Characterization of *Leishmania* species by using Isozyme analysis

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Abstract: A prospective study was done on 96 patients with Cutaneous Leishmaniasis (CL) attending to Al-Karamah Teaching Hospital in Iraq, during a period from November, 2014 to January, 2015. All samples were investigated for *Leishmania* amastigotes by Giemsa-stained smear were gave positive 68 (70.8%); however the Novy MacNeal Nicolle (NNN) culture led to the growth of promastigotes in all samples 60 (62.5%). Also the characterization of the parasites by Isozyme analysis revealed 100% positive. Our results showed that the highest number of patients 41 (42.7%) was in age group (21-30) years old and 51 (53.2%) of CL patients were had wet lesions while 45 (46.8%) had dry lesions, most of them in arm. The statistical analyses were carried out with Minitab version.

Keywords: Cutaneous leishmaniasis, Isozyme, Culture, Human

1. INTRODUCTION:

Leishmaniosis comprises a variety of syndromes caused by members of the protozoan parasite *Leishmania* (Kinetoplastida: Trypanosomatidae), which are transmitted to mammal hosts by the bite of infected phlebotomine sandflies belonging to the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World) [1]. Various forms of clinical manifestations of human leishmaniosis have been described and divided into three major clinical entities: visceral leishmaniosis (VL, kala azar), cutaneous leishmaniosis (localised, diffuse or disseminated CL, oriental sore, uta, pian bois, chiclero’s ulcer) and mucocutaneous leishmaniosis (MCL, espundia) [2].

*Leishmania major*, *L. tropica* and sometimes *L. infantum* are the causative agents of cutaneous leishmaniasis in Old World. Most of the cases of CL occur in Afghanistan, Algeria, Saudi Arabia, Brazil, Iran, Iraq, Syria, and Sudan [3]. In Iraq, two species are present: *L. tropica*, the agent of anthroponotic cutaneous leishmaniasis (ACL), and *L. major*, the agent of zoonotic cutaneous leishmaniasis (ZCL). Both ACL and ZCL were reported as causative agents of leishmaniasis in Iraq, but ACL is found mainly in suburban areas [4]. In the last two decades, biochemical techniques, most notably isozyme electrophoresis (IE), provided an effective and reliable tool for characterization of *Leishmania* isolates [5-8]. This work aimed to throw light on prevalence rate of cutaneous leishmaniasis in Iraq and assessment of their epidemiological and clinical aspects.

2. MATERIALS & METHODS:

2.1 Population study

This study was carried out during the period from October 2014 to February 2015 in Al-Karamah teaching hospital of Kut city, Iraq. A total of 96 skin samples were taken from patients with CL. All patients were divided into four age groups.

2.2 Parasitological Examination

Smears were prepared from skin scrapings of the edge of the ulcer, then fixed in methanol and stained with Giemsa and examined under the microscope with a 40X lens and with a 100X oil immersion lens. If at least one intra- or extracellular amastigote with a distinctive kinetoplast was found the smear was declared positive. When no amastigotes were seen after 15 minutes of inspection, the smear was declared negative. Many of the patient smears were double checked, the observations were in concordance.

2.3 Culture

The lesions and the adjacent normal-looking skin around them were cleaned, sterilized with 70% ethanol, and allowed to dry. A small amount of the scraped tissue was inoculated on the liquid phase of Novy-McNeal-Nicolle (NNN) medium...
(10% of rabbit blood) medium. The culture was incubated at 25°C and examined for parasite growth by the inverted microscope and also light microscope every 4 days until promastigotes were seen or up to one month before being discarded as negative. The cultures were made at least in duplicates for each case [9].

2.4 Isozyme Electrophoresis
The parasite was submitted to Isozyme Electrophoresis on cellulose acetate plates. Running conditions and revelation techniques were derived from Dujardin et al., (1996) [10]. Each sample was mixed with a hypotonic enzyme stabilizer, maintained during 30 min on ice, centrifuged for 2 min at 3500×g and then immediately run for electrophoresis. Each 16 ml aliquot allowed the survey of as many as 12 different enzyme systems, including additional analyses for control and further verifications.

2.5 Statistical Analysis:
The suitable statistical method was used in order to analyze and assess the results by using T-test in Minitab version [11]. The comparison of significant (P-value) in any test were: S= Significant difference (P<0.05), HS= Highly Significant difference (P<0.01), and NS= Non Significant difference (P>0.05).

3. RESULTS & DISCUSSION:
RESULTS
Table 1. show the prevalence of positive cases of CL by using different diagnostic methods. The highest infection (100%) appeared by using isozyme analysis while the lowest infection (62.5%) appeared by culture on NNN medium. The isozyme profile of 96 cases of cutaneous leishmaniasis recovered in the present study showed two distinct patterns [12]:

1- Banding pattern identical to that obtained with the reference strain of L. major.
2- Banding pattern identical to the reference strain of L. tropica.

Regarding age distribution, CL have been reported high prevalence (42.7%) in age group (21-30) years old and the wet lesions more frequently (53.2%) than dry lesions (46.8%) (Table 2).

DISCUSSION:
The diagnosis of CL classically relies on microscopic examination and in vitro cultivation. These classical methods require the presence of a relatively high number of viable or morphologically intact parasites; this may pose a problem particularly in the chronic phase of CL where parasite levels in skin lesions are very low. In contrast, an isozyme approach is both sensitive and specific [13,14]. The present study showed higher sensitivity and specificity of isozyme analysis (100%) than Giemsa-smear (70.8%) and culture on NNN medium (62.5%) in identification of Leishmania species and with agreement of results were recorded by other researchers[13-15]. The negative samples may have belonged to the lesions that have received antileishmanial treatment or the old lesions with spontaneous healing progress. This is in agreement with previous report [16], also with Kumar et al. who indicated that direct microscopy or parasite culture alone detected respectively 65.5% and 48.2% of the positive samples [17].

The majority of the CL cases (42.7%) in age groups (21-30) years old, which may be due to several factors, such as outdoor activities and sleeping outdoors. The most likely reason is an increase in human- sandfly contact because high activity. These results were in agreement with previous reports indicating more exposure as a result of educational and occupational situations [18,19]. Ulcerative wet type lesions were present in 62%, while the nodule dry type lesions were present in 38%. These observations are in agreement with those reported from Iraq[20], Iran [21], Colombia[22], Pakistan[23], and Afghanistan [24].

CONCLUSIONS:
This study revealed that Isozyme profile is a reliable method for identification of Leishmania species and can applied in epidemiologic investigations in Iraq.
### Table 1. Comparison among three methods in diagnosis of CL cases

<table>
<thead>
<tr>
<th>No. of Examined</th>
<th>Giemsa-smear</th>
<th>Culture on NNN</th>
<th>Isozyme analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>68 (70.8%)</td>
<td>60 (62.5%)</td>
<td>96 (100%)</td>
</tr>
</tbody>
</table>

### Table 2. Age distribution of CL cases according to the type of ulcer

<table>
<thead>
<tr>
<th>Age groups (Year)</th>
<th>+ve cases</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wet</td>
<td>Dry</td>
</tr>
<tr>
<td>10 and less</td>
<td>8 (8.3%)</td>
<td>6 (6.2%)</td>
</tr>
<tr>
<td>(11-20)</td>
<td>14 (14.6%)</td>
<td>16 (16.7%)</td>
</tr>
<tr>
<td>(21-30)</td>
<td>23 (23.95%)</td>
<td>18 (18.75%)</td>
</tr>
<tr>
<td>(31-40)</td>
<td>6 (6.25%)</td>
<td>5 (5.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>51 (53.2%)</td>
<td>45 (46.8%)</td>
</tr>
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<table>
<thead>
<tr>
<th>P-value</th>
<th>C.S</th>
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<tbody>
<tr>
<td>0.01</td>
<td>Significant</td>
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REFERENCES:


