Molecular epidemiological study of cutaneous leishmaniasis in the east north of Iran

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ARTICLE INFO

Objective: To identify and study the epidemiology of Leishmania species isolated from patients with leishmaniasis by PCR method in Sabzevar, Khorasan Razavi Province, Iran.

Methods: Aspirated samples from the lesions of 86 patients with confirmed leishmaniasis used for direct smear preparation. The samples are prepared and cultured in the Novy–MacNeal–Nicolle and Roswell Park Memorial Institute–1640 culture mediums. After extraction of DNA by using phenol–chloroform, parasite kintoplast DNA gene amplification was done by using PCR. The electrophoresis pattern of each species was compared with standard species of Leishmania tropica (L. tropica) and Leishmania major (L. major).

Results: Results of PCR patterns of kintoplast DNA gene suggested that two types of L. tropica and L. major isolated from leishmaniasis patients in Sabzevar and their findings indicated that both species of parasites, L. tropica and L. major are prevalent in Sabzevar.

Conclusions: According to this information, Sabzevar can be divided in two focal points of dry and dry–wet in terms of leishmaniasis, while it was previously known as the dry focal point. Likewise a significant relationship observed between Leishmania species to time distribution, type of lesion, amount of parasites and infection districts.

1. Introduction

Cutaneous leishmaniasis is a parasitic infection that is caused by various species of Leishmania. The disease occurs due to Leishmania inoculation to human skin via the bite of sand fly infected with promastigotes of the parasite. After infection, promastigotes enter into macrophages[1–3]. In macrophages, promastigotes convert to amastigotes which will induce nodules after proliferation and gradually the progressive lesion turns into papules and vesicles. After the lesion got well, it leaves a scar. Clinically, the lesions are divided into dry and wet forms[4–7]. Leishmania major (L. major) and Leishmania tropica (L. tropica) are responsible for wet and dry forms of leishmaniasis, respectively. There are different reservoirs for these parasites. Mice and wild-type rodents are reservoirs of L. major, while human and dog (accidental hosts) are as reservoir hosts of L. tropica[8].

The disease is considered as a public health problem in some parts of Iran, particularly Sabzevar in Khorasan Province (Figure 1). Since reservoirs of the disease are
different in the two species of parasites, in order to eradicate the disease, the species need to be identified. All forms of cutaneous leishmaniasis have the same morphology and it is not possible to differentiate species of *Leishmania* by clinical signs or microscopic methods. Among the existing approaches, molecular methods, such as PCR are very sensitive and rapid for identification of infectious agents. Since Sabzevar is one of the important focal points of the disease in Iran, we decided to study the epidemiology of cutaneous leishmaniasis using PCR method there[9-12].

2. Materials and methods

In this study 86 biopsies were collected from cutaneous leishmaniasis suspected persons in Sabzevar from May 2010 to May 2011. Biopsy was done with a scalpel under the lesion through picking up the macrophages. Two slides of each patient’s lesions were prepared and stained with Giemsa. For amastigote cultivation, aspiration was performed from lesion using an insulin syringe containing 0.5 mL sterile saline buffer. The stained slides were examined by microscope with 40× and 100× magnification for observing amastigotes. If amastigotes were observed, the sample obtained from aspiration of the lesion would be entered into the Novy–MacNeal–Nicolle medium. To reduce microbial contamination potential, 250 to 500 IU of penicillin and 2 mg streptomycin per milliliter of culture was added to Novy–MacNeal–Nicolle medium. The culture mediums were incubated at 25 °C. After 3 to 4 d, the wet slides were prepared from the culture medium for observation of the promastigotes forms of parasites. Promastigotes with flagella were counted using a Neobar slide. If they reached one to two million per milliliter, they would be transferred to Roswell Park Memorial Institute–1640 culture medium containing fetal bovine serum to mass cultivation of promastigotes. After proliferation adjusted (1 000 promastigotes per milliliter) in culture medium, the promastigotes were centrifuged and washed with phosphate buffered saline for DNA extraction. DNA extraction was performed with phenol–chloroform method[13–15].

