

## Pathological Study of Cutaneous Leishmaniasis in Al-Diwaniya province

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### الخلاصة

تم دراسة 42 مريض سريريا قسموا الى 25 (59.5%) ذكور و 17 (40.5%) إذ استند التشخيص على الفحص السريري والعلامات السريرية حيث وجد ان المرضى مصابين بمرض اللشمانيا الجلدية، ووجد ان معظم المرضى يعانون من آفات جلدية متعددة (64.3%) والبعض يعاني من آفة واحدة لكل مريض (35.7%)، عدد الآفات لكل مريض تراوح بين آفة واحدة و 12. معظم الآفات تركز على وجه المصابين بنسبة (40.5%).

الآفات درست نسيجياً حيث شوهد ان الطبقة المتقرنة والخلايا الجلدية في مكان الإصابة كانت متأكلة او ساقطة غير موجودة في مكان التقرح، او على شكل قشور مع دم متخثر كذلك تم استئصال خزعة من نسيج الجلد (10 نماذج) وتصيغه بواسطة صبغات الهيماتوكسلين والايوسين وتم فحصه بواسطة المجهر حيث شوهد تجمع طفيلي اللشمانيا بطورها اللاسوطي Amastigotes فضلا عن تجمع الخلايا المناعية مثل الخلايا اللمفية وخلايا العدلة وخلايا البلعم الكبير وهي تبتلع الطفيلي.

كذلك في هذه الدراسة، تم سحب (11) نموذج من افرازات الآفات بواسطة محقنة لغرض الفحص المباشر وزرع الطفيلي حيث تم عمل مسحات مباشرة لغرض تصيغها بواسطة صبغة الكمزا قبل عملية الزرع للطفيلي وأظهرت النتائج الحالات الموجبة لنتائج زرع الطفيلي حيث انه نما بشكله المسوط promastigote في 63.4% من الحالات المزروعة على وسط (3N) بينما كانت النتيجة موجبة في 72.7% لنفس الحالات المزروعة على الوسط ثنائي الطور biphasic medium و محلول لوك Luck's solution.

### Abstract:

In the present study, 42 patients were observed, as cutaneous leishmaniasis, from 1 September 2012 until the end of August 2013. They divided to 25 (59.5 %) males and 17 (40.5%) females, and the diagnosis was depend on clinical examination, pathological lesions and parasitic isolation. Lesions were classified depending on number, so the patients who have multiple lesions were (64.3%), while (35.7%) have single lesion, the number of lesions per patient ranged from one to 12 lesions. Most of the lesions were located on face (40.5%), but some lesions were on the face & hand (21.4%), while few were found in hand and back (2.4%). Histopathologicaly, the cutaneous lesions were observed; keratinized layer and epidermal cells were necrosed or not present over the ulcer, Skin biopsies were taken, fixed with formalin, sectioned, and stained with hematoxyline and eosin stain (H & E stain). Microscopic examination, showed accumulation of amastigote, inflammatory cells such as neutrophils and lymphocytes with macrophages engulf amastigotes. Eleven samples of aspirated blood were collected by fine needle, for making direct Giemsa-stained smears before the cultivation, 72.7% of the cases were positive to the presence of *leishmania tropica*. There are three culture media were used in this study, biphasic medium, NNN medium, and lock's solution; 63.4% of cases were positive in NNN medium, and 72.7 % were positive in both of biphasic media and Lock's solution.

## Introduction

Leishmaniasis has been an old public health problem in South-West Asia and the Arab World reported from time immemorial as the pharaohs ruled in Egypt and Assyrians in Mesopotamia (Iraq) [1], [2]

In old world, cutaneous leishmaniasis caused by *L. major*, *L. tropica* and *L. aethiopica*. In New world, it caused by *L. Mexicana* and *L. braziliensis* complexes. Majority of cutaneous leishmaniasis cases occur in Afghanistan, Brazil, Iraq, Iran, Peru, Saudi Arabia and Syria, with 1-1.5 million new cases reported annually worldwide [3]. Two species are present in Iraq: *L. tropica*, agent of anthroponotic cutaneous leishmaniasis (ACL) *L. major*, agent of zoonotic cutaneous leishmaniasis (ZCL) [4]. The parasite exists in two morphological forms: the non-flagellated amastigote (3-5  $\mu\text{m}$  in diameter) living intracellular in macrophages of the mammalian host, and the flagellated promastigote (15-30  $\mu\text{m}$  in length, plus the flagella), living extracellular in the intestinal tract of the sand fly-vector [5]. The culture form of *Leishmania* parasites morphologically identical to that present in the sand fly vector [6]. Fever, headache, weight loss, loss of appetite and varying hematological abnormalities were common features associated with various forms of presentation of cutaneous leishmaniasis [7].

## Materials and Methods:

This study was carried out at medical microbiology department of Medical College, Al-Diwaniya, over period of (12) months, from first of September 2012 until the end of August 2013. 42 patients of all age groups and both sexes observed and diagnosed as cutaneous leishmaniasis, among all patients; age range was (1 year-71 years). Diagnosis was based on history (patients coming from endemic areas, persistence of lesions), cutaneous lesions (nodules, plaques, ulcers) and response to specific antileishmanial treatment (pentostam). Duration of lesions varied from 4 weeks to 6 months.

## ➤ Direct Smear Examination and Cultivation the Parasite:

### ➤ Making Direct Giemsa Smear:

The sample from the cutaneous lesion taken by aspiration with fine needle as the following steps: (1) the lesion and skin around the lesion was disinfected by 70% ethanol. (2) Sterile syringe of 1 ml contain 9 ml of sterile normal saline was used to inject the fluid intradermal through intact skin in to the active red border of the lesion. (3) Aspirate the injected fluid as the needle draw back until the bloody stained fluid aspirate. (4) Small amount of aspirated fluid was taken and placed on clean glass slides, then fixed by using absolute methanol and then Giemsa stain applied for 10 minutes to making direct Giemsa smear before the culturing, Amastigote diagnosed as round or spherical shape with kinetoplast [8]. The aspirated fluid were injected to tubes, which contain **the culture media** [10 ml of biphasic media, 8-10 ml of NNN medium, and 5 ml of lock's solution].

## ➤ Preparation the Media for Cultivation the Parasite:

### ➤ Biphasic medium: [9]

Nove and Mac Neal used it for the first time in 1904; this medium was used for cultivation and continuation of promastigotes stage of *leishmania spp.*, it has composed of two phases, one is a solid phase and the other is liquid phase, these phases are:

➤ **Solid Phase:** Kagan and Norman (1970) prepared it from the following composition: brain heart infusion (33.3 gram), D-glucose (8.0 gram), agar (16.0 gram), distill water (900 ml), difibrinated rabbit blood (100 ml), streptomycin sulfate (0.2 gram) and crystalline penicillin 200, 000 I.N.U.

➤ **Procedure:** (1) All solid constituent except the antibiotic and glucose has been dissolved in Distill water and justify the pH to 7.4 and sterilize the solution in autoclave (121 °C) for 15minute. (2) Blood had been aspirated from by cardiac puncture using

sterile syringe of 10ml and put it under sterile condition in sterile tubes contain small glass bole then shack the blood for three minute to separate the fibrin. (3) The sterile solution had been cooled to 50°C then added the antibiotic and glucose which sterilized by filtration, and Defibrenated rabbit blood. distributed (8-10) ml of this media in sterile plain tubes of 25ml till solidify, all tubes incubated in 37°C for 24 hours to ignore contaminated tubes and the sterile tubes were kept in 4°C till use [9].

➤ **B. Liquid phase (Lock's solution):**

It was prepared it from the following **composition:** NaCl (9.0 gram), KCl (0.42 gram), CaCl<sub>2</sub> .H<sub>2</sub>O (0.32 gram), NaHco<sub>3</sub> (0.2 gram), D-glucose (2.0 gram), Streptomycin sulfate (0.2 gram), Crystalline penicillin (200,000 I.N.U).

➤ **Procedure:** All constituent except antibiotic and glucose has been dissolved in one Letter of distill water and justified the pH to 7.5. Sterilization of the solution in autoclave (121 °C) for 15minute then cooled of the solution until 50°C and adding the antibiotic and glucose, which has been sterilized by filtration, and was kept in 4°C until use. For Biphasic media preparation, 1-2 ml of lock's solution added to the tubes that contain the solid media [10].

➤ **Nove- MacNeal- Nicolle (NNN) medium:**

Kagan and Norman (1970) prepared it from the following **composition:** NaCl 6 gram, agar (14 gram), distill water (900 ml), defibrenated rabbit blood (100 ml), and gentamycin (50 mg).

➤ **Procedure:** All solid constituent except the antibiotic and blood has been dissolved in distill 'water and justify the pH to 7.4 and sterilize the solution in autoclave (121 °C) for 20 minute, the sterile solution

had been cooled to (48-50) °C. Then added the antibiotic and blood and distributed (8-10) ml of this media in sterile plain tubes of 25ml till solidify, all tubes incubated in 37°C for 24 hours to ignore contaminated tubes and the sterile tubes were kept in 4°C till use. Blood had been aspirated from rabbit by cardiac puncture using sterile syringe of 10ml and put it under sterile condition in sterile tubes contain small glass bole then shack the blood for three minute to separate the fibrin [9].

➤ **Lock's solution:**

It is the liquid phase of biphasic medium; it is composed of the same chemical components and prepared by the same procedure of [10]. Nevertheless, it employed without the solid phase of biphasic media.

**The biopsy: [11]**

The biopsy was taken from the peripheral part of a skin lesion within the inflammatory active outline in the form of elliptical sections measuring approximately 1 cm, all biopsies directly, were placed in 10% formalin and sent for histopathological laboratory at Al-Diwaniya teaching hospital

**Processing and Staining with**

**Hematoxyline and Eosin :**( 1) Fixation the biopsy by 10 % formalin. (2) Dehydration by ascending alcohol (70%, 90%, and 100%). (3) Embedding the biopsy in the paraffin wax. (4) Sectioning by the microtome, the sections were transferred to glass slides. (5) Clearing by incubation of the slide and washing by xylol. (6) Staining the slides by hematoxyline and eosin (H&E) stain. (7) Mounting by DPX with cover slides. (8) Examination the slides by light microscope.

**Results:**

➤ **Number of lesions and duration of disease:**

The clinical sings observed in 42 patients whom attended to hospital, the number and

percent of lesions quite differ from case to another; some patients suffer from one lesion (15 patients, 35.7%), while others suffer from more than one lesion (27, 64.3%); only one case was suffer from 12 lesions. The duration

of disease before attending to hospital varied from less than one month (2.4%) to 6 months (97.6%), only one case attended to hospital, have duration 20 days. All patients had

responded for the pentavalent antimonials treatment antileishmanial treatment (pentostam), table (1).

**Table (1) show lesion number and duration time before attending to hospital**

| Lesions No. |       | Duration time before attending to hospital |       |                 |      |                |       |
|-------------|-------|--------------------------------------------|-------|-----------------|------|----------------|-------|
| One lesion  |       | More than one                              |       | Less than month |      | Month and more |       |
| 15          | 35.7% | 27                                         | 64.3% | 1               | 2.4% | 41             | 97.6% |

➤ **Distribution the sites of the lesions:** Different clinical lesions were observed as nodule, crusted lesions and sometimes ulcers. Skin lesions concentrated on face, arm, leg,

neck and back of the Patients (figure 1). There were (40.5%) of the lesions on the face, as well as (21.4%) on face and hands while on the hands only (19.1%); table (2).



**figure 1: (1-A) Boy 12 years old with cutaneous lesion on the nose, (1-B) Girl nine year old has a lesion on the right cheek with dry crust, (1-C) Heavy infection of CL on face of one year old, the lesions were nodular with crust. (1-D) Cutaneous ulcer on the arm of man 25 years old man, (1-E) Cutaneous lesions on legs, (1-F) Woman 55 years old has scar of cutaneous leishmaniasis on her left hand.**

**Table (2) show the distribution of the lesions on some parts of body**

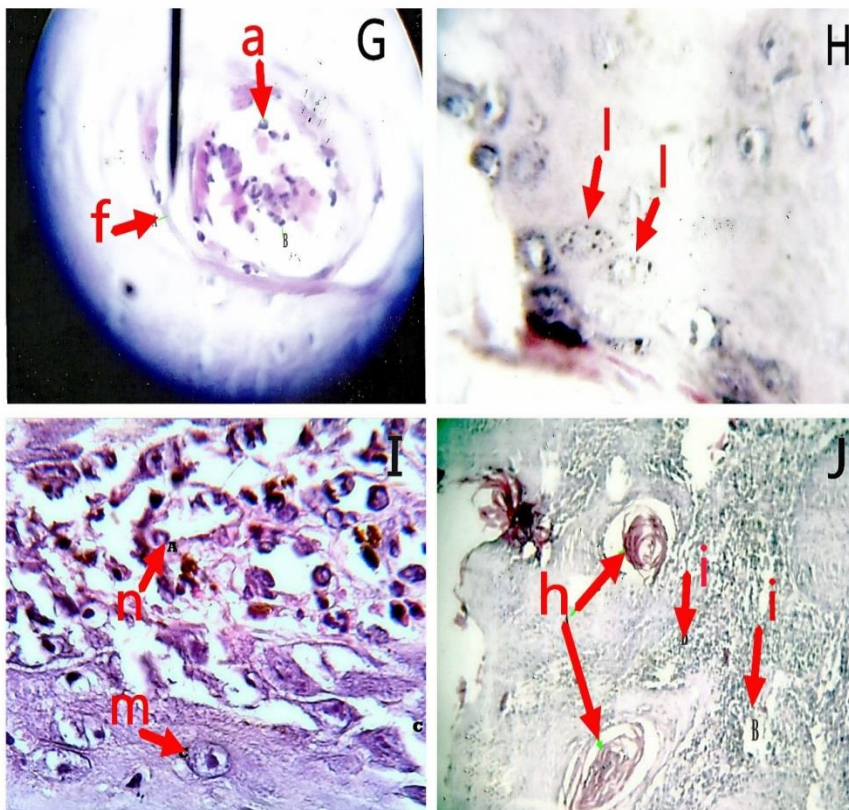
| Face |      | Face& hand |      | Hand |      | Hand & leg |     | Hand &back |     | Nose only |     | Leg |     | Neck |     | Total |   |
|------|------|------------|------|------|------|------------|-----|------------|-----|-----------|-----|-----|-----|------|-----|-------|---|
| No.  | %    | NO.        | %    | No.  | %    | No.        | %   | No.        | %   | No.       | %   | No. | %   | No.  | %   | No.   | % |
| 17   | 40.5 | 9          | 21.4 | 8    | 19.1 | 3          | 7.1 | 1          | 2.4 | 1         | 2.4 | 2   | 4.7 | 1    | 2.4 | 42    |   |

➤ **Histopathological Changes:**

Keratinized Layer and epidermal cells necrosed and not present over the ulcer lesion, only crusts of coagulated blood;

; figure (2)

Microscopically infiltration of infiltratory cells and fibrous tissue with accumulation of amastigote, Hair follicles empty without hair, As well as macrophages engulf some amastigote



**figure 2; 2-G show cross section in cutaneous ulcer circulated by fibrous tissue (f) with hyaline degeneration and presence of amastigotes stage (a) in the lesion 40 X, H.E stain. 2-H show macrophage Langerhans cells (l) engulf the parasite near ulcer, 40 X H.E stain, 2-I show some inflammatory cells such as neutrophil (n) with macrophages (m) engulf some amastigotes (C), 40X H.E, 2-J show cross-section in skin of patient with cutaneous leishmaniasis. Hair follicle (h), without hair bulb, only keratinized material in the center, accumulation of inflammatory cells (i) 10 X, H.E stain.**

➤ **Direct examination and Culture:**

**Direct smears** were done from secretions before cultivation and stained with Giemsa stain, then examined by light microscope (Figure 4-k).

Examination of specimens from 11 CL patients, the parasite (promastigote) detected in 63.4% of tested specimens, while 36.4% were negative, table (3).

The specimens **cultured on media**, the media that used in this study include NNN media, biphasic media and lock's solution (the liquid phase of biphasic media).

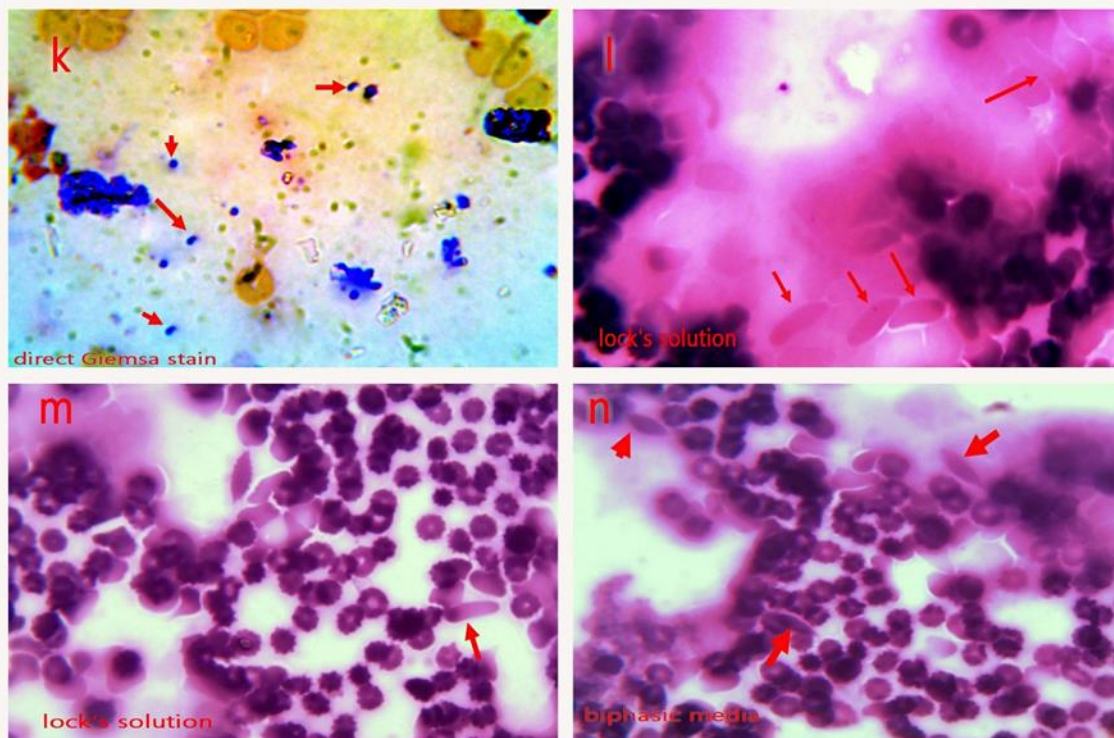
The growth of parasite of cutaneous leishmaniasis on NNN media and biphasic

media appeared after 7-9 days of inoculation and incubation at 26 c<sup>0</sup>, while cultivation on lock's solution was appeared after 9-11 days of inoculation time at 26 c<sup>0</sup>, the parasites was noticed in all media that were used in this study.

In the NNN media the growth noted in 63.4% of culture, while it is not appear in 36.6%, nevertheless the growth was positive in 72.7% of specimens cultured on both of biphasic media and lock's solution, while the results was negative in 27.3% in those two media; figures (3-l), (3-m), (3-n) and table (3).

**Table (3) show the direct and cultivation results**

| Test     | value | Direct Giemsa | Media      |                 |                 |
|----------|-------|---------------|------------|-----------------|-----------------|
|          |       |               | NNN medium | Biphasic medium | Lock's solution |
| Positive | No.   | 7             | 7          | 8               | 8               |
|          | %     | 63.4          | 63.4       | 72.7            | 72.7            |
| Negative | No.   | 4             | 4          | 3               | 3               |
|          | %     | 36.6          | 36.6       | 27.3            | 27.3            |



**Figure 3; (3-k) show direct smear for secretion collected from the lesion show amastigote of leishmania, (3-l) and (3-m) show promastigotes of Leishmania in lock's solution, (3-n) show promastigotes of Leishmania parasite on biphasic media.**

**Discussion:**

In this study most patients suffered from more than one lesion (64.3%) while, only (35.7%) were suffered from single lesion, this findings was very close with results of [14] who recorded that single lesion was found in (36%) of all cases. In addition, [12] tacitly, showed that (53.6%) of patients presented with multiple lesions. Contrary to the findings of this study [14] found that the majority of patients have only one lesion (80.6%). While, [15] found (68.4%) of CL patients were had only one lesion, in comparison, with results of [16] showed that half of the patients (50%) have one lesion. In regarding to the results of this study highest number of lesions in one CL patient, in this study, was 12 lesion and this was very close to results of [13] who found 10 lesion in one patient. Yet, [17] who recorded 41 lesions, which is the highest number of lesions among all researches.

➤ **Duration of The disease:** In the present study, almost all CL patients (97.6%) were attended to hospital after duration of disease more than one month (2–6 months). All patients responded to the antileishmanial treatment and that was confirming to the CL leishmaniasis; that is mean of duration of disease ranged from (1-6) months and this in tacit manner, so close with findings of [18] who demonstrated that The average of disease duration was  $4.8 \pm 2.4$  months. While this findings were less than the range which observed by [19] who observed duration of lesions varied from 4 weeks to 12 months. The lesions were clinically grouped into early (duration less than 2 months) and late lesions (duration more than 2 months).

➤ **The types of Lesions:** In this study, the lesions were as nodules, ulcer and crusted lesions; but diffuse lesions were not present. Typical cutaneous lesion start as small erythematous papule, which changes to plaque, or nodule, which later develop to an ulcer [21]. The types of lesion found in the study in agreement with [22] who

➤ **Number of Lesions of CL and Duration of The disease:**

demonstrated that CL patients were had nodular lesions (dry type), and ulcerative lesions (wet type). In addition, the results of this study tacitly, the same morphologically of [13] who showed that the majority of cases were presented as the wet type and the dry type (late ulcerative) form, the remaining had been presented as chronic lupoid cases and diffuse leishmaniasis was not found.

➤ **Distribution of Lesions on The body:** In the present study, most patients have lesions on more than one anatomical site of the body, most of the lesions were found on the face (61.9%) then on arms and hands (50%). The lesions were more frequently on the legs (4.7%), while other parts of the body were infected; (just one case of infection was in the trunk). Hot weather play important factor in distribution of infection because some people prefer to sleep out their rooms, so they become in direct contact with insect. In addition, most of the people (especially the children) leave their faces, hands and legs without cover, exposed to the bites of sand flies [22]. Regarding, the anatomical sites of lesions, the observations of this study so close with [22] who showed that in more than 60% of cases, the lesions were on the face. In addition, the result agree with [13] who showed that the face was the most effected part (64.3%), also [21] found that lesions much less frequently on the trunk and this agree with the present study. The same, [24] agreed with findings of this study in that the face was the most effected part in the body While the observations of [13] slightly, contrasted the results of the present study; found that the higher proportion (57%) of lesion where located on the upper limbs then faces (47%) and lower limbs (15%). [16] showed that lesion site on hand (62.8%), leg (10.2%), face and head (8.8%), hand and leg (10.2%), other sites (8%), and this observations contrast the observation of current study. [21] explained that most lesions were present on exposed area of the

body and this match the observations that noticed in present study.

#### ➤ **Microscopically Pathological Changes:**

In this study, examining for biopsies done under different light microscopic powers, different changes noted in the structure of skin and its layers. Thickening of keratinized layer and accumulation of inflammatory cells in dermis layer, fibrous tissue infiltration with hyaline degeneration and presence of amastigotes in lesions, also hair follicle without bulb noted. Macrophage engulf the parasites and inflammatory cells such as neutrophils and lymphocytes were present. These observations of current study were tacitly in line with previous studies, according to previous study conducted in the Saudi Arabia;

In study in Pakistan, [26] the results concerning the histopathological observations showed distinctive histopathological features were grouped as dermal reactions and epidermal changes. In dermal reactions features were the presence of *leishmanial tropica* (LT) bodies, plasma cells infiltrate with or without necrosis, granuloma formation, giant cells, lymphocytes, epitheloid cells and polymorphonuclear leukocytes most common epidermal changes were hyperkeratosis and acanthosis. In some cases atrophy and pseudo-carcinomatous hyperplasia was seen, this findings tacitly in agreement with current study. In study conducted on rhesus monkeys, which appears to be very similar in progression and resolution of skin to that observed in human, showed that distinct histopathological patterns were observed in different lesions at biopsy. However, there was predominance of lymphocytes and or macrophage in inflammatory infiltrate [26] and this findings were also in a tacit manner, in agreement with the present study.

#### **Cultivation The parasite:**

##### ➤ **Direct Giemsa Stain:**

In the present study, amastigotes stage of the parasite detected in (63.4%) of direct

smears stained with Giemsa stain. This result was so close to results of [18] who found that smears were positive in (63.2%) of samples and negative in (36.8%). As well as, in regard the direct examination of smears were less than results obtained from [22] who demonstrated that (73%) of the cases were positive. In comparison with the result of the current study, were higher than the results exhibited [14], who revealed amastigotes in (59.4%) of samples, as well [16] did the same, where they showed (55.5%) positive cases.

##### ➤ **Cultivation of Parasites on Media:**

There are three types of media were employed in the present study; NNN media, biphasic media and Lock's solution. Regarding the time required for getting positive culture was appeared after 7-9 days in NNN media and biphasic media, while the growth seen in lock's solution after 9-11 days of incubation at 26 C°. [19] showed that culture on NNN medium yields positive results after 1-3 week and this observation without being expressed in words, agree with the present study's findings in regard the time needed for appearing positive result. Culture usually requires 3 to 10 days to grow and sometimes more with some leishmania New World species [27]. [18] explained that cultures from (59.2%) of patients were identified as positive by the first week, culture from (10.5%) of patients were identified as positive at 14 days and 21 days no new positive results. In NNN media, the positive culture was noticed in (63.4%) of cultured tubes, while the positive culture was observed in (72.7%) in tubes cultured with biphasic media and Lock's solution. [18] showed result approximated to the present study, where cultures from (69.7%) of patients and identified as positive (the promastigotes observed) on biphasic media. [22] recorded less percent of positive cultures (43%) of cases were positive in NNN media culture. While, [13] showed much lesser percent where only (29.7%) of CL patients were positive on media. All this variations in percents of culture of researchers because any experiment deepened on its



circumstances and its environmental factors with variations of parasitic strains.

### Conclusion:

- Diagnosis of cutaneous leishmaniasis by direct smear is the fastest and easiest method for diagnosis the parasite.
- Multiple lesions were the highest percent (64.3%), while the single lesion was 35.7%.
- Most lesions were on the face and few numbers were present on the back.
- White blood cells are concentrated in the place of lesions and decreased in the peripheral blood (leukopenia).

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