Previous studies with regard to Cutaneous Leishmaniasis have come with great information. In spite of that, we still have few gaps that need to be addressed, in which this study covers through: -A cytomorphologic study of the different manifestations seen for the amastigote form in cutaneous Leishmania. -A review of the application of the microscopic method in cutaneous leishmania diagnosis. -Neutrophils role in cutaneous leishmania. -Wither the Amastigote form is the only form found in humans infected with cutaneous leishmania. -The pathological features of cutaneous leishmania. Origin and Properties of the Mononuclear Cells (with tail) seen in the Cutaneous Leishmania smear. -An approach for cutaneous leishmania treatment. This book is prepared for those in the medical field who are interested in digging through new concepts and findings in Cutaneous Leishmaniasis. The book may a breakthrough for new ideas.

Mohammed Wael Daboul - (D.D.S) Doctor in Dentistry - Damascus University 1985 - Master degree in Science /Biology - University of Bridgeport 1991. - MT(ASCP) 1992. - Laboratory Medicine Specialty Degree - Syria 1998. - An Editorial Board member(Our Dermatology On Line Journal since 2010).

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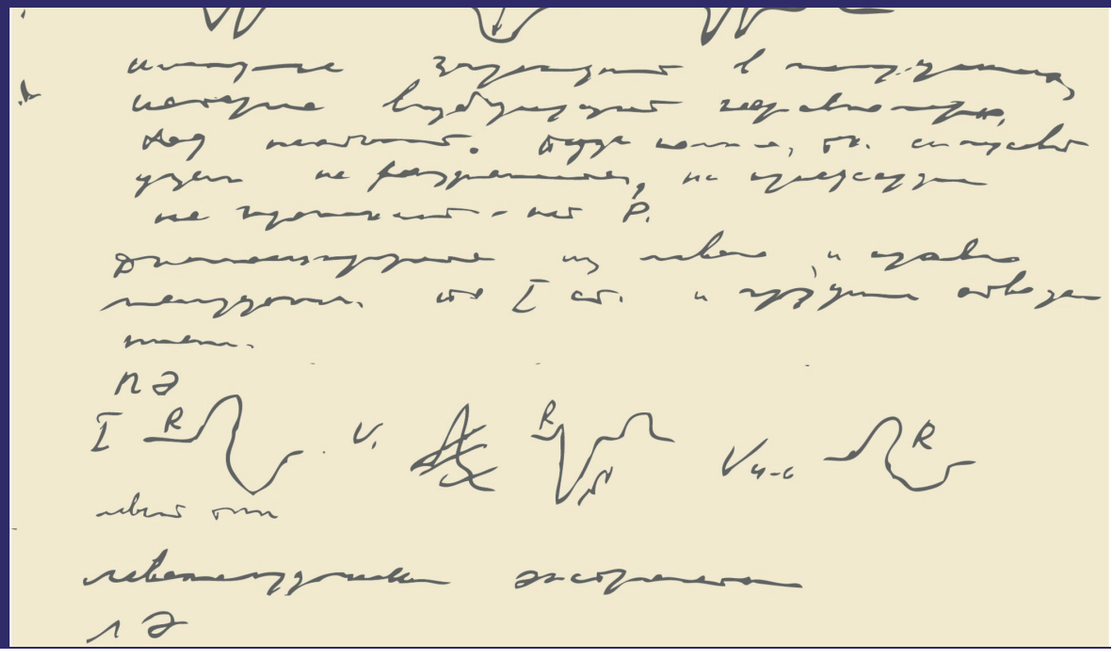
Cutaneous Leishmaniasis



Daboul

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Cutaneous Leishmaniasis. A New Concept

A THESIS TO CONFER Ph.D DEGREE IN PARASITOLOGY

Mohammed Wael Daboul,
DDS, MSc, MT(ASCP)
Laboratory medicine specialist

اللايشمانيا الجلدية في قراءة جديدة

الدكتور محمد وائل ديسر دعبول

بسم الله الرحمن الرحيم

وقل رب زدني علما

Table of Contents

Cutaneous Leishmania . A New Concept

Introduction: Cutaneous Leishmaniasis is an endemic disease, residing in the mediterenian area (1). Once in a while, new foci are discovered in close locations to the original ones (2). The disease is caused by the Leishmania parasite 1* , which is transmited by an infected sand fly 2* in its promastigote form. During its meal, the sandfly transmits the promastigote into the victom skin, where it pentrates the skin layers. There, it loses its flagella. The parasite then, is phagocytosed by the macrophages and transformes into the amastigote form. The amastigote form multiplies within the macrophage, (3) 3* multinucleated giant cells 4*, Langrhanse cells and the dendritic cells5* in the skin tissues. Residing in the macrophage, the amastigote form is considered an obligatory intracelluler parasite (5). The lesion appears in the skin as a dirty ulcer with volcanic edges (6). It expands reaching 3-4 cm in diameter 6*. Towards healing, the lesion shrinks, terminating with a small scar which lasts for life long 7*. The lesion may be manifested in one or two locations of the skin and rarely, the infection may be seen at more than two different locations in the skin of the same patient (8) 8*. With leishmania infection, the infected patient develops a permanent immunity. In Africa during epidemics, it has been recognized that some of the natives do inoculate their unexposed individuals with leishmania secretory fluid in a hidden area to gain immunity (9). Other studies expressed the possibility of the disease transmittance among infected drug users who share same needles. The disease also, can be transmitted by congenital transmission to the fetus (10). Diagnosis is done by different methods, of which, is the serologic (11), the culture methods (12), the complicated methods like (PCR) Polymerase chain reaction methods and the classical method by direct microscopic smear. The microscopic method is considered the gold stander method for leishmania diagnosis, with its limitations (14). The sensitivity of the microscopic method is considered low (less than 70%) (15) when detecting the LD (Leishmania Donavan) bodies 9* within the phagocytes, (which is the principle of the microscopic method in diagnosing

leishmania.). Previous studies expressed that, a total disappearance of the amastigote from the smear may occur during the disease course 10*. In spite of that, the disease episode continues for months later before the heal finally is achieved (16). It is worth noting that the healing is not a complete one but it is manifested with a permanent scar. The direct microscopic study divided cutaneous leishmaniasis cytomorphology into two pathological forms. The first is the moistened lesion, where macrophages are loaded with amastigotes engulfed in, besides increased number of lymphocytes and the plasma cells that are seen in the screen 11*. The second is the dry nodular type where, a granulomatous infiltrate consisting of less lymphocytes, epithelioid cells and multinucleated giant cells with hairy fibroid structures and less macrophages infected with the amastigotes are noticed (1) 12*. The studies went in deep details into many of the genetics of the different strains of cutaneous leishmania (17). The researchers studied the relationship between the parasite and the immune system including the disease initiation and progress and the development of immunity in the human host (18). The studies elaborated on the role of each of the immune cells in disease etiology and lesion progress (19), and also, the correlation between this disease and the immuno-deficiency factors whether acquired or inherited (20). The studies went through the effect of environmental changes on leishmania spreading (21), and the different methods applied to control the disease and stop its spread, in addition to the treating remedies. This in brief, is a summery of what previous studies have come with in regard to cutaneous leishmaniasis. In spite of that, we still have few gaps that need to be addressed which include:

1- Recent studies coming from Jordan and Palestine (2) revealed that new foci of leishmania are discovered in the region. In Syria, there are signs indicating that cutaneous leishmania is spreading from its base, which is Aleppo city towards the southern part of Syria. Our first aim, in this study is to follow up the spread of the epidemic in Damascus city and if possible, to identify the foci through a pioneer study that needs other detailed studies to follow up.

2- The direct microscopic smear method is considered the simplest, cheapest and the less complex one. But the sensitivity of this method is limited. It does not exceed 70%. It is now almost a century since the scientists (Leishman- Donovan) discovered this method of leishmania identification. The principle of the method is to detect the amastigote forms (LD bodies) inside the macrophages as a way to diagnose the infection. Since its discovery, no revision or modification to the method was done. It is of most important to implement studies aiming to develop this type of method to elevate its sensitivity and to increase its specificity, so it becomes the most reliable method especially, in those locations were poorest people are residing and leishmaniasis is endemic. In that way the majority of the population are benefited from such a test and positive cases, previously unreported are now to be discovered and reported with the new procedure.

3- Going through detailed microscopic studies for many cases of the disease revealed the appearance of figures not reported before. Applying those figures in the microscopic method for diagnosis of cutaneous leishmaniasis could be useful in increasing the sensitivity and specificity of the microscopic method for better diagnosis.

4- The Polymorphonuclear leucocytes role in cutaneous leishmania is still not fully understood. Most studies concentrated on the role of the different phagocytes and macrophages being the host cells for the parasite to multiply in. The studies also concentrated on the lymphocytes being the cells involved in immunity establishment by having a complex relation with the phagocytes which contribute to the disease progress and control. Those studies have neglected the neutrophils role in such a disease, which, may be of same importance of the phagocytes or the lymphocytes. The neutrophils are known as being the first line of defense in cases of acute infection, and that those cells have the most important role in the process of the foreign microorganism phagocytosis and elimination. No other cell in human body including the phagocyte can compete with the neutrophils in such a role. One of the principles of this research work is to pinpoint some of the basis of that role.

5- All the previous studies belongs to cutaneous leishmania agree on:

a- The promastigote form but not the amastigote form is the one that initiates the illness, transmits the disease and causes the infection. It multiplies in the sand fly victor.

The sand fly, when having the blood meal from the infected host, carries with it the amastigote form, which transforms into the promastigote form in the sand fly got, and there it completes its life cycle.

b- Once the promastigote form is transmitted into the human skin during the next meal, it is phagocytosed by the macrophage where, the flagella is lost and the promastigote is converted again to the amastigote form. It multiplies inside those macrophages until filling the cytoplasm.

c- The amastigote form is considered by all previous studies as an obligatory intracellular parasite that cannot survive the harsh extracellular environment. But still, and according to the previous studies, once the amastigote is released to the outer cellular fluid, it should be re- phagocytosed by an other macrophage to continue its life cycle. So it is assumed that the amastigote cannot be seen but rarely, in the extracellular fluid and in a limited number; once the phagocytes membrane is ruptured and the amastigote is released.

d- During that time, the sand fly consumes the infected blood meal from the skin lesion taking the amastigote with it to complete its life cycle in the sand fly got.

By considering that the promastigote form and not the amastigote form is the one that causes the infection, and that this form is not present in the infected human skin, then how could it be possible to explain the disease transmission among the infected drug users who share the same needles, or to explain the inoculation mechanism of the disease transmission among the natives who sake immunity, or to explain the congenital transmission of the disease to the fetus?

An other important inquiry applies questioning the sand fly role as being the only sight for the parasite to complete its life cycle by transformation into the promastigote form in the sand fly got?

If the promastigote form, contrary to what previous studies came with, is not the one that causes the infection with leishmania, then the causing factor in that case must be the amastigote form. And the question in such case should be: is the amastigote form an obligatory intracellular parasite like what previous studies told? In other words being an obligatory intracellular parasite and in order to transmit the infection, should the amastigote be carried out within its host phagocyte into the new infected position in the skin of the same patient? or could the amastigote survive in the extracellular media under certain circumstances?

e- The studies expressed that, later, during the disease process, the amastigote form and its phagocytosing cells tend to disappear from the smear without giving an explanation to the reason of the disappearance. This raises a question about the causing factor disappearance while the disease itself resumes for months to come.

f- The studies also pointed that cutaneous leishmaniasis terminates by a scar formation without a complete healing of the skin. This also raises a question; why not a complete cure as long the infecting agents (the amastigote forms) disappeared from the screen?

6- The histopathological studies showed that the disease has two main features: the wet ulcerative lesions where mainly infected macrophages and abundant of lymphocytes and plasma cells are seen, and the dry nodular lesions where there was a tendency to form granuloma with epithelioid cells and less lymphocytes and plasma cells and abundance of hairy fibroid structures. Again, is that considered enough to express all the pathological features of cutaneous leishmaniasis during the disease process toward healing?

7- the pathological studies revealed the importance of the role the lymphocytes and plasma cells play in cutaneous leishmaniasis, without giving a detailed cytomorphological study of those strains of lymphocytes, which are seen under the microscope and participate in the disease process.

8- It is important after that broad study for cutaneous leishmania to direct our effort looking for some kind of remedies which can be effective in curing or at least reliving the disease. Most traditional treatment are still not well effective. Finding a

new substance which is more effective is the goal of the last pioneer study done in this thesis.

The purpose of this work is to provide some answers for those queries through the following titles which go further deep into the details:

1- A study of 16 cases of cutaneous Leishmania in Damascus.
2- A cytomorphologic study of the different manifestations seen for the amastigote form in cutaneous Leishmania.
3- Application of the microscopic method in cutaneous leishmania diagnosis.
4- Neutrophils role in cutaneous leishmania.
5- Is the Amastigote Form the Only Form Found in Humans Infected With Cutaneous Leishmania?
6- The pathological features of cutaneous leishmania.
7- The Origin and Properties of the Mononuclear Cells (with tail) seen in the Cutaneous Leishmania smear.
8- Toward an approach for cutaneous leishmania treatment.

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Note:

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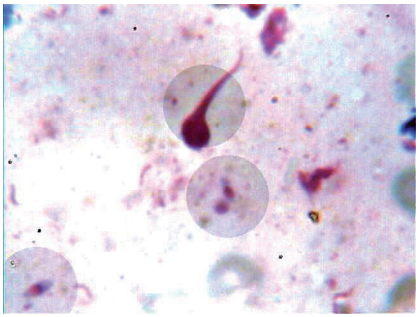
Cutaneous leishmania. A New Study



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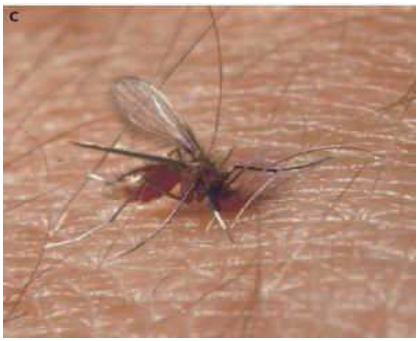

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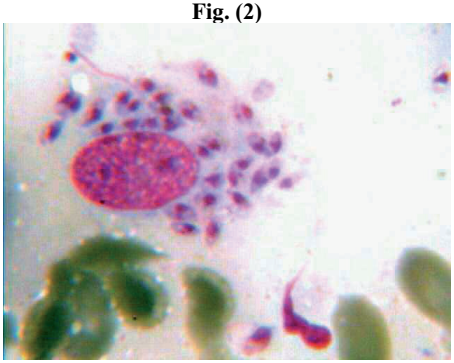

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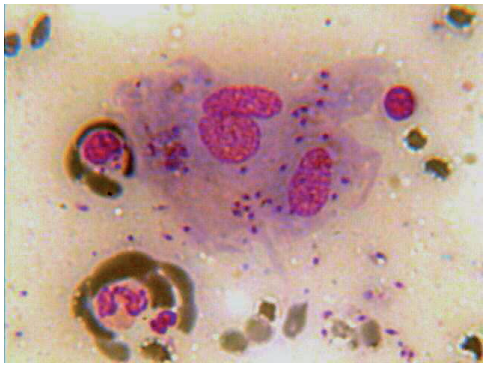

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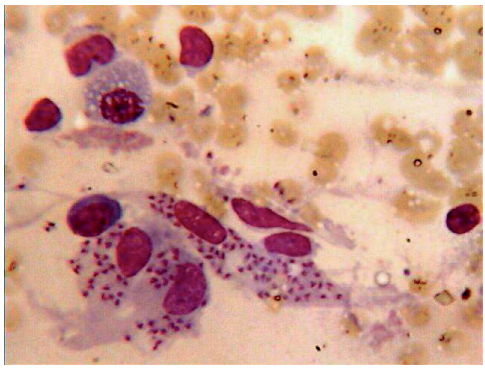

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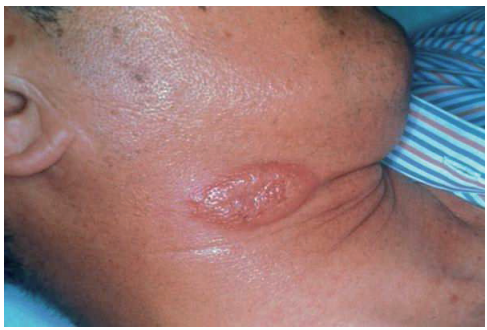

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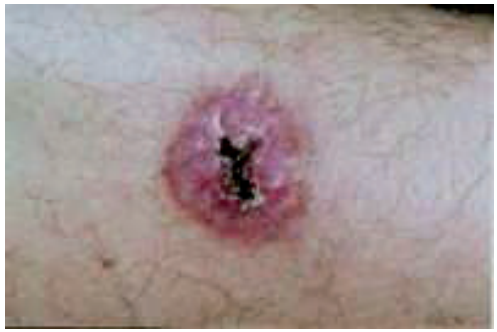

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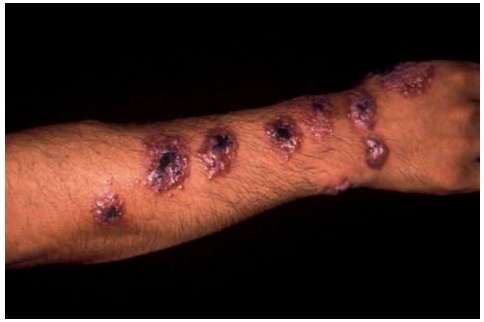

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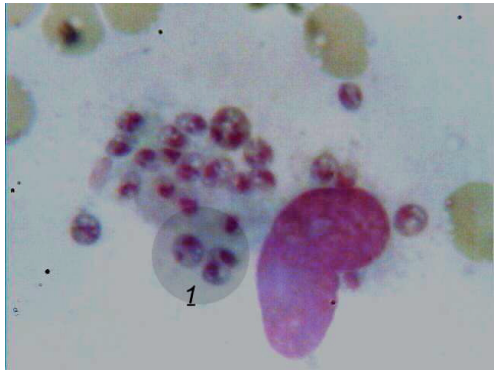

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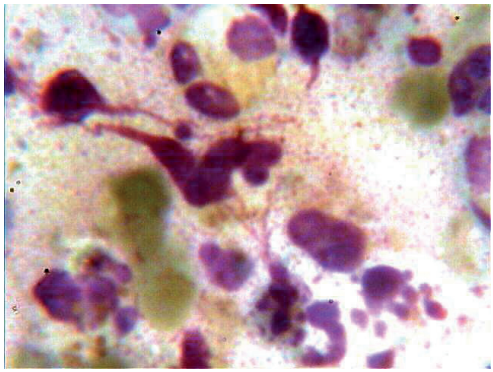

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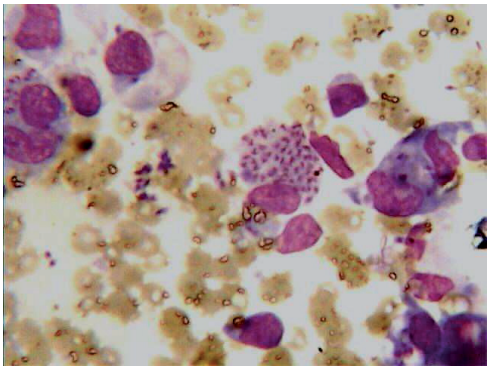

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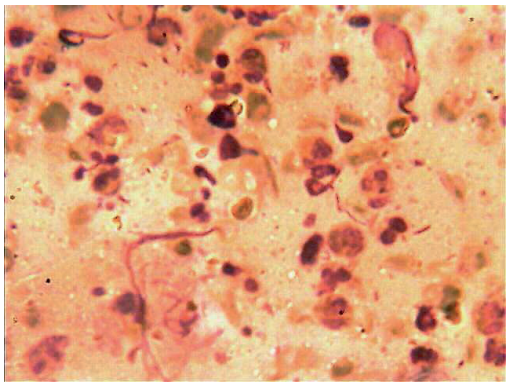

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1- A study of 16 cases of cutaneous Leishmania in Damascus

Abstract:

Cutaneous leishmaniasis is endemic in the Mediterranean area. Recent studies coming from Jordan and north of Palestine (5) tell that Several new foci are reported for the first time indicating the spread of the disease in the region. Our follow up Of leishmania spread in Syria lead us to the discovery of new foci in Damascus city. On performing our microscopic examination for the 16 referred specimen, very interesting discoveries were noticed. . Contrary to Previous literature in which, all agreed on the fact that the promastigote form is only found in the victor (Sand fly), We were able to identify the flagellate (promastigote or Trypomastigote like parasite form) in side the tissues taken from the lesion of human skin. Not only that but we found that this flagellate, at a later stage of the disease, is producing fiber like structures. That in itself explains the fibrous nature of the inflammatory reaction at the disease sight.

Introduction:

It is well documented that, Mediterranean area is one of the endemic areas for cutaneous Leishmania. Aleppo city was historically known as a focal area for this disease. Many names were given to the cutaneous lesion in this part of the world like (the stick of Aleppo), (Oriental sore), (Yearly sore)(8) Till the mid 80s of last century, most cases being acquired in Syria were located in Aleppo city and the surrounding suburban area. Since then, new cases have been discovered towards the southern of Aleppo. And since the year 2000, we came to observe cases of cutaneous Leishmania in Damascus city and the urban area. We have received in our laboratory since 2004 until the time of this paper, 16 clinically diagnosed cutaneous Leishmania cases sent by dermatology consultants for laboratory diagnosis.

Cutaneous Leishmania literature review:

Cutaneous leishmaniasis (CL) is endemic in over 70 countries. The yearly incidence is estimated at (1, 500, 000) cases. CL can be divided into *Old World* and *New World* forms. Old World CL (also known as Aleppo, Baghdad, or Delhi boil; Kandahar, Lahore, or Oriental sore; and Biskra button) is found in widely scattered parts of Asia, Africa, and Europe and in much of the Middle East, especially in the Jordan Valley, Sinai Peninsula, Iran, Iraq, and eastern Saudi Arabia (1). In foci where man is believed to be the sole reservoir (anthroponotic foci), epidemics are linked to human migrations from rural to poor suburban areas; in zoonotic foci, where mammals are the reservoirs, epidemics are related to environmental changes and movement of non-immune people to rural areas (3).

Most infections exist as zoonoses amongst wild animals, such as rodents and dogs, and are most prevalent in rural or forest areas. Whilst man is usually an incidental host, such infections are by no means uncommon – in endemic areas up to 9% of the healthy population may have positive leishmania skin test - indicative of an earlier, often a symptomatic, infection (4).

Over 90%of the Cutaneous leishmaniasis occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil and Peru, However, CL often occurs in specific pockets – not only of place but also in time – for example Delhi boil affected 40,000 people in the early 1940s but is rarely seen in Delhi today (4)**.** The geographical distribution of CL is mainly determined by the sand fly vectors (Phlebotomus sp and Lutzomyia sp). They live in dark, damp places, and are relatively weak flyers, with a range of only 50 meters from their breeding site. Unlike mosquitoes, they fly silently and their small size (2-3mm long) allows them to penetrate mosquito nets. They are most active in the evening and at night .

The parasite exists in 2 forms, the amastigote form and the promastigote form. The amastigote form occurs in humans, whereas the promastigote form occurs in the sand fly and in artificial culture.

Amastigotes: are spherical in shape, only about 2.5 to 5 µm in diameter, and are contained within a parasitophagus vacuole within a macrophage. There is a

prominent nucleus and kinetoplast, and the cytoplasm is vacuolated and contains lysosomes. The outer membrane has a polysaccharide component but there is no surface coat.

Promastigote: The promastigote is a similar in structure, apart from the prominent flagella. The surface membrane has binding site molecules such as glycoproteins, and manose receptors have also been detected. These are important in the uptake of the promastigotes by the macrophages. Antibodies in the host serum bind to the promastigotes and facilitate uptake and entry into the macrophage. The macrophages having Fc receptors on their surface. No morphological differences can be seen between species. When seen in a stained blood smear the amastigotes within the macrophages are referred to as LD bodies (Leishman-Donovan bodies) (7). In all forms of leishmaniasis the presence of amastigotes within the cells of the mononuclear phagocytic system remains the hallmark of the disease, although at times they may be difficult to detect (4).

Only the female sand fly transmits the protozoan, infecting itself with the *Leishmania* parasites contained in the blood it sucks from its human or mammalian host. During a period of 4-25 days, the parasite continues its development inside the sand fly, where it undergoes major transformation into the promastigote form. A large number of flagellate forms (promastigotes) are produced by binary fission. Multiplication proceeds in the mid gut of the sand fly, and the flagellates tend to migrate to the pharynx and buccal cavity of the sand fly. A heavy pharyngeal infection is observed between the sixth and ninth day of an infected blood meal. A bite during this period results in the spread of leishmaniasis (2).

Following the bite, some of the flagellates entering the circulation are destroyed while others enter the cells of the reticuloendothelial system. Here they change into the amastigote form. The amastigote forms also multiply by binary fission, with the multiplication continuing until the host cell is packed with the parasites and ruptures, liberating the Amastigotes into circulation. The free Amastigotes then invade fresh cells, thus repeating the cycle and, in the process, infecting the entire

reticuloendothelial system. Some of the free Amastigotes are drawn by the sand fly during its blood meal, thus completing the cycle.

Dermotrophic species, such as *L. major*, usually remain close to the inoculation site, causing cutaneous disease. Any spread of dermotrophic species tends to be late, and only to adjacent skin (producing satellite lesions). (4).

Microscopically:

In early cutaneous lesions, the dermal infiltrate is mainly made up of macrophages filled with amastigotes. There are relatively few lymphocytes and plasma cells. As the lesion develops more lymphocytes and plasma cells appear and the superficial dermis becomes more edemic. The overlying epidermis becomes hyperkeratotic and subsequently breaks down to form an ulcer covered with a coagulum of hyperkeratotic debris, dried exudate, dead cells and a mixture of live and dead organisms. Over the following months, there is a gradual decrease in the number of amastigotes and macrophages, leaving a granulomatous infiltrate consisting of lymphocytes, epithelioid cells and multinucleate giant cells. At this stage it may be difficult or impossible to detect organisms in H&E, or Giemsa, stained sections. Most patients have 1 or 2 lesions, usually on exposed sites, varying in size from 0.5 to 3 cm in diameter.

Old World CL is caused by several Leishmania species, including Leishmania major, *Leishmania tropica*, and *Leishmania infantum.)* . The progression of the disease is similar for these species. The first sign is a small erythematous papule that may appear immediately following a bite from a sand fly but usually appears 2 to 4 weeks later. The papule slowly enlarges over a period of several weeks and assumes a more dusky violaceous hue. The lesion eventually becomes crusted in the center. When the crust is removed, there is a shallow ulcer, often with a raised and indurated border. The ulcer is typically painless. After approximately2 months, the peripheral spreading stops and the lesion gradually tends to heal leaving eventually a permanent scar.

New studies coming from Jordan and north of Palestine indicate that several new foci are reported for the first time indicating the spread of the disease (5).

Purpose of the study:

1- The discovery of new foci in the surrounding states requires a follow up of the spread of cutaneous leishmania inside the country (Syria) to be implemented.

2- Most of the studies about leishmania express that there are two types for the parasite, the intracellular type (Amastigote) and the extra cellular one (Promastigote). And according to most papers, the amastigote type is the one found inside the macrophage in the smear taken from the lesion in human, where as the promastigote form is the one found principally in the Sand fly.

Depending on that, it is assumed that the promastigote form can not be recovered in any smear taken from the exposed area of human skin, because once the promastigote inters the skin, it converts into the amastigote form which is a compelled intracellular form found inside the macrophage. That is why the, microscopic method for cutaneous leishmania diagnosis after staining with different stains (Giemsa, Wright, H&E) is limited to the detection of the amastigote forms inside the phagocytes.

In this study we test the above mentioned hypotheses whether it true that the promastigote is absent in the specimen taken from the lesion.

Material and methods:

Samples: samples were collected from the 16 patients referred to the laboratory in the form of microscopic slides from the lesion area of the skin. Two slides were prepared from each lesion, and if in case there was more than two exposed areas

in the same patient, we chose the two more edematic lesions and took couple of slides from each one.

Specimen are collected as follows:

1- Slit skin smears: We cleaned the area with 0.9% saline. Squeeze the edge of the lesion between thumb and forefinger and made shallow 1 mm slits through skin to dermis with a scalpel then scrape the edges to make slide smears, then made smears as thin as possible. Air dry, fix in methanol, and stained with Wright stain.

2- Dermal scrapings: We scraped dermis with scalpel along the necrotic lip with a scalpel, obtaining as much tissue as possible, then making thin smears on slide. Air-dried, fix in methanol, and stained with Wright stain.

3- Microscopic photos are taken for each case and numerated as such: Case one (1p), case one photo one (1p1), case 2 (2p), till (16P). A comparison is made between the microscopic features of the parasite in our photos and some parasite reference photos numerated e.g. (1,2,3) without the letter (P).

Results:

Table 1 shows the geographic source of the samples from Damascus and the suburban area surrounding it.

Location	Maza	Kadm	Midan	Mokhaiam & Sayeda Zainab	Daryya	Moaddamia & Suhnaya	Kodsya &Barada valley	Horan	Masaken Barza
# of cases	2	1	1	2	2	2	4	1	1

The results in table (1) indicate that different locations in Damascus city became foci for such an epidemic and it seems that Kodsya area is the most hit by this parasite. (4 cases).

Table (2) classifies the cases in accordance with the number of lesions clinically seen in each patient.

# of lesions	1	2	3	4	9
# of cases	6	5	2	2	1

From table two we find that most of the infected cases had only 1 or 2 skin lesions while only one case had 9 different lesions at different locations.

Table 3 shows the amastigote form appearance in the intra or extracellular position:

Amastigote form	located in the intracellular space alone	Located in the extracellular space alone	Appearance of the amastigote with no promastigote like form	Amastigote appearance in or out side the Macrophage
# of cases	0/16	1/16	4/16	8/16
Percentage %	0	6.25	25	50

Table three reveals that the amastigote form can be recovered only in 50% of the disease cases and is missed by the other 50%. The amastigote is recovered alone with no other presumed forms in only 25% of the cases referred. From the table also, the amastigote form can not be recovered only intracellularely (0 out of 16) while in one case the amastigote is recovered in the extracellular fluid without being simultaneously found in the intracellular area.

Table (4) The appearance of the (Promastigote) forms in the microscopic smears of the patients.

Promastigote like	Present alone	Available in	Associated with the

forms)	in the smear	the smear	amastigote form
# of cases	0/16	7/16	2/16
Percentage	0	43.75	12.5

Table 4 shows the presence of the presumed promastigote like form in 43.75% of the cases and is associated in 12.5% with the amastigote form.

Table (5) the Trypomastigote like forms presence in the smears from patients infected with cutaneous Leishmania.

Trypomastigote like forms	Seen in the smear	Associated with the amastigote form	Associated with promastigote like form	Present alone in the smear (with no other forms)
# of cases found	12/16	4/16	7/16	3/16
Percentage	75	25	43.75	18.75

From the figures in table 5, the trypomastigote like form is present in75% of the cases referred. It is associated with the amastigote form in 25% of the cases and with the promastigote form in 43.75% of the cases, and found alone in 18.75% of the cases referred.

Table (6) The percentage of each form of the parasite present in the smears:

Forms of leishmania parasite	Trypomastigote like form	Amastigote form	Promastigote like form
Percentage	75%	50%	43.75%

Discussion:

From table 1. We find that cutaneous Leishmania is spreading through the western and the southern parts of Damascus city. Most cases are found in Kodsaya and Barada Valley and that may be due to elevated humidity, which gives the sand fly better environmental living conditions. Additionally, One case was found in Horan valley, which is located far in southern part of Syria. That means that leishmania spread is continues towards the south, or there may be some foci present in the southern part of Syria which are connected to the foci discovered in Erbed (Jordan) and north of Palestine (5). Our research work is limited to both clinical screening and microscopic testing of the cases for the diagnosis and detection of the disease. It is of great importance to follow up this work with other laboratory procedures in order to identify the parasitic strains discovered, which is beyond the scope of this study.

From table 2. it is clear that, in most of the time, the number of exposed areas found in each patient is one or two, but only in one case, it was noticeable that 9 positions were infected. That was the case of a child presented to us with lesions in both arms and face, neck, shoulder, and the head of foot. Some locations were inflamed and moist; others were dry and close to heal, which indicate that some of the lesions were infected earlier than others.

Table 3 shows that in 25% of the cases where the amastigote appeared in the smear, was associated with either the promastigote like or the Trypomastigote like organisms seen in the same smear.

Table (4) and the associated photos (see Photos attached) show the appearance of extra cellular organisms having flagella extending out and a clear nucleus in side. We compared the microscopic photos of these organisms found, with reference photos taken by scanning electron microscope and normal microscope (see photos), and came to the conclusion, that the organisms in both the photos were almost identical from the cytomorphologic point of view. (See the documenting photos for the different parasites mentioned in tables (3,4,5,6) attached at the chapter end).

The promastigotes like organisms were detected in 43.75% of the smears of the 16 patients. They were associated with the amastigote type in the same smear in 25% of

the cases. The promastigote like form was associated with an other flagellate form discovered which is (Trypomastigote like form, see Medical parasitology edition 3) in 44% of the cases(6).

Table (5) and the photos attached show the appearance of microorganisms in the extra cellular fluid look like Trypomastigote (6). Those Trypomastigote like organisms were found in 75% of the studied cases of leishmania. That is even 25% more than the amastigote appearance in the smears. The appearance of the Trypomastigote like form without the amastigote appearance was in 50% of the cases in the smears. That means that if we combined both the amastigote and Trypomastigote in our microscopic method of the parasite detection in the smear, the sensitivity of the microscopic method to detect the leishmania will rise into 100% compared with the clinical diagnosis.

Referring into the literature review, all agree that two forms of the parasite are present, the intracellular amastigote form found in the vertebrate host, and the promastigote form predominately found in the insect vector. Amastigotes*:* are spherical in shape, only about 2.5 to 5 µm in diameter, and are contained within a parasitophagus vacuole within a macrophage …When the parasite is seen in the smear, the amastigote form is the one found inside the macrophage and called LD bodies.

It is clear through what was mentioned and through the photos attached (and about other 200 photos studied, from all the 16 specimen received and reviewed from patients having the parasitic disease) that the results this study came with, are in contradiction with the information in the articles reviewed. The photos revealed the following:

1- The appearance of the amastigote in a noticeable quantity spreading in and out of the phagocytes in the extracellular fluid means that such form is not only an obligatory intracellular parasite, but it multiplies inside the macrophage, and once filling the cytoplasm of the macrophage, the macrophage membrane ruptures and the

amastigote takes its way to the outer cellular media where it stays there for a considerable time.

2- Previous articles and research papers did not give a clear explanation in regard to the mechanism of the disease process. The microscopic studies do not explain where the amastigote organisms disappeared from the smear, and accordingly, they cannot provide an explain for the cause of and the principle of the disease progress and exacerbation in the skin lesion during the upcoming months of the disease progress, though, the causing organisms presumably had disappeared. It is quite illogical to watch the disappearance of all the macrophages carrying the amastigotes and the amastigotes themselves from the lesion and watch the disease in progress for many months to come. The scientific answer for that dilemma is an absolute presence of the causing factor in the lesion site. Previous researches acknowledged the disappearance of such causing factor because they considered the amastigote as being the only parasite form to appear in the lesion in human. The noticeable existence of amastigotes in the extra-cellular fluid after being released from the macrophage with this huge number, and then their disappearance, together with the infected macrophages later, from the microscopic smears while the disease persists, directs us to the suggestion that there must be some cytomorphological and physiological changes which emerged in the amastigote forms released, in order to adopt the extracellular media. These changes are manifested with the gradual conversion of the amastigotes to promastigote or trypomastigote like forms. (Photos attached clearly indicate the flagellate existence in the extracellular fluid at different sizes according to their growth rates). Some look like (candle flame) with 3 microns in length. Others are more developed where the head only is about 7 microns in length.

An important phenomena which was not clearly elucidated in the previous literature during the disease progress of cutaneous Leishmania, is the reason behind the continual fibrous inflammatory reaction without healing, with the absence of the

presumed cause (which is the amastigote) from the lesion. The explanation is that, either the causing organism or one of its components must be present in the lesion that stimulates that inflammatory reaction. Most studied cases of leishmania we had, were microscopically free of secondary infections, whether microbial, or fungal, and usually antimicrobial or antifungal treatment was administered to the patient with no cure before his referral to us. That will reject any secondary factors as a cause, and consider an inner leishmania factor as the real cause in such fibrous reaction. This reaction may be compared from the histological point of view, especially with the lymphocyte and plasma cell presence in the lesion, with the immunologic reaction against a foreign implant.

Towards the disease end, the microscopic photos, reveal that these promastigote or trypomastigotes like organisms, during the disease progress continue producing the flagella where it becomes more thickened and elongated matching in shape the fibers produced by the fibroblasts. It is quite possible that such fibroid substances may have concealed the parasite from being identified by the microscope in the infected tissues at that point. Through the microscopic follow up, the attached photos reveal that what were suspected as fibroblasts and fibrocytes are in fact those more grown promastigotes or trypomastigotes like organisms producing the fiber like materials. It seems that those remaining foreign fibers at the last stage towards healing, will explain the incomplete cure of the skin in the area with a permanent scar formation.

The discovery of the promastigote or Trypomastigote like parasite in human in the extra cellular fluid makes mankind as a host for this parasite where it is able to complete all its life cycle. The victor (sand fly) job by the fact presented will be just transforming of the disease and the promastigotes multiplication. In addition to that, the presence of the flagellate in human, is a way to explain the self-reinfection with the parasite in an other location of the skin like the case presented with multiple lesions in different skin locations. It is untrue that all the infected lesions in that case are due to the insect bite because each lesion was at a different progression stage.

That makes the explanation is the reinfection by self transmittance through etching the infected area and then re-etching and inserting the carried promastigote like form into the new location. This explanation could be expanded to the native Africans who inoculated their uninfected people to gain immunity, or the cases of infected drug users who share same needles

Last:

Damascus city and the suburban area around it have become a new focus and an inhabitance for cutaneous leishmania. The parasite is heading to the southern part of Syria from both north and south as, new foci are discovered in Jordan and north Palestine. Follow up should be attained, and more research work must be done to identify the new parasite strains discovered.

This study, which has proved that the promastigote like form of leishmania must be present in the extracellular fluid of mankind who is infected with the parasite, requires follow-up detailed studies to support it and to achieve a re-understanding of the life cycle of such a parasite in accordance with the findings the research results will introduce.

Upon the completion of work, an exudate was received from the submandibuler area requesting an Acid Fast bacillus detection. After the stain with (Ziehl Neelsen stain), no acid-fast bacillus was seen but the slide showed a clear match of its component with the organisms seen in the leishmania cases. By following the case history of the patient, we found that the patient resides in Almohajereen district of Damascus and that the lesion shape was clinically matching cutaneous leishmania. Microscopic photos taken for both the Ziehl Neelsen and Wright stain are attached.

A study of 16 cases of cutaneous Leishmania in Damascus

In summary:

The purpose of our study was to follow up the spread of Leishmania epidemic in Syria and to detect the presence of focal locations in Damascus city and the urban areas around it. It has been confirmed that the parasite became an inhabitant in Damascus city and around it and its spread, is in progress toward the southern part of Syria. There is a clear evidence that cutaneous leishmaniasis infection is spreading fast among the population in Damascus City, which should bring attention that the disease might turn into an epidemic in the near future

During our investigation about the parasite by the microscopic method using the Wright stain procedure, we found that 50% of the cases clinically diagnosed as cutaneous leishmaniasis, could not be diagnosed by microscopic examination, through trying to detect only the presence of the amastigote form of the parasite, whether inside the macrophage or out side in the extra cellular media. This is the principle of the traditional method being used to detect the parasite and confirm the diagnosis of the lesion. That means that, 50% of the clinically diagnosed cases as positive for cutaneous leishmaniasis and in according to the microscopic procedure mentioned above, will be missed as (Falls negative). In addition to that, most researches having considered, as a scientific fact, that only the amastigote form is an obligatory intracellular parasite seen in infected human but not the promastigote form. the promastigote form is found only in the Sand fly. This fact makes any explanation of the disease mechanism and its progress mysterious and unclear, where a progressive inflammatory disease is present with the disappearance of the causing organism at some stages of the disease. Additionally: most researches did not give a clear explanation in regard with the fibrous inflammatory reaction of the skin that lasts for months after the amastigote and its phagocyte disappearance from lesion. Further, no clear interpretation to the life long scar lasting is available although all patients become disease free after one year.

All that meant to suggest that the causing organism or one of its component must be present in the lesion inside the inflammatory cutaneous layers, may be in a different form than the amastigote, which can adapt the extracellular life.

Going back into over thousand photos from 32 slides taken from 16 patients, we could identify at 75% of the cases investigated, the presence of microorganisms in the extra cellular fluid which do not match any of the known blood cells or blood components. They are as small as 3 micron in diameter when associated with the amastigote form in the same slide, till 8 micron for their body alone after growth, with flagella like organ attached to it. The head is shaped at different shapes, some like candle flame or rounded heads or flowing spindle shape continuing with the flagella. When we compared these forms with reference photos taken by means of general microscope or by scanning electron microscope or normal electron microscope, the organisms discovered where exactly matching the promastigote seen in those photos. following up these discovered forms of promastigote or Trypomastigote like organisms during the disease course, we found that towards the end of the disease, the flagella part of the parasite tends to become more thick and it turns into a fiber like structure which may hide the parasite itself from being discovered. The ending fiber like structure will explain clearly the end point of the cutaneous leishmaniasis disease episode by forming the fibrous inflammatory reaction and that is the cause of healing with a permanent scar. That means that, the human body and tissues have recognized the fiber like structure produced the parasite in the lesion sight as a foreign body and the immune system produced its defense against it which is manifested by

the lymphocytes and the plasma cells reaction and then, towards the end caused the formation of that permanent scar in the sight after the heal.

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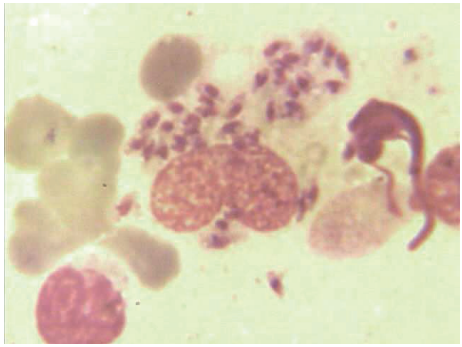
8- Fawzy Hassany Medical Parasitology second addition (Syria) 1993-1994

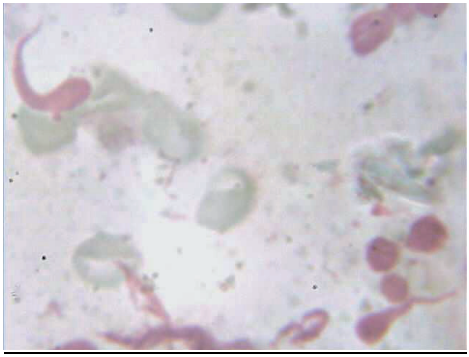
Images of study (1)

A study of 16 cases of cutaneous Leishmania in Damascus

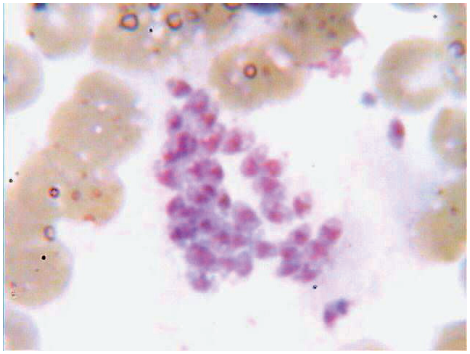
(Sand Fly) *1



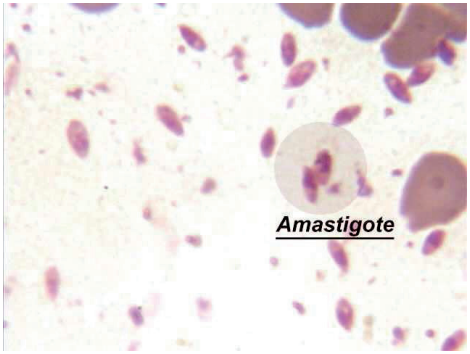
(LD bodies Amastigotes) X 1000 *2



(Promastigotes) X 1000 *3



(Extra cellular amastigotes) X 1000



(Extra cellular amastigotes) X 1000

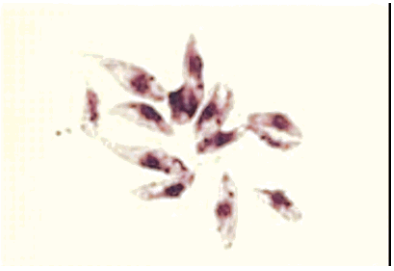
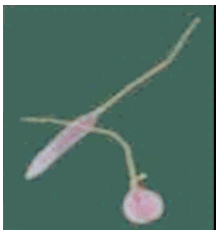


Photo (1) Reference for the promastigote L. microscope



(2) Reference. Promastigote Photo with SE microscope

(3) Reference. Promastigotes Photo with SE microscope

(4) Reference. Promastigotes Photo with SE microscope

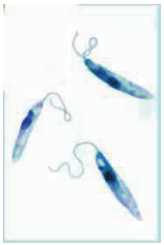
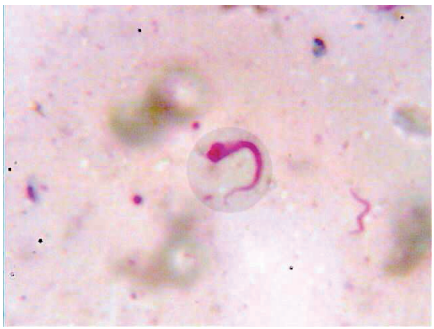
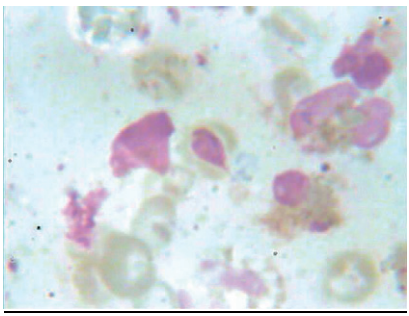


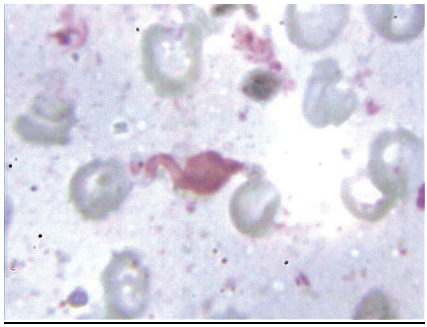
Photo (5) Reference for the promastigotes L. microscope

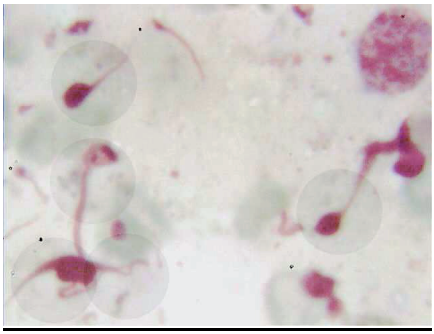
The following images are for the promastigotes taken during our study

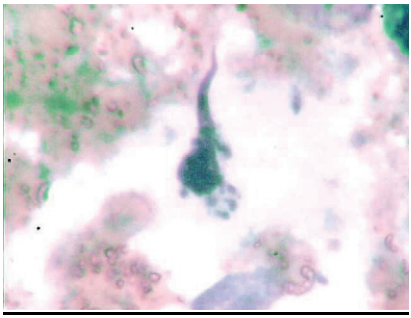
Promastigotes and Trypomastigotes



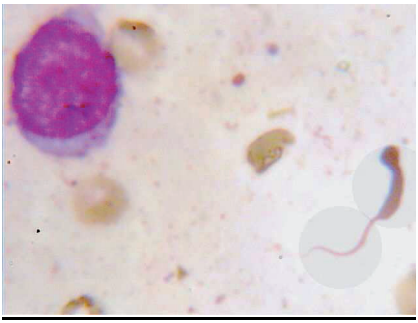


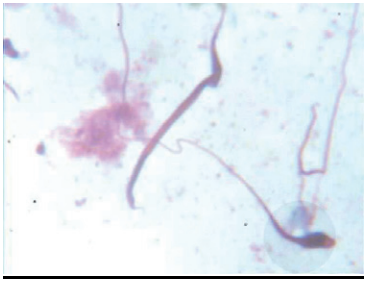


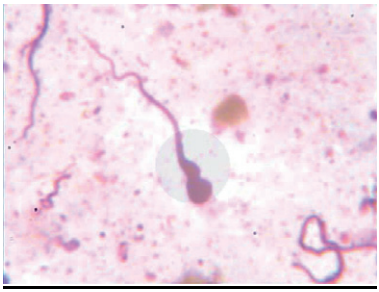




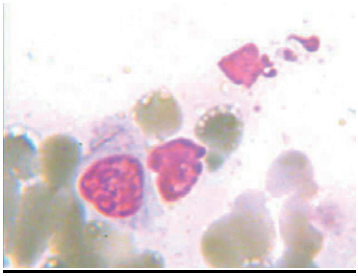
Conversion of the promastigotes into fiber producing promastigotes:

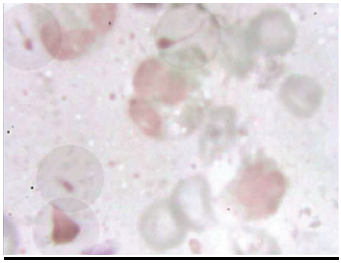


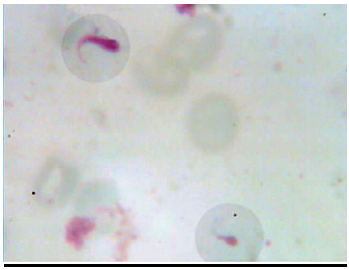




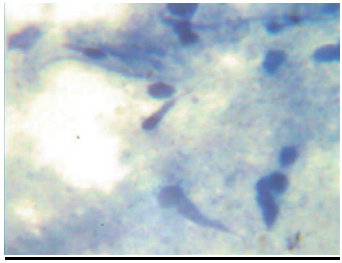
Conversion of the candle flame like parasite into fully developed promastigote:

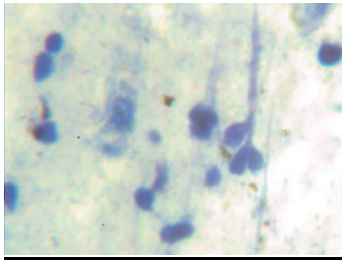


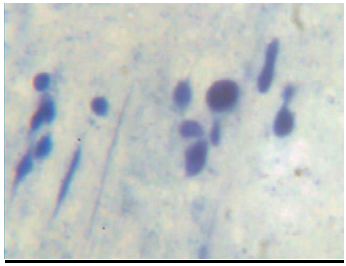




Promastigotes with (Ziehl Neelsen stain)







2 -A cytomorphologic study of the different manifestations seen for the amastigote form in cutaneous Leishmania

Abstract:

Introduction and objectives: Although the specificity of the microscopic method by identifying the LD bodies within the infected macrophage from the skin lesion was very high, its sensitivity (30%) remains limited. The purpose of our study is to work on elevating the sensitivity of the microscopic method by not only identifying the LD bodies within the macrophage, but by checking the amastigote appearance in its different cytomorphologies in both the intra cellular macrophage and the extra cellular fluid of the infected area.

Materials and methods: 37 cases of clinically diagnosed cutaneous leishmaniasis were studied after staining the microscopic slides taken from the presumed infected area with Wright stain.

The study was divided into two parts:

Part one: Defining the different microscopic cytomorphologies of the amastigote forms and later, classifying the amastigote forms according to their common microscopic features into specific classification. Part two: Studying and calculating the appearance of the amastigote forms in each and every case in either the intra or extra cellular fluid and comparing them.

Results: the oval in shape and spindle in shape amastigote types are found in the same rate (43% of the cases referred). Where as the round shape amastigotes are present only in 5 % of the cases referred, and the overall amastigote appearance of any type was at about 49% of all the 37 cases referred to us. 49% of the cases referred show the appearance of a smooth and spreading type of chromatin inside the amastigotes, and other 49% show the amastigotes with a condensed and or polarized chromatin in one pole of the amastigote. A third type of the amastigote is seen with only condensed chromatin and total absence of the cytoplasm (Candle flame-shape). Overall, the appearance of the amastigote of any of the mentioned above features constitutes 49% of the cases referred to us. The amastigote is seen at 41% of the

cases referred inside the macrophage, and 46% of the cases referred are having the amastigote present in the outer cellular compartment. In 38% of the cases referred, the amastigote is shown sharing both the intra and extra cellular compartments, while an overall appearance of the amastigote in the cases referred was 49%.

In conclusion: Our data showed that an additional 20% sensitivity and more specificity is gained by the microscopic method making the method more competitive with other procedures and to be considered as the gold standard method for such leishmaniasis diagnosis.

Introduction:

Since 1996, we followed up the microscopic method for cutaneous leishmania identification in both Ministry of health hospitals at Saudi Arabia and later here in Syria. Although the specificity of the above mentioned method by identifying the LD bodies within the infected macrophage from the skin lesion, was very high, its sensitivity (30%)(1) remains limited.

The purpose of our study is to add more impulse to the microscopic method by not only identifying the LD bodies within the macrophage, but by checking the amastigote appearance in its different cytomorphologies in both the intra cellular macrophage and the extra cellular fluid of the infected area.

Literature review:

Leishmaniasis is an infection caused by a protozoan parasite belonging to genus Leishmania. The majority of infections with Leishmania are of the cutaneous type. Other types include visceral, and mucocutaneous Leishmania (2).

Cases of Leishmaniasis have been reported on every continent except for Australia.

The disease is endemic in areas in the tropics and subtropics such as Central and South

America, southern Europe, Asia, the Middle East, and Africa. It is estimated that approximately

10% of the world's population or approximately 350 million people are at risk of contracting this

Infection. Worldwide there are 2 million new cases of Leishmaniasis reported annually.

4related to the

AIDS epidemic, global warming, and other environmental factors (3).

Two forms of the parasite are present, the promastigote form found in the insect vector and the intracellular amastigote form found in the vertebrate host.

Amastigotes: are spherical in shape, only about 2.5 to 5 µm in diameter, and are contained within a parasitophagus vacuole within a macrophage. When seen in a stained blood smear the amastigotes within the macrophages are referred to as LD bodies (Leishman-Donovan bodies).

Considering cutaneous leishmania, in peripheral blood smear the amastigotes are seen inside the circulating monocytes and neutrophils. Histological examination of the lesion revealed extensive subcutaneous lymphohistiocytic inflammation with clusters of amastigote within histiocytes.

Definite diagnosis of cutaneous leishmaniasis is based on the isolation of the causative organism by smear and culture or its identification in tissue section (1).

Due to lack of sensitivity of such traditional diagnostic tests, (4,5), some researchers have adopted different techniques to enhance finding the L.D bodies by the traditional microscopic smear method, an example for that: A-Thick film, by producing a single straight leukocyte edge when making a peripheral smear. B- By centrifuging citrated blood and withdrawing the sediment, which then is smeared, dried, and stained (6).

Going through the literature above, all the studies assume the presence of the amastigotes in the intra cellular space of the macrophage, while our approach to the smear method presumes the amastigote presence not only in intra cellular macrophage but in the extra cellular fluid as well.

Materials and methods:

37 cases of clinically diagnosed cutaneous Leishmaniasis were sent by consultant dermatologists to the laboratory for confirmation. All the cases obtained were numerated and couple of slides were taken from each patient from the infected area.

Samples were collected as follows:

Slit skin smears: The area was cleaned with 0.9% saline or 70% alcohol. We squeezed the edge of the lesion between thumb and forefinger and made shallow 1 mm slits through skin to dermis with a scalpel then scraped the edges to make slide smears, then smears are made as thin as possible, air dried, fixed in methanol, and stained with Wright stain.

Dermal scrapings: We Obtained 2-4 scrapings from different areas of the lesion. We scraped dermis along the necrotic lip with a scalpel, obtaining as much tissue as possible, then making thin smears on slide, air dried, fixed in methanol, and stained with Wright stain.

Microscopic photos were taken for the causing factor featured (the amastigote) in each related case for confirmation and documentation.

The study was divided into two parts:

- Part one: Defining the different microscopic cytomorphologies of the amastigote forms by identifying them first and then describing the differences in the cytomorphology of the amastigotes noticed among all the cases and later, classifying the amastigote forms according to their common microscopic features into specific classification which is shown later in two tables prepared for that purpose, taking into accounts the following criteria:

A- The general microscopic shape of the amastigotes as a whole. (Round, oval or spindle) where the round ones are globular in shape and the oval amastigotes are wider than the spindle in shape-ones, which are thinner and usually have more dense chromatin inside.

B- The microscopic shape, localization and the density of the amastigote chromatin.

Part two: Studying and calculating the appearance of the amastigote forms in each and every case in either the intra or extra cellular fluid and comparing them.

Results:

Table one: reveals the total count of the amastigotes appearance in all the cases referred according to each of the cytomorphologic categories: (Oval, Spindle, Round)

Table (1)

Amastigote form presence	Oval shape	Spindle shape	Round in shape (Globular)	Amastigote of any of the three kinds
# Of cases	16/37	16/37	2/37	18/37
Percentage %	43%	43%	5%	49%

On table one: we see that the oval in shape and spindle in shape amastigote types are the most predominant in our samples and they are found in the same rate (43% of the cases referred), (photos 1,4,5). Where as the round shape amastigotes are less found, (photos 2,6). They are present only in 5 % of the cases referred, and the overall amastigote presence of any type was at about 49% of all the 37 cases referred to us.

Table two reveals the total count of the amastigotes appearance in all the cases referred according to each of the chromatin morphologic categories: (a-Chromatin Smooth and spreading, b-Chromatin condensed and or polarized, c-Chromatin condensed with cytoplasm disappearance (Candle flame shape).

Table (2)

Amastigote form presence	Chromatin Smooth and spreading	Chromatin condensed and or polarized	Chromatin condensed with cytoplasm disappearance (Candle flame shape)	Amastigote of any of the three kinds
# Of cases	18/37	18/37	15/37	18/37

Percentage %	49%	49%	40%	49%

From table two we find that the chromatin by itself is another feature to be considered when looking to the amastigote cytomorphology. 49% of the cases referred show the presence of smooth and spreading type of chromatin inside the amastigotes (see photo 3,4,5), and other 49% show the amastigotes with condensed and or polarized chromatin in one pole of the amastigote while the cytoplasm remains in the other pole (see same photos). A third type of the amastigote is seen with only condensed chromatin and total absence of the cytoplasm (photos 3,4). We called it (Candle flame-shape). Overall, the appearance of the amastigote of any of the mentioned above features constitutes 49% of the cases referred to us.

Table three shows The Amastigote form presence in the intra cellular macrophage or extra cellular fluid:

Table (3)

Amastigote form	Intra cellular amastigote	Extra cellular amastigote	Amastigote presence in the intra cellular or extra cellular fluid or in both locations	Amastigote Present in both intra and extra cellular fluid similtinuasly
# Of cases	15/37	17/37	18/37	14/37
Percentage %	41%	46%	49%	38%

As shown in the table above, the amastigote is seen at 41% of the cases referred inside the macrophage (photos 1,6), and 46% of the cases referred are having the amastigote present in the outer cellular compartment (photos 2,3,4,5). In 38% of the cases referred to us, the amastigote is shown sharing both the intra and extra cellular compartments, while overall appearance of the amastigote in the cases referred was 49%.

Discussion:

Most of the investigators for Leishmaniasis have mentioned in their papers the microscopic method of diagnosing the leishmania disease. They related on the appearance of the LD bodies within the macrophage as the standard method for assuming the infection with the disease. In fact, referring to table three, we found that in the extra cellular compartment, the amastigote type of leishmania is noticed in 46% of the cases referred, which exceeds the 41% of the cases where we found the LD bodies within the macrophage. By that, obviously, researchers missed a considerable number of cases being considered as falls negative. Our attached photos (2,3,4,5) approve clearly such an amastigote presence. The amastigote presence in the extra cellular compartment can be easily identified by normally trained technician and does not require a big effort or sophisticated instruments to locate it. Identification of the amastigotes in the extra cellular fluid not only will add to the method sensitivity but to its specificity as well. Due to our confirmation of the 38% of the cases where the amastigote has been found both in the intra and extra cellular compartments, the specificity is elevated, (Go back to table 3).

In our research above, identification of the amastigote in the extra cellular fluid has added 5% more to the microscopic test sensitivity and even more to its specificity.

Going back to table one and photos (1,2,3,4,5,6) attached, it is obvious that there are three different shapes of the amastigote form, the round, the spindle, and the oval forms. When locating the amastigotes whether inside the macrophage or outside in the fluid, we should consider the presence of any one of those forms. Our data in the table declares that the oval and the spindle shape forms are more present in both the intra and the extra cellular areas of the infected skin at (43%) rate. Occasionally at 5% rate we come through the round type of the amastigotes in or outside the macrophage (see photo 2). Being able to differentiate and identify the different forms will add more to the sensitivity of our microscopic procedure.

Depending on the chromatin appearance within the amastigote, (Table two) and photos (3,4,5) we classified the chromatin manifestations into the three mentioned in the table above. Photos (3,.4,.5) reveal the smooth, thin chromatin usually

spreading in the central location of the amastigotes, while once the chromatin becomes more condensed, it assumes a polar position. Both the shapes are found at the same rate 49% of the cases. Another interesting feature shown is the chromatin condensation alone with the cytoplasm disappearance. It is restricted to the outer cellular fluid and we could not locate it in side the macrophage. We called it (candle flame shape) as a general name. Photos (3,4)

It seems that such chromatin manifestation has something to do with the amastigote life cycle. But with the amastigote appearance at 46% rate in the outer cellular fluid which is more than the 41% rate of its presence in the intracellular macrophage (see table three). It is absolutely clear that such amastigote appearance in the outer cellular fluid is beyond the original facts and all the previous studies indicating that the leishmania in human is an obligatory intracellular parasite. (1).

On applying a new method to the laboratory work some of the most important roles are:

1- it should be easy to be performed by the general personal working in the field.

2- it should be cost effective. (7).

Our add to the microscopic method fulfilled both the roles in the best manner.

We recommend the following considerations to be added to the microscopic procedure and be implemented:

1- Consider looking for the amastigote form not only within the macrophage or the intracellular area but in the extra cellular fluid as well.

2- Familiarize yourself with the different cytomorphologies of the amastigote forms and look for them in the intra and extra cellular space.

3- Be familiar with the chromatin distribution within the amastigote and the (candle flame shape).

By those recommendations, our data showed that an additional 20% sensitivity and more specificity are gained by the microscopic method making it more competitive with other procedures and to be considered as the gold standard method for such leishmaniasis diagnosis.

In conclusion:

Previous studies on Leishmaniasis in general and cutaneous Leishmania in particular revealed that, identification of the parasite by microscopic smear is limited to only 30% of the infected cases. The method microscopically investigates the presence of only LD bodies (leishmania Donovan Bodies) within the infected macrophage. Our protocol in defining the different shapes of the amastigotes and localizing them in both the intra and extra cellular fluid of the infected skin has added almost an additional 20% sensitivity and more specificity for the traditional microscopic method and made the diagnosis of cutaneous Leishmania at almost 50% rate of the infected cases that are clinically diagnosed, making the smear method more competitive with other diagnostic procedures.

Images:

Image (1) : See the intra cellular amastigotes within the macrophage with (1) the oval shape appearance. (X 1000).

Image (2) : See the extra cellular amastigote in the round shape (X 1000).

Image (3) : See the three different shapes of the amastigotes according to the chromatin features:
(1) The chromatin is centrally localized smooth and spreading in an oval shape amastigote. (2) The chromatin is condensed, polarized in spindle shape amastigote. (3) the candle flame condensed chromatin with the cytoplasm loss. (X 1000)

Image (4) : See the extra cellular amastigotes according to their A- shapes: (1) Oval shape. (2) Spindle shape. B- Chromatin features: (1) Smooth uncondensed and spreading. (2) Condensed and polarized. (3) Candle flame shape. (X1000)

Image (5) : See the extra cellular amastigotes according to their A- shapes: (1) Oval shape. (2) Spindle shape. B- Chromatin features: (1) Smooth uncondensed and spreading. (2) Condensed and polarized. (X1000).

Image (6) : See the round shape intra cellular amastigote (1) (X 1000).

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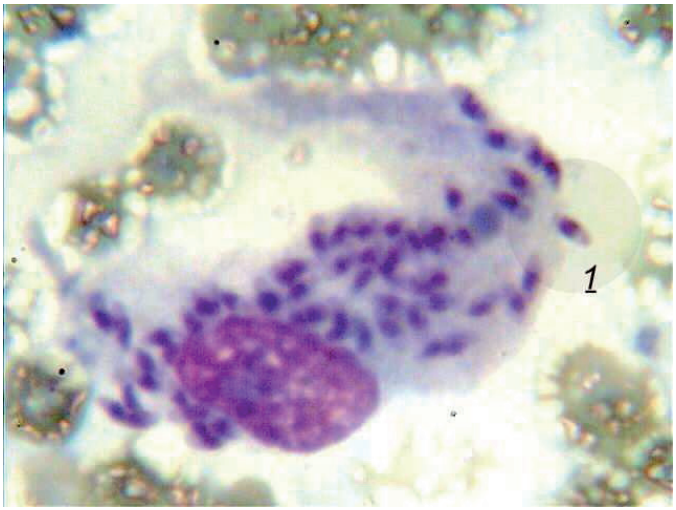

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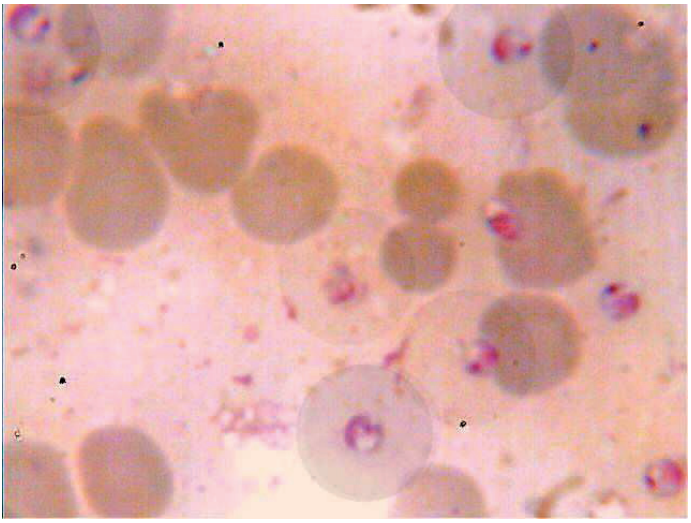

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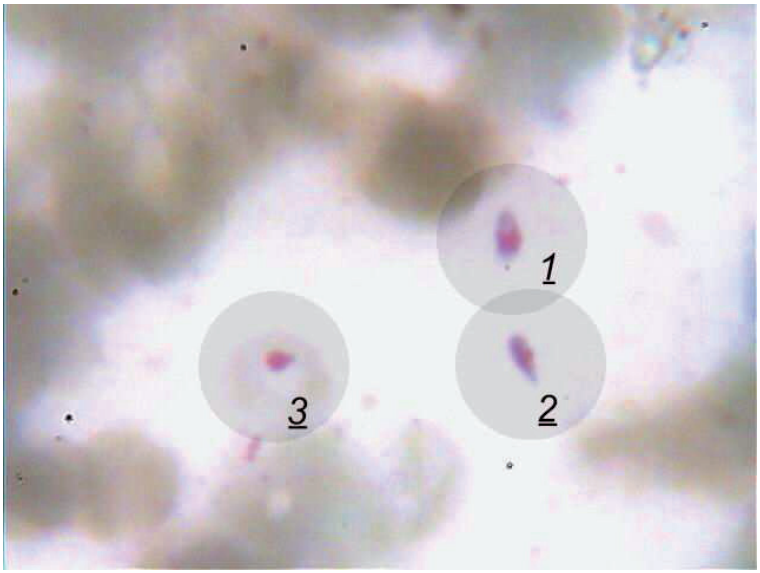

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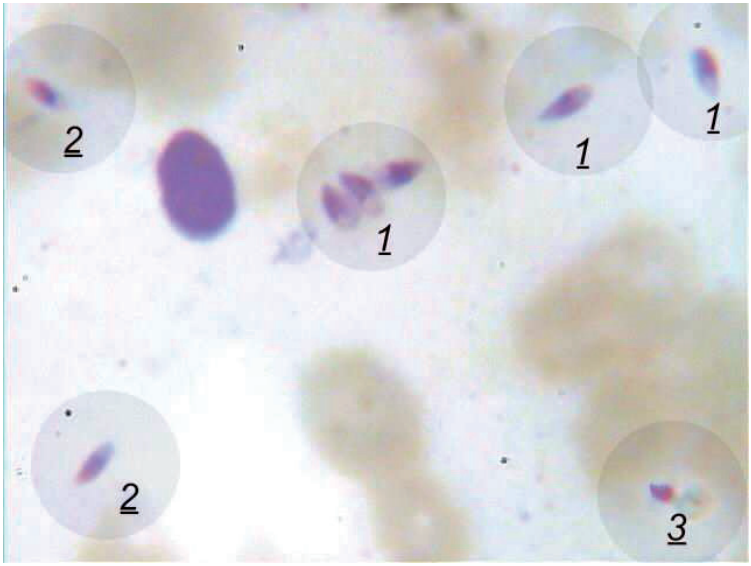

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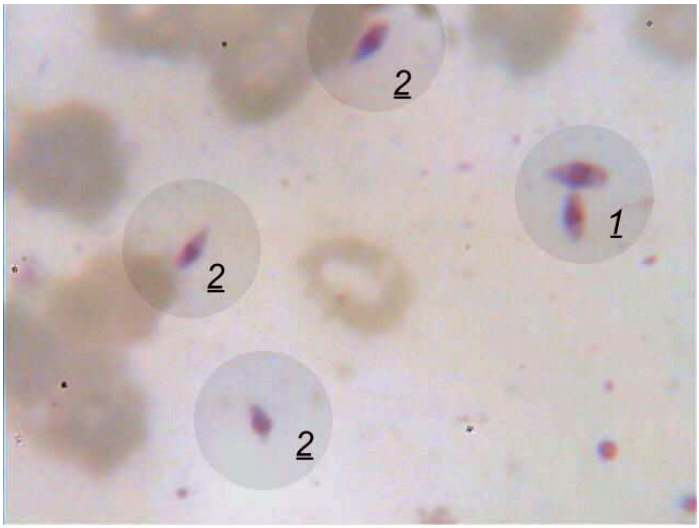

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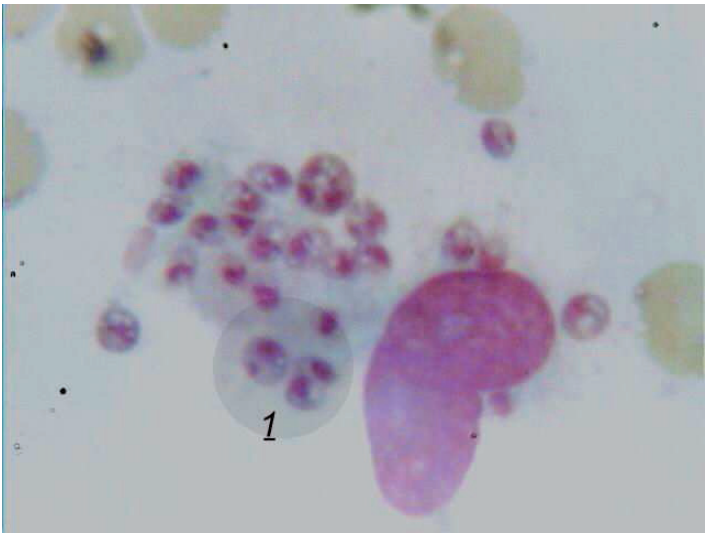

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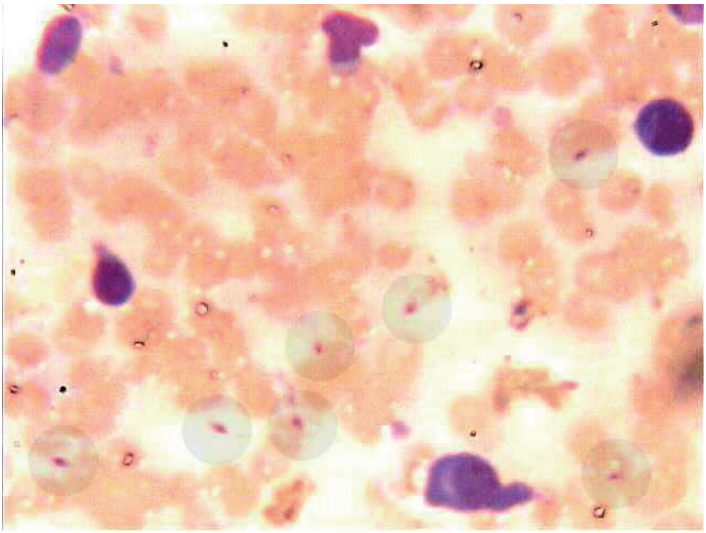

Image (7)

3- Application of the microscopic method in cutaneous leishmania diagnosis

Abstract:

Introduction and objectives: Cutaneous leishmania is spreading fast.
This study aims at developing the microscopic method to achieve a full detection of all positive cases of leishmania.
Methods: 50 human cases have been studied by applying microscopic smears stained with Wright stain. Microscopic photos were taken for the presumed unfamiliar figures.
Results: Mononuclear cells with tails are present at a rate of (98%). They are associated with LD bodies in 50% of the cases. The polygonal figures and the spherical forms are present at the same rate (60%) and are associated with LD bodies in 24% of the cases. The small promastigote like forms are seen at a rate of (76%) and are associated with LD bodies in 26% of the cases. The giant promastigotes like forms are present in (80% of the cases) and are associated with LD bodies in 28% of the cases. Candle flame forms are present in (40% of the cases) and are associated with the LD bodies in 21% of the cases.
Discussion: It is applicable to use those discovered figures in diagnosing cutaneous leishmania

Introduction:

The Mediterranean area is considered one of the most common places that cutaneous leishmania inhabits (1,2,3). Cutaneous leishmania was classified according to its spreading area into two classes:
Old world leishmaniasis: which includes Afghanistan, India, Pakistan and the Middle East countries including Syria and south of Turkey.

New world leishmaniasis: They inhabit South American countries. (1,3).

More than two million new cases of leishmania are discovered every year. (4). In addition to that, more than 350 million individuals are prone to leishmania infection (5). It is highly important to find a suitable, manageable with high sensitivity method for leishmania diagnosis in order to identify those new discovered cases and provide the best available treatment.

The direct microscopic method for cutaneous leishmania secretion is considered one of the simplest methods used to detect any possible infection. This method is considered as a reference

method for many sources (5,6,7). The microscopic method detects the presence of (LD) bodies

Leishman Donovan bodies within the macrophages and multi nucleated giant cells or Langerhan's cells in the skin lesion (8). (LD) bodies are nothing but the amastigote forms. They appear inside the cytoplasm of those mentioned cells in a spherical or a spindle like shape containing a nucleus and having a dimension between 2-4 microns in diameter. The principle of the microscopic method depends on the appearance of these LD bodies in the intracellular space for those cells. The presence of those LD bodies will confirm the diagnosis. While absence of those bodies may exclude the diagnosis with the infection. This diagnostic method has been discovered by both the scientists (Leishman- Donovan) more than a hundred years ago. Since that discovery, no developmental work or modification has ever been made on the procedure. Though it is a simple one, it is considered less sensitive and can at best, detect about 60-70% of the infected cases. That means that over 30% of cutaneous leishmania infected cases cannot be detected by this microscopic method and are considered as falls negative.

The purpose of this study is to search the possibility of developing and improving this traditional microscopic procedure so that we are able to detect the additional 30% of the undiagnosed cases where the LD bodies do not appear in the microscopic slides.

Procedures and methods:

50 cases have been studied, 43 males and 7 females who attended the laboratory between April 2006 and July 2008. Samples were obtained from the secretions of what was previously diagnosed by consultant dermatologists as cutaneous leishmania infection. Samples were collected as follows:

1- Slit skin smears: The area was cleaned with 0.9% saline or 70% alcohol. We squeezed the edge of the lesion between thumb and forefinger and made shallow 1 mm slits through skin to dermis with a scalpel then scraped the edges to make slide smears, then made smears as thin as possible, air dried, fixed in methanol, and stained with Wright stain.

2- Dermal scrapings: We Obtained 2-4 scrapings from different areas of the lesion. We scraped dermis along the necrotic lip with a scalpel, obtaining as much tissue as possible, then making thin smears on slide, air dried, fixed in methanol, and stained with Wright stain.

In the microscopic study, we looked for the unfamiliar cytomorphologies in the smears, when they repeatedly appear in the different infected lesions, that could be of a diagnostic significance. Microscopic photos for confirmation and documentation were produced for those suggested potential figures featured in each related case. A classification was later made for those different figures and cytomorphologies according to their percentage of appearance among the different smears in a related table.

Results:

The attached table indicates the count and the appearance of such unfamiliar figures and their percentage of appearance in the smears. The table indicates the association of those figures with the LD bodies (the amastigote forms) in the same smear.

Total count of the cases	Mononuclear cells with tail	Multi formed polygons	Spherical forms measure 2-7 microns	Small promastigote like forms <12 microns	Large promastigote like forms>14 microns	Candle flame forms
50	49	30	30	38	40	20

Percentage	98%	60%	60%	76%	80%	40%
Association with LD bodies	25/50	12/50	12/50	13/50	14/50	11/50
Percentage	50%	24%	24%	26%	28%	22%

From the table above, it is clear that some new, distinct, cytomorphological figures have been discovered. Those new figures were associated with the infected lesions in more than 50% of the cases. The discovered figures are much different in their morphology than the well-known blood components and the skin tissues. That includes cells like fibroblasts, which appear in normal cases. Here is a brief description for those figures while a detailed description is found in the study (The pathological features of cutaneous leishmania (9).

Mononuclear cells with tail: The percentage of the appearance of those cells in the smears as noticed from the table above in leishmania infection is as high as (98%) figure (1). The cytoplasmic tail is elongated in some cases up to more than 20 microns in diameter (figure 2). Both figures 1,2 show the different cytomorphologic manifestations of those cells. It is important to emphasize the amastigote appearance in the same photos as a confirmatory clue. That proves the possibility of using those new discovered figures to elevate the sensitivity and the specificity of the traditional microscopic procedure in detecting cutaneous leishmania.

Polygons multiformies: Those morphologies are 3-8 microns in diameter. They appear with different sides looking in a way like the neutrophil lobe (figure 3). The association of such figures with the amastigote form (LD bodies) is in about 24% of the cases.

Spherical forms: They are spherical in shape, very dense, having no surrounding cytoplasm. The dimension of such forms is between 2-8 microns. They appear in 60% of the studied cases of leishmania. They are associated with the LD bodies in 24% of the cases.

The small flagellates: Those small flagellates look very much in shape like the promastigote form of the leishmania (figure 7 shows the different morphologies that this flagellate microscopically appears in the smears). Small flagellates appear in 76% of the infected cases and are associated with amastigote forms in 26% of the cases.

The big flagellates: They look like the small flagellates in shape but their size is huge. They can reach 20-30 microns in diameter (figure 5). They appeared in 80% of the studied cases and were associated with LD bodies in 28% of the cases studied.

The candle flame forms: They are formed of chromatin mass with a tiny tail protruding out. Their dimension is 2-4 microns (figure 6). They show up in the smears in 40% of the studied cases. They are associated with the LD bodies in 22% of the cases.

Discussion:

The organisms with their different cytomorphologies presented above represent a distinguishing mark that could be utilized in diagnosing cutaneous leishmania, especially in cases where the (LD bodies) are totally absent from the microscopic smear. When applying the traditional microscopic reference method that most laboratories rely on, they only detect the LD bodies in the maximum of 70% of the infected cases. It is not yet possible to detect the disease in the other 30% of the positive cases when the LD bodies disappear from the smear. According to many studies (1-6), it seems that at a later stage of the disease process of cutaneous leishmania, the disappearance of such LD bodies together with the macrophages that are infected with, is a fact. The disappearance of such LD bodies then, cannot be explained by the decrease in the sensitivity of the microscopic procedure. It is more or less, due to a real disappearance of such amastigote form during the disease progress. Our study found that in many disease cases of leishmania, even at that stage of the disease process with the (LD bodies) disappearance, the clinical signs continue to show a sharp inflammatory reaction with a deep ulceration, and for a long period still to go (9).

The appearance of such discovered forms of organisms (the mentioned above) in the smear, and their association at different rate with LD bodies in the same smear, is a proof that those cytomorphologic figures are evidence for cutaneous leishmania. And thus, they could be utilized as a diagnostic material for detecting cutaneous leishmania especially in cases when (LD bodies) are not found in the smear . Regardless of those organisms origin, it is obvious that such organisms do not match in their morphology any of the well known cellular components or organelles that are present in the blood or the skin tissues. And because it might still be possible to find these organisms associated with other types of disease, and until we are sure that they are specific for cutaneous leishmania, it is suggested for cutaneous leishmania diagnosis, to consider, not to depend on the appearance of only one type of those organisms in the smear. When LD bodies are absent, it is more applicable to confirm the presence of at least three different types of those organisms in the same smear. In case (LD bodies) are found in the smear, no need then for looking for those organisms.

In conclusion:

This study has added a good bonus for the traditional microscopic method in diagnosing cutaneous

leishmania. The old method that was used to detect only the presence of LD bodies in the smear is a hundred years old method. It very much lacks sensitivity. And with the fast spread of the disease in third world countries and with limited resources, it is hoped for an easy, non-costy, fast and reliable method for detecting cutaneous leishmania. The microscopic method when adding those discovered figures and modifications to it, is capable enough to do the job with a 100% sensitivity achievement and almost equal specificity.

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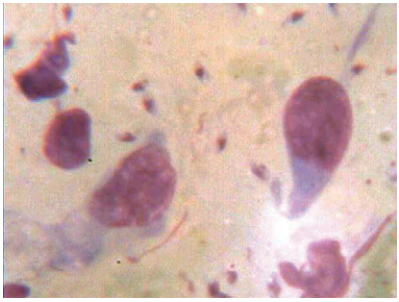

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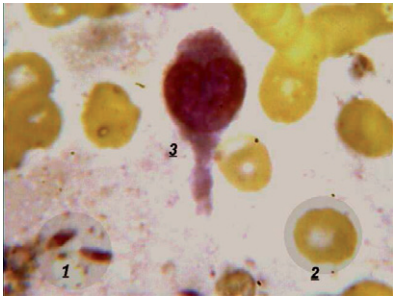

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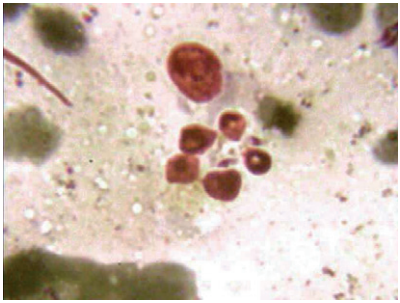

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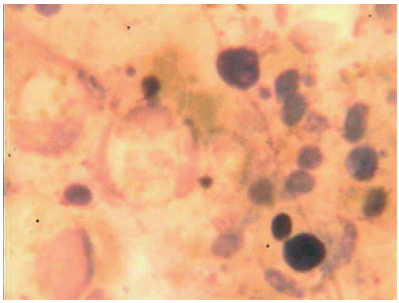

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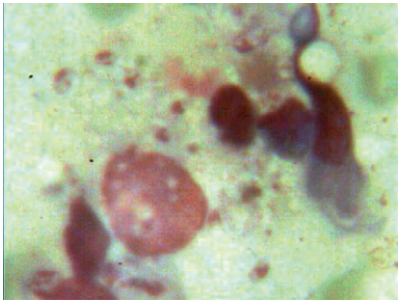

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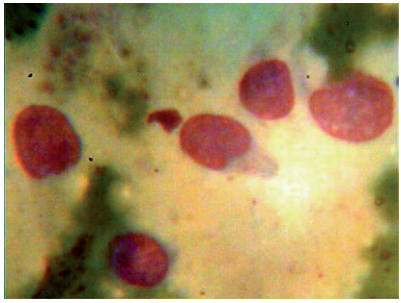

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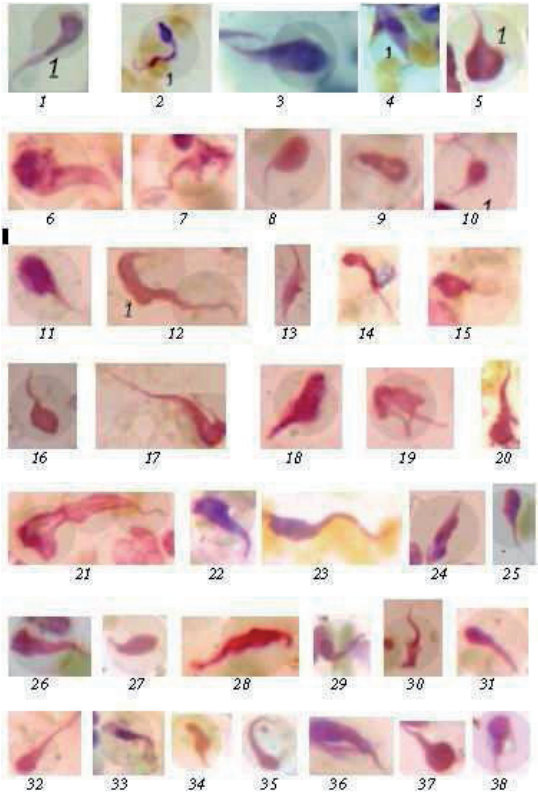

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4- Role of neutrophils in cutaneous leishmania

Abstract:

Background: Neutrophils are always present in the cytomorphologic process of the leishmania disease. Their role is still not fully understood.

Methods: A study was done on 56 cases of clinically diagnosed cutaneous leishmaniasis by means of microscopic examination in order to define the presence of the neutrophils as being: a- (+++) when they are the major predominant cells and count >%35 in the smear among macrophages and lymphocytes. b- (++) minor cells present counting between 10- 35%. c-As (+) when such neutrophils are less than 10% of the total count.

Results: Category a- (+++) was present in 4/56 cases at (%7) rate and category b- (++) in 20/56 cases studied at (%36) rate, while category c- (+) was noticed in 32/56 cases at (%57). Only in category (a) (+++): cytomorphologically, the neutrophils appeared to practice their known phagocytosing role in eliminating the amastigotes released in the extracelluler fluid from those ruptured phagocytes of the injured tissues.

Conclusion:

In a later stage of the disease process, neutrophils seem to have a strong role in leishmania elimination through their function of amastigote phagocytosis, dissociation and digestion.

Introduction:

Millions of Cutaneous Leishmania infections in humans are reported worldwide. Cutaneous leishmaniasis is endemic in over 70 countries. The yearly incidence is estimated at (1,500,000) cases (1).

Polymorphonuclear neutrophils have been reported to participate in the early control of Leishmania infection (2,3). Early in the infection the neutrophil cells crucially

contributed to parasite killing. A rapid and sustained neutrophilic infiltrate at localized sand fly bite sites have been observed by using dynamic intravital microscopy and flow cytometry. Invading neutrophils efficiently captured Leishmania major parasites early after sand fly transmission (4). Studies showing that PN (Polymorphonuclear neutrophils) are present in vivo in areas of Leishmania destruction or can kill the parasites in vitro have suggested that these cells might be involved in the inhibition of the parasite multiplication. Neutrophils also, appear to play a major role in the development of protective immunity (5,6). A recent report demonstrated that interaction of L. major-infected macrophage with dying neutrophils from C57BL/6 mice induced parasite killing mediated by neutrophil elastase and TNFα production from neutrophils (5).

Most of these studies have been concentrating on the neutrophils effect during the very early stage of cutaneous leishmaniasis, while in the late stages of cutaneous leishmania; non-has been mentioned about the neutrophils role. The pathological features in the later stages of the disease indicated a gradual decrease in the number of amastigotes and macrophages, leaving a granulomatous infiltrate consisting of lymphocytes, epithelioid cells, and multinucleated giant cells. At this stage it may be difficult of even impossible to detect the amastigotes in H&E, or Giemsa, stained sections (7). In a dry nodular type of lesion, there is a tendency to form granuloma with less lymphocytes and scanty plasma cells (8). As can be noticed, the studies above have not claimed any neutrophilic roles or even neutrophils appearance in the pathological features during late stages of the disease process while they mentioned the amastigotes disappearance without connecting it with the cause or giving any reason behind this disappearance.

This study aimed at cytomorphologically investigating the appearance together with the phagocytosis function of such neutrophils on the amastigotes from human infected lesions with cutaneous leishmania at a later stage of the disease process.

Materials and Methods:

In the approach to find out the role that neutrophils play in cutaneous leishmania, a study was done on 56 cases of clinically diagnosed cutaneous leishmaniasis between Oct 2006 and Feb 2008. 50 (90%) of the patients were males and 6 (10%) were females. The study was done by means of microscopic examination in order to define the presence of the neutrophils as being: a- the major predominant cells (+++)>%35 of the different cells microscopically present in the smear including macrophages and lymphocytes. b- as minor counting cells (++) in the smear when they constitute between 10- 35% of the different mentioned cells seen under the microscope. c- When such neutrophils are less than 10% of the total previous cells seen under the microscope and in such case, they are considered as (+). 2 microscopic slides were prepared from each patient, stained with Wright stain and studied under the three mentioned categories. Fifty fields were studied in each slide and the mean of neutrophils, phagocytes and lymphocytes count was calculated per field with a magnification (40*10).

Results:

Table 1 shows the number of cases of neutrophils appearance over the total number of studied cases according to the following categories (the percentage of neutrophils count /total neutrophils, phagocytes and lymphocytes count in the microscopic slides: category A- neutrophils >35%, B- neutrophils 10-35%, C- neutrophils <10%)

Categorized neutrophils according to their mean percentage in the field	>35% (+++)	10-35% (++)	<10% (+)
The number of cases of neutrophils appearance among the studied cases	4/56	20/56	32/56
The percentage among the cases	7%	36%	57%

It appears from table (1) that the neutrophils are mostly present in the highest concentration among the three cell types, neutrophils, lymphocytes and phagocytes

in only 7% of the cases studied. While in 93%, the neutrophils where not the actual predominant cells that appeared in the slides. In 57% of the cases studied the neutrophils were rarely seen <10%.

Table 2 shows the relation between the amastigotes appearance in the smear and the neutrophils concentration: (neutrophils count / the total neutrophils, phagocytes and lymphocytes count) in the smear.

Amastigotes	heavily concentrated in the extra cellular fluid	Intracellular presence	Not seen
appearance cases	4/56	23/56	29/56
The percentage	7%	41%	52%
Neutrophils appearance	(+++)	(++) or (+)	(+)
Neutrophils concentration	>35%	0-35%	<10%

It is clear from table (2) that when the amastigotes are heavily concentrated in the extracellular fluid, the neutrophils become more concentrated in the field while when the amastigotes are intracellularely present with very mild appearance in the extra cellular fluid, the neutrophils are less counted, (0-35%) and when the amastigote disappears from the screen, the neutrophils tend to become even less in the microscopic slide (<10%).

Discussion:

All the cases referred were clinically diagnosed by expert dermatology consultants as cutaneous leishmaniasis. Concerning the disease stage, the cases in this study were chosen in a random manner according to their referral from the clinic and no definite disease stage was previously determined. So the cases presented could be at any disease stage. The cases were classified according to their pathological and clinical features: meaning that, when the patient clinically appears with a skin ulcer measuring 2-5 cm in diameter with a wet exudates and the pathological features show a dermal infiltrate mainly made up of macrophages filled with amastigotes,

lymphocytes and plasma cells, then we are in an early stage of the disease process. While when the lesion clinically appears smaller in size dry and nodular with dried exudates and the microscopic features show a decrease in the number or a disappearance of the amastigotes and their macrophages, leaving a granulomatous infiltrate consisting of epithelioid cells, multinucleated giant cells, less lymphocytes and scanty plasma cells (7,8), then we are talking about a later stage of the disease process. As long as the cases were clinically manifested and investigated by the dermatologist before being referred to the laboratory for diagnosis, then non of the cases should have been in the very beginning of the disease process (as presented in studies 2,3,4) because in the very early stage of the disease and after the sand fly transmits the parasite to the host, most studies indicated that no immediate clinical symptoms appear (7,8), then the neutrophils function if microscopically shown up in this particular study, must have been a sign in a later stage of the disease.

It appears from the photos attached that once the neutrophils are becoming the predominant cell type >35% in the field, the function of the neutrophils on phagocytosis of the amastigotes shows up. (Figure 1,2). When the neutrophils are in less concentration, the phagocytosis process of the amastigotes does not appear in the screen (Figure 3). An association is well defined between the increase in neutrophils appearance and the amastigotes presence in the extracellular fluid as shown in table (2)(figure4). An other association exists between the neutrophils process of phagocytosis to the amastigote and the amastigote being heavily released into the extracellular fluid (figure 1,2). From the photos presented, the neutrophils are shown phagocytosing the released amastigotes in the extracelluler fluid in the infected lesions. In fact neutrophils, are best specialized in the phagocytosing process, while macrophages are considered less efficient than PMNs (Polymorphonuclear neutrophils) at killing foreign microorganisms, and the mechanisms of killing are not as well understood (9). Accordingly, one can assume that those PMNs must have a strong role to play in phagocytosing and destroying many of the amastigotes released after membrane rapture of the infected macrophages. Otherwise, it is not possible to explain the amastigotes disappearance from the infected tissues after their release in

that huge number into the extra cellular fluid later in the healing process of cutaneous leishmania (7). It is obvious that once the amastigotes are released from the ruptured phagocytes in to the extracelluler fluid at a late stage of the disease process, they are considered by the neutrophils as foreign microorganisms (a potential hazard). The amastigotes become within those PMNs command and operation, and a kind of an acute phase reaction is established. An attraction of more neutrophils takes place. That causes an increase in the neutrophils concentration in the damaged area. The neutrophils start the process of phagocytosis to those released amastigotes. That of course will participate in amastigotes elimination and so far a disease control.

In conclusion:

Our study has declared that the polymorphonuclear cells are at no time absent from the disease process of cutaneous leishmaniasis in any of its stages. While the previous studies assured the role of the PMNs in the early stages of the disease process, this study concentrated on the PMNs role in the later stages of the disease. The neutrophils were highly effective in phagocytosing the amastigotes released from ruptured phagocytes to the extracellular fluid after they became under those PMNs operation . The PMNs effectively established their role by phagocytosing the amastigotes and that was associated with the amastigotes later disappearance from the screen. This fact comes in agreement with most leishmania studies as they emphasis that in the late stages of the disease process, the amastigote forms disappear from the screen.

Figures:

Figure 1- Amastigotes are engulfed within the neutrophile.

Figure 2- An increase number of the neutrophils in the screen with phagocytosis activity.

Figure 3- Less neutrophils and more lymphocytes and no phagocytosis function.

Figure 4- The amastigotes released in the extracellular fluid with an elevation in the number of the neutrophils and the phagocytosis activity.

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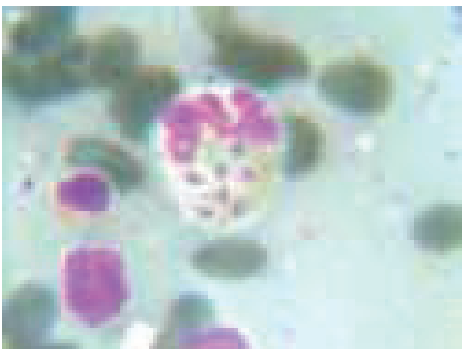

Fig. 1

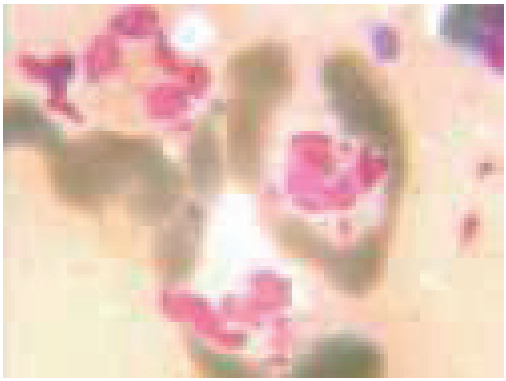

Fig. 2

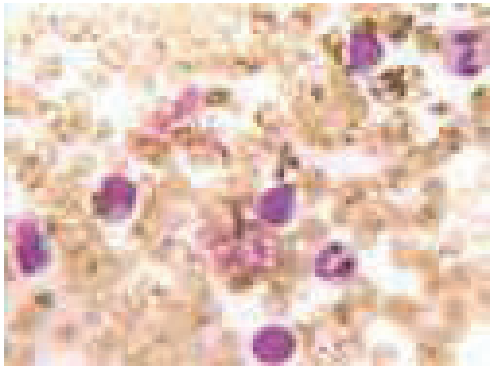

Fig. 3

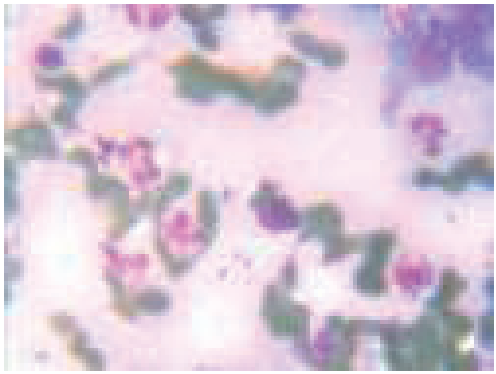

Fig. 4

5- Is the Amastigote Form the Only Form Found in Humans Infected With Cutaneous Leishmania?

Abstract:

Background: Previous studies of cutaneous Leishmania revealed that 2 forms of the parasite are present: the intracellular amastigote form found in the vertebrate host, and the promastigote form predominately found in the insect vector.

Methods: Samples were collected from the lesion area of the skin from 42 patients referred to the laboratory and microscopic slides were prepared.

Results: Out of 42 cases, only 1 (2.4%) showed the presence of the amastigote intracellularely alone but not in the extracellular area, and 2 (4.75%) showed the presence of the amastigote in the extra cellular but not in the intracellular location. In 20 of 42 cases (48%), we observed the amastigote presence in the intracellular or extracellular area. Out of all 20 cases in which the amastigote was recovered, we were able to identify the amastigote in 17 cases (85%) in the intracellular and extracellular locations simultaneously. Additionally, out of all 42 cases referred, the amastigote form alone, with no promastigote associated, was microscopically recovered in 10 cases (24%).

Conclusion: Contrary to previous studies, we found that in the human body cutaneous Leishmania does not act as an obligatory intracellular parasite. Leishmania is intracellular and extracellular parasite that infects first the mononuclear phagocyte. There, it remains in the form of amastigote and

multiplies by binary fission, and when released from the mononuclear phagocyte, after membrane rapture to the extracellular fluid, the amastigote transforms into some promastigote-like form in a progressive sequence. At a later stage, this promastigote like organism continues its development and transforms again to produce pseudofiber, leaving the lesion location at the end with a

permanent scar.

Introduction:

In 1997, we received the first challenging specimen referred from a consultant dermatologist at a hospital belonging to the Ministry of Health in Saudi Arabia for cutaneous leishmania identification by the microscopic smear method. Clinically, the lesion looked typical for the dried type of cutaneous leishmania with a painless ulcer and a raised, indurated margin and a necrotic base. On performing the general method for microscopic exam and staining with Wright stain, we were unable to identify the Leishman-Donovan (LD) bodies within the macrophages, which is the principle of the microscopic diagnosis of cutaneous leishmania. Afterwards, for the next 10 years in practice, we had several cases like this, and we failed to demonstrate the amastigote presence in almost 50% of the cases by microscopic smear.

The purpose of our study was to microscopically identify any extracellular form of leishmania present in human lesions and, if found, to describe the new form microscopically and its transformation sequence on a step-by-step basis with photo support.

Literature Review:

The group of diseases known as the leishmaniasis are caused by protozoa of the genus *Leishmania*. These are present in 3 different forms: visceral leishmaniasis (VL), Mucocutaneous leishmaniasis, and cutaneous leishmaniasis (CL). The visceral form (kala-azar) is the most severe form of the disease and, if left untreated, is usually fatal. mucocutaneous leishmaniasis is caused by *Leishmania braziliensis* or related New World species, and parasites may disseminate to the oral and nasopharyngeal mucosa.

Cutaneous leishmaniasis is the least severe. It is endemic in over 70 countries. The yearly incidence

is estimated at (1,500,000) cases. Over 90% of the cutaneous leishmaniasis occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru. The geographical distribution of CL is mainly determined by sand fly vectors (*Phlebotomus* sp and *Lutzomyia* sp). They live in dark, damp places, and are relatively weak flyers, with a range of only 50 meters from their breeding site. They

are most active in the evening and at night.1 It is agreed among *Leishmania* researchers on the following:

1) In vertebrates, including human host, *Leishmania* species are obligate intracellular parasites of mononuclear phagocytes2,3;

2) There are 2 forms of the parasite: the amastigote form, which is an intracellular one specifically found in the vertebrate host including human, and the promastigote form, predominately found in the insect vector and not found in human4; and

3) Demonstration of the amastigotes and not the promastigote form in lesions plays an important role in the diagnosis of cutaneous leishmaniasis.5,6 Following a sand fly bite, some of the flagellates, once in circulation, enter the cells of the reticuloendothelial system. There, they transform into amastigote forms. The amastigote forms then multiply by binary fission within the macrophage
until the host cell is packed with the parasites and ruptures, liberating the amastigotes into circulation. Then the free amastigotes invade fresh cells, thus repeating the cycle. In the process, some of the free amastigotes are drawn by the sand fly during its blood meal, thus completing the cycle.2

Pathology of the Disease:

According to Hepburn, "over the following months, there is a gradual decrease in the number of amastigotes and macrophages, leaving a granulomatous infiltrate consisting of lymphocytes, epithelioid cells, and multinucleate giant cells.
At this stage it may be difficult or even impossible to detect organisms in H&E, or Giemsa, stained sections." In the clinical features, he writes, "Most patients have 1 or 2 lesions, usually on exposed sites, varying in size from 0.5 to 3 cm in diameter. There is, however, considerable variation: Some lesions do not ulcerate, others develop sporotrichoid nodular lymphangitis. Most lesions heal over months or years, leaving an atrophic scar."1 An interesting question arises: Why is not healing complete without permanent scar?

Referring to Sharquie and colleagues, "The morphology of LD bodies (amastigotes) in smears assumed mainly spindle shape, other morphological forms like barrel,

safety pin and umbrella like were noticed, while the morphology in histopathological sections were rounded with a nucleus and kinetoplast. However, in some sections spindle shape form similar to smear morphology was detected. LD bodies were seen in histopathological sections in 30% of patients. Other histopathological features were mainly abundant of lymphocytes and plasma cells in the wet ulcerative lesions while in dry nodular types there was a tendency to form granuloma with less lymphocytes and scanty plasma cells."7 That raises a very interesting question, which is: where did the amastigotes and their macrophages disappear that caused the LD bodies to be seen in histopathological sections in only 30% of patients? And as long as the lesions continue over months to a year with no amastigote presence (which means the disappearance of the causing agent of the disease took place), is it possible that any kind of amastigote transformation has happened and a new form was missed by microscopic smear exam?

Methods of Transmission:

The predominant mode of transmission is the bite of a sand fly; however, there are also uncommon modes of transmission through congenital transmission, blood transfusion, and, rarely, through inoculation of cultures.2 In some regions, natives inoculate their children in a site normally not visible to protect the child from getting disfiguring scars latter in life.8 Having had other modes of transmission aside from the bite of a sand fly raises our doubts about the absoluteness of the sand fly vector for the parasite to complete its life cycle, and thus assuming the possibility of the promastigote form to be present in the extracellular fluid of the infected vertebrate.

Materials and Methods

Since 1999, we have received 42 patients referred to us from consultant dermatologists clinically diagnosed with cutaneous leishmania for confirmatory laboratory diagnosis. Our method of identification was parasite diagnosis by microscopic examination of the skin lesion, which remains the "gold standard" with its usual limitations.

Samples

Samples were collected from patients referred to the laboratory as microscopic slides from the lesion area of the skin. Two slides were prepared from each lesion. In case we had more than 2 exposed areas in the same patient, we chose the 2 more edematic lesions and took a few slides from each, stained with Wright stain.

All the smears for each and every case were reserved and numerated as such: Case one (1c); case one slide one (1c1), case 2 (2c), and so forth. Comparison was made among the microscopic features of the parasite in the different slides as follows:

1) A study was done for counting the amastigote appearance in the intracellular or extracellular location in all cases.

2) Another study was done to identify and compare the manifestations of the different shapes of the amastigotes seen in the extracellular fluid among the cases referred.

3) Then a study was done for locating the presence of any type of suspicious organism appearing different than any of the blood or the human dermal tissue components.

4) A morphologic comparison was made on the discovered flagellates among the different smears taken from the different cases referred to us.

5) Another morphologic comparison was made between the presumed fiber-forming promastigote form found, and fibroblasts in skin biopsy specimen stained with H&E stain to rule out the difference.

Six hundred documentary microscopic photos were taken for the presumed parasite forms discovered for confirmation, documentation, and to compare them with other reference photos

for the parasite in both its amastigote and promastigote forms.

Results

Table 1 shows the amastigote form presence in the slides intracellular or extracellular.

Amastigote form	Intra cellular alone	Extra cellular alone	Only amastigote present with no promastigote form	Amastigote Present in or out side the Macrophage
# of cases	1/42	2/42	10/42	20/42
Percentage %	2.38	4.75	24	48

Table 1 reveals that out of 42 cases referred to the laboratory, microscopically, only 1 case (2.4%) showed the presence of the amastigote intracellularely alone but not in the extracellular area; 2 cases (4.75%) showed the presence of the amastigote in the extracellular but not in the intracellular location; and in 20 cases (48%), we microscopically observed the amastigote presence in the intracellular or extracellular area. Out of the 20 cases in which the amastigote was recovered, we were able to identify the amastigote in 17 cases (85%) in the intracellular and extracellular locations simultaneously. Additionally, in all 42 cases referred, the amastigote form alone—with no promastigote associated—was microscopically recovered in 10 cases (24%).

Table 2 shows other forms found in the microscopic slides of the 42 patients.

Promastigote like forms	Seen in the smear	Associated with the amastigote forms	Present alone in the smear (with no amastigote forms)
# of cases found	32/42	10/42	22/42
Percentage	76	24	52

Table 2 reveals that out of 42 cases referred to the laboratory, microscopically, the promastigote-like form was found in 32 (76%) of the cases referred, and the amastigote
form was recovered associated with the promastigote-like in 10 cases (24%) of the total. The promastigote form was recovered alone without the amastigote combination in 22 cases (52%).

Discussion:

Table 1 indicates that the amastigote forms are almost available in both intracellular and extracelluler fluid similtinuasly every time the amastigote form is found in the smear. That presence in the extracelluler fluid ascertains the amastigote ability to survive in the extracelluler environment not necessarily the same as inside the macrophage, but at least as a bridge for transformation into the promastigote-like form. That leads to the conclusion that in humans, the amastigote form, contrary to the previous studies, is not an obligatory intracellular parasite which cannot survive in the extracelluler environment as long as we see it in both locations at almost the same rate. From Table 2 we concluded that the images (Images 1–12) have proved the presence of other forms apart from the amastigote form in the extracelluler fluid. We called these forms "promastigote-like" as a general term, and, according to their developmental stage and relying on the photos taken from the slides, we classified them in the following order:

1)Once outside the macrophage, the amastigote form becomes like an ova containing the promastigote embryo. The nucleus is usually centralized and the surrounding cytoplasm has a spindle-like shape and the chromatin is smooth (Images 1, 2, and 5).

2)Inside the amastigote, the nucleus starts to take a polarizing position and the cytoplasm appears in one side of the amastigote. At the same time , the chromatin shows more condensation (Images 1 and 2).

3)This developing embryo becomes larger in size and more condensed, assuming the candle flame shape while the cytoplasm disappears (Images 1 and 8).

4)The embryo continues to grow with a small tail protruding outside (Images 1, 7 and 8).

5)The tail continues growing, taking the flagella shape together with the whole organism increasing in size from 2 to 3 microns to approximately 4 to 6 microns starting to assume a small promastigote shape (Images 7 and 8).

6)In this stage, the promastigote becomes fully mature with dimensions approximately 8 to12 microns in diameter (Images
3–6 and 8).

Sharquie and colleagues wrote, “The morphology of LD bodies (amastigotes) in smears was mainly spindle in shape, other morphological forms like barrel, safety pin and umbrella like were noticed.”7 In fact, they described the transforming stages of the amastigote to promastigote-like but were not able to identify the transformation significantly.

7)Transformation into fiber-forming promastigote stage: In this stage the promastigote-like form starts transforming into fiber production where the flagella becomes more condensed, thickened, and enlarged to about 40 microns or more in length (Image 10). We may also see the fiber formation not only from the flagella position but also from the opposite side (Image 12), or occasionally at different poles (Image 11). This fiber structure formation in the derma may react like a foreign graft and explain the inflammatory fiber granulomatous immune reaction associated with the lymphocytes, mononuclear cells, and variable number of plasma cells (graft rejection-like reaction). Once again, Sharquie and colleagues in their description, “Other histopathological features were mainly abundant of lymphocytes and plasma cells in the wet ulcerative lesions while in dry nodular types there was a tendency to form granuloma with less lymphocytes and scanty plasma cells.”7 They were able to describe the histopathological condition of the disease without connecting it with the etiology, which is the presence of the promastigote in the wet lesion and the presence of fibers and fiber-forming parasites in the dry nodular type of lesions. Our findings

indicate that we could see a mixture of both histopathological features he mentioned at different rates, in different smears according to a certain stage of the disease case.

8) The last phase, conversion to fibers: Here the fibers are loaded, elongated, and the parasite nucleus becomes thinner, smaller, thready, and more condensed in the middle, along with the fiber continuing from both sides (Image 9-12). It is like the parasite embalms itself. At this point, few lymphocytes and plasma cells are seen and, clinically, the lesion is dry and close to healing and forming a permanent scar.

The formation of the post-healing lifetime scar is justified by the production of these types of pseudo-fibers in the lesion by the parasite itself, and not the human fibroblasts. Apparently, those remaining foreign fibers formed and inserted in the last stage of the lesion healing, will stimulate and become a focal area of permanent immune inflammatory reaction, and thus explains in part the incomplete cure of the skin in the area with a permanent scar formation.

Some researchers went through this phenomena. Hepburn wrote, “There is, however, considerable variation: some lesions do not ulcerate, others develop sporotrichoid nodular lymphangitis. Most lesions heal over months or years, leaving an atrophic scar.”1Without detecting the reason of this lifetime scar.

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Is the Amastigote Form the Only Form Found in Humans Infected With Cutaneous Leishmania? (Photos)

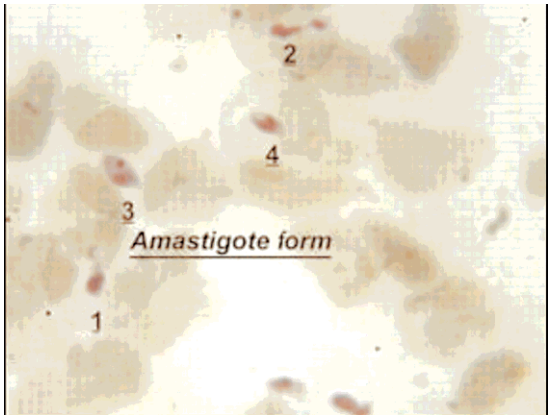
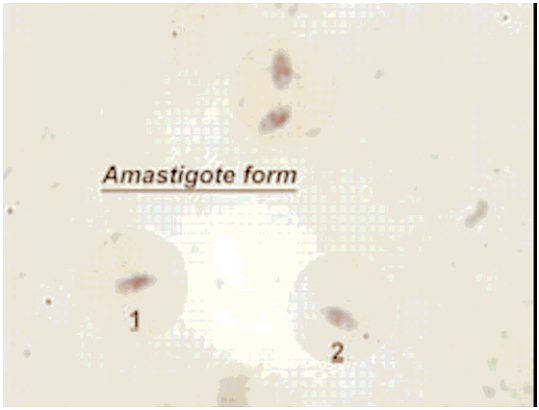


Image 1



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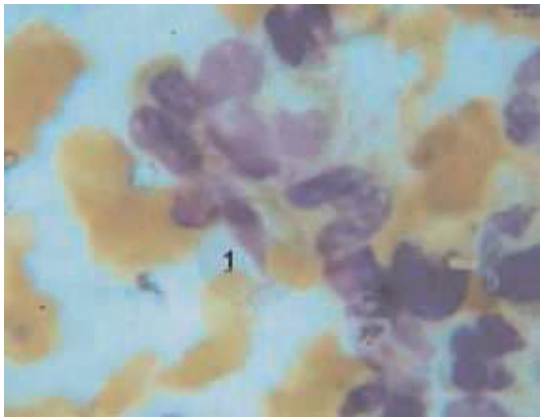

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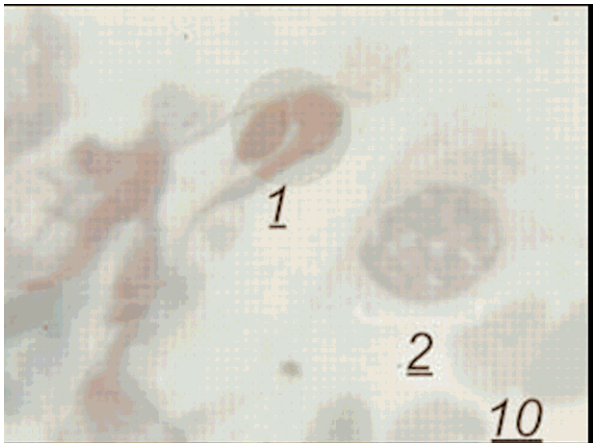

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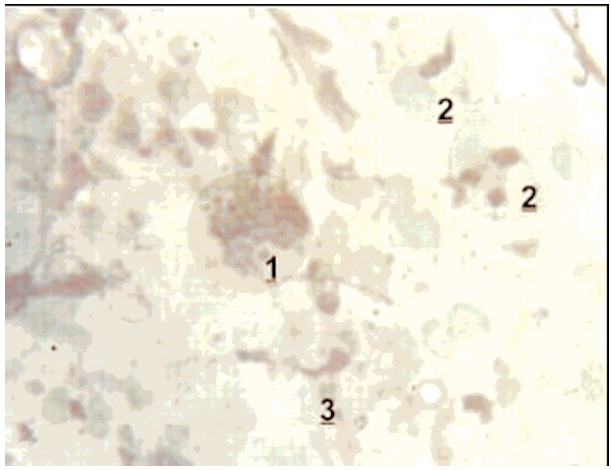

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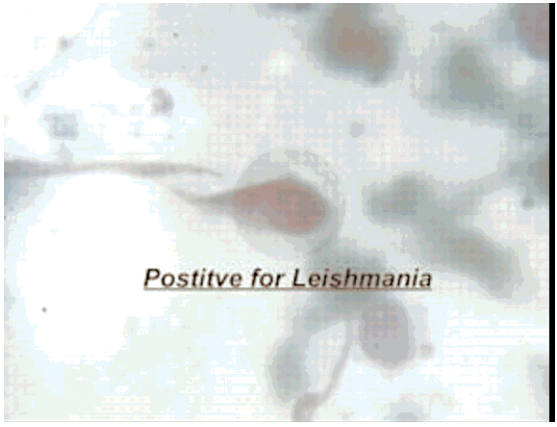

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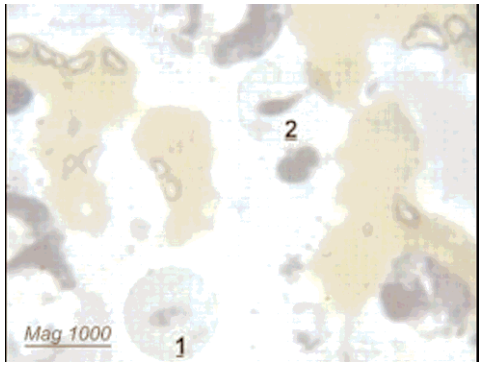
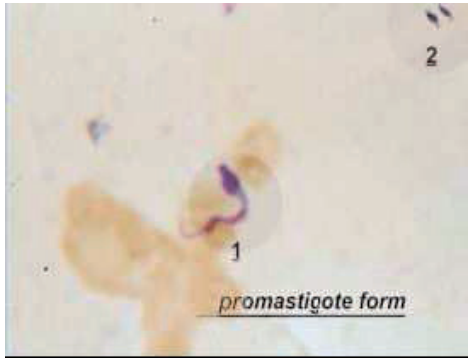


Image 7



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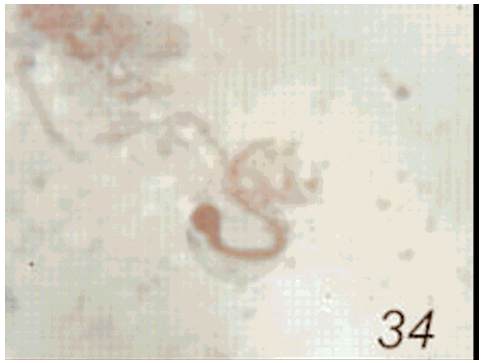

Image 9

Image 10

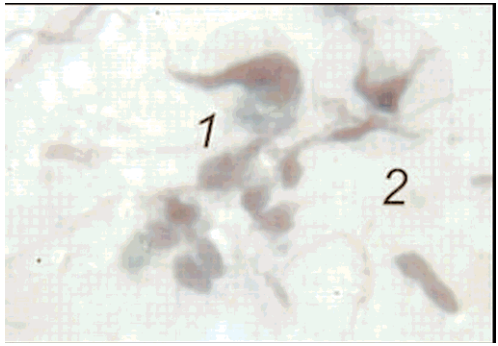

Image 11

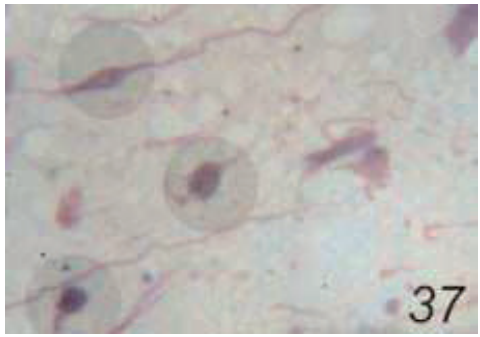

Image 12

6-The pathological features of cutaneous leishmania

Abstract :

Background: With the new discovery of the promastigote form and the fiber producing promastigote form in the infected lesion in human with cutaneous leishmania (1), it became important to reconstruct the pathological features of the disease process considering the new findings.

Methods: 42 cases of cutaneous leishmania referred to the laboratory in Damascus between January and October 2007 for microscopic diagnosis and simultaneously rearranged in series according to the approximate time of the lesion appearance. A reconstruction model of the pathology of the disease sequence was established.

Results: Lymphocytes (with tails) are microscopically present in 41 out of the 42 (98%) of the cases referred. Amastigote form microscopically is present in 20 cases out of 42 (48%). Promastigote and fiber forming promastigote microscopically are present in 32 out of the 42 cases (76%). Candle flame form appears in 21 out of 42 in (50%) of the cases and the spherical and polygon forms appear in 25 out of the 42 cases referred at 59% rate

Conclusion: Neutrophils have a role in eliminating the amastigotes released to the outer cellular fluid. Some subgroups of lymphocytes by converting into giant promastigote like forms have a role to play in the disease process by directing the process into fiber formation and hence controlling the causing factor (the different parasite forms present) and through that eliminating the disease.

Introduction:

Studies of leishmaniasis that were done before lack many of the pathological features of the disease process. Many pathological events are either unclearly elucidated or uncertain or overlooked with out being fully explained. That made the whole pathology of the disease process somewhat ambiguous and unclear.

Cutaneous leishmaniasis presents as a skin ulcer at the site of the sand fly bite and generally heals spontaneously with a scar within three to six months.

Histological examination of the cutaneous lesion sometimes reveals extensive subcutaneous lymphohistiocytic infiltrate with clusters of amastigote within histiocytes (2). Other times it shows granulomatous inflammation with histiocytic infiltrate (3). Other histopathological studies indicate lymphocytes and plasma cells abundance in the wet ulcerative lesions. The overlying epidermis is hyperkeratotic and subsequently break down to form an ulcer covered with dried exudate, dead cells and a mixture of live and dead organisms (4). Through the disease process, over the following months, there is a gradual decrease in the number of amastigotes and macrophages (4), leaving a sporotrichoid granuloma with less lymphocytes, epithelioid cells, multinucleated giant cells and scanty plasma cells in dry nodular lesions (5), (6), (7).

From the immunopathologic point of view: The extent of the disease manifestation is a combination of the parasite pathogenesis and the immune host response. The interactions between the parasite virulence factors and the cell- mediated immunity is not fully understood (8). A study of post-kala-azar dermal leishmaniasis declared that there is an abundance of CD4, which closely interacts with Leishmania antigen present (9).

An other study proposes the presence of three groups of antigenic determinants in the parasite: First group is the invasive / evasive determinants. They help the parasite to establish infection in the host.

Second group is the parasite pathoantigenic determinants. The immune response against such determinants results in immunopathology causing the disease symptoms to appear. Third group is the vaccine determinants: When the immune system interacts with those determinants they lead to parasites elimination. A hypothetical model was constructed assuming that the disease virulence is due to interaction between the host's immune system and the leishmania parasite determinants. Recent works revealed the existence of T-cell epitopes in leishmania cytoplasmic molecules elucidating protective immunity (10).

Such studies mentioned above, assume the amastigote as the only form present in vertebrates and hence, it summarizes the whole process of the disease of cutaneous leishmania accordingly. Our findings (1) reveal that beside the amastigote, other forms of the parasite are present and missed by those studies. These findings will add more light to the pathology of the disease process.

Additionally, the pathological features under the microscope have to be a reflection to the immunopathologic interaction between the parasite and the host.
Unfortunately going through the literature presented, we do not see such a reflection. In fact, all what is seen is a mix up of deficient pathological elements timely misarranged describing the whole set of the immunopathologic interaction during the disease course.
The purpose of our study was to reconstruct the pathologic features with the aid of the images in the right sequence by adding the new pathological figures discovered and the new data presented (1) and locating them into their right position of the sequence on step by step basis. This will contribute to better understanding of the pathology of the disease.

Material and methods:

42 cases referred to the laboratory in Damascus since January 2007 from consultant dermatologists as being clinically diagnosed as cutaneous leishmaniasis.
From each lesion couples of slides were obtained and stained with Wright stain.
A study was done to identify the presence of the lymphocytes with tails among the different samples and cytomorphologically to compare them with each others in the cases referred and with typical forms of lymphocytes as controls.
An other study was done to identify and compare the different cytomorphologies of the parasite forms (amastigotes, promastigotes, candle flame shapes, polygon forms, spherical forms and fiber forming forms) found and to define their appearance percentage among the different samples.

The approximate time of the lesion appearance at the time of the sample collection was investigated through patient inquiry.

With the time aid of the lesion appearance connected with the pathological features for all the 42 samples simultaneously rearranged in series according to that approximate time and studied, a reconstruction model of the pathology of the disease sequence was established.

Confirmatory microscopic photos were taken for documentation.

Results:

Table 1_ Appearance of the lymphocytes (with tails)

Total number of samples investigated	42
Total appearance of the lymphocytes (with tails)	41
Percentage of total appearance of the lymphocytes(with tails)	98%

From table-1- we found that the lymphocytes (with tails) are present in 98% of the cases. 41 out of the 42 samples referred are having this type of lymphocytes microscopically showing up. The appearance of such lymphocytes among the different samples is not in the same density. That was related to the disease stage when the sample was collected in the lab.

Table 2_ Appearance of Amastigotes, Promastigotes, Fiber forming promastigotes, (Candle flame) and (Polygons and spherical forms).

Appearance of	Amastigote	Promastigote	Fiber forming Promastigote	Candle flame	Spherical& Polygons
Total appearance	20/42	32/42	32/42	21/42	25/42
Appearance percentage	48	76	76	50	59

Table –2- reveals that in 20 cases out of 42 (48%) the amastigote microscopically is present while the appearance of the promastigote and the fiber forming promastigote is equal. They are present in 32 out of the 42 cases (76%). The candle flame form appears in 21 out of 42, (50%) of the cases and the spherical and polygon forms appear in 25 out of the 42 cases referred at 59% rate.

The reconstruction model of the disease pathology:

Phase one (The lag phase): The disease pathology starts when the infected sand fly during its meal transfers the parasite to the cutaneous area of the human skin. A (lag) incubation period extends from one day up to several months (11). During which, the promastigote type of the parasite enters the subcutaneous area where it loses its flagella and is engulfed by the macrophage or the phagocyte which plays an important role. The parasite assumes its multiplication within that cell while the macrophage starts the recognition process.

Phase two: The second phase after the lag phase is (the intracellular amastigote appearance phase) where inside the macrophage; the amastigote begins to show up multiplying and filling the cytoplasm of the phagocytes (Figure 1). Synergistically, the macrophage plays a role as an antigen presenting cell, which present the amastigote antigen in its surface to other cells of the immune system meaning the B, and T lymphocytes and natural killer cells. Microscopically, the lymphocytes are noticed in large numbers surrounding the infected macrophages at this point (Figure 1). Once the parasite is recognized by such cells, the lymphocytes morphologically and microscopically seem to behave somewhat different than the usual lymphocytic immune reaction. The usual immune reaction in cell-mediated immune response is characterized by T cell activation in to helper, suppressor or cytotoxic T lymphocytes, which morphologically show if at all, mild to moderate enlargement changes in those T lymphocytes. The B-lymphocytes usually are increased in size converting last to plasma cells with the nucleus positioned at one pole of the cell. In both cases, morphologically, there are no cytoplasmic protrusion or extension changes out of those lymphocytes. Here, may be due to its unique distinction as

being the only parasite that infects the macrophage and utilizes it for its own multiplication, major morphologic changes take place on a time basis within the lymphocytes. A polarization of the nucleus and an elongation of the cytoplasm within the lymphocytes are recognized. Later, the nucleus becomes more condensed and the cytoplasm becomes like a pale blue tail protruding out from the cell. Afterwards, the nucleus becomes even more condensed like a plain dark piece and it is impossible at that stage to microscopically differentiate any of its nucleus parts. Here the cytoplasmic tail becomes even thinner and more elongating while the whole size of what is believed to be a lymphocyte becomes overall smaller. Then, the cytoplasmic elements inside the lymphocyte tail disappear leaving it like a flagella looking sheath while the condensed nucleus appears in the other pole. The new-formed structure looks in shape very much like a giant type of the promastigote parasite. From the photos taken for those changes, a schematic diagram of the lymphocytes conversion in a sequence was built up (Figure1A). In this stage also, macrophages with the amastigotes multiplying inside are markedly noticed. Many of the macrophages become giant in size and some multinucleated cells appear with the amastigotes showing inside their cytoplasm. These phagocytes together with the different lymphocytes constitute the core microscopic features of our second stage (Figure 1). Interestingly enough, many of the macrophages seen tend to form elongating cytoplasmic tails protruding out. This phenomenon is called (The tail phenomena) (Figures 2,10). In this stage neutrophils are occasionally seen.

Reaching the end of this stage, the macrophages membrane tears out and the amastigotes are released in a large number into the extra cellular fluid leaving other microscopic features the same (Figure 3).

Phase three: (The promastigote appearance phase): In this phase, the already released amastigotes are present in the extra cellular fluid, which stimulate an acute phase inflammatory reaction characterized by neutrophils accumulation for phagocytosing and amastigote killing. Other figures present are decrease numbers of the lymphocytes, plasma cells, and what are known as the lymphocytes with tail, and the presence of what looks like giant promastigotes. We may also see a reducing

number of macrophages with the amastigotes inside. At this stage, in the extra cellular fluid, the amastigotes appear at different shapes from round to oval and to spindle while the chromatin inside is either spreading or condensed and taking a polar position. Interestingly, candle flame figures erupt out of those amastigotes, taking the spindle shape with a tail protruding out. At a later time on their development, some of those candle flame figures become polygons in shape reaching in maximum the RBCs size. They become difficult to differentiate from regular lymphocytes. Other growing parasites from the candle flame appear round in shape reaching also the RBC size. Some of those candle flame figures and the amastigotes too are phagocytosed by the neutrophils. We may later see many destroyed neutrophils as a result of such parasitic neutrophilic interaction (Figure 4). From that time on, some of those candle flame figures that have the spindle shape and the tail become more enlarged taking the shape of small promastigotes. Later on, the developing promastigotes are manifested at many different cytomorphologies (see figure 2A indicating the amastigote transformation into promastigote) making this phase by large, the promastigote appearance phase

Phase four: (The amastigote disappearance phase): At this phase the neutrophils have already consumed the extra cellular amastigotes, so the amastigotes are rarely seen in the extra cellular fluid while the macrophages and the multinucleated giant cells with the intracellular amastigotes tend to disappear, leaving the screen at the end of this phase with few neutrophils and plasma cells and few of the different lymphocytes including the mononucleated cells with tail and the lymphocytes originated giant promastigotes and increased number of the parasitic promastigotes and polygon figures. (Figure 5).

Phase five (The fiber formation phase): In this phase the parasitic polygons together with the lymphocytic giant promastigotes and the parasitic promastigotes start gathering and establishing the core elements for fiber production (Figure 6). Those parasitic promastigotes begin producing thick fiber structure from their flagella area until they are terminated with a thinning nucleus in a middle of a whole fiber (Figure 7).. By this way, those formed parasitic promastigotes are involved in fiber formation

and got embedded in the center of hairy like fiber elements which cause the disease to be controlled by controlling the causing factor which is the parasite and hence terminate the illness (Figure 3A illustrates the fiber formation on a step bases) .

Discussion:

One may question the credibility of such lymphocytes (with tails) whether being originated from lymphocytes or from the parasite during its development sequence. We had this dilemma in mind. But when going back through the disease process, it was found that the lymphocytes (with tails) appeared early on phase two and that is much earlier before the amastigote had transformed in to any promastigote form. The appearance of the polygon forms was declared in the middle of phase three. Those polygonal forms are the only forms, which may look like and may be morphologically confused with lymphocytes but they appeared later within the disease process. Additionally, the only figures that are seen in the start of the disease process are those macrophages and the different lymphocytes. So one can assume that the mononucleated cells(with tail) were developed from the lymphocytes.

Second, the question of the origin of the giant promastigotes from those lymphocytes (with tails): From the diagram attached (Figure 1A) and hundreds of photos taken (Figures 9,10), we can be sure that such conversion from the lymphocyte (with tails) to the giant promastigote took place.

But the question is what role does this conversion play in the disease sequence?

Our hypothesis suggests that from the immunologic point of view, first, the morphology of those mononucleated cell and macrophages (with tails) is unique, that we are uncertain of any such transformation in the morphology of either lymphocytes or macrophages like this has occurred in any other condition. Second, the parasitic promastigote produced by the amastigote seems to be so large for the neutrophil to phagocytose. In such case, the alternative process of the disease control seems to be in the direction of the parasite surrounding and embalming by hairy fiber formation. Here, an immunologic interaction takes place among the three figures the parasite in one hand and the macrophages and some subgroup of the lymphocytes in the other

hand. This subgroup of the lymphocytes becomes activated and is converted to lymphocytes (with tails). In their conversion process to a huge size promastigote, lymphocytes (with tails) had an obvious role to play. That role could be an educational role for the parasitic promastigote towards producing the fiber like materials so that the disease will control itself.

Other studies totally overlooked the neutrophils role in the disease process. In fact neutrophils, are best specialized in phagocytosing process while macrophages are considered less efficient than PMNs (polymorphonuclear neutrophils) at killing bacteria, and the mechanisms of killing are not as well understood (12). Accordingly, one can assume that those PMNs must have a strong role in phagocytosing and destroying the amastigotes released after membrane rapture of the infected macrophages (Figure 8). Otherwise, it is not possible to explain the amastigotes disappearance from the infected tissues after their release in that huge number into the extra cellular fluid.

In conclusion:

Researchers always relied on one fact that the only type of parasite present in the vertebrate including human being infected with leishmania is the amastigote form and they formulated all their pathological and immunologic understanding of the disease process on such an incomplete fact (2,3,4,5)

The presence of such amastigote and promastigote forms together in the vertebrate may modify our understanding to the pathology, life cycle and hence our approach for controlling and treating the disease. Talking about the disease, it not only is the cutaneous type of leishmaniasis but the visceral leishmaniasis (VL) as well as the mucocutaneous leishmaniasis. All the three parasite types share similar morphology. And though, this study did not check the presence of the promastigotes in the other tow types meaning, the mucocutaneous and the visceral leishmaniasis due to the limitation of resources, the similarities in morphology among those different parasites make them not even possible to differentiate their types by mean of microscopic exam (4). That permits us to predict similarities to our findings in cutaneous leishmaniasis in both types of parasites infection. Those similar findings

are the appearance of such promastigotes at their different stages of development starting from their amastigote transformation into promastigote form and ending with fully mature promastigote appearance inside the infected location of the human host.

Our new discovery with respect to leishmania parasites in general through the cutaneous leishmania species in particular allowed us the following advantages:

1- A better understanding for the disease process in its all stages.
2- A better understanding to the pathology of the disease and the macrophages, neutrophils and lymphocytes role in it.
3- A 100% sensitivity and high specificity for leishmania detection by the microscopic method.
4- A better understanding of the parasite life cycle and the role of both the victor and the host.

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The pathological features of cutaneous leishmania Photos

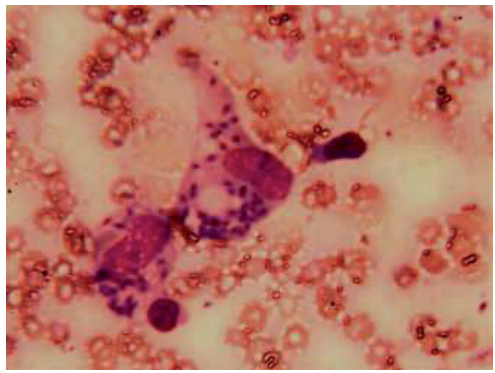
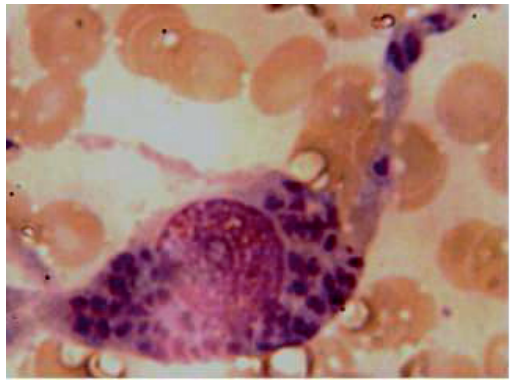


Fig. (1) X 400



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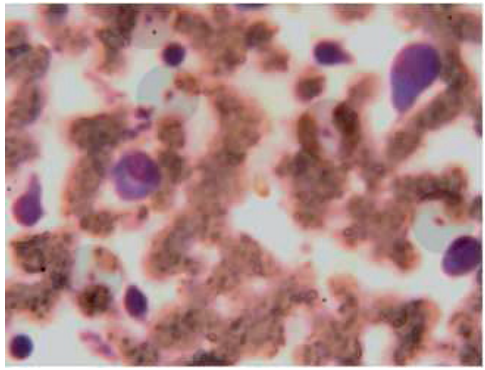

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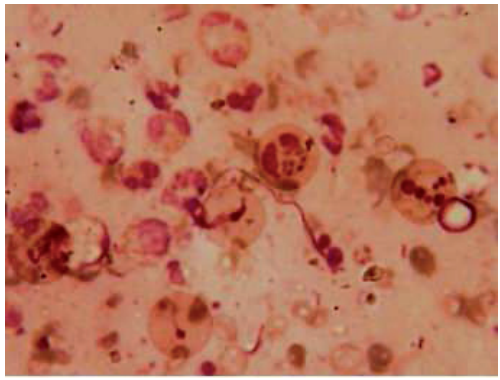
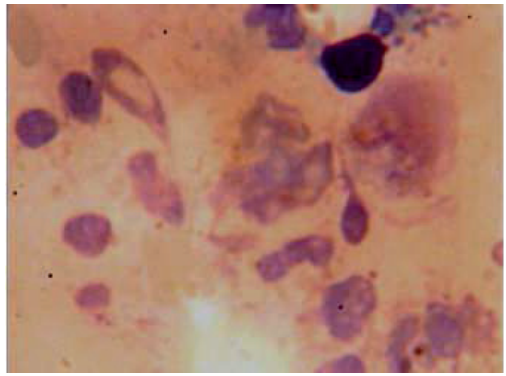


Fig.(4) X 400



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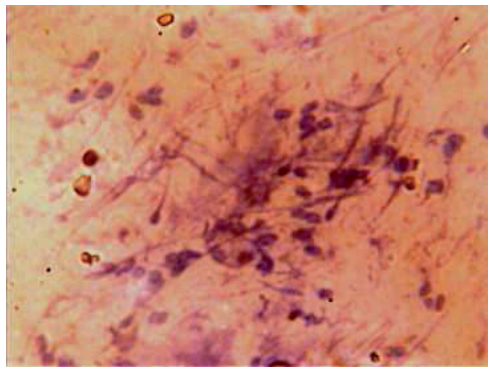


Fig.(6) X 200

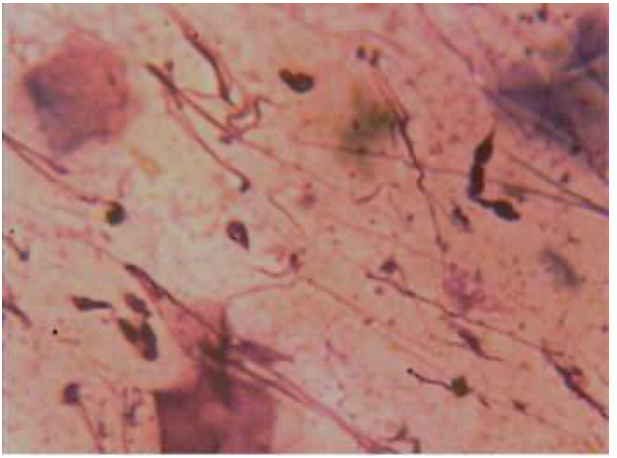


Fig. (7) X 400

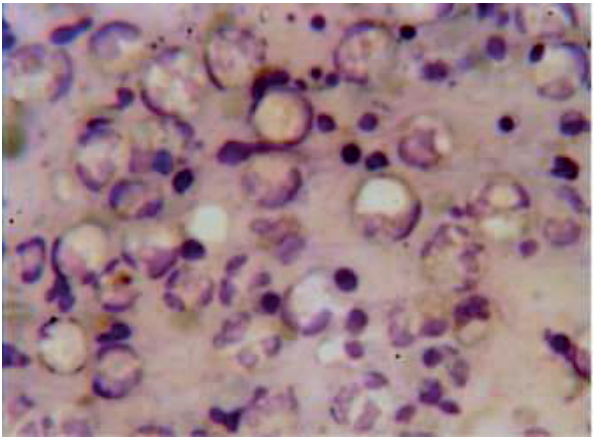
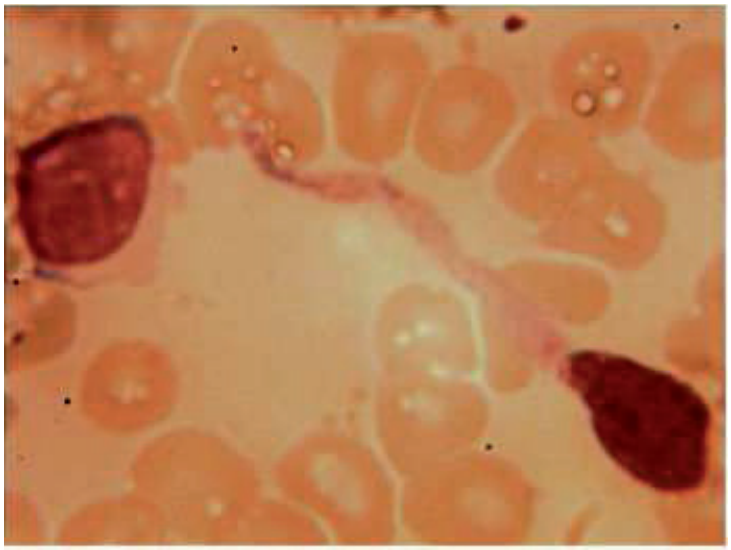


Fig.(8) X 400



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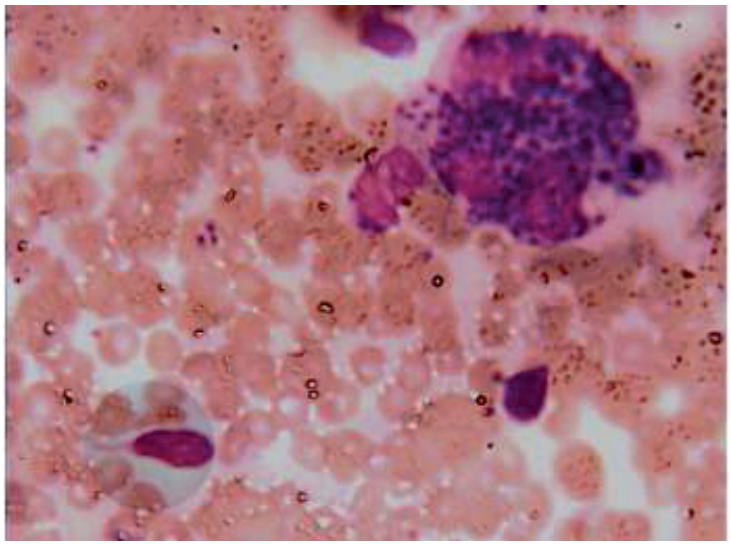

Fig. (10) X 400

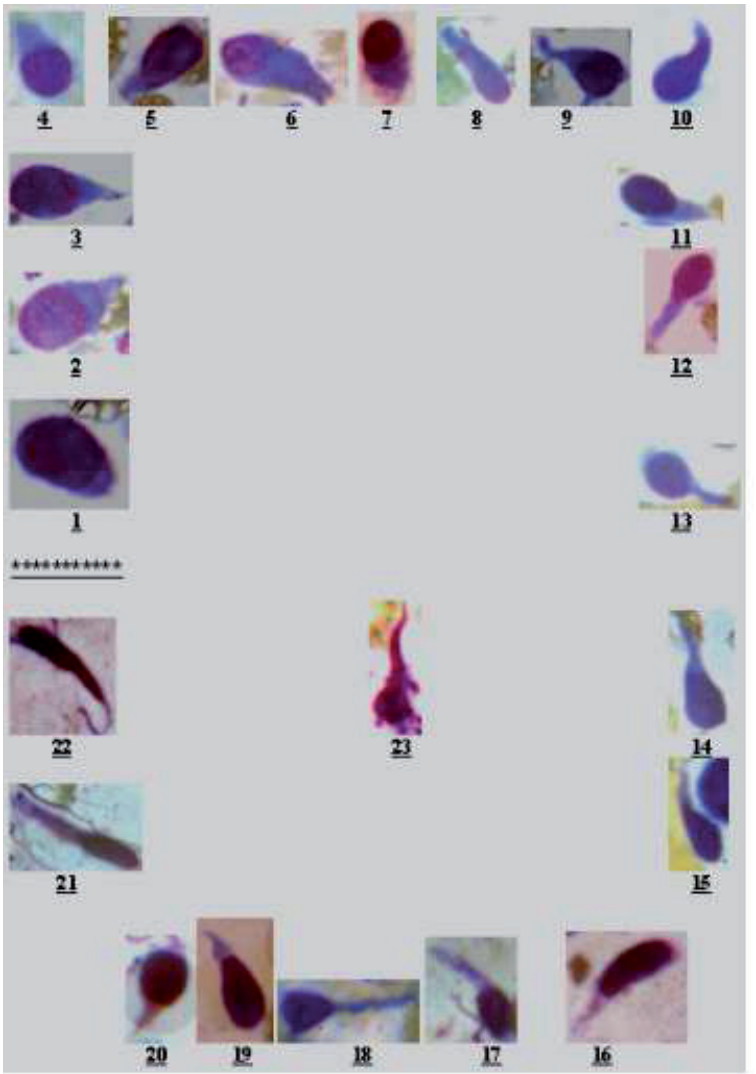

Fig. (1A)

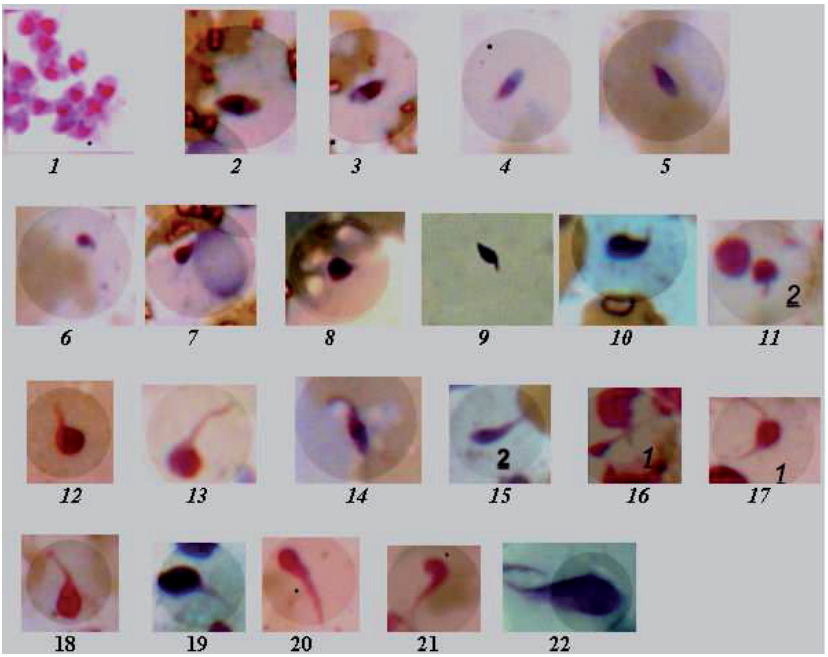

Fig. (2A)

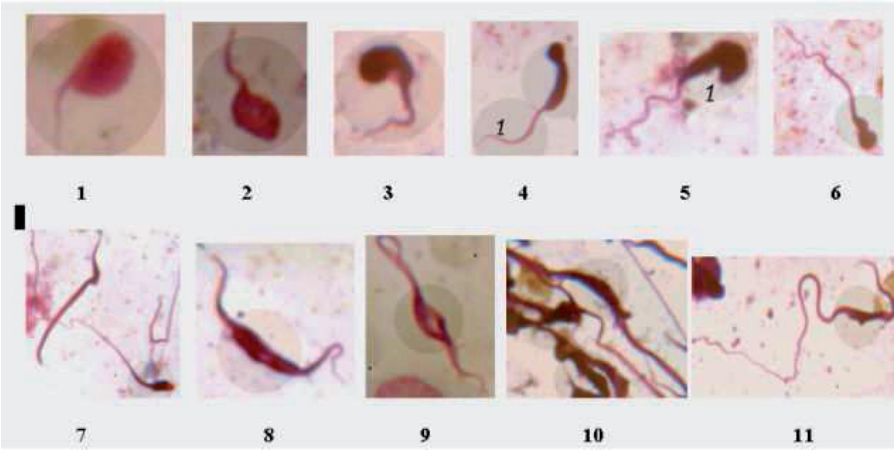

Fig. (3A)

7- The Origin and Properties of The Mononuclear Cells with Tails in Cutaneous Leishmania

Introduction and objectives: Cutaneous leishmania attacks the epithelium and the derm. It causes a dirty ulcer filled with dead tissues, cells and fluids(1). The parasite components are also present. The disease attracts the defending cells represented by macrophages, granulocytes(2), lymphocytes (fig.1), and plasma cells (fig. 2).

The observer of the disease process microscopically will recognize the appearance of the lymphocytes and plasma cells all a long the disease course(3). Lymphocytes and plasma cells have the same origin.

Lymphocytes are produced in the bone marrow from pluripotent stem cells. They fall into two main groups with different functions, T cells and B cells. The two groups are indistinguishable by traditional Romanowsky staining. T lymphocytes are responsible for cell mediated immunity. The final stage of B lymphocyte maturation (in bone marrow and lymph nodes) is plasma cells, whose nuclei often show radial bars, and whose basophilic cytoplasm layer is always wide. B lymphocytes and plasma cells are characterized by their ability to produce antibodies (immunogobulin), they are responsible for “humoral” immunity.

With the disease, cutaneous leishmaniasis, all the small monocytes seen under the microscope were classified under the categories lymphocytes or plasma cells(4). The purpose of the work was to apply an intense microscopic study to the lymphocytes seen in cutaneous leishmania and reclassify them. When a distinct cell is identified the origin of this cell is defined and then, its role in cutaneous leishmania is to be declared.

Methods: The studied sample consisted of 50 patients, males and females who had cutaneous leishmania. In this study, microscopic slides were prepared from the skin lesion secretion and stained for the direct smear with Wright stain and for the histological samples with HE stain (Heamatoxilin-Eosin) and later with LCA stain (which is an immuno-chemical stain specific for lymphocytes) and CD3 (an immuno-chemical stain specified for T-lymphocytes). A comparative

cytomorphologic study was conducted to those mononuclear cells with cytoplasmic protrusion among the different preparations with the different staining methods in order to confirm the presence of any resemblance or match among those presented cells in the different preparations. Microscopic photos were done for those presumed cells for results confirmation and documentation.

Results:

Table (1) indicates the incidence of the mononucleated cells (which look like lymphocytes) in the smears stained with Wright stain, according to their different cytomorphology.

Total # of cases studied	Incidence of the mononucleated cells (normal lymphocytes)	Incidence of the (Plasma cells)	Incidence of the mononucleated cells with tail
50	50	50	49
100%	100%	100%	98%

Table (2) indicates the results of the study comparing the cytomorphology of the mononucleated cells with tail under the different staining procedures and their match.

mononucleated cells with tail	Wright stain	H&E stain	LCA stain	CD3 stain
Degree of matching	+++	+++	+++	++++

The study revealed that three cell types are present: the first is the classical lymphocyte, which was present in 50/50 of the specimen prepared. The second was the plasma cell, though its concentration in the specimen was low when compared to the lymphocytes; it appeared in 50/50 of the prepared samples as well. The third was the mononuclear cell, which looks like the lymphocytes but with a cytoplasmic protrusion erupting out as a tail, with a different length among the different cells (fig.

3,4). Those cells appear in 49/50 of the specimen studied. The Immunohistochemical stain for the lymphocytes in general (LCA), was positive for the normal lymphocytes, those monocytes with the cytoplasmic protrusion and the giant promastigote-like cytomorphologic figures, which are found in the cutaneous leishmania smear indicating that all those figures are derived from lymphocyte origin (fig. 6).

Both the cells and the giant promastigote like forms also took the CD3 stain, a specific stain for (T) lymphocytes (fig. 7). A high degree of cytomorphologic matching was noticed among the mononuclear cells (with tails) when comparing the smears with the different stain.

Discussion: The presence of a new type of cells looking like lymphocytes with a cytoplasmic protrusion was proved. This protrusion may increase in length to have a tail appearance giving the impression of a giant promastigote-like (fig. 8,11). These cells were present in 98% of the studied cases. That makes these cells a defined cytomorphologic figures featuring cutaneous leishmaniasis. On defining the origin of those cells, three cases among the 50 were chosen and studied histologically after staining them with H-E (fig. 5) and later with (LCA) the immunochemical stain for the lymphocytes in general. As expected, both the lymphocytes and the cells with cytoplasmic protrusions or tails were positive for the stain. That concludes that those cells with cytoplasmic protrusion or tails are originated from lymphocytes. In order to define the type of lymphocytes those cells were derived from, an other stain was used for the three specimens above, which is the (CD3) stain for the T specific lymphocytes. It appeared that (the cells with tail) were also positive for such a stain. This concludes that these cells are derived from the T lymphocytes. Lewis observed in lymphocyte locomotion, that the cell first extends a pseudopod separated from the rest of the cell by a groove that encircles the cell body. Eventually, as the groove deepens, the nucleus is pushed forward through the constriction ring, giving rise to the classic shape of the motile lymphocyte, resembling a hand mirror or pear. The advancing front is occupied by the nucleus, which is separated by a deep constriction from the rest of the cytoplasm that trails behind, forming the handle of the mirror.

The cytoplasmic tail is called the *uropod.* Lewis in his previous observation was taking about the exact same mononucleated cells
with tails we are discussing.
The tail in some of those cells may be elongated up to 20 micron in diameter, which make their whole cytomorphology looks in morphology, like the parasitic promastigote but with a very large size as seen in Wright stain(fig. 8). This giant promastigote form was positive for both LCA (fig. 9) and CD3 (fig. 10).
Coming into the role those mononucleated cells with tail in cutaneous leishmaniasis, after the amastigotes disappearance from the lesion (5), through the function of neutrophils phagocytosis, those mononucleated cells with tails become the major active cells, indicating a chronic phase inflammatory reaction(6).
T lymphocytes, play major roles in cell-mediated immunity along with macrophages(7). They protect against intracellular pathogens, and are involved in delayed type hypersensitivity reactions, and transplantation rejection(8). Those mononucleated cells with tails being of lymphocytic origins and are specifically derived from T lymphocytes must play the same role the original T cells play.
On the other hand cutaneous leishmania, is a chronic type of disease which lasts for a year or more. Then it is reasonable to say that those lymphocytes with tail in the disease last episode, microscopically are shown to act forming nests-like, hunting for and capturing the parasite organelles within the lesion area of the skin and trapping them in those nests aiming from that, to control the disease. The sporotrichoid granulomatous nature (3) of the lesion towards the disease end, with a permanent scar formation, may then be partially explained by the function of those cells with their elongated irrupting tails.

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The Origin and Properties of The Mononuclear Cells with Tails in Cutaneous Leishmania Photos

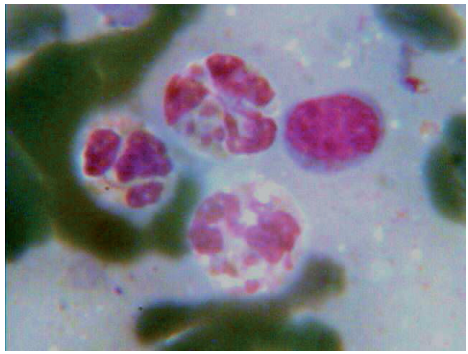
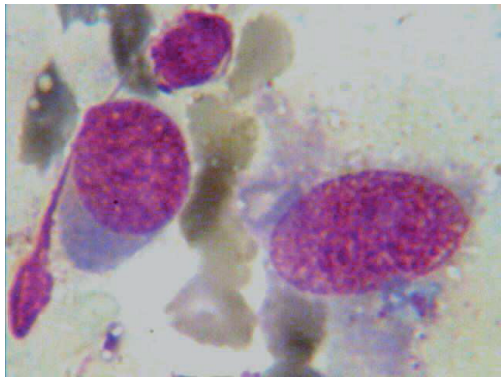


Fig.**(1)**



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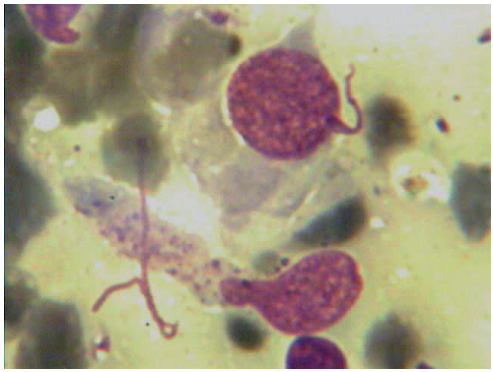

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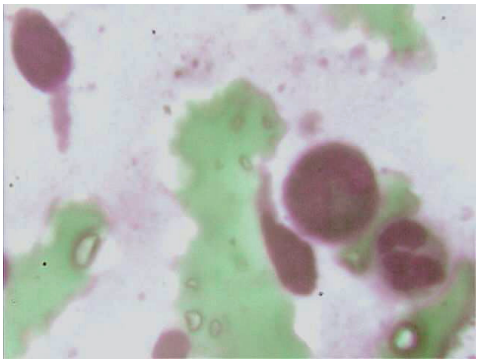
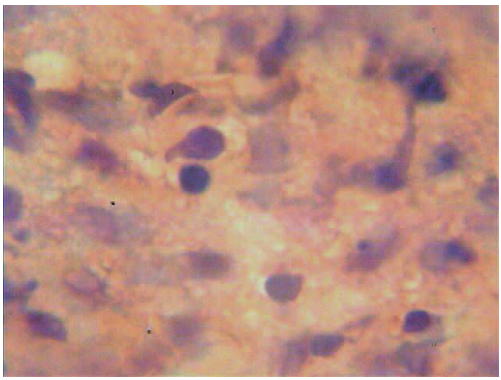


Fig.**(4)**



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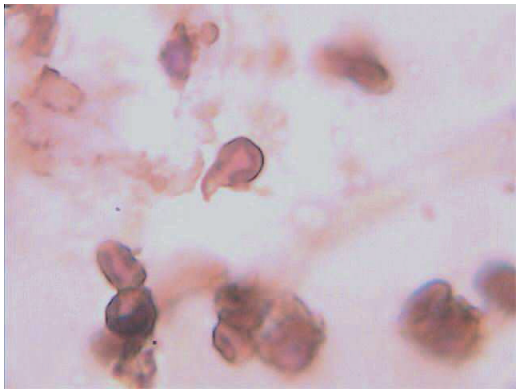

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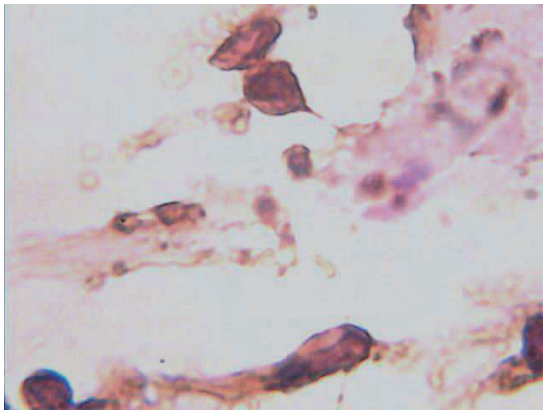
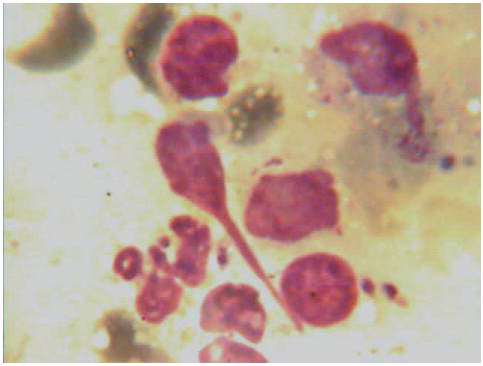


Fig. **(7)**



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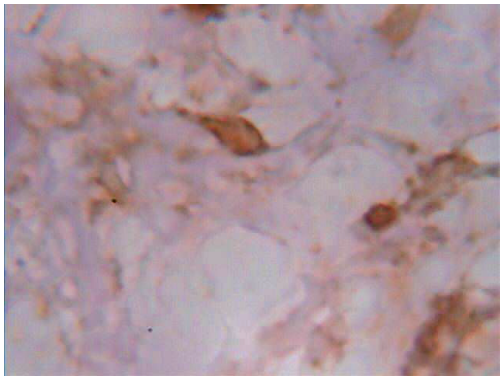

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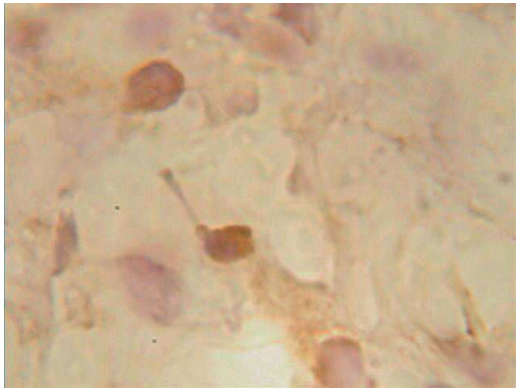

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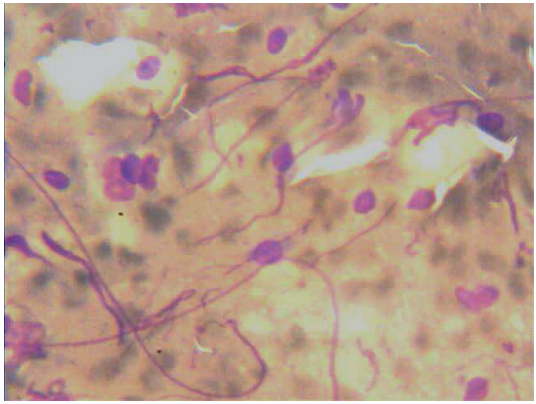

Fig. (11)

8-Toward an approach for cutaneous leishmania treatment

Abstract::

Introduction: Most drugs being used for cutaneous leishmania treatment are still non well effective, extremely expensive, risky with side effects, more invasive and relapses still occur. The purpose of the study is to achieve more understanding of cutaneous leishmania disease and through that to try an other form of treating substance that can provide better results with less damages to the tissues.

Methods: Seven patients infected with cutaneous leishmania were chosen for DAB-1 application. Clinical as well as microscopic study and follow-up with documenting photos for the lesions, indicating the starting point for the cases before treatment initiation, and the disease development after DAB-1 application was accomplished.

Results: Before treatment, the lesions size was between 1.8-7 cm. Cases were inflamed and ulcerated. After 8 days of treatment, inflammation shrank. After 16 days, the lesions and the ulcers decreased into almost half their size. 24 days post treatment, inflammation began disappearing and epithelial islands continued to grow inside the ulcers filling a considerable part of them. By the end of day 32, ulcers were covered with a continuous layer of epithelium, and heal is achieved after two to three months of treatment.

Conclusion: The study proved that DAB-1 is capable of healing leishmania in 6-8 weeks after application and is compared favorably to the other traditionally used drugs. DAB-1 could be a breakthrough in cutaneous leishmania treatment.

Introduction:

Many studies have indicated that the drug of choice for treating cutaneous leishmania is Sodium antimony gluconate (Pentostam) (1). It is applied intramuscularly in case of multiple lesions. In a single lesion, it is usually injected into the ulcer margin (2). The healing process of the lesion usually takes between 14 to 16 weeks and in some cases even more. The heal is usually completed with a scar formation. If present in a delicate location like in the face, the lesion can cause a bad defect and deformation

for the patient. Although it is the drug of choice, Pentostam induces side effects (3). Even with its application, Pentostam cannot induce a complete heal with a perfect epithelization. The final result in the lesion in-position is a permanent scar formation. Follow-up studies in south-western Europe, using pentavalent antimonials, show a positive response in 83% of cases. However, 52% of patients relapse within a period of one month to three years(4) .

Other drugs being used for cutaneous leishmania treatment are paromomycin, Amphotericin B, Fluconazole and Pentamidine, but relapses still occur with a risk of side effect and the drugs remain extremely expensive.

DAB-1 on the other hand, is a topical ointment, which is applied topically to the ulcer lesion. It is produced from natural substances and obviously, has no side effects.

The purpose of this study is to measure the DAB-1 effect on cutaneous leishmania by tracking both the clinical and the cytomorphologic effect, and checking the healing process over a period of two to three months.

MATERIAL And METHODS:

Seven patients infected with cutaneous leishmania and presented with clear lesions were chosen for DAB-1 application. Before applying the ointment, couple of microscopic slides were prepared from each lesion stained with Wright stain and tested for all the suspected pathological features including the parasite form found in the intra or extra cellular space. Clinical as well as microscopic photos for the lesions beginning with the starting point for the cases before treatment initiation were taken for documentation.

Each lesion was cleaned with normal saline and dried with gauze. The ointment is added to a sterile gauze and applied directly over the lesion. The ointment dressing was changed once every day for 24 consecutive days. After that and for the next 24 days, the ointment dressing was changed once every three days. Every three days couple of photos for each lesion were taken to mark the clinical signs of the healing process. At 8,16, 24,32,45,60 days interval, other couple microscopic smears were prepared from each lesion, stained with Wright stain and tested to identify the

cytomophologic development of the cure process. Documentary microscopic photos were taken for the cytomorphologic features for more detailed study and analysis.

Results:

Table 1 shows the clinical features present for each lesion before the ointment application.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Lesion size	7 cm	3 cm	5 cm	3 cm	1.7 cm	4 cm	1.8 cm
Inflamed	+++	++	+++	+/-	+/-	++	-
Ulcerated	+++	-	+++	++	-	++	-

Note: (+++) Highly inflamed, or ulcer> 2cm in diameter. (++) Moderately inflamed or ulcer 1-2 cm in diameter.(+/-) indicates mild inflammation. (-)No inflammation or nodular type with no ulcer.

Table 2 shows the clinical features development for each lesion after 8 days of the ointment application.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Lesion size	6 cm	2 cm	4 cm	2 cm	1.3 cm	3 cm	1.5 cm
Inflamed	++	+	++	+/-	+/-	+	-
Ulcerated	+++	-	++	++	-	++	-

Table 3 shows the clinical features development for each lesion after 16 days of the ointment application.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7

Lesion size	4 cm	2 cm	1.5 cm	2 cm	1 cm	2 cm	1 cm
Inflamed	+	+	+	-	-	+	-
Ulcerated	++	-	++	+	-	++	-

Table 4 shows the clinical features development for each lesion after 24 days of the ointment application.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Lesion size	3 cm	1 cm	1 cm	1 cm	0.8 cm	1 cm	0.5 cm
Inflamed	+	-	+	-	-	+	-
Ulcerated	+	-	+	-	-	+	-

Table 5 shows the clinical features development for each lesion after 32 days of the ointment application.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Lesion size	3 cm	1 cm	1 cm	1 cm	0.6 cm	1 cm	0 cm
Inflamed	-	-	-	-	-	-	-
Ulcerated	-	-	-	-	-	-	-

Table 6 shows the cytomorphologic features present in the slides for each lesion before the ointment application.

Cytomorphologic figures appearance	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Phagocytes with intracellular amastigotes	-	++	++	-	++	+	-
Extracellular	-	++	+++	-	+	++	-

amastigotes							
Promastigotes	+++	+	+	++	++	++	++
lymphocytes	++	++	+++	++	+	++	+
Neutrophils	++	-	+	+	-	++	-

Note: (+++) > 5 cells or leishmania configurations seen per HPF. (++) 3-5 cells or leishmania configurations seen per HPF. (+) 1-3 cells or configurations in average seen per HPF. (-) No cells or leishmania elements seen.

Table 7 shows the cytomorphologic features present in the slides for each lesion after 8 days of the ointment application.

Cytomorphologic figures appearance	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Phagocytes with intracellular amastigotes	-	-	-	-	+	-	-
Extracellular amastigotes	-	-	-	-	+	-	-
Promastigotes	+++	++	+++	+	+++	+++	+
lymphocytes	+	++	+	+	+	+	+
Neutrophils	++	++	+	++	+	++	+

Table 8 shows the cytomorphologic features present in the slides for each lesion after 16 days of the ointment application :

Cytomorphologic figures appearance	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Phagocytes with intracellular	-	-	-	-	-	-	-

amastigotes							
Extracellular amastigotes	-	-	-	-	-	-	-
Promastigotes	+	+	+	+	++	+	-
lymphocytes	+	-	+	+	+	+	-
Neutrophils	+++	++	++	+	++	+	-

Table 9 shows the cytomorphologic features present in the slides for each lesion after 24 days of the ointment application.

Cytomorphologic figures appearance	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Phagocytes with intracellular amastigotes	-	-	-	-	-	-	-
Extracellular amastigotes	-	-	-	-	-	-	-
Promastigotes	+	+	+	-	+	+	-
lymphocytes	+	+	+	-	+	+	-
Neutrophils	+	+	-	-	+	+	-

Table 10 shows the cytomorphologic features present in the slides for each lesion after 32 days of the ointment application :

Cytomorphologic figures appearance	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Phagocytes with intracellular amastigotes	-	-	-	-	-	-	-

Extracellular amastigotes	-	-	-	-	-	-	-
Promastigotes	+	+	-	-	-	+	-
lymphocytes	+	-	+	-	-	-	-
Neutrophils	+	+	-	-	-	+	-

Tables 1-5 show the clinical features present for each lesion before and after DAB-1 application on time bases.

It is clear from table 1 that the size of the lesions is between 1.8 and 7cm when cases were referred . All the cases except one were clinically inflamed. Four of the cases were ulcerated and the other three were not ulcerated (Figures 1A, 1B).

After 8 days of treatment with DAB-1, the first sign of healing process to show in table (2) is the decrease in the size of the lesion characterized by the inflammatory reaction in a range between 16-33%. The inflammation also showed a clear reduction in its virulence while a decrease in the ulcer size appeared in only one case with the ulcer decreased about 0.4 cm in its size (Figures 2A, 2B).

After 16 days of treatment and according to table (3) each lesion was reduced in size to about half. The inflammation virulence was further decreased in almost every case and the inflammation disappeared from two additional cases. All ulcers were reduced in size to less than 2 cm in diameter. Isolated islands of epithelial cells started to show up inside the ulcers (Figures 3A, 3B).

By the end of day 24 of treatment, as seen in table (4) all the cases were reduced to less than half their original size. The ulcers and the inflammation totally disappeared from 4 of the cases. Mild inflammation and small ulcers with less than 1 cm in diameter were seen in the three left cases. The epithelial islands continued to grow inside the ulcers filling a considerable part of them. (Figures 4A, 4B).

By the end of day 32 of treatment, looking back to table (5), a relief in the inflammatory reaction was noticed and the ulcers disappeared from all the studied cases. Ulcers were covered with a continuous layer of epithelium. The size of the lesions continued to be the same as was the case in day 24 but with the disappearance

of the clinical disease signs (Figures 5A, 5B). On day 45, the lesion area continued in process toward a normal looking skin(Figures 6A, 6B). And on day 60, the lesion looked almost close to normal with a remote inflammation (Figures 7A, 7B). After 75 days of treatment initiation, the skin started to appear normal (Figures 8A, 8B), and after three months completion, the skin in the area turned into normal looking (Figures 9A, 9B)

Tables 6-10 show the cytomorphologic features present for each lesion before and after DAB-1 application.

In table 6 we notice the cytomorphologic features before DAB-1 application. In four of the cases, the intracellular and extracellular amastigotes were present together with the infected macrophages in the smear. Promastigote- like figures and lymphocytes appeared in all the seven studied cases at different concentration, indicating the chronic nature of the disease. Neutrophils were present in 4/7 cases. (Figures 1C, 1D).

After 8 days of treatment as appears in table 7, a disappearance of the amastigotes and their infected macrophages from the smears appeared in 6/7 cases, while the promastigote like forms continued to appear in all the cases. Under the microscope, a decrease in lymphocytes count and elevation in the neutrophils count appeared in each case. The presence of such increase number of neutrophils is a sign of the disease conversion from a chronic to an acute inflammatory reaction (figures 2C, 2D).

By day 16 as shown in table 8, there was a total disappearance of the infected macrophages together with the amastigotes from all the cases. Neutrophils continued to appear with a little less count in all cases but one, where a complete disappearance of all the disease microscopic cytomorphology was noticed. Promastigote like forms appeared in 6/7 of the cases but in less concentration. Lymphocytes were present in less count. Larger area of immigrating epithelium was noticed. (figures 3C, 3D).

In day 24 (table 8): A less concentration of all the microscopic disease figures including promastigote like forms, Lymphocytes and neutrophils was noticed with a

total disappearance of all the microscopic signs in two of the cases. More epithelial cells were present. (figures 4C, 4D).

In day 32 very few promastigote like forms were still present in three of the cases and few lymphocytes and neutrophils were seen in the smear. Overall, other smears appeared almost like normal blood smears with few migrating epithelium. (figures 5C, 5D).

Discussion:

One of the important things to be illustrated in this study is the total disappearance of the amastigotes form both in the intra and the extracellular reign together with their engulfing phagocytes. It is interesting to see the lesion still clinically hyper active and inflamed with the disappearance of such important disease figures. Other studies have observed this same phenomenon (5). One answer for that dilemma is that the amastigote form is not necessarily the only influential factor during the whole process of the disease course, or the amastigote might have been active at one stage and then disappeared from the lesion. That contradicts the previous basic understanding of the disease process. It is clear from all previous data and literature presented, that the amastigote form is the main acting player and causing organism in cutaneous leishmania disease process. According to Hepburn NC who summarizes the general previous understanding of the aspects of the disease: (In all forms of leishmaniasis the presence of amastigotes within the cells of the mononuclear phagocytic system remains the hall mark of the disease, though they sometimes may be difficult to detect.) (6). Indeed, the true fact is that the amastigotes at certain time of the disease are impossible to detect. The disappearance of such giant cells infected with the amastigote form while the disease process both clinically and cytomorphologically is still in action, indicates that such phagocytes, giant cells, macrophages and monocytes, at specific point of the disease process become resistant to be infected with the amastigotes. Literature tells (the amastigote forms multiply by binary fission, within the macrophage until the host cell is packed with the parasites and ruptures, liberating the amastigotes into circulation-then the free amastigotes invade fresh cells, thus repeating the cycle) (7). In contrary with the

previous concept, those amastigotes released into the extracellular fluid at a specific stage, become unable any more, to invade the adoptive phagocytes or replicate within those macrophages. This inability, is explained by an immune interaction between the host immune system and the leishmania parasite determinants (8) which causes a development of resistance in the phagocytes against the parasites. This is in the disease course, where we notice the disappearance of the phagocytes with the intracellular amastigotes from the smear.
Tables 7,8,9 and 10 confirm the disappearance of the amastigotes from the smear at that point.
As noticed in this study, DAB-1 ointment worked favorably on hastening the disappearance of the amastigotes from the lesion when compared to the disease in its natural process.

According to the traditional definition: *(*Leishmania are intracellular parasites that infect the mononuclear phagocytes. Leishmania are obligatory intracellular parasites) (7). This definition means that extracellular amastigotes cannot survive the harsh extracellular environment. Literature did not explain the way the expelled amastigotes disappear after being released from the infected macrophage. As we see clinically (by going to tables 7-9), the disease in that stage is deeply active and still not yet healing. It is clear then that, the extra cellular amastigotes must have been able to find a way to survive in the extra cellular fluid (9) and to grow from such an inactive form (the amastigote form) which is an ova like form into the active form which is the flagellate a (promastigote like form) (10). The promastigote form then that replaces the amastigote is the one that is developed and becomes the active form penetrating the subcutaneous tissues and causing the real signs and symptoms of the disease at that stage.

Coming to the pathology of the disease, according to Hepburn: (Over the following months, there is a gradual decrease in the number of amastigotes and macrophages, leaving a granulomatous infiltrate consisting of lymphocytes, epithelioid cells and

multinucleate giant cells. At this stage it may be difficult or even impossible to detect organisms “pointing to the amastigote form” in H&E, or Giemsa, stained sections.)(6). As shown in tables 2,3,4,6,7,8 and9, the lesion at that stage is still clinically deeply inflamed with large ulcer and exudates. In fact, Those clinical and pathological signs still represent an active form of the disease process. Here we notice clearly, the appearance of many cytomorphologic forms that have been developed through the process of amastigote transformation. Those forms are Promastigote and fiber forming promastigote like forms, candle flame forms, the spherical and polygon forms (11). All those forms are active forms of the parasite inside the tissue, and at that point, they are the ones causing the pathological inflammatory signs (fig. 1D, 2C,2D, 3C,3D, 4C). After the amastigotes are released from the infected macrophages to the extracellular space, the fetus erupting from the amastigote will develop in a series of steps starting from a candle flame shape into a mature promastigote like form (12). Those active forms of the parasite will never turn back or transform in side the host vertebrate into the amastigote form again. By that the amastigote forms disappear from the smear and what is left is destroyed by the function of neutrophils (13) as shown in tables (7,8,9). Once the parasite is in its active form in the body, which is the promastigote form, and without drugs interference, the immune system represented by the lymphocytes will further react to control the flagellate type of the parasite by introducing T type lymphocytes with a protruding tail that is capable to form a base for a nest- like; that will trap those flagellate type of parasites and terminate the disease with a permanent scar formation(11). That comes in agreement with the statement written by Hepburn: (There is, however, considerable variation: some lesions do not ulcerate, others develop sporotrichoid nodular lymphangitis. Most lesions heal over months or years, leaving an atrophic scar.) (6).

In most cases of treatment using the different types of drugs and injections like Sodium antimony gluconate (Pentostam) or Pentamidine, the disease will heal leaving a permanent scar behind it. Relapses still occur and the drugs remain

extremely expensive. That is not the case with DAB-1 product. As seen from the follow-up smears, DAB-1 has a stimulatory effect on the epithelium. This study shows that from day 16 of DAB-1 application (fig 3A, 3B, 3C 3D), the epithelial cells become active and regenerate moving from the edges forward into the ulcer center, forming isolated islands. In day 24, the islands gather and start covering the whole ulcer and by day 32, one whole layer of epithelium covers the full ulcer (fig 4A, 4B, 4C, 4D). DAB-1 and according to the cytomorphologic figures seems to be active in killing the parasite. It is obvious that the parasite count was decreasing from the time of DAB-1 application. Not only that, but DAB-1 seems to have a chemotactic effect on neutrophils. As we notice from tables 7 & 8, the neutrophils increased in number in the lesion after 8 days of DAB-1 application and continued to increase by time. The fiber forming promastigotes with their fibers are left to the action of those neutrophils to degenerate and get red of. By the end of the disease process, the connective tissues with the upper layer of the epithelium will rejuvenate forming a normal cutaneous tissue with no scar left (figs 4D, 5A,5B, 5C, 5D). Follow up of one case up to 2 years after treatment with DAB-1, declares no relapses observed .

In conclusion :

DAB-1 is capable of healing leishmania in 6-8 weeks after application and is compared favorably to any other used drugs where the healing process may take between 14 to 16 weeks and in some cases even more.

DAB-1 has the power to stimulate the epithelium regeneration, migration and multiplication. it is more effective than the other applied drugs in generating normal tissues for cosmetic reasons.

DAB-1 has a strong anti-parasite effect on leishmania. This can be confirmed by the parasite disappearance from the smears within time.

DAB-1 seems to have a stimulatory effect on neutrophils function, as they act on the sporotrichoid nodular fibers produced by the parasite reducing the effect of the scar formation.

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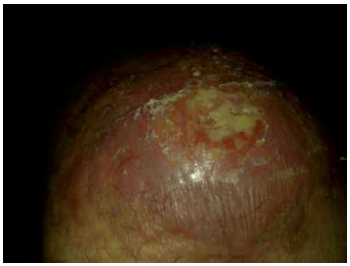


Fig. 1A

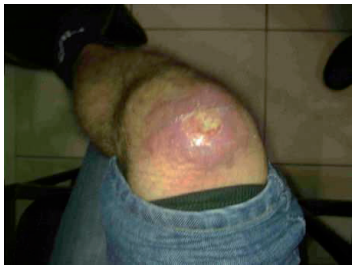
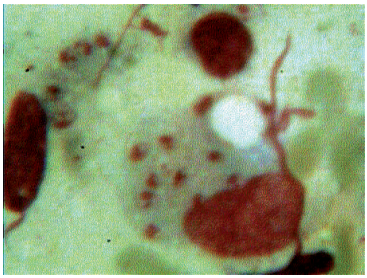


Fig. 1B



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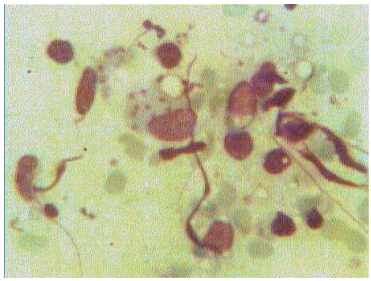

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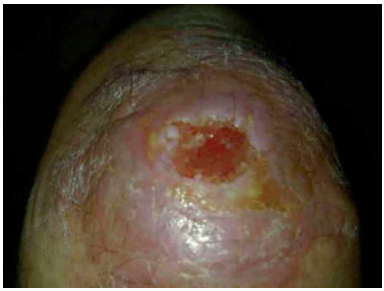

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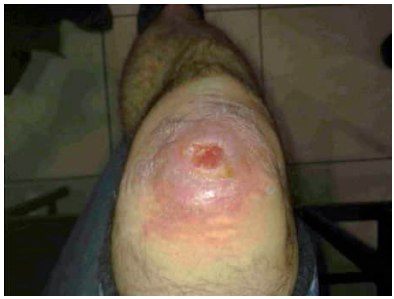

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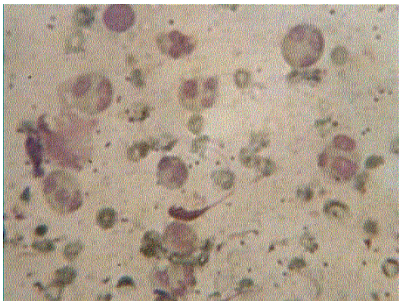

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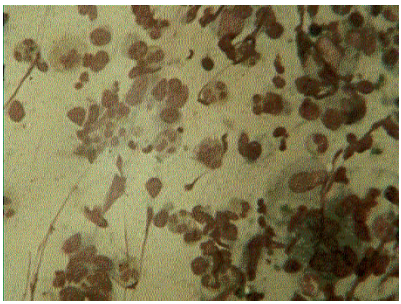

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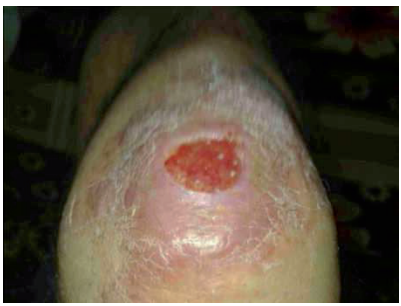

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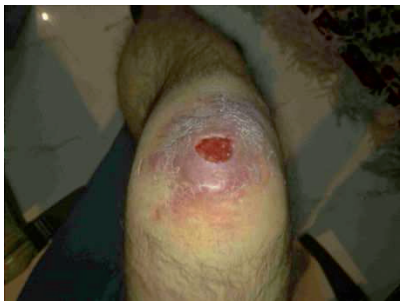

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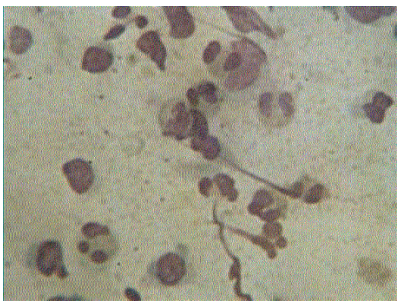

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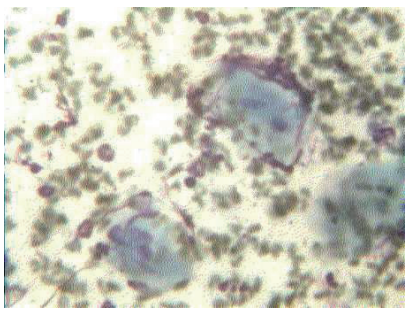

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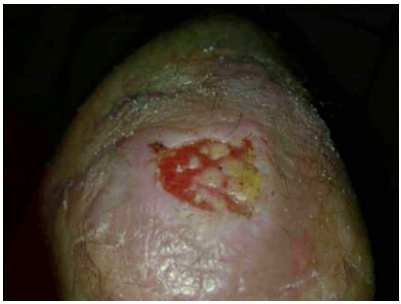

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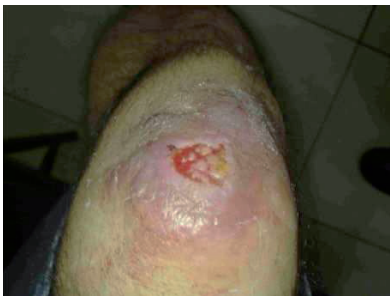

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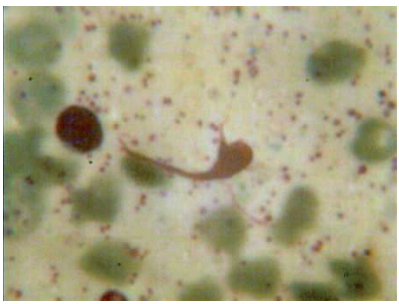

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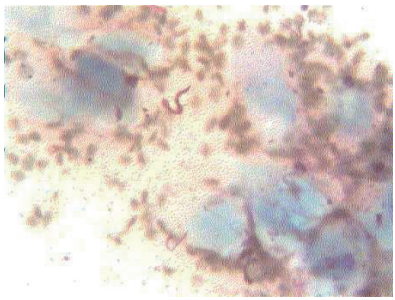

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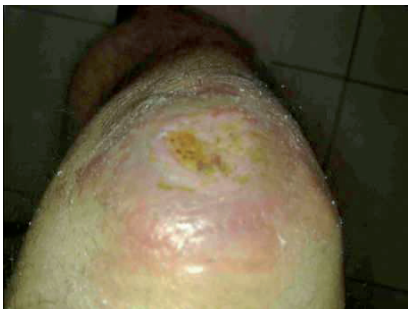

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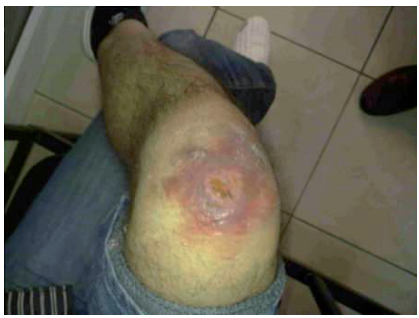

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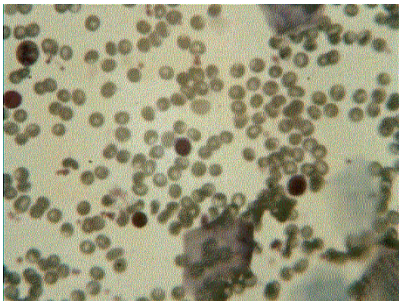

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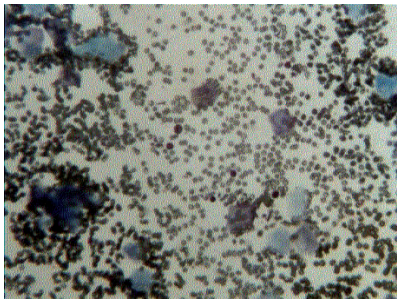

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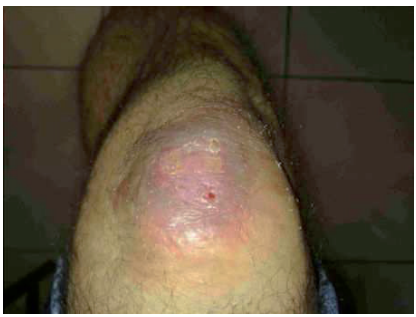

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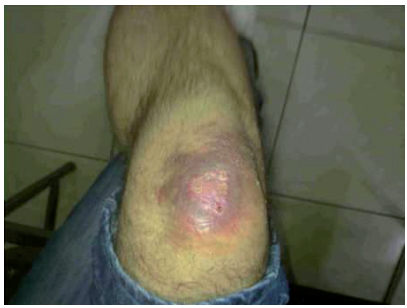

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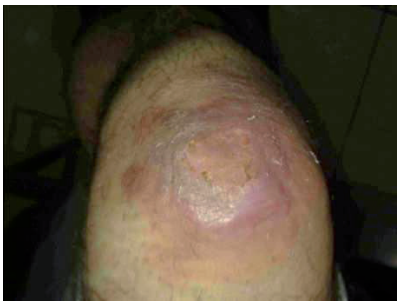

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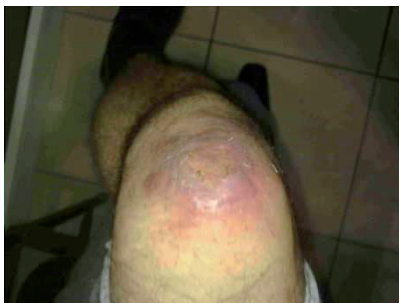

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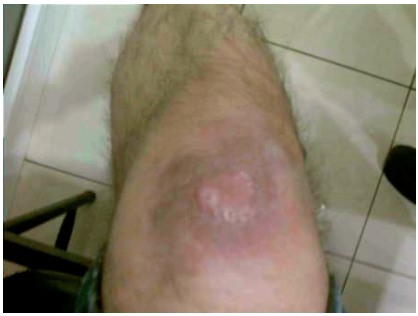

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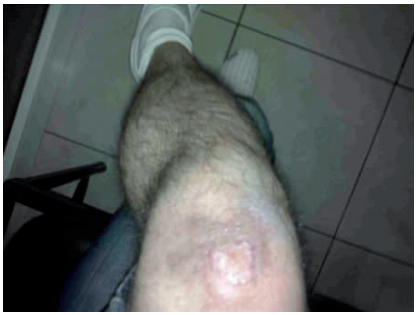

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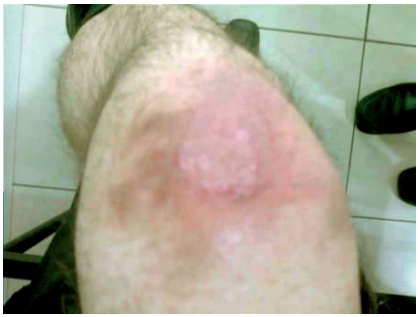

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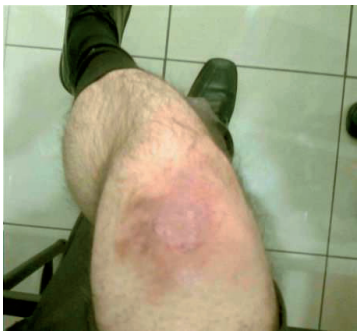


Fig. 9B

Primary Health Care & Family Medicine

Definition of Primary Health Care:

Practical, scientifically sound, and socially acceptable method and technology; universally accessible to all in the community through their full participation; at an affordable cost; and geared toward self-reliance and self determination
(WHO & UNICEF, 1978).

Primary health care is primary care applied on a population level. As a population strategy, it requires the commitment of governments to develop a population-oriented set of primary care services in the context of other levels and types of services.

Primary Health Care exists to provide high quality and cost effective promotive, preventive, curative and rehabilitative health care services for the community, in a comprehensive bio-psycho-social approach in line with international standards.

Primary Care:

Primary Care is the provision of integrated, accessible health care services by clinicians who are accountable for addressing a large majority of personal health care needs, developing a sustained partnership with patients, and practicing in the context of family and community. (1996 IOM Report on the Future of Primary Care.) 1*

Primary Health care is one of the first levels of contact of individuals, families, & community with the national health system in many countries. It is a way of achieving health for all. It should be conceived as an integral part of any country's plan for socioeconomic development.

A call for transformation of health services into family medicine based services, was emphasized by WHO in lieu with the international, regional institutions trends.

Characteristics of Family Medicine/General Practice:

…. Seek appropriate consultation when indicated.

…. Provide continuity of care throughout the patient's lifetime.

…. Represents a synthesis of knowledge from other medical disciplines plus contributions from Family Medicine.

…. View the whole patient as a unique individual as a person.

…. Identify the patient's problems within the context of family, environment and culture.

…. Manage the majority of these problems – especially those which occur frequently.

…. Counsel & promote health education (patient and peers).

…. Mindful of the cost effectiveness related PHC services.

Main Challenges faced by the primary health systems in devastated locations in EMR:

- Poverty & lack of access to basic services.

- Programs sustainability.

- Insufficient Political commitment.

- Weak linkages between PHC and mobilized community.

- Inadequate distribution of resources (human and non-human).

- Deteriorated physical infrastructure- water-sewage systems & environmental degradation. 2*

- Limited institutional and human resources capacity building, and lack of an electronic health information system.

- Moving obstacles from hospital oriented health system to primary health care (health professionals, community, etc.)

- Inadequate care continuity - personnel, pathway, appointment.. 3*

Social Determinants of Health:

The social determinants of health refer to both specific features and pathways by which societal conditions affect health and that potentially can be altered by informed action. 4*

SDH in Conflict and post-conflict emergencies:

Conflict affects the most vulnerable, exposes fault lines in society. It is Likely to increase inequities between those who can, leave and those who cannot and are forced to stay. Those the last, lose major parts of human rights: security, food, shelter, social interaction, health care etc. Conflicts are always associated with breaches of medical neutrality and so far progression from stress to distress and disease.5*

Equity in Health and Health Services:

It is the absence of systematic and potentially remediable differences in one or more aspects of health across population groups defined geographically, demographically, or socially.

Equity Research is not the same as Social Determinants Research.

• Social determinants research assumes an individual model of health.

• Equity research assumes a population model of health.

CDC and PHC: mutual benefit:

-The control of communicable diseases plays a vital role in the achievement of "health for all"

- Integrating communicable diseases prevention and control programs with PHC is essential to ensure:

– Better access to prevention and treatment services.

– Equity in distribution of the services.

– Sustainability of services.

Strengthening Communicable Diseases Control through PHC:

-Case detection: District level lab technicians on detection of CD.

- An effective drug supply and Standardized treatment: District center keeps and maintain CD records and provide necessary treatment.

- Provision of vaccination: PHC centers are the main vaccination delivery points.

- Monitoring and evaluation system: Keeping and maintaining diseases and immunization records.

Contributing to health system strengthening through CD Control:

-Building human capacity through basic training, periodic in-service training, supervision and follow-up.

-Enhancing better utilization of human resources: multi-purpose health workers.

- Improving community participation.

- Immunization and Control of Vaccine-Preventable Disease. 6*

Linking Immunization activities to PHC:

1- Vaccination is basically provided through the PHC services.

2- Reaching Every District (RED) approach: emphasis on:

– Reaching all villages with at least 4 times a year with regular routine immunization services.

– With Fixed and/or Outreach and/or Mobile activities.

– Optimizing utilization of available resources & integrating with other PHC interventions.

- Active community participation in planning immunization sessions, implementation, monitoring.

HIV/AIDS Achievements through CDC and PHC correlation between 1990 & 2008:

Before 1990s: No strongly established national programmes and very strong stigma & discrimination was present .

After 2008: A clear improvement in the problem of stigma & discrimination and national programmes in all countries of the region was established.

Constraints and Challenges:

1-Communicable diseases still responsible for around one third of all deaths and one third of all illnesses in the EMR.

2- Almost all these deaths and illnesses are preventable.

3-The tools needed are well known.

4- Effective and equitable use of the available tools is facing major challenges.

5– Inadequate coverage of health care delivery services.

6– Difficult to reach population in certain areas with complex emergency.

7– Inadequate and rapid turn over of trained human resources.

8– Weak community participation.

9– Lack of coordination of NGOs (National Government Organizations) and involvement of the private sector.

Many achievements, have been attained. However Cutaneous Leishmania could be a challenging example presented as a model for Constraints in achievements in Syria and may be in other parts of the Mediterranean area :

In spite of the achievements, few undermined communicable diseases are spreading faster and they are almost becoming as an epidemic in certain parts of the EMR. As for an example, Cutaneous Leishmaniasis in Syria:

Cutaneous Leishmaniasis is an endemic disease in over 70 countries. The yearly incidence is estimated at (2, 000, 000) cases. Residing in the mediterenian area, Aleppo city was historically known as a focal area for this disease7*.Once in a while, new foci are discovered in close locations to the original ones 8*. Till the mid 80s of last century, most cases being acquired in Syria were located in Aleppo city and the surrounding suburban area. Since then, new cases have been discovered towards the southern of Aleppo (Table 1&2). Since the year 2000, we came to observe cases of cutaneous Leishmania highly concentrated in Damascus city and the urban area (Table 3 and the diagram).

Table (1) Total number of patients diagnosed with cutaneous leishmania between 2005 - 2009 in Syria

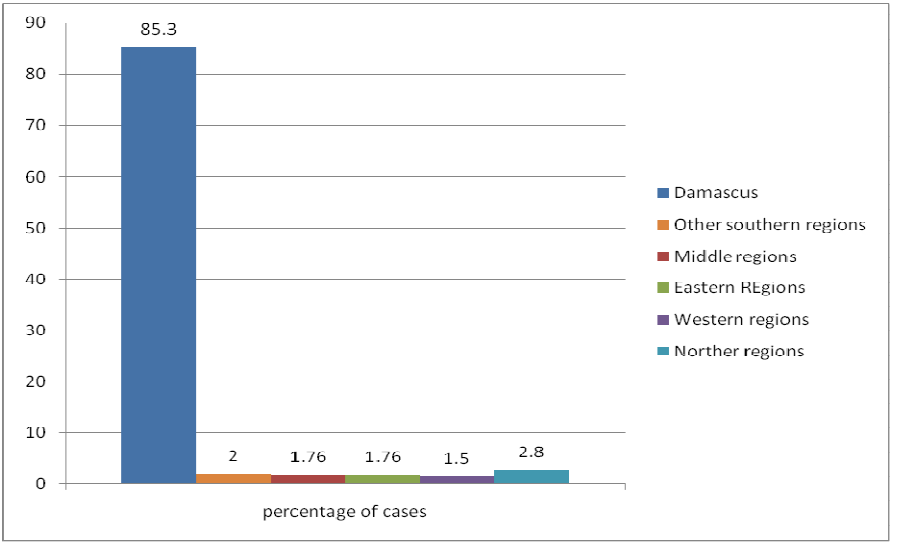
Total number of patients	1478
Number of females	454
Number of males	976
Number of undefined gender	48

Table (2) Distribution of identified cases according to the regions in Syria

City	Number of patients	Region
Hims	7	Middle
Hama	19	Middle
Lathikiyah	7	North West
Al Hasaka	7	North East
Idlib	9	North
Ar rakka	7	East
Al Suwaida	5	south
Al Qamishli	5	North East
Al Qunaitirah	6	South
Banias	6	North West
Al Sheikh Mountain	1	
Jablah	3	North West
Daraa	19	South
Deir Al Zur	7	East
Tartus	6	west
Aleppo	33	North
Unclassified	71	
Damascus	1261	South

Table (3) The Disease incidence according to the region

Region	City	Number of patients	Total NUmber	percentage
Middle	Hims	7	26	1.76 %
Middle	Hama	19		
East	Al Hasaka	7	26	1.76 %
East	Ar rakka	7		
East	Al Qamishli	5		
East	Deir Al Zur	7		
West	Jablah	3	22	1.5 %
West	Banias	6		
West	Tartus	6		
West	Lathikiyah	7		
Region	City	Number of patients	Total Number	Percentage
South	Al Suwaida	5	31	2 %
South	Al Sheikh Mountain	1		
South	Al Qunaitirah	6		
South	Daraa	19		
North	Aleppo	33	42	2.8 %
North	Idlib	9		
South	Damascus	1261		85.3 %
	Unclassified	71		4.8 %



A Diagram representing the Disease incidence in percentage according to the region

The disease is caused by the Leishmania parasite, which is transmited by an infected sand fly in its promastigote form. During its meal, the sandfly transmits the promastigote into the victom skin, where it pentrates the skin layers. There, it loses its flagella. The parasite then, is phagocytosed by the macrophages and transformes into the amastigote form. The amastigote form multiplies within the macrophage, multinucleated giant cells, Langrhanse cells and the dendritic cells in the skin tissues 9*. Residing in the macrophage, the amastigote form is considered an obligatory intracelluler parasite 10*. The lesion appears in the skin as a dirty ulcer with volcanic edges 11*. It expands reaching 3-4 cm in diameter. Towards healing, the lesion shrinks, terminating with a small scar which lasts for life long 12*. The lesion may be manifested in one or two locations of the skin and rarely, the infection

may be seen at more than two different locations in the same patient 13*. With leishmania infection, the infected patient develops a permanent immunity.

Lack of the disease control and the fast spread of the disease towards becoming an epidemic is an example of the challenges for PHC program in EMR.

Going back to the constraints and challenges presented above it is recognized that with regard to Cutaneous leishmania disease specifically:

1- This illness is almost preventable by controlling both the sand fly vectors (Phlebotomus sp and Lutzomyia sp) and its human or mammalian host.

2- The tools needed are well known: Controlling the victor sand fly growth and multiplication is achieved by controlling the living conditions and restoration of the damp places in the zoonotic foci, where the victor can survive, or by eradication of the victor itself. Treating the patient is achievable through the PHC and family medicine measures by first diagnosis of the disease with the laboratory personal and then treatment implimentation by family medicine team work.

As for the other challanges: 3- 4 – 5- 6- 7 and 8: That there is a shortage in service delivery in case of cutaneous leishmania due to the upper constrains is a fact. However, one important factor to shed the light on, is the emphases on the role of Laboratory Medicine in diagnosing and identifying both the disease and the causing factor. In addition to that, no one could under estimate the achievements of the laboratory medicine for PHC through both the discovery and invention of new techniques and practical methods for detecting and identifying the causing factor for different communicable diseases. In case of cutaneous leishmania, as for an example, a broad study of the disease was done 14*. Many papers in that regard were presented some of them published in EMR magazine 15*. Those studies came with new diagnostic features for the microscopic smear method which could be applied for fast and easy diagnosis

16*. Unfortunately since the papers publication, no further follow up or any measures were taken. This of course can cause a delay toward controlling the disease and may contribute sometimes to the exaggeration and the fast spread of it. The importance of the discovery in that regard is that new cytomorphologic figures are found in case of cutaneous leishmania which could be of very important diagnostic values. If those figures were considered, the sensitivity of the microscopic method in detecting the parasite will be dramatically elevated from maximum 60% up to a 100%, and the specificity is elevated as well. Considering those findings on time, could be of importance in closing some of the gabs of constraints and challenges that have been mentioned above, especially with the favorable cost effectiveness of the discovered methods and the easiness of human resources to be trained with the procedure. If the newly discovered diagnostic methods were considered and applied, an adequate coverage of health care delivery services with regard to laboratory testing is easily achieved. And due to the procedure simplicity, it could be very easy to reach population in certain areas with complex emergency. Being a cheap procedure the community participation is most likely to be strong.

Strategic Innovation: "Community-based initiative"

There is a true need to go for a phrase that can be shared by all. Setting a clear goal is the key. This goal reflects a paradigm shift of health authorities (that people themselves could be relied upon). It is valuable to give more importance to social measure and integrate technical with social measure, a process innovation that enables people to take actions in aspects of health matters. E.g. prevention, promotion, treatment, rehabilitation, health security. In that matter it is of importance to assign roles of various players accordingly. Enhancing leadership means people's leadership which will hopefully overcome the resistance to change17*. This Strong capacity and leadership must be at all levels:

– Scale up community-based interventions based with trained community workers

– Full involvement of the private sector and NGOs.

– Functional inter-sectoral mechanism

– Integration between different programmes specially in the cross cutting issues

Let's work together ! We can make the difference.

Evidence for Family Medicine

• Family Medicine training resulted in less referrals and ones of higher quality in Thailand (Jaturapatporn, 2006)

• Faculty family physicians had improved continuity of care, communications and enablement when compared with general doctors and residents in Thailand (Jaturapatpron, 2007)

• Teamwork within PHC is associated with better health outcomes and rewarding professional experience in the US (Solheim, 2007)

• Research suggests that primary care teams with better cohesiveness leads to better patient health care outcomes and patient satisfaction (Grumbach, 2004)

• Review study suggests that trained nurses can achieve as good health outcomes for patients as doctors (Laurant, 2005)

• Female health workers in Pakistan have been successfully trained in primary care and reduced infant and child mortality (Barzgar, Sheikh et al, 1997).

• Community-based primary care workers trained in psychological therapy improved rates of depression among mothers in rural Pakistan (Rahman, 2008, Lancet Series)

• Health workers with a shorter duration of training performed as well as and often better than those with a longer training in assessing, classifying, and managing episodes of routine childhood illness (Huicho, 2008, Lancet Series).

Primary health care oriented countries

• Have more equitable resource distributions

• Have health insurance or services that are provided by the government

• Have little or no private health insurance

• Have no or low co-payments for health services

• Are rated as better by their populations

• Have primary care that includes a wider range of services and is family oriented

• Have better health at lower costs 18*

Conclusions:

1. Primary Care (Personal Care) as a part of Primary Health Care (Community-oriented Primary Care) can improve health.

2. Family Medicine as a specialty of Primary Care can play an important role in delivering Community-oriented Primary Care

3. Although the goal of developing a cadre of family doctors to participate and lead the advance in community-oriented. Primary Care is great, it is achievable.

4. Countries and regions should share their experiences and resources to achieve this goal.

5. Careful distribution of resources is important.

6. High technology medicine is important but low technology medicine gets the right patient to the right high technology at the right time.

6- Underestimation of the role of the laboratory medicine and the personal in PHC team work should be reconsidered.

7- Follow-up of the new discoveries of the techniques and procedures in medical technology fields for diagnosis, and their application must be undertaken more serious.

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Promastigote existence in infected lesions of cutaneous leishmania

Abstract:

Leishmaniasis is an endemic parasitic disease in 88 countries. It is widely distributed throughout the world, caused by vector-borne, obligate, intracellular hemoflagellates of the genus *leishmania*. The parasite continues its life cycle transforming to promastigote in the midgut of the sandfly vector and is transmitted to the human host in the form of promastigote via the bite of the sandfly. Other less encountered forms of transmission are because of a laboratory accident, direct person-to-person transmission, organ transplant and blood transfusion. There is evidence that leishmaniasis may be transmitted either in utero or during the peripartum period. The promastigote form is considered the primary organism of disease transmission between the vector and the host. By not having a chance to continue its life cycle and transform into promastigote within the vector sandfly when considering the many different routes of transmission other than the sandfly bites, it is reasonable to assume an alternative possible existence of the promastigote form of the parasite in the infected lesion of cutaneous leishmaniasis in human host. The information presented below indicates that a real transformation of amastigote to promastigote form occurs within the human host cutaneous lesion in the extracellular fluid after the macrophage membrane eruption and the amastigote release. New techniques are recommended for future studies to confirm these findings including real-time Polymerase chain reaction (PCR) and applying the immunohistochemistry techniques using a novel monoclonal antibody (mAb) against the parasite flagellate (promastigote form) cell wall component.

Introduction:

Leishmaniasis is a parasitic disease widely distributed throughout the world, which is endemic in the tropical and subtropical regions of 88 countries. About 2 million cases are estimated to occur annually worldwide, most of them presenting the cutaneous or mucosal form[1]. Leishmaniasis is caused by vector-borne, obligate, intracellular hemoflagellates of the genus *leishmania*. The various species of *leishmania* are transmitted by the bite of sandflies belonging to the genus *Phlebotomus*. Sandflies acquire the amastigote directly from infected skin or by ingesting parasites circulating in the blood of the reservoir host. In the sandfly, the amastigote transforms into a flagellate promastigote that is infective to humans. After inoculation of the promastigote into a human host, the organism enters macrophages or histiocytes that are present in dermal tissue causing the infection where it is transformed back into amastigote form, an obligatory intracellular parasite who divides by binary fission. After multiplication, daughter amastigotes reinvade neighboring histiocytes [2].

Although the parasite most commonly is transmitted via the bite of the sandfly vector, it may also be transmitted as a result of a laboratory accident, direct person-to-person transmission, organ transplant, blood transfusion and inoculation. In addition, there is evidence that it may be transmitted either in utero or during the peripartum period, while keeping in mind in all those modes of transmission the presumed missing promastigote form [3,4].

Other than the sandfly bite, considering the many different routes of transmission motioned above where the promastigote form that is primarily the source of infection to humans and the one supposed to transmit the disease and according to the information available in literature is presumably absent from the infected lesion while the intracellular amastigote is supposed the only kind to be found in human host.

Given the apparent paucity of research specifically addressing this topic, the purpose of this manuscript is to shed the light from the scarce studies available on the

alternative presumed existence of the flagellate promastigote form that is dismissed, in the infected lesion of cutaneous leishmaniasis in human host.

Literature review:

Studies questioning the correct assessment of the mode of spread of cutaneous leishmaniasis:

So far, only sporadic reports have described cases where the cutaneous *leishmania* parasite has spread to the blood stream and is circulating and carried by blood leukocytes from one organ to another. One study reported that *leishmania* was detected in peripheral blood samples from a striking proportion of blood donors living in the Mediterranean area [5]. However, the general impression has been that these cases are atypical because cutaneous leishmaniasis is usually a localized lesion of the skin so it is highly unexpected to detect the amastigote form which is the only assumed kind found in human in the peripheral blood samples among all those blood donors. Included among these unusual cases are situations in which healed scars of the Aleppo button recurred several years later. With the amastigote disappearance from the lesion towards healing [6], this places a big question mark on the real cause of recurrence. In such instances, the source of the parasite was thought to be residual organisms limited to the skin lesion without further distinct details [7].

Cruz and colleagues have reported that Leishmania spp. were detected by PCR in 32—52% of the syringes discarded by Spanish injecting drug users. That indicates that the transmission occurred directly from human-to-human without the sandfly bite injection of an external promastigote. Moreover, they note shared restriction fragment length polymorphisms in roughly 20% of the samples tested, which suggests that clones of leishmania may spread through the sharing of needles [8]. That clearly reveals that the disease transmission occurred by the only presumed existed parasite in the lesion the amastigote form. This may question the basic fact that the promastigote is the one who primarily transmits the disease among different hosts.

Susceptibility to cutaneous *leishmania* can be greatly influenced by malnutrition, immunosuppression (e.g. HIV) and, host genetic background. The advances made during the last few years in the chemotherapy of neoplasias, in the use of transplants and in the therapy of autoimmune diseases have led to the reactivation of protozooses under these conditions, thus confirming the opportunistic and residual characters of these pathogens. Intracellular parasites are known to persist lifelong in mammalian hosts after the clinical cure of the disease, but the mechanisms of persistence are poorly understood [9]. It is uncertain whether *Leishmania* parasites ever disappear after clinical cure of cutaneous leishmaniasis. The results suggest that clinical cure of American Cutaneous Leishmaniasis (ACL) is rarely associated with sterile cure [10]. In experimental cutaneous leishmaniasis, live parasites have been demonstrated in various strains of mice after clinical cure by chemotherapy. It has been shown that mice experimentally infected with L. major that recover from infection still harbor infective parasites many months later [11]. In the mouse model of leishmaniasis, the persistence of even a low number of parasites in the skin after healing has been shown to maintain the host as a long-term reservoir of infection for vector sandflies. In human ACL, the persistence of *Leishmania* parasites after treatment and clinical cure has been demonstrated by the detection of DNA in peripheral blood and scars of patients. This persistence has also been described for other Leishmania species [10]. In most of these cases the disease recurrence is related to self-transmittance due to the existence of a residual parasite without proper definition whether promastigote or amastigote form.

Studies questioning the correct assessment of the cellular processes involved in cutaneous leishmaniasis:

Recently, it was demonstrated that the human neutrophils were activated by *L. amazonensis* infection or its lipophosphoglycan (LPG) through LTB-4 production, that cooperates with degranulation of neutrophils and killing of parasites [12]. Besides, the release of neutrophil-derived extracellular DNA containing antimicrobial peptides is an important neutrophil function that contributes to parasite

killing [13]. Such killing to the parasite occurs in the extracellular space which is not supposed to be the appropriate environment for the amastigote to survive, hence comes the necessity for an existence of a different form of living parasite able to survive extracellularely.

Control of *Leishmania* infection involves a vigorous Th1-depdendent cellular immune response. Therefore, the observation of the opportunistic character of this protozoosis during the last few years was no surprise, with this parasite causing infectious disease in immunosuppressed patients, particularly those infected with HIV [14].

Leishmania and HIV coinfections have been reported in 35 out of the 88 countries in which leishmaniasis is endemic. It has been demonstrated that AIDS and leishmaniasis, can interact in a vicious cycle of mutual aggravation. With AIDS, various other opportunistic infections such as tuberculosis or systemic mycoses are found in co-infected patients, demonstrating the marked immunosuppression generated in these individuals. In that particular case, *Leishmania* infection may possess some similarities with chronic infectious diseases such as TB. primarily both sharing the coninfection with HIV.

In summary, in infected lesions in humans, the protozoa is characterized by the following: being able to survive in the extracellular fluid, can cause the disease transmission and later recurrence, can be recovered in the peripheral blood, and is able to remain hidden silent for long time. In addition to that and most importantly, these protozoa can multiply, completely survive and transmit the disease without the sandfly involvement or engagement.

The information presented above lead us to the conclusion that the presumed parasite existed in the infected human lesion and caused the disease recurrence or transmission has more or less the same features and characteristics of the promastigote form that exists in the sand fly and transmits the disease after the bite than the obligatory intracellular amastigote.

Promastigote survival and the role of glycoconjugates:

Leishmania parasites are capable to survive and to proliferate due to the protection conferred by unique glycoconjugates present on the parasites' cell surface or secreted. Most of these specialized molecules are members of a family of phosphoglycans or of glycosylinositol phospholipids. The major surface glycoconjugate of all *Leishmania* promastigotes is a molecule called lipophosphoglycan (LPG). LPG enables the attachment of the parasite to the sandfly midgut lectin in the epithelial cells, which enables the parasite to persist in the gut during excretion of the digested blood meal (procyclic promastigotes). While During metacyclogenesis of L. major promastigotes, the LPG undergoes extensive modifications allowing the parasite to detach from the midgut and migrate towards the insect's mouthparts.

Inside the mammalian host infective (metacyclic) promastigotes display an increased resistance to lysis induced by the complement system in the blood serum. (LPG) and secreted proteophosphoglycan (PPG) glycoconjugates play a key role in protecting the promastigote against non-specific host defense and in interacting with host serum components. The longer LPG molecules expressed on metacyclic promastigotes may contribute to their resistance to serum by preventing access of the C5b-9 membrane attack complex to the promastigote membrane. LPG has a role for in protecting the parasite from digestion by lysosomal enzymes. Because LPG repeating units are highly effective in scavenging hydroxyl radicals and superoxide anions, it has been proposed that they may protect promastigotes from these toxic oxygen metabolites generated during the oxidative burst. All of that could strengthen the promastigote preference over the amastigote in the process of survival within the extracellular fluid of human host outside the infected macrophage[15] .

Discussion:

Determining the mode of transmission of cutaneous *leishmania* disease either by the sandfly bite as a vector or by other human-to-human mode excluding the sandfly bite has an important impact in re-understanding the parasite life cycle and declaring the role of both the vector and the host. Congenital, reactivation of *leishmania* after

HIV infection, re-infection with leishmania among drug users sharing the needles, organ transplants and inoculation are non-vector of cutaneous leishmania disease modes of transmission. While literature tells that the promastigote develops and transforms from the amastigote form primarily inside the vector sandfly gut and that leishmania disease transmission occurs primarily by the promastigote form of the parasite through penetration of the skin tissues after the sandfly bite. That does not seem the case in above motioned disease transmission modes [3]. The above facts of disease transmission excluding the sandfly bite may question the vector sandfly obligatory role in the parasite life cycle through amastigote to promastigote transformation [16].

Going back to the articles reviewed in order:

-Having the cutaneous *leishmania* parasite spread to the blood stream and is circulating by blood leukocytes from one organ to another is not a usual case when the amastigote form is supposed to be the one circulating in the blood. Note that the author here did not define the amastigote by name but reported it as (*leishmania* parasite). Being an obligatory intracellular parasite, the amastigote is usually localized inside the macrophage and does not spread to the blood stream. The report here does not agree with the basic understanding of cutaneous *leishmania* disease pathology.

-The study reported that leishmania was detected in peripheral blood samples from a striking proportion of blood donors living in the Mediterranean area.

By revealing that striking proportion, that means that the vast majority of infected individuals with the disease are presenting the amastigote in their peripheral blood contrary to the disease basic scientific concept as being a localized lesion. Again note that authors referred to the parasite without naming it amastigote. The general impression has been that these cases are atypical. This is obviously due to the contradiction between the original scientific concept and the observation. Considering the promastigote existence in the host that may better declare the condition. The amastigotes though can be found outside of macrophages when they

are released from them in order to infect other macrophages, that existence is a remote and a transient one and for only a limited period. Amastigotes unlike promastigotes are not equipped with defense mechanisms to resist the harsh environment in the extracellular fluid. They can not specifically resist the lysing function of the complement system of the body fluid. That beside other factors is a key issue in the destruction of the amastigotes and their disappearance once outside the infected macrophage. While the promastigote existence with the protecting LPG and PPG glycoconjugates against non-specific host defense prevents the interaction with host serum components and protects against the access of the complement mediated lysis through the action of C5b-9 membrane attack complex. Whether procyclic or metacyclic promastigote form is the one transformed and existed in the host is still yet to be revealed.

Non-infective procyclic promastigotes of all Leishmania species are extremely sensitive to fresh serum. L. major procyclic promastigotes rapidly activate the complement cascade via the alternative pathway, with deposition of covalently bound C3b on the parasite surface. While metacyclic promastigotes display an increased resistance to lysis. It is logical to assume that the transformed promastigote form existed in the vertebrate host in its specifications is more like the metacyclic one but that matter is yet to be resolved [15]. See the diagram.

- Aleppo button recurrence was reported as an unusual case due to the amastigote disappearance from the lesion towards healing. With the amastigote disappearance the recurrence must have taken place by a residual parasite reactivation supposedly in this case an other transformed type.

-In the case of Leishmania spp detected by PCR in 32—52% of the syringes discarded by Spanish injecting drug users, the transmission of the disease could be achieved by inoculation which is atypical as being a rare type of an additional mode of transmission. This may open the question wide on the misconception of the limited existence to the amastigote only in the vertebrate host, and from there turning in to the true role of both the vector and the host.

-In case of shared restriction fragment length polymorphisms in roughly 20% of the samples tested noted in those drug users sharing needles, the data in this study in particular, wouldn't allow any doubt of sandfly-promastigote transmission exclusion of the disease and confirms the direct inoculation, raising the question of the promastigote existence within the infected tissues.

-The acceptance of the fact that immunosuppression (e.g. HIV) and the chemotherapy of neoplasias in the use of transplants and in the therapy of autoimmune diseases have led to the reactivation of protozooses under these conditions. This is a true positive addition to the understanding of the disease concept revealing the opportunistic and residual characters of the pathogens and admitting that the pathogen in the host is capable by himself to reinitiate the disease . Having an opportunistic and residual characters, the parasite entitled to, is unlikely to be the amastigote form. It must be other transformed form. Opportunistic means being able to reactivate the disease conditions once the circumstances change in favor of the parasite. Such reactivation must involve the promastigote flagellate whose primary task is disease transmission or reactivation.

-The neutrophil function with degranulation and DNA release taking place in the extracellular fluid is considered as a sign of the extracellular existence of the protozoa in the host, hence the reaction of the neutrophils is correspondent to that.

-By having the parasite causing infectious disease the question goes why in immunosuppressed patients, particularly those infected with HIV? The answer is because *leishmania* infection involves a vigorous Th1-depdendent cellular immune response. Th1 helper is mainly affected by the HIV virus and evidently, the cellular immune response is disturbed. But how could that effect the disease reestablishment? It is due to the opportunistic character of this protozoa, meaning its ability to reactivate itself within the tissues and produce the disease again. That concludes that a residual organism is the one that reproduces the disease and that is out of the amastigote capacity.

-The residual nature of the parasite inside the host in the mouse model of leishmaniasis study together with its infectivity, illustrate an eulogistic character of the protozoa as it is capable of both transmission and reestablishment of the disease taking the role of both the amastigote and the promastigote.

Putting all the above facts together, with the sandfly role exclusion, one may conclude that either the disease transmission among humans takes place through an undiscovered promastigote existence hidden somewhere in human skin lesion, or contrary to the information available, the disease transmission could be achieved by the amastigote form as being the only type of leishmania parasite presumably exists in human [17,18]. Still in such case, an important question arises which is: if it is the amastigote then how could such an obligatory intracellular parasite transmit the disease into an other host? Could it be a transmission as a whole of the infected macrophage containing the infecting intracellular amastigotes together to the new host? The delicacy of the condition may not allow such transmission to take place this way. Even with this presumption, the amastigote in its form is still incapable to transmit the disease because once out side the infected cell, and being transient in a harsh aggressive environment, it cannot survive long as literature tells [19,20]. Being an obligatory intracellular parasite within the tissues of humankind, the amastigote form of the parasite lacks the capacity and not suited technically, functionally or physically to transmit the disease into another host unless otherwise precisely transformed into promastigote form. In order for the disease to be transmitted then, the promastigote form must exist for such transmission to take place. That concludes that some kind of real transformation of the amastigote to promastigote form must have taken place in human cutaneous infected lesion [21]. See the diagram.

At a later stage while the disease itself is still in process and no complete cure yet, the disappearance of the amastigote form (LD. Bodies) from the microscopic smear with the persistence of the parasite as demonstrated by the DNA detection in the scars as declared by the American Cutaneous Leishmaniasis (ACL) study may again

strongly suggest the existence of the parasite in a transformed and modified morphology that was dismissed or could not be identified by the smear conventional microscopic procedure [6].

Fibroid processes compatible with healing were found in the scars, although residual inflammatory foci were observed in some samples. This result is in agreement with a systematic histopathological studies concluding that clinical cure did not always coincide with histopathological cure. Follow-up of this condition for over 15 years, the data in hand supported with many microscopic images concludes that a real transformation of amastigote to promastigote form occurred within the lesion in the extracellular fluid after the macrophage membrane eruption and the amastigote release [21,22,23] (Images 1 to 6).

In his study " Wild Gorillas as a Potential Reservoir of Leishmania majorJ Infect Dis." Ibrahim Hamad et-al confirmed the existence of the promastigote type of the parasite in stool acquired from wild Gorillas. In his words " Next, fluorescence in situ hybridization was performed to visualize L. major parasites in fecal samples from the gorillas. Both promastigote and amastigote forms of the parasite were found. This work strongly suggests that wild gorillas carry pathogenic Leishmania parasites." [24].

Laura Quin˜onez-Dı´az, et-al in her experimental study titled " Effect of Ambient Temperature on the Clinical Manifestations of Experimental Diffuse Cutaneous Leishmaniasis in a Rodent Mode.” came with very interesting findings supporting and proving the existence of the promastigote form in the vertebrate host. She wrote “Promastigote forms on the skin surface could preclude the detection of Leishmania because this parasite form is not expected by physicians or laboratory workers, and is not easily detected because it is embedded in squamous epithelium. In fact, doubt has recently emerged as to whether the amastigote is the only form of Leishmania present in cutaneous leishmaniasis (Daboul 2008), as was seen during a review of slides prepared from cutaneous lesions, where in 22/42 cases promastigote forms were observed, which is consistent with our findings. In fact, parasite forms are not

easily distinguished because they are often incorporated into scales or bound to hair, where immunofluorescence is important to visualize them." [25].

Our findings and the studies presented here strongly suggest that the amastigote form at certain stage of the disease process is released from the infected macrophage into the extracellular fluid [23]. There in the extracellular fluid, a survival process with a stepwise transformation of many of the released amastigote forms into promastigote form is attained [21]. The mechanism of such transformation is similar to what happens within the gut of the sand fly (Images 7 to10). See figure 1.

This corroborates the persistence of an apparent infection by various *Leishmania* species usually associated with cutaneous leishmaniasis. This phenomenon is particularly important in view of the increasing coexistence of AIDS and leishmaniasis. It is relevant to investigate whether immunocompromised, clinically cured patients can act as reservoirs of leishmaniasis in areas of active transmission.

Coetaneous Leishmaniasis and Mycobacterium Tuberculosis**:**

It has been noticed that *leishmania* parasite infection with its chronic sequel characterized at the end stage with scar formation and no full recovery of the cutaneous tissues, in many aspects may share similarities with Mycobacterium tuberculosis infection. Both can survive for a long time in the body of a healthy host, causing no symptoms after primary infection. Years later, M. tuberculosis within the old foci can be reactivated in some TB carriers, leading to symptoms of post-primary tuberculosis. While in case of cutaneous leishmaniasis, *leishmania* parasites present in the skin scar and by the aid of sand fly bite (or by the other modes of transmission including immuno-suppression effects) will lead to reinfection or recurrence and resuming the parasite life cycle.

During the asymptomatic period, M. tuberculosis is believed to survive in old foci of infection, while the parasite in case of cutaneous leishmaniasis embalms itself within the scar tissues in the skin [21]. Yet these foci in case of TB are uniformly negative by culture and acid-fast staining, which are the conventional methods to detect mycobacteria. Similarly, the *Leishmen-Donovan* method, which is the conventional

microscopic method to detect the parasite in case of leishmaniasis, becomes at the later stages of the disease process negative for the *LD* bodies [6].

The precise location and amount of M. tuberculosis in these foci in case of primary TB, or the *leishmania* parasite persistence in case of the terminating stage of cutaneous leishmaniasis therefore, are not well understood.

In cutaneous leishmaniasis, one explanation for the reason behind this negativity in identifying the parasite by microscope is that the microscopic method has its limitation and detects only the LD. Bodies within the macrophage, which is the principle of such microscopic method for diagnosing cutaneous leishmaniasis. Since the time of *Leishmen-Donovan* and over hundred years ago, a concrete perception which later converted into a form of adoption and never questioned or reviewed again was accepted revealing that only the intracellular amastigote exists in human host. While in-fact, certain amounts of *L*. parasites that remain have already lost their normal morphology (the amastigote form) and transformed into a flagellate like structure in the histological sections of the old lesions, but missed by the classical microscopic method [23]. Otherwise, how could we explain the disappearance of the amastigote form together with the infected macrophages in many reported cases at a later stage of the disease while the disease continues to be in process for long time to come as such a disease process with the absence of an infecting cause? Towards healing, *leishmania* lesions seldom contain amastigote forms of parasites, they contain few transformed parasites.

CONCLUSION:

Here we report that in case of cutaneous leishmaniasis with the scar formation, a mild granulomatous lesion with fibroid structure is a well-known characteristic of the disease progress towards healing. This fibroid structure in the lesion is characterized by the existence of the promastigote parasite flagellate embedded in many different shapes and morphologies [23]. This promotes that the end case scar lesion is most likely to be positive for the flagellate parasite genome if tested by the different other lab diagnostic procedures. New techniques are recommended for

future studies confirming the existence of the flagellate including checking specifically for the (promastigote form) parasite during the time of the amastigote disappearance from the microscopic smear at a later stage of the disease process. This can be done by performing real-time Polymerase Chain Reaction (PCR) which is considered a highly sensitive and specific technique for detection of the of *leishmania* parasite in the scar tissues of old cutaneous lesions. It may also be possible to perform the immunohistochemistry techniques using a novel monoclonal antibody (mAb) against the parasite (promastigote form) cell wall component[26]. That requires in the future a thorough follow-up with the application of both techniques.

It is important to note that the new findings presented have a strong impact on the clinical side. The implications of such findings on the disease diagnosis is valuable. Considering the promastigote existence in the smear method, this will add a lot to the procedure indices, reaching almost 100% of sensitivity and specificity and making the procedure one of the most valuable in diagnosing leishmaniasis. Prevention is an other aspect that these findings effect. The discovery of the residual existence of the parasite for long in the host and its role in disease recurrence should direct the authorities attention to take measures for prevention especially with its ability to cause both the recurrence and the transmission. Our findings have also its impact on the disease treatment. Unfortunately, the findings tell that to date and with the repeated recurrence of the disease after long time of treatment, that indicates that most of the drugs in-use are not able yet to provide a permanent cure for the disease and more studies in that direction to achieve curable drugs are highly needed.

The risk of reactivation of latent leishmaniasis from these skin scar old fibroid lesions should always be considered especially with the sand fly existence in the endemic arias.

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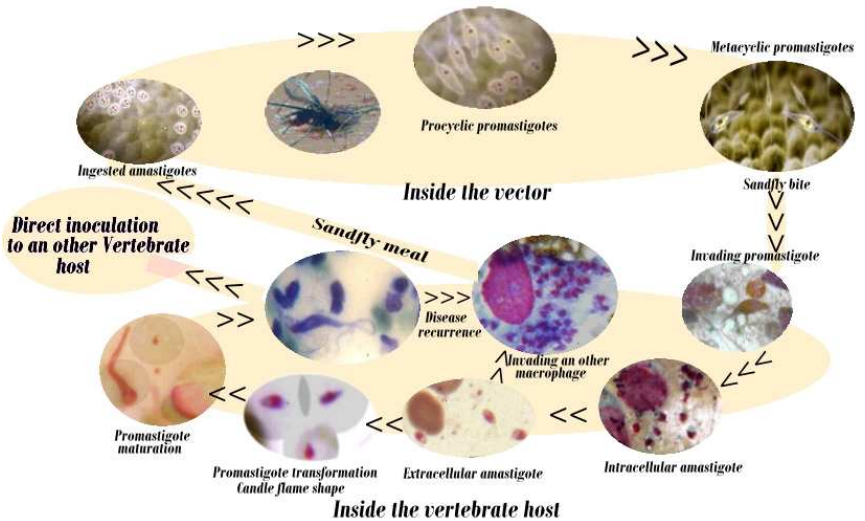
27- The photos were taken from a video titled "The life cycle of leishmania in the insect vector" and produced by Dirceu E. Teixeira, Marlene Benchimol, Juliany C. F. Rodrigues, Paulo Henrique Crepaldi, Paulo F. P. Pimenta and Wanderley de Souza. Support: UFRJ, IBCCF, IBqM, FAPERJ, CECIERJ and INMETRO. Published 14/10/2013

Table 1:

Differences between traditional (e.g., sand fly) and alternative (e.g., human host). routes of transmission

route Alternative Traditional route

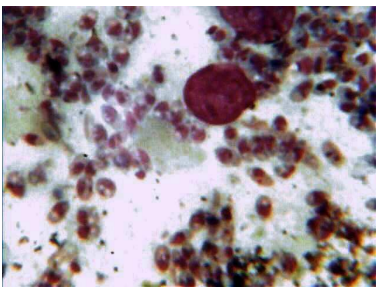
Vector (sandfly) involvement	**No vector involvement**
Procyclic and metacyclic generation	**Transformed form of promastigote**
Transmission by metacyclic form	**Transmission with transformed form**
Amastigote ingestion from the host into the sandfly vector.	**Amastigote stepwise transformation to promastigote**
Metacyclic promastigote skin penetration	**Direct inoculation or latent reactivation**
Different parasite strains may be involved	**One strain is mainly involved**



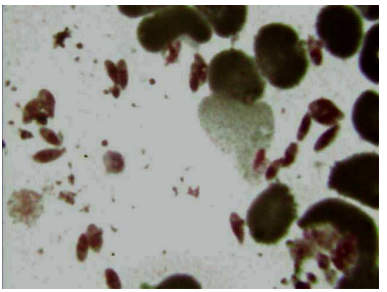
Cutaneous leishmania parasite life cycle

Figure 1

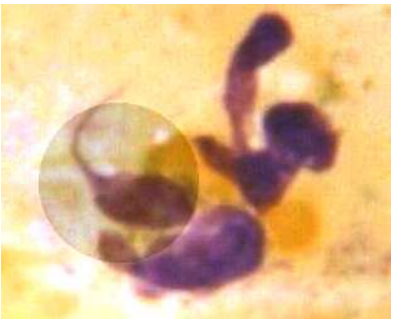
Images showing the transformation of the amastigote form in the extracellular fluid of human skin lesion to a flagellate like promastigote form. (Wright stain)



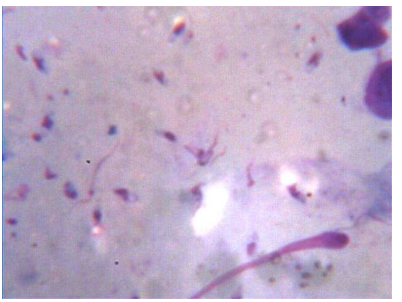
Mag (1000) The amastigotes intra and extracellular. (1)



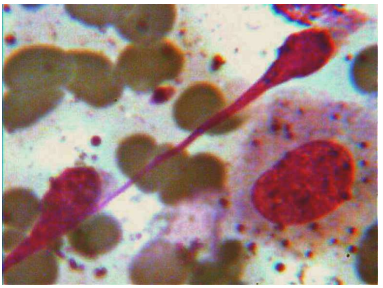
Mag (1000) Extra cellular amastigotes. Some presenting transformation changes.(2)



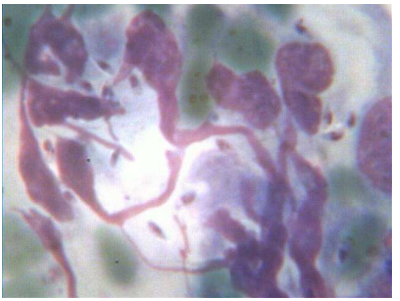
Mag (1000) Extra cellular promastigote like flagellates.(3)



Mag (1000) showing Amastigotes One flagellate with elongating tail. (4)

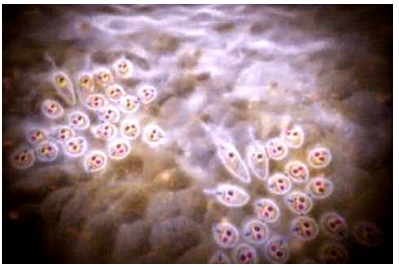


Mag (1000) showing promastigote like flagellates and amastigotes.(5)

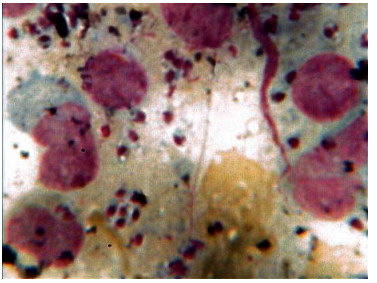


Mag (1000) A flagellate with fibroid elongating tail (6).

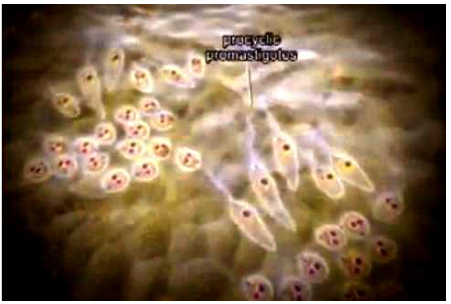
A microscopic comparison in cytomorphology between the parasite appearance and transformation in both the mid got of the sandfly victor and the extracellular fluid of the skin infected lesion in human.



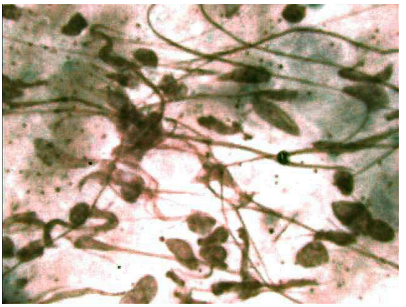
Mag (1000) The amastigote appearance in the midgut of the Sandfly (7)



Mag (1000) The amastigote appearance in the cutaneous tissues of infected lesion from human. 8



Mag (1000) the promastigote transformation within the the sandfly (9)



Mag (1000) The promastigote transformation in the infected midgut of lesion. in human skin (10)