2.1. Kintoplast DNA (kDNA) amplification by PCR

A pair of primers for conserved sequences of *Leishmania*, kDNA including forward: (5’ TCG CAG AAC GCC CCT ACC 3’) and reverse: (5’AGG GGT TGG TGT AAA ATA GG3’) were
designed. Amplification of kDNA using these primers led to 600 bp and 800 bp bands for *L. major* and *L. tropica*, respectively[16].

PCR for amplification of kDNA gene was performed according to the following stages (Table 1). A total of 20 µL reactions were prepared by using 2 µL 10x PCR buffer adjusted to 1.5 mmol/L MgCl2, 0.2 mmol/L of each dNTP (Invitrogen, Carlsbad, CA, USA), 20 pmol of each primer, 1 IU Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 5 ng of DNA template. Two positive control (*L. major* strain MRHO/IR/75/ER and *L. tropica* strain MHOM/IR/01/yaza) and a negative control sample were used[17].

Table 1

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>First denaturation</td>
<td>95.0</td>
<td>300</td>
</tr>
<tr>
<td>31</td>
<td>Denaturation</td>
<td>94.0</td>
<td>30</td>
</tr>
<tr>
<td>31</td>
<td>Annealing</td>
<td>60.6</td>
<td>60</td>
</tr>
<tr>
<td>31</td>
<td>Extension</td>
<td>72.0</td>
<td>60</td>
</tr>
<tr>
<td>1</td>
<td>Final extension</td>
<td>72.0</td>
<td>300</td>
</tr>
</tbody>
</table>

3. Results

3.1. Leishmania species

The results showed from a total of 86 isolates, 32 samples (37%) were *L. tropica* and 54 samples (63%) were *L. major* (Figure 2).

3.2. The mean size of the lesions

Most of isolates had 11–20 mm lesions and a few of them had lesions with more than 40 mm in size (Table 2). There was not any significant relationship between the size of lesions and the parasite species (*P*>0.05).

Table 2

<table>
<thead>
<tr>
<th>Mean size of lesions (mm)</th>
<th>Number</th>
<th>Percent</th>
<th>Number</th>
<th>Percent</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–10</td>
<td>10</td>
<td>11.8</td>
<td>22</td>
<td>25.5</td>
<td>32</td>
<td>37.3</td>
</tr>
<tr>
<td>11–20</td>
<td>16</td>
<td>18.8</td>
<td>25</td>
<td>29.0</td>
<td>41</td>
<td>47.8</td>
</tr>
<tr>
<td>21–30</td>
<td>5</td>
<td>5.9</td>
<td>6</td>
<td>6.8</td>
<td>11</td>
<td>12.7</td>
</tr>
<tr>
<td>&gt;40</td>
<td>1</td>
<td>1.1</td>
<td>1</td>
<td>1.1</td>
<td>2</td>
<td>2.2</td>
</tr>
<tr>
<td>Sum</td>
<td>32</td>
<td>37.6</td>
<td>54</td>
<td>62.4</td>
<td>86</td>
<td>100.0</td>
</tr>
</tbody>
</table>

3.3. Type of lesion

The comparison between the two species revealed that among *L. tropica* species dry type is dominant, while the highest frequency is related to secretory type in *L. major* species. Moreover, 8 patients had two types of lesions on their body (Table 3). These information indicated a significant relationship between the type of lesions and parasite species (*P*<0.05).

Table 3

<table>
<thead>
<tr>
<th>Type of lesions</th>
<th><em>L. tropica</em></th>
<th><em>L. major</em></th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percent</td>
<td>Number</td>
</tr>
<tr>
<td>Dry</td>
<td>22</td>
<td>25.5</td>
<td>10</td>
</tr>
<tr>
<td>Secretory</td>
<td>8</td>
<td>9.3</td>
<td>21</td>
</tr>
<tr>
<td>Purulent</td>
<td>0</td>
<td>0.0</td>
<td>17</td>
</tr>
<tr>
<td>Two types of lesions</td>
<td>2</td>
<td>2.4</td>
<td>6</td>
</tr>
<tr>
<td>Sum</td>
<td>32</td>
<td>37.2</td>
<td>54</td>
</tr>
</tbody>
</table>

3.4. Correlation between Leishmania species and season

In patients with *L. tropica* infection, the highest frequency was in the spring with 12 cases and the lