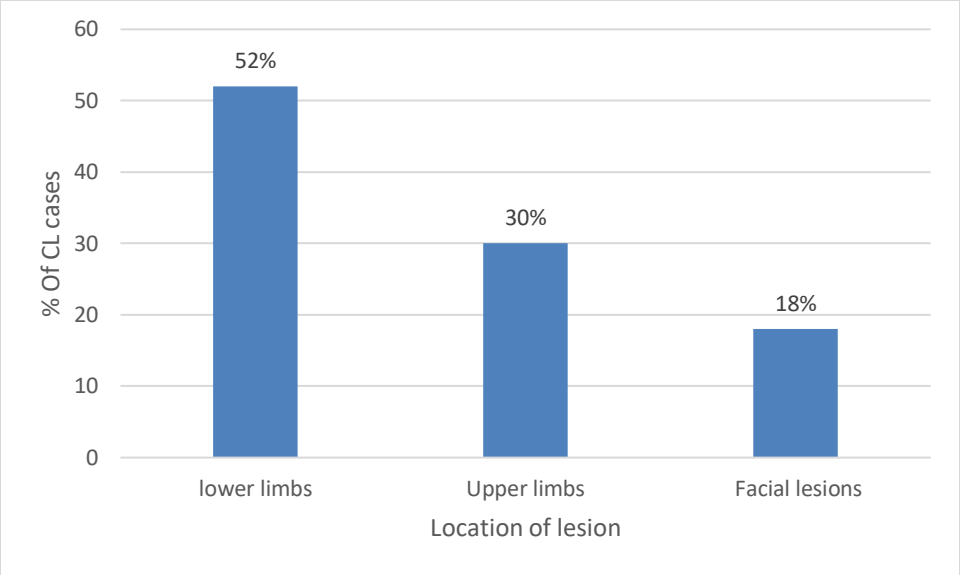

Figure 3 Location of cutaneous leishmaniasis lesion

Size of Lesions: 58% of lesions were ≤4 cm, while 42% were larger than 4 cm.

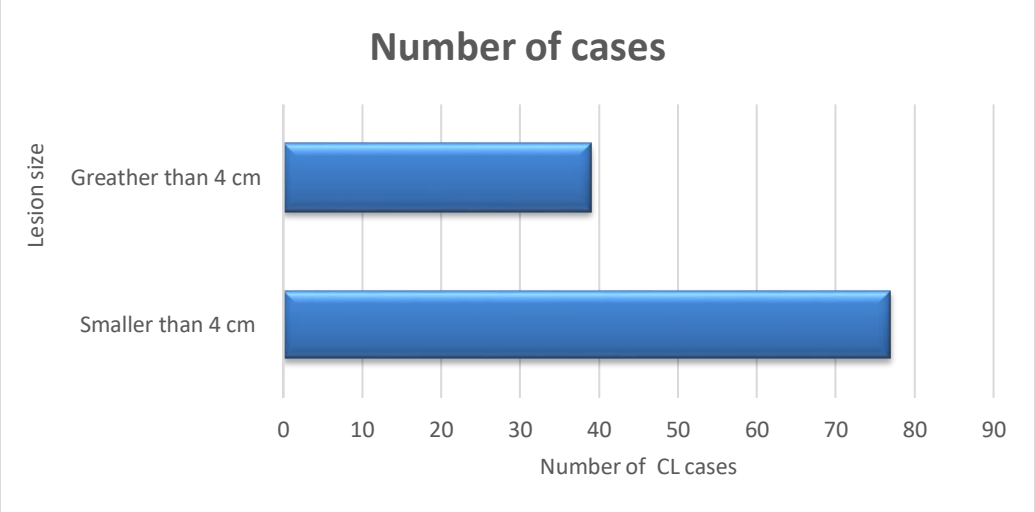

Figure 4: Lesion size of cutaneous leishmaniasis cases

Monthly variation: The graph depicts a fluctuating pattern of cutaneous leishmaniasis cases across the ten months presented (March 2024 to December 2024). There are periods of higher case numbers interspersed with periods of lower numbers (Figure 5).

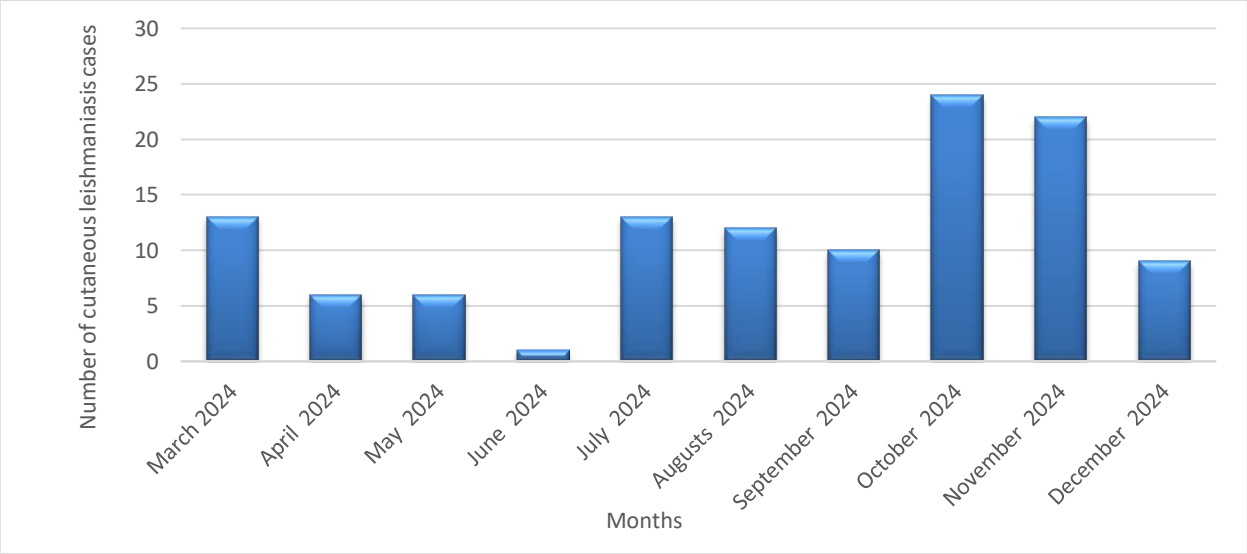

Figure 5: Monthly variation of Cutaneous leishmaniasis

PCR and Microscopic Test Results:

PCR result showed that 104 (89.66%) were positive for Cutaneous Leishmaniasis. 12 (10.34%) were negative for both PCR and microscopy. All the samples which were positive by microscopy were also positive by PCR. Out of 53 microscopic negative samples, 12 (22.64%) were positive by PCR. A statistically significant difference was found between PCR and microscopy method for diagnosis of CL in Libya ($\chi 2 = 15.93$, $p = 0.0001$).

Table 1. Comparison between Microscopic and PCR for the diagnosis of Cutaneous Leishmaniasis

PCR	Microscopy (+)	Microscopy (-)	Total	Statistic
PCR (+)	63	12	104	$\chi 2=15.93$ P- value: <0.0001
PCR (-)	0	41	12	
Total	63	53	116	

Microscopy has high sensitivity (100%) but lower specificity (77.4%). PCR's positive predictive value (84%) and negative predictive value (100%) suggest high diagnostic accuracy. The results suggest that PCR is a reliable diagnostic tool for detecting Leishmaniasis. Number of positive test results among actual positives (PCR + and Microscopy +) = 63, while Number of negative test results among actual positives (PCR + and Microscopy -) = 12, Sensitivity = TP / (TP + FN) = 63 / (63 + 12). The microscopy test's sensitivity is approximately 84%, indicating that 84% of actual Leishmaniasis cases (PCR +) were correctly identified by microscopy, and 16% of actual cases were missed (false negatives).

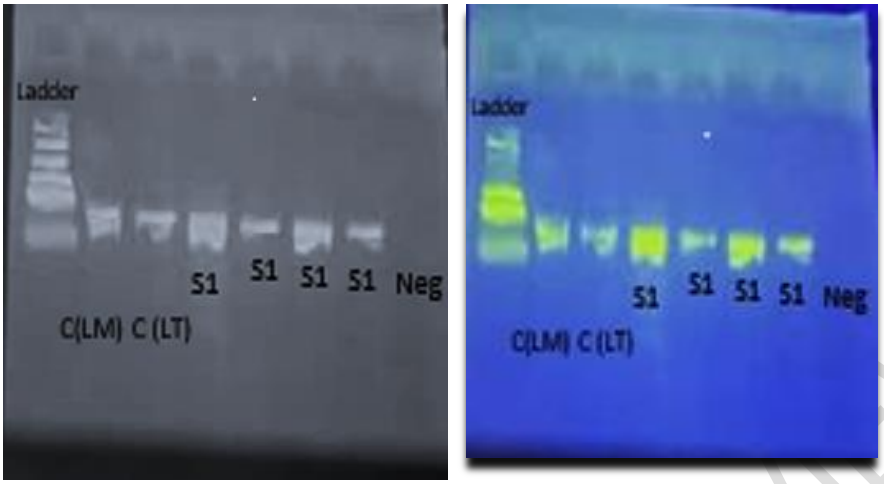

Figure 6: The gel picture for Amplified DNA products were confirmed with 1% agarose gel and visualized under ultraviolet (UV) light after staining with Syber glod nucleic acid gel stain.

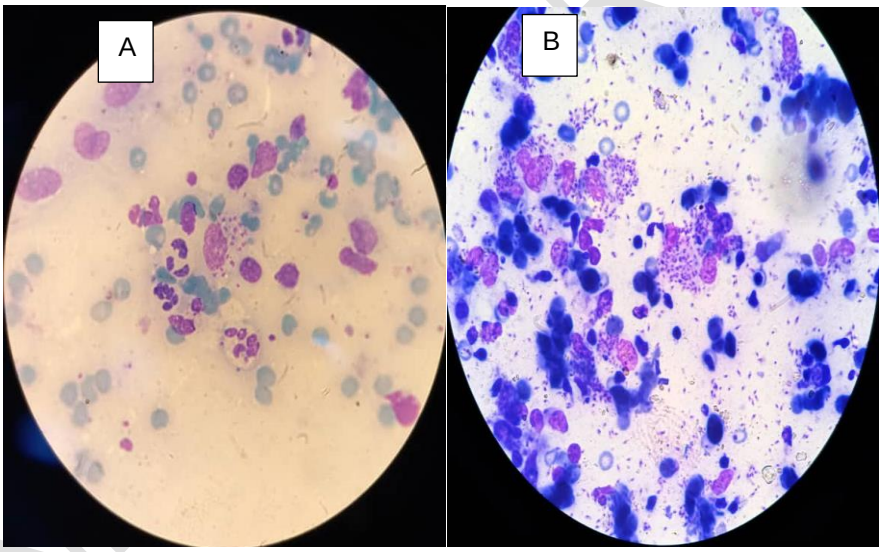

Figure 7 the picture of microscopic slid for Amastigote inside the macrophage (A), and outside the microphage (B)

Correlation Between Lesion Size and Test Results:

Patients with larger lesions (≥ 4 cm) had a significantly higher likelihood of positive PCR results compared to those with smaller lesions (OR = 2.5, 95% CI: 1.5-4.2, $p < 0.01$). Similarly, microscopy was more likely to be positive in patients with larger lesions (OR = 1.8, 95% CI: 1.1-3.0, $p < 0.05$). Lesions on the lower limbs and upper limb showed higher rates of PCR positivity (59% and 35%, respectively) compared to facial lesions (19%).

Geographical Distribution:

The bar graph in **Figure 8** illustrates the distribution of cutaneous leishmaniasis cases across various municipalities. A significant disparity is observed, with some municipalities reporting a

substantial number of cases, while others have very few or none. "Gharyan" and "Kikla" stand out with the highest number of reported cases. Conversely, a large proportion of municipalities exhibit low case numbers, with many reporting only a single case or even no cases. This uneven distribution suggests potential variations in environmental factors, socioeconomic conditions, and human behavior across these municipalities, which may influence the transmission and spread of the disease.

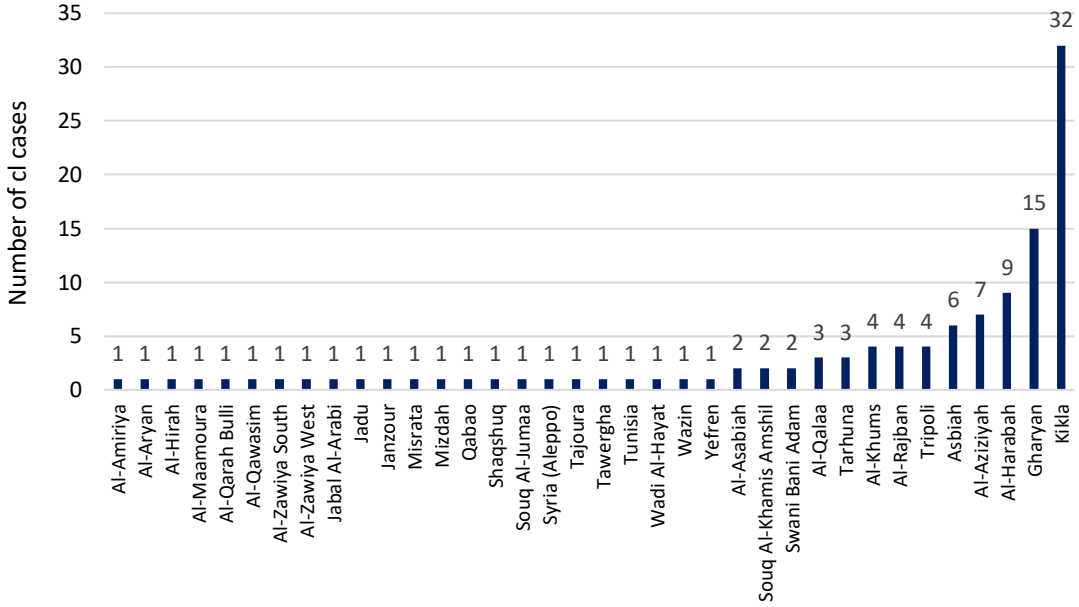

Figure8: Municipalities and Cutaneous Leishmaniasis Cases Data

The study provides valuable insights into the epidemiology, clinical presentation, and diagnostic methods of cutaneous leishmaniasis (CL) in Libya. The findings align with and expand upon previous research, highlighting key trends and regional variations in the disease. The demographic analysis revealed a relatively balanced gender distribution, with a slight predominance of male cases (males 83 vs. 33 females). This is consistent with other studies in the region, which have shown that males are more frequently affected due to their higher engagement in outdoor activities, such as farming and night duties, which increase exposure to sandfly bites(9). The age distribution, with the majority of cases occurring in adults aged 20-50 years, is also consistent with previous findings, suggesting that occupational and environmental exposures play a significant role in disease transmission(10).

Lesions were predominantly found on the lower limbs (46%), followed by the upper limbs (28%) and face (18%). This distribution aligns with other studies in the Mediterranean region, where exposed body parts are more susceptible to sandfly bites(11). The size of lesions also correlated with diagnostic outcomes, as larger lesions (≥4 cm) were more likely to yield positive PCR and microscopy results. This could indicate that larger lesions are either more advanced or more likely to be infected with secondary pathogens, necessitating prompt and accurate diagnosis(12).

The study highlights the superior sensitivity of PCR over microscopy for diagnosing CL, with 89.66% of cases testing positive by PCR compared to 54.31% by microscopy. This finding is consistent with other studies that have demonstrated the higher sensitivity of molecular methods, particularly in cases where parasite load is low or lesions are chronic(12). The statistically significant difference between PCR and microscopy ($\chi^2 = 15.93$, $p < 0.0001$) underscores the importance of incorporating PCR into routine diagnostic protocols, especially in regions where CL is endemic (13).

The geographical distribution of CL cases revealed significant disparities, with Gharyan and Kikla reporting the highest number of cases. This uneven distribution may be attributed to variations in environmental factors, such as the presence of sandfly vectors and rodent reservoirs, as well as socioeconomic conditions that influence healthcare access and disease control measures(14). The high prevalence in these areas aligns with previous studies that identified Al-Jabal Al-Gharbi province as a major endemic focus for CL in Libya (15).

The findings of this study are consistent with earlier research conducted in Libya and other Mediterranean countries. For instance, a study in Al-Jabal Al-Gharbi province found that males and individuals engaged in outdoor activities were at higher risk of CL, with lesions predominantly affecting exposed body parts (9). Similarly, molecular studies have identified *Leishmania major* and *Leishmania tropica* as the primary causative species in Libya, with *L. major* being more prevalent in rural areas and *L. tropica* in urban settings. The seasonal peak of CL cases between November and January, observed in this study, also aligns with the known activity patterns of sandfly vectors in the region(11).

The study underscores the need for targeted public health interventions in high-prevalence areas like Gharyan and Kikla. Enhanced surveillance, vector control measures, and public awareness campaigns could help reduce the disease burden. Additionally, the integration of PCR into diagnostic protocols can improve case detection and management, particularly in chronic or low-parasite-load cases(11).

4. CONCLUSION

This study provides critical insights into the epidemiology and diagnosis of CL in Libya, reinforcing the importance of molecular diagnostic methods and highlighting regional variations in disease prevalence. These findings can inform future research and public health strategies aimed at controlling and preventing CL in endemic regions.

CONSENT

As per international standards or university standards, patient(s) written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

The study was approved and confirmed under the rules and regulations of research in the NCDC Tripoli-Libya. Therefore, at commencing the research point, ethical approval and authorization were issued while referring to the hospital

Laboratory tests for diagnosis

Parasitological diagnosis A search for amastigotes can be performed using light microscopy to directly examine the biopsy specimen, scraping or impression smears subjected to Giemsa staining.

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