Cutaneous Leishmaniasis Characterization in the Center Part of Iran; Is that an Emerging Disease

Zahra Safari, Mojtaba Saadati, Mohammad Doroudian, Sayed Mostafa Hossaini, Mehrdad Hashemi and Saeed Rezai zarchi

Faculty of Engineering and Science, Department of Biology, IHU, Tehran, Iran.  
Applied Biotechnology Research Center, Baqiatallah University of Medical Sciences, Tehran, Iran.  
Islamic Azad University, Central Tehran Branch, Young Researchers Club, Tehran, Iran.  
Young Researchers Club, Science and Research Branch, Islamic Azad University, Tehran, Iran.  
Department of Genetics, Islamic Azad University, Tehran Medical Branch, Tehran, Iran.  
Department of Biology, Payam-E-Noor University, Yazd, Iran.

ABSTRACT

Background & Objectives: Cutaneous leishmaniasis (CL) is endemic in different parts of Iran and has long been recognized in most provinces of Iran. The present study was conducted to determine the prevalence of cutaneous leishmaniasis in patients referring to laboratories in Yazd Health Center.

Materials and Methods: This study was conducted on 67 samples collected from infected cases of leishmaniasis, Yazd Health Center, during the 2 years period from 2006 to 2008. Initial variable included age, sex, duration of infection, place of residence and number of lesions that all were recorded in an information data. Results: total number of 67 infected persons 29.8 percent were female and 70.2 percent male. The highest prevalence rate (29.8%) was in 20-30 years old. More than one active lesion was seen in 32.8% of individuals. The rang of the commonly affected site of the body were foot (43.28%). Both dry (35.82%) and wet (64.18%) types of lesions were observed that indicated presence of more than one strain of the parasite. Conclusion: Yazd is an endemic region of the cutaneous leishmaniasis in center of Iran. The clinical finding pattern belonged to different endemic strains of L. major and L. tropica in Ardakan and Yazd indicates that the possible transmission of infection from Isfahan to this area.

Key words: Cutaneous leishmaniasis; Endemic; Yazd-Iran; Leishmania major; leishmania tropica.

Introduction

Leishmaniasis is caused by parasitic protozoa of the genus Leishmania. Humans are infected via the bite of phlebotomine sandflies, which breed in forest areas, caves, or the burrows of small rodents. About 12 million people in 88 countries are infected annually, with a number of 350 million people considered as high risk population, world wide. It is estimated that out of 1-1.5 million cases of recorded documentary, 90 % occurred in six countries: Iran, Saudi Arabia, Syria, Afghanistan, Brazil and Peru [1,2]. Different species of Leishmania are classified by their geographical distribution, clinical symptoms, epidemiology, sand fly vector and/or animal reservoirs [3,4]. Diagnosis of leishmaniasis can be made on the basis of clinical and epidemiological studies; the confirmation of the disease is however depending on the demonstration of the parasite in the specimens to avoid any misdiagnosis [4,5]. It is also important to confirm the species of the parasite in acute cases to evaluate the prognosis and to choose a proper treatment of the disease [6,7,8]. Until recently, the major criteria in the diagnosis of leishmaniasis were based primarily on clinical feature, microscopic examination of the stained tissue smears and/or culture of promastigote derived from the specimens [7,8]. Direct detection of the parasite is feasible by the microscopic examination of the giemsa-stained tissue smear and in-vitro culture of the parasite. Even though, the microscopic examination of tissue samples are rapid, cheap and easy perform, due to the small number of the parasites, they are not sensible tests [4,9]. Culture
techniques are however considered as the most sensitive tests, but they are sophisticated, time consuming and need a professional technical work and they are harboring the risk of contamination for Lab personnel [4,10]. Furthermore, the sensitivity and specificity of such conventional techniques was compared to PCR in detecting leishmania [11]. Diagnostic PCR assay using the internal transcribed spacer 1 (ITS1) region of the rRNA genes (40-200 copies) and the splice leader mini-exon (SLME) (100-200 copies) have been introduced as a sensitive technique in detecting cutaneous leishmaniasis (CL) [9,12,13,14].

Materials and Methods

Samples:

This study was a descriptive study performed, by a simple sampling during a 2 years period from 2007 to 2008, in the Yazd Health Center, Iran. The work was conducted on 67 samples collected from suspected cases of leishmaniasis, were examined by Gimsa Stain method and microscopic examination, in vitr culture and species-specific kDNA-PCR assay. Then, they were completed for the infected cases including the information about age, sex, places of residence, number and the site of lesions. In this study every body with amastigote forms in smear was considered as an infected person. Information was gathered, classified and analyzed.

Culture of the Specimens:

The cutaneous lesions and surrounding areas were cultured on N.N.N. medium before being incubated at 25 °C for one week. The cultures were checked during every two days interval to reach the log phase of the growth. The positive cultures were then confirmed by presence of promastigotes which finally sub-cultured into RPMI medium [15,16].

Extraction of DNA from the Clinical Samples:

The tissue samples were transferred into 1.5 ml eppendorf tubes before being lysed in 250 µl of lysis buffer (containing 50 mM NaCl, 10 mM EDTA, 1% SDS 50 mM Tris-HCL). The final concentrations for SDS and proteinase K were 5% and 200 µg ml⁻¹, respectively. RNase was also added at the final concentration of 100 µg ml⁻¹ (incubated with the solution at 37 °C for 30 min.).

The samples rotated in a hybridization oven at 56 ° C and digested overnight. The samples were gently been mixed for 5 min. After centrifugation of the samples at 10,000 rpm, the upper layers were collected in new eppendorf and equal volume of chloroform was then added and centrifuged for 1 min. The upper phase was removed and precipitated with 50 µl of 3 M sodium acetate (pH 6.0) and 500 µl cold Isopropanol (kept in -20 °C), followed by storage on ice for 1 hour. The specimens were subsequently centrifuged at 10,000 rpm for 5 min at 4 °C. The Isopropanol layer was then removed following by washing the DNA pellet in 200 µl of 70% ethanol. After another centrifugation for 10 min. the ethanol phase was removed and the vacuum dried DNA was resuspended in 30 µl DNA free water. It was finally aliquoted and stored at -20 °C for further use [15,16].

PCR assay: Primer set: a primer set designed to amplify a 120 bp fragment of the mini circle present in K DNA of the members of leishmana was as follow:

\[ MLTRF: \text{5'- ggg tag ggg cgt tct gcg aa- 3'} \]
\[ MLTRR: \text{5'- cgc act att tta cac caa ccc c-3'}. \]

The PCR mixture (25 µl) was prepared using; 2 µl PCR buffer, 10 mM Tris HCL (pH~9.0), 2 mM Mgcl₂, 50 mM Kcl, 0.01% gelatin, 25 µg ml⁻¹, 20 mM DNTP, 1 µl DNA Taq polymerase and 1 µl DNA template. The DNA amplification was carried out with initial denaturation at 94 °C for 6 min followed by 35 cycles of denaturation at 94 °C for 30s, annealing at 50 °C for 1 min., extension at 72 °C for 30s and final extension at 72 °C for 7 min. The amplified fragment was finally run on 0.1 µg ml⁻¹ 1% agarose gel and visualized under UV transluminator [17].

Statistical Analysis:

The Fisher and McNemar tests were used to determine any statistically significant differences in disease prevalence between females and males, frequency of the cutaneous lesions for each patient and Frequency of the sampling according to the duration of infection. The data were analysed using a statistical package.

Results:

A total of 67 samples were taken from cutaneous lesions. Table 1 has shown the frequency of different age groups. The highest rates were found in the age group of 20 to 30 years old.

Analysis of the ulcers distribution in the body showed, 43.28 percent on the legs as the most affected location, 38.8 percent of the lesions were found on the hands 10.44 percent on the face, and 7.46 percent on the other site of the body (Table 2). Distributions of the cutaneous lesions for each patient are detailed in Table 3.

From the all infected patients, 45 (67.16%) had only one lesion with the high frequency rate, 13 patients (19.40 %) 2 lesions, 7 persons (10.44 %) 3 lesions and 2 (2.98%) had 4 or more active lesions. The proportion of the samples according to the sex was 70.14% to 29.85%, men to women, respectively (table 4).
The percentage of the sampling according to the duration of the infection is shown in table 2.

Additional category was also designed according to the number of the cutaneous lesions per each patient (table 5).

The lesions were observed to be of two types. In 67 cases, 43 (64.18%) showed wet type of lesions characterized by exudates, redness and inflamed margins and the remaining 24 (35.82%) were of dry and nodular type covered by crust (table 6).

Further characterisation of the causative Leishmania, using species-specific kDNA-PCR amplification on the isolates, resulted in the generation of a 120 bp DNA fragment in positive samples (Fig. 1). The minimum number of the parasite which we could finally detected by PCR technique around 30 parasites in the reaction mixture.

Discussion:

Cutaneous leishmaniasis, which cause skin sore, is considered as one of the endemic disease in most geographic areas in Iran. The disease is widespread among the tropics and subtropical areas which mainly resulted from the traveling of non-immunized infected people to the free areas and also its complicated epidemiological nature of the disease. Skin lesions are frequently occurring in the uncovered parts of body such as upper limbs and face [18,19]. In an earlier report from Sri Lanka, external surfaces of the limbs were mainly affected compared to any other parts of the body and also this was more common in males [18,20]. The similarities of the Iranian life style in covering habits showed that none of the patients were affected with the cutaneous lesions below the waist or above the knees. Dedet et al., have also reported an association between the dressing custom [18]. However, in our study, on of the patient has shown the lesions on the scalp which is unusual site, here and elsewhere.

Tissue culture in detecting leishmaniasis is time consuming, expensive and need some technical expertise.

Muco-cutaneous lesions may appear and remain for a period of few months or years, the lesions which are problematic in the diagnosis of leishmaniasis. In these cases, PCR is considering as a reliable technique. However, using the molecular techniques in diagnosing the muco-cutaneous lesions has also been previously addressed . In the South America, a modified technique by employing water bath instead of PCR assay to identify the vectors and reservoirs and to choose a treatment protocol have been developed. Such techniques may dramatically improve the epidemiological aspects of leishmaniasis [21]. Using molecular techniques may be of great values in distinguishing the parasite in scraping skin lesions compared to the microscopical smears [22,23,24]. However, sampling procedures should be performed according to the techniques developed by [25]. The sensitivity and specificity of PCR in detecting the parasite in dermal lesions were 100% [22]. Proteinase-K digestion followed by a phenol chlorophorm extraction method is currently considered as classical extraction method to detect leishmania [26,27,28,29,30,31,32]. Kinetoplastic specific primer has also been selected because of their higher sensitivity to detect the parasite. The aim of using a direct PCR procedure was to detect a single parasite in clinical specimens. Previous studies on the purified DNA of the reference strains have confirmed a high sensitivity by employing AB primers compared to single parasite. Furthermore, in the PCR using KDNA primers AB we were able to distinguish L. major and L. tropica infections. Harris et al., 1998 estimated that 1 pg of DNA was equal to 10 parasites [32,33,34,35,36]. Ramirez et al., and Weigle et al., have underlined the lack of an ideal standard gold technique to detect cutaneous leishmaniasis poses some problems in estimating the sensitivity of PCR.

However, in our study, the infection was simultaneously confirmed by few diagnostic tests in which one of the positive culture specimens was negative in all duplicate PCR assay, two samples were positive only by duplicate PCR assay, while all other specimens were considered positive by using at least two techniques.

In the present study, we have obtained three positives out of 67 samples using culture. The low sensitivity of culture might be due to the poor technical approaches and application of disinfectants prior to the sampling [22,36,37]. Some how, PCR was not able to detect the parasite from the biopsy materials which prepared for staining. This might be due to the presence of inhibitory stuff such as chemicals presented in the medium. The higher level of sensitivity on fresh biopsy materials was comparable to those obtained by other molecular techniques targeting the same locus which was 94% (37) or different multicopy genes such as kinetoplastic DNA which was recorded as 75.7% to 100% [22,38,39,40]. Miniexon sequences with the record of 89.7% 41, the gyp63 with the percentage of 85% 42 and the hsp70 locus with the rate of 100 43.

Here, by using a PCR-RFLP targeting the ITS region, we obtained a better sensitivity results than those reported by Marfurt et al., which was 51.7% [41,42].

In conclusion, use of this method for the diagnosis of the NW cutaneous leishmaniasis is highly recommended especially in mucosal or chronic cases [38,40,43].
**Fig. 1:** Gel electrophoresis of products of PCR with kDNA primer using the phenol chloroform extracted samples. Lane 1-6: PCR with G1 primers (120 bp), Lane 7: positive control, Lane 8: Negative control (no template), Lane 9: 100 bp ladder (Fermentas).

**Table 1:** Percent of different age groups examined in the present study.

<table>
<thead>
<tr>
<th>Age groups (years)</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>5</td>
<td>7.46</td>
</tr>
<tr>
<td>11-20</td>
<td>11</td>
<td>16.41</td>
</tr>
<tr>
<td>21-30</td>
<td>20</td>
<td>29.85</td>
</tr>
<tr>
<td>31-50</td>
<td>16</td>
<td>23.88</td>
</tr>
<tr>
<td>51-60</td>
<td>9</td>
<td>13.43</td>
</tr>
<tr>
<td>61-78</td>
<td>6</td>
<td>8.95</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 2:** Frequency of the sampling according to the duration of infection.

<table>
<thead>
<tr>
<th>Duration of infection</th>
<th>Number of patient</th>
<th>percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than one month</td>
<td>16</td>
<td>23.88</td>
</tr>
<tr>
<td>One month</td>
<td>10</td>
<td>14.92</td>
</tr>
<tr>
<td>2 month</td>
<td>13</td>
<td>19.40</td>
</tr>
<tr>
<td>3 month</td>
<td>11</td>
<td>16.41</td>
</tr>
<tr>
<td>4-5 month</td>
<td>8</td>
<td>11.94</td>
</tr>
<tr>
<td>6-12 month</td>
<td>9</td>
<td>13.43</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 3:** Analysis of the ulcers distribution in the body.

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of patient</th>
<th>percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hand</td>
<td>26</td>
<td>38.8</td>
</tr>
<tr>
<td>Leg</td>
<td>29</td>
<td>43.28</td>
</tr>
<tr>
<td>Face</td>
<td>7</td>
<td>10.44</td>
</tr>
<tr>
<td>Other site</td>
<td>5</td>
<td>7.46</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 4:** Category of patients according to the different sex groups.

<table>
<thead>
<tr>
<th>Location</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yazd</td>
<td>26 (38.8%)</td>
<td>12 (17.91%)</td>
<td>38 (56.7%)</td>
</tr>
<tr>
<td>Ardakan</td>
<td>21 (31.34%)</td>
<td>8 (11.94%)</td>
<td>29 (43.28%)</td>
</tr>
</tbody>
</table>

**Table 5:** Frequency of the cutaneous lesions for each patient.

<table>
<thead>
<tr>
<th>Number of lesion</th>
<th>Number of patient</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>One lesion</td>
<td>45</td>
<td>67.16%</td>
</tr>
<tr>
<td>Two lesion</td>
<td>13</td>
<td>19.40%</td>
</tr>
<tr>
<td>Three lesion</td>
<td>7</td>
<td>10.44%</td>
</tr>
<tr>
<td>Four lesion or more</td>
<td>2</td>
<td>2.98%</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>100%</td>
</tr>
</tbody>
</table>
Table 6: Types of lesion.

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>Number of patient</th>
<th>percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry</td>
<td>24</td>
<td>35.82</td>
</tr>
<tr>
<td>Wet</td>
<td>43</td>
<td>64.18</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>100</td>
</tr>
</tbody>
</table>

Acknowledgments

We thank the leishmaniasis staff of the Yazd Research Center for assistance in the collecting of samples from suspected person. We are also grateful to all staff of Molecular Parasitology Lab in Pasteur Institute of Iran.

References


