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Clinical, Parasitological, and Anatomopathological Characteristics of Cutaneous Leishmaniasis Cases at a Tunisian Hospital

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ABSTRACT

Introduction:

Cutaneous leishmaniasis (CL) is the most common vector-borne disease in Tunisia. Confirmation of the diagnosis is based on direct examination (DE) of the dermal fluid, supplemented, if necessary, by PCR. Occasionally, differential diagnoses of CL require anatomopathological examination.

Objective: The aim was to study the clinical features and report the results of different diagnostic methods of cases of CL diagnosed in a Tunisian university hospital.

Methods:

This was a cross-sectional study that included patients followed at the dermatology department of Charles-Nicolle Hospital for CL, retained in view of parasitological and/or anatomopathological and/or molecular arguments.

Results:

Among 120 patients consulting for suspected CL, the diagnosis was confirmed in 45 of them. In 5 cases, LC was discovered incidentally by pathological examination. In all, 50 cases were selected. Women were statistically more affected than men (p=0.001). The average age was 35. The average duration of lesions (4.9 months) was statistically associated with a positive diagnosis (p=0.012). Most patients infected in the north of the country had single lesions, whereas all those with multiple lesions were infected in the center or south (p<0.001). The DE was positive in 74% of cases.

Pathological examination revealed CL in 15 patients. PCR was performed in 10 cases and confirmed the diagnosis in four. Agreement between DE and PCR was poor.

Conclusion:

CL remains a frequent parasitosis in Tunisia. The differential diagnoses are multiple, which implies the diversity of confirmation methods.

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Résumé

Introduction

La leishmaniose cutanée (LC) est la maladie vectorielle la plus fréquente en Tunisie. La confirmation du diagnostic repose sur l'examen direct (ED) du suc dermique complété au besoin par une PCR. Parfois, des diagnostics différentiels de la LC justifient le recours à l'examen anatomopathologique**.**

Objectif : étudier les caractéristiques cliniques et rapporter les résultats de différentes méthodes de diagnostic de cas de LC diagnostiqués dans un CHU tunisien.

Méthodes :

Il s'agissait d'une étude transversale ayant inclus des patients suivis au service de dermatologie de l'Hôpital Charles-Nicolle pour LC, retenue devant des arguments parasitologiques et/ou anatomopathologiques et/ou moléculaires.

Résultats :

Parmi 120 patients consultant pour suspicion de LC, le diagnostic a été confirmé chez 45 d'entre eux. Dans 5 cas, la LC a été découverte fortuitement par l'examen anatomopathologique. Ainsi, au total, 50 cas ont été retenus. Les femmes étaient statistiquement plus atteintes que les hommes (p=0,001). L'âge moyen était de 35 ans. La durée moyenne des lésions (4,9 mois) était statistiquement associée au diagnostic positif (p=0,012). La majorité des patients contaminés au nord du pays présentaient des lésions uniques, alors que tous ceux ayant des lésions multiples ont été contaminés au Centre ou au Sud (p<0.001). L'ED était positif dans 74%. L'examen anatomopathologique a trouvé un aspect cadrant avec une LC chez 15 patients. Une PCR, réalisé dans 10 cas, a confirmé le diagnostic chez quatre parmi eux. La concordance entre l'ED et la PCR était médiocre.

Conclusion :

La LC demeure une parasitose fréquente en Tunisie. Les diagnostics différentiels sont multiples ce qui implique la diversité des méthodes de confirmation.

Key-words: Leishmaniasis, cutaneous, parasitological diagnosis, histology, PCR, Tunisia

Mots-clés :

Leishmaniose, cutanée, diagnostic parasitologique, PCR, histologie, Tunisie

INTRODUCTION

Leishmaniasis is a tropical disease caused by parasitism of the mononuclear phagocyte system by flagellated protozoa of the genus *Leishmania*. They are transmitted by dipteran insects of the genus *Phlebotomus* (*Ph*) in the Old World and *Lutzomyia* in the New World (1). This vector has a crepuscular and nocturnal activity and is present throughout the year in intertropical areas and during the summer in temperate regions. The skin is the primary portal of entry, resulting in three main clinical forms: cutaneous leishmaniasis (CL), cutaneous-mucosal leishmaniasis and visceral leishmaniasis (2).

In Tunisia, CL is the most common vector-borne disease with three nosogeographic forms: sporadic CL, chronic CL, and zoonotic CL (ZCL), posing a public health problem. Although CL is self-curable and does not have a life-threatening prognosis, it can cause considerable aesthetic, and psychosocial inconvenience (3).

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CL is often suspected based on epidemiological features (geographical origin of the patient, residence) and lesion's appearance. The diagnosis is confirmed by detection of the parasite in the dermal fluid. However, this test can be falsely negative, hence the importance of molecular techniques. Sometimes, the diagnosis of CL is not considered as a first option due to atypical clinical forms. In fact, CL can mimic both infectious (tuberculosis...) and malignant (cutaneous lymphoma...) diseases for which a skin biopsy is the first option (4).

The objectives of our work were to study the clinical aspects of CL, and the contribution of different methods of positive diagnosis.

METHODS

Study population

We conducted a descriptive cross-sectional study at the Parasitology-Mycology Laboratory of the Charles-Nicolle Hospital in Tunis (CNH) over a 3-year period. We included patients from the Dermatology department of the CNH with a confirmed diagnosis of CL by parasitological, molecular and/or anatomopathological examination. However, patients whose diagnostic and therapeutic management was not provided by the Dermatology Department of CNH were not included.

Sampling

Each patient underwent one or more collections from suspicious lesions. A form was completed containing data from the interview (age, sex, address, geographical origin and/or history of residence in CL endemic areas during the previous year, date of appearance of the lesion…), clinical examination of the lesions and the results of the direct exam (DE). Consent has been obtained from patients.

Sampling for DE was performed at the Parasitology-Mycology Laboratory of CNH. A few millimeters of dermal incision were made under the adherent crust or on the raised edge at the periphery of the lesion, until serous fluid was obtained.

Dermal fluid sampling for possible molecular study was performed at the Parasitology-Mycology Laboratory of the Pasteur Institute of Tunis, in case of negative DE and/or negative anatomopathological exam with strong epidemiological and clinical suspicion of CL. Serum samples were collected with a swab. They were placed in Eppendorf containing 200 microliters of sterile physiological water and stored at -20°c.

A 3 mm skin biopsy was taken from the edges of the patient's lesion for histopathological examination at the Dermatology Department of CNH as a first-line approach when the clinical appearance suggested one or more differential diagnoses of CL, or a second-line approach after negative DE with strong epidemiological and clinical suspicion of CL, and when PCR was negative or could not be performed (due to the excessive cost of this method).

Sample processing

Microscopic DE was performed at the CNH Parasitology-Mycology Laboratory. Smears were made on slides with dermal fluid and stained with May-Grünwald-Giemsa. Positivity was defined by the detection of parasites in intra or extra-macrophagic amastigote form under a light immersion microscope (magnification x 100). Histopathological examination was performed at the CNH Pathology Laboratory. Tissue samples were fixed in 10% formalin buffer, dehydrated in graded ethanol, cleared with xylene, impregnated with paraffin, sectioned at 5 mm thickness, stained with haematoxylin and eosin. Periodic acid-Schiff staining was performed when a granulomatous reaction was observed with haematoxylin and eosin. Molecular diagnosis was performed at the Parasitology-Mycology Laboratory of the Pasteur Institute of Tunis using real-time PCR. Dermal fluids were subjected to DNA extraction using the commercial kit "QIAamp DNA Blood Mini Kit Qiagen®". The primers used for amplification targeted kinetoplastic DNA.

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In conclusion, a diagnosis of CL was confirmed if the microscopic DE or histopathological examination or PCR was positive. If all three techniques were negative, the diagnosis of CL was excluded.

Statistic analysis

The monitoring of all data, as well as the statistical analysis, was conducted using SPSS 25 software.

The comparison of percentages from two independent samples was done using the Pearson Chi-square test, and in case of invalidity, the Fisher's test was employed. The comparison of means was performed using the Student's T-test if the variable followed a normal distribution. Otherwise, non-parametric tests were used. The significance threshold (p) was set at 0.05.

The concordance study between two qualitative variables was carried out using the Kappa concordance index. Its interpretation according to the scale proposed by Landis and Koch is illustrated by table 1.

Table 1: Interpretation of the Kappa concordance index

Kappa concordance index	Degree of agreement
< 0	Very bad
0,01 – 0,20	Bad
0,21 – 0,40	Poor
0,41 – 0,60	Moderate
0,61 – 0,80	Good
0,81 – 1,00	Excellent

RESULTS

- During the study period, 120 patients attending the dermatology department were sampled for suspected CL. Their mean age was 34.5 ± 21.8 years, ranging from 6 months to 86 years. Most patients with CL lived in the north-eastern region of the country (86%). The governorate of Tunis was the most represented (41%).

- Cutaneous leishmaniasis was confirmed in 50 patients, considering all diagnosis's method:

- The diagnosis was confirmed by positive DE and/or histopathological examination and/or PCR in 45 patients (38% of consultations).
- In 5 cases, the diagnosis was confirmed incidentally by histopathological examination of skin lesions suggesting other differential diagnoses.

Epidemiological and clinical profile of patients with confirmed CL

The sex ratio of the patients was 0.61, and the difference between sexes was statistically significant (p = 0.003).

Their mean age was 35 ± 23.2 years [9 months- 71 years]. There was a predominance of children and young adults under 20 years old, but the association with these age groups was not statistically significant (p=0.07) (Figure 1).

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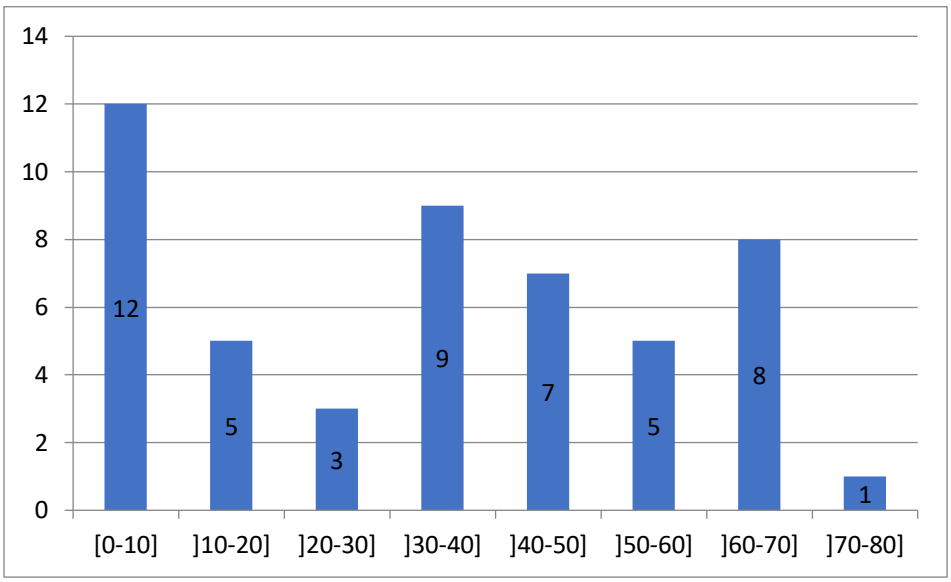

Figure 1: Distribution of patients with CL according to age group (in years)

Considering the patient's place of residence and travel to a CL-endemic area, contamination probably occurred in Tunisian north in 52% of cases, center in 26%, and western south in 22%. The binary logistic regression model showed that the difference in distribution according to likely contamination regions was statistically significant (p=0.007).

The mean time from lesion onset to consultation was 4.9±4.7 months [three weeks-two years]. The duration of lesion development was statistically significantly associated with lesion positivity (p=0.012).
Most lesions were located on the extremities (n=35, 70%: Figure 2). Facial lesions were observed in 12 cases (24% of cases: Figure 3,4), including one case involving the nasal mucosa (Figure 4).

The mean number of lesions was 2.1±2.2 [1–12 lesions]. Thirty-one patients had a single lesion (62%). The majority of those contaminated in the north had single lesions (77%). All patients with multiple lesions (≥3 lesions) were contaminated in the central or southern regions (14 patients). The difference in the number of lesions according to geographical origin was statistically significant (p<0.001).

Ulcerative-crusty lesions were the most frequently observed (58% of cases: Figure 5). However, the association of this appearance with the diagnosis of CL was not statistically significant (p=0.27).

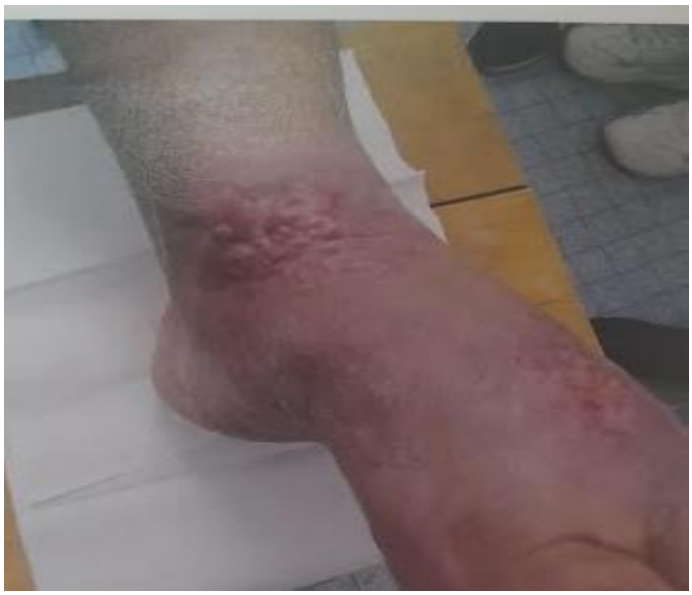

Figure 2: Vegetative lesion of cutaneous leishmaniasis on the foot

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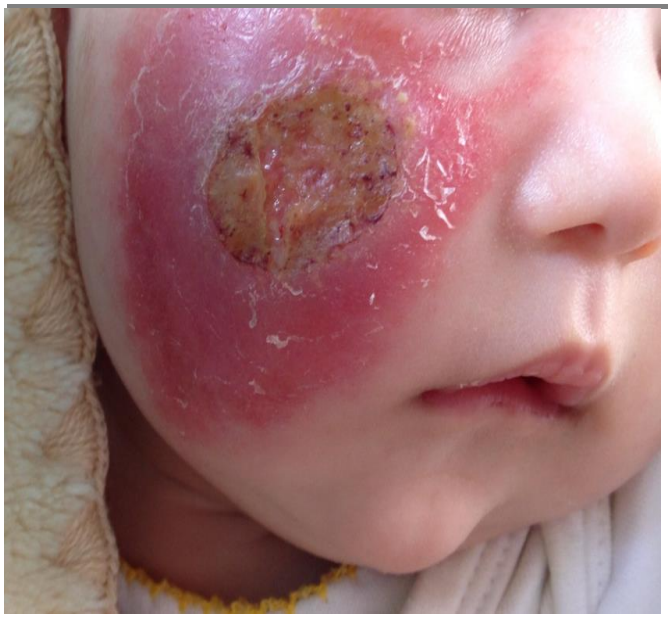

Figure 3: Ulcerative-crusted lesion on the face of an infant"

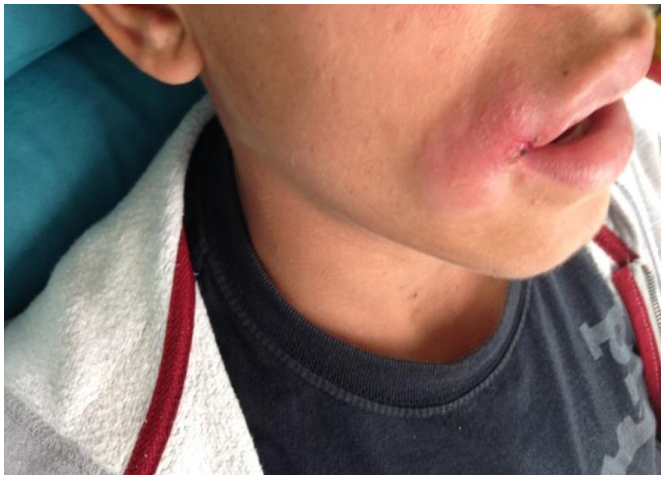

Figure 4: Erythematous lesion on the cheek extending to the labial mucosa

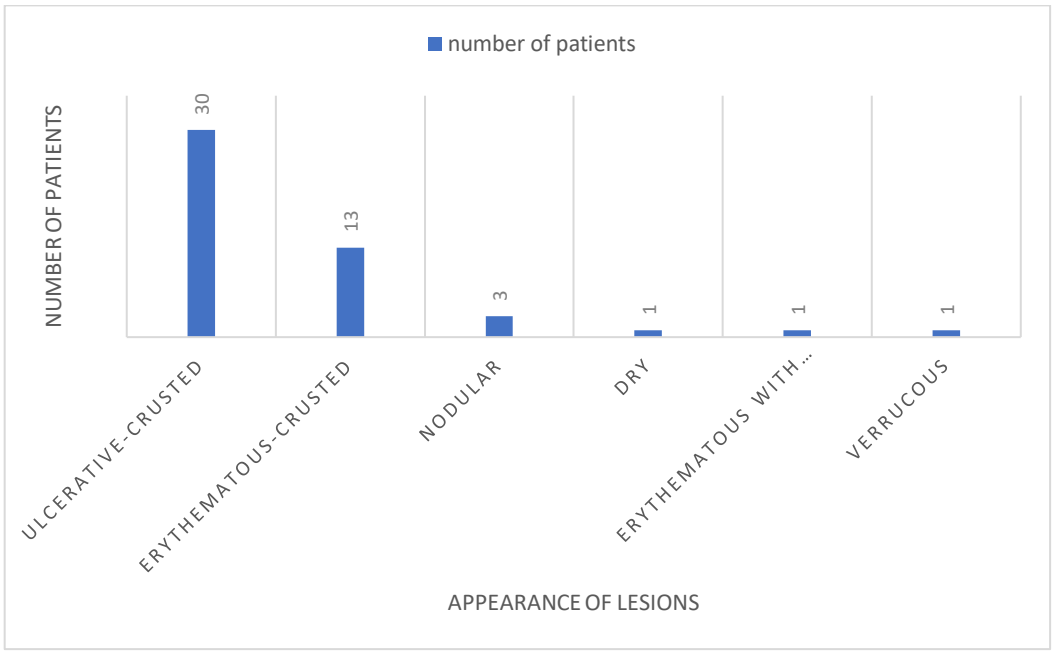

Figure 5: Distribution of patients according to the appearance of lesions

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Results of different method of diagnosis

Direct examination

Direct exam was performed for all patients consulting for suspicious CL (120 patients). For one patient, it was performed as a second-line procedure in the presence of a biopsy suggestive of a CL). Amastigote forms (figure 6) were observed in 34 lesions (28.1% of the group of patients who underwent dermal smears). For the 50 patients with CL confirmed, the DE was performed in 46 cases (with a positivity rate of 74%).

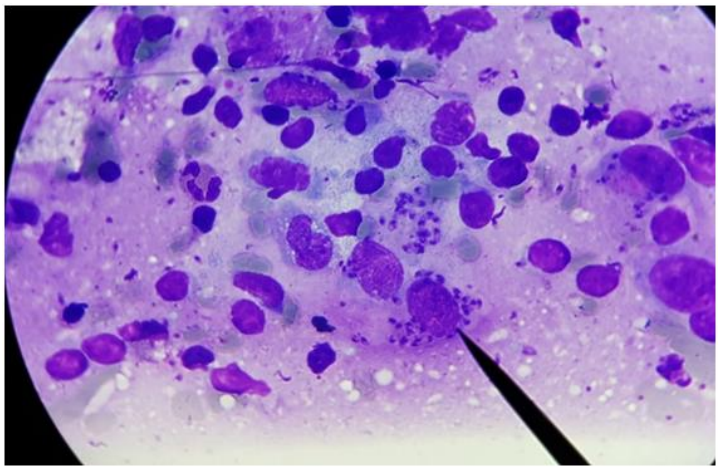

Figure 6: Intra-macrophagic amastigote forms of *Leishmania sp* (Magnification *100) (Parasitology-Mycology Laboratory, Charles Nicolle Hospital, Tunis)

Histopathological Examination

Histological signs in favor of CL were identified in 15 cases. In 5 cases, this exam was performed as a first-line diagnostic tool for lesions suggestive of dermatoses other than CL.

Granulomatous changes in the dermis were identified in 7 cases (figure 7). Two of them had basophilic bodies in the cytoplasm of histiocytes, suggesting the presence of parasites. Inflammatory infiltrate rich in lymphocytes, histiocytes and eosinophils without granuloma formation were observed in 8 cases.

The diagnosis of CL was supported, by using at least one of the following criteria: positive PCR or DE, or good progress with treatment.

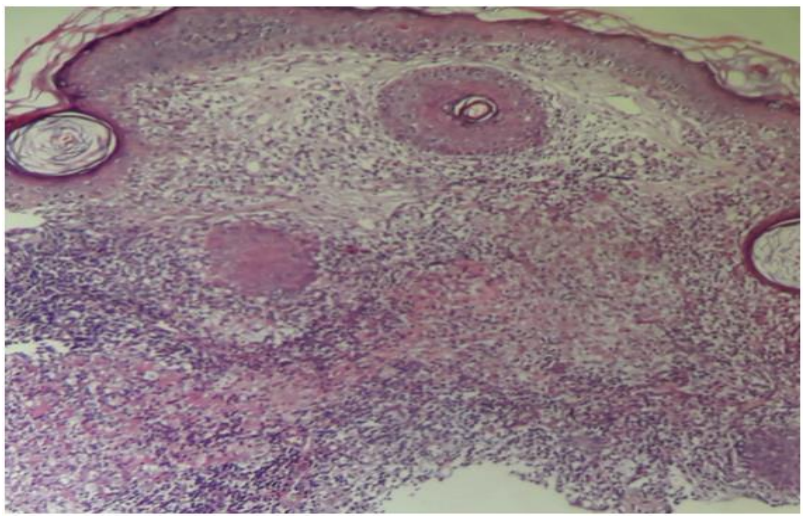

Figure 7: Microscopic examination of skin biopsy: Granulomatous leishmaniasis - Dense dermal infiltrate rich in lymphocytes with epithelioid granulomas (Hematoxylin Eosin100) (Pathology Department, Charles Nicolle Hospital, Tun

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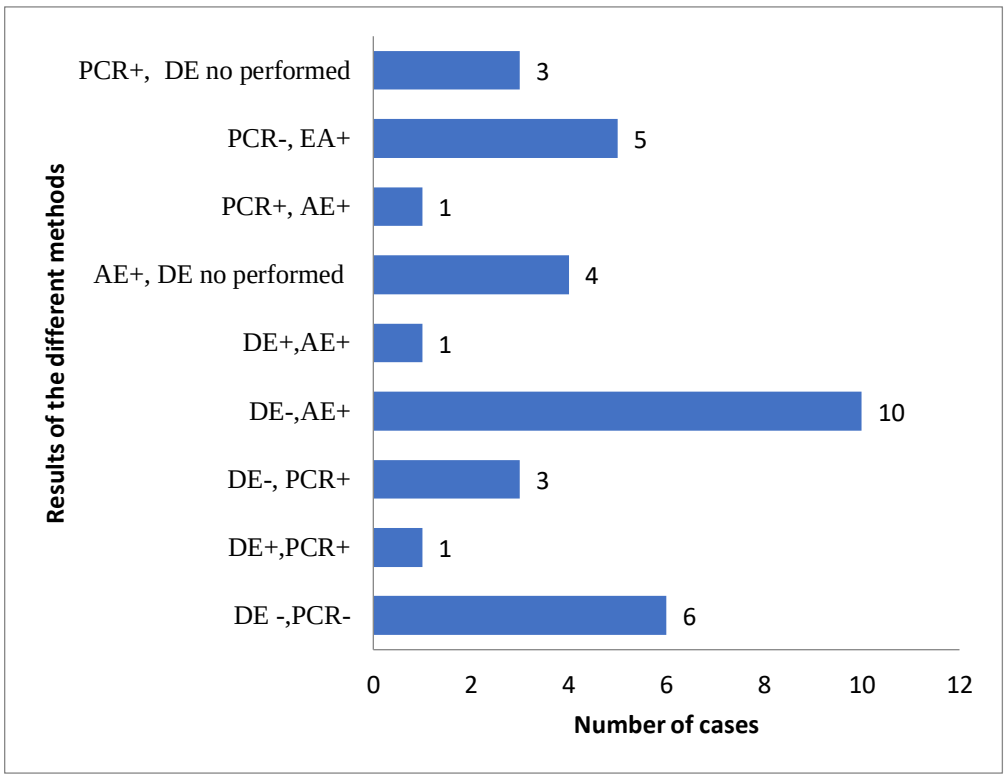
Molecular diagnosis:

PCR was conducted in case of negativity of DE with presence of a strong epidemio-clinical suspicion (5 cases), or to confirm the results found by the anatomopathological examination (1 case). It was positive in four out of 10 patients.

Comparison between different diagnostic methods

The results of the different diagnostic methods are illustrated in figure 8.

No concordance could be established between the anatomopathological examination and the other examinations because we only have the number of positive biopsies. The concordance between DE and PCR was poor (kappa=0.28) (p=0.4).



DE: Direct Exam, AE: Anatomo-pathological Exam

Figure 8: Results of the different methods of diagnosis

DISCUSSION

Epidemiological study

Cutaneous leishmaniasis is a vector-borne zoonosis, classified by the World Health Organization as a neglected tropical disease (5). More than 90% of cases come from Afghanistan, Iran, Saudi Arabia, and Syria in the Old World, and Brazil and Peru in the New World (6,7). It has been emerging in North Africa since the early 1980s, with a significant increase in the incidence of cases and a geographical expansion (3,8). The incidence exceeds thousands of cases each year in Algeria, Libya, Morocco, and Tunisia (3).

In our series, over a period of three years, 50 cases of CL were confirmed. Our study does not reflect the true prevalence of the disease, since most of the patients included live in Grand Tunis. In the literature, the governorates most affected by ZCL are Kairouan, Sidi Bouzid, and Gafsa (9,10). Similarly, the governorates of Tozeur, Tataouine, Sousse, and Mahdia have been seriously affected in recent years (11,12). Currently, contamination was spread throughout the entire Tunisian territory (8,11).

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In our study, the frequency of confirmed CL cases was statistically higher in women (62%). This aligns with other Tunisian studies (13,14,15), in contrast to foreign studies where a male predominance was noted, especially in Morocco (16), Iran (17), and Brazil (18). A predominance of children and young adults under 20 years was observed in our series, with an average age of 35 years, as well as other studies in Tunisia (9,11,14,15), and Sicily (19). Positivity in children suggests active transmission of the infection (19). It is well-known in historical foci of ZCL that young children bear the heaviest burden of the disease, "native" adults are often being immunized by previous infections or repeated contacts with the parasite. Furthermore, the areas where the proportion of adults is particularly high indicate both acute transmission of the parasite and, more importantly, the absence of protective immunity in the population (14).

Clinical study:

CL is characterized by a chronic course. In the study, the average duration from lesion appearance to consultation was 4.9 months. This long delay is often due to the slow progression of lesions, which can persist for months or even years before healing, leaving flat, hypopigmented scars (9,14,16). The variability in lesion duration is influenced by host and parasite factors, with *L. major* potentially causing more rapid manifestations (20).

All discovered areas can be affected by CL (21). According to literature (8,9,11,20), the extremities and the face were the predominant locations. The preferential localization on the extremities, associated with multiplicity, is a suggestive of ZCL (14). The involvement of the face favors sporadic CL (20). These differences could be secondary to properties of sandfly species, transmitting *L. infantum* (responsible for sporadic CL), which might be more attracted to the CO2 emitted by respiratory pathways (8). Additionally, some population behaviors, such as using blankets to cover the body except the face during nocturnal coolness in the North, could also explain these data, mainly among young children (8,9).

Nasal mucosa involvement was reported in only one patient and associated with cutaneous lesion. It usually occurs by contiguity from a cutaneous focus, less commonly by direct sandfly bite on the mucosa. These locations should not be overlooked, especially considering a history of staying in an endemic area. Unlike New World mucocutaneous leishmaniasis, forms observed around the Mediterranean basin are characterized by the absence of mutilating lesions and a good response to conventional therapies (21).

The number of lesions, in our study, ranged from one to 12, with an average of two lesions per patient. The multiplicity of lesions is indicative of ZCL. The correspondent vector *Ph papatasi*'s inability to obtain a sufficient blood meal in a single bite may result in multiple bites and, consequently, multiple lesions (9).

Clinical polymorphism of lesions in our study, confirm the results reported in the literature (1,8,11). The predominance of the ulcerative-crusty form, also noted in other studies in North Africa (13) and Tunisia (1), is explained by the frequency of CL caused by *L.major*. This form is characterized, as described earlier, by often multiple lesions, localization in uncovered regions, especially on the extremities, and short evolution duration. However, genetic diversity analysis of *L. major* isolates has revealed a high level of polymorphism (43%) among them (22).

Other clinical aspects have been observed, including erythematous lesions (18%). This is thought to be related to sporadic CL (8). In the literature, numerous clinical presentations are possible in CL, such as impetigo-like, vegetative, lupoid, pseudotumoral, psoriasiform, ulcerative, lymphangitic, and nodular forms.

Then, the diagnosis of CL is thus suggested by the painless nature of the lesion, its location in exposed areas, the absence of itching, general symptoms, and satellite lymphadenopathy, a history of residence in an endemic area, chronicity, and resistance to antibiotic treatments.

Diagnostic methods

CL can be confused with other co-endemic diseases. A correct diagnosis is crucial for applying the appropriate treatment. Regarding laboratory methods, there is a lack of a gold standard for the diagnosis (23).

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Three confirmation techniques for CL were used in this study: microscopic examination of dermal smears, histopathological examination of skin biopsies, and real-time PCR. These techniques were not performed for all patients, making it difficult to deduce their sensitivity or specificity. However, our approach reflects the real-life situation in hospitals working with available resources while trying to ensure adequate diagnosis for proper patient management.

DE was performed initially in 120 patients and secondary in one case. It allowed the detection of the parasite in 74% of diagnosed CL cases, likely another Tunisian study (9). However, this rate varies among studies and countries, ranging from 43% to 83.8% (table 2).

Table 2: Prevalence of positive direct exam among diagnosed cutaneous leishmaniasis cases: Results of several studies

Study	Country	Year	Number	Prevalence of positive direct exam
Belhadj and al (24)	Tunisia	2005	105	83,8%
Bensoussan and al (25)	Palestine	2006	92	74,4%
Neffati and al (26)	Tunisia	2009	299	60,6%
Mouttaki and al (27)	Morocco	2014	58	43%
Yildiz and al (28)	Turkey	2017	62	71,4%
Hamouchi and al (29)	Morocco	2018	114	71%
Tebrouri and al (9)	Tunisia	2019	347	78,3%
Our study	Tunisia	2020	125	74%

DE appears to be the best test for diagnosis as it is economical, easy, rapid, and safe (1). It should be prescribed as a first intention before any biopsy, especially since it has high specificity by showing the amastigote forms of *Leishmania* (1,21). However, its sensitivity varies depending on the number of parasites, quality of the dermal fluid sampling, and microscopist's experience. This explains the disparities between different studies (30,31,32).

Resorting to dermal aspirate culture could compensate for weakly negative results, although this technique is not always available.

Histopathology is usually performed in atypical presentations or when DE is negative (33). The presence of a granulomatous reaction, with a high percentage of macrophages and multinucleated giant cells, is a crucial suggestive indicator for the diagnosis of CL in patients living in endemic areas with clinically suggestive CL lesions (2). Moreover, non-ulcerated or atypical CL is characterized by a mononuclear inflammatory infiltration of the dermis, predominantly composed of lymphocytes followed by macrophages with discreet parasitism (34).

CL presents a broad spectrum of histologically expression, and can mimic other inflammatory and neoplastic diseases (35). Immunohistochemical analysis could support the diagnosis of CL. CD1a expression by *L. major*, *L. tropica*, and *L. infantum* amastigotes has been demonstrated (34). It is a valuable tool that facilitates the diagnosis of leishmaniasis and studies the cellular immune response developed during infection (36). It allows for a better visualization of amastigotes compared to hematoxylin-eosin for CL diagnosis (2).

The introduction of molecular biology techniques such as PCR has added greater sensitivity in the diagnostic (37). PCR was not performed for all patients in our series, creating a selection bias.

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The quantitative PCR, used in our study, has better sensitivity than qualitative PCR. However, the choice of primers seems to play a role in the sensitivity of the technique (28). The kinetoplastic DNA, the target used in our study, is the most commonly identified in the literature for the diagnosis of CL (9).

PCR is also effective in determining the species of the parasite. The interest in species identification is mainly epidemiological (3,12). Other molecular methods have been developed, including Loop-Mediated Isothermal Amplification, which could be used as a first-line molecular test for early diagnosis and rapid management of CL cases in public health programs (38). However, it remains a costly technique that may not be available in all laboratories, especially in developing countries.

In our series, histopathological examination favored a diagnosis of CL despite negative DE or PCR. It can be explained by the presence of inhibitors such as hemoglobin, bacterial DNA, especially when the concentration of the target DNA is very low. This problem could be resolved by using PCR inhibitor controls.

It seems that the parasitic load is inversely proportional to the duration of evolution, indicating the superiority of histopathological evaluation for chronic forms in the course of healing (39).

Three cases negative by DE were revealed through PCR in our study. One patient among them had Glucantime injections before the dermal smear, which could be the cause of the false negativity of the DE. In a comparative study between DE and PCR, Culhaand al. concluded that PCR should be used for samples with suspected chronic CL that are negative by DE (40). Thus, there is a divergence among different diagnostic methods, limiting the choice of the "Gold Standard" to define a case of CL (37).

CONCLUSIONS

CL remains an emerging parasitic disease in Tunisia. Clinical polymorphism is important. The diagnosis, suggested by epidemiological data, is sometimes hindered by the cost of certain diagnostic methods, notably PCR.

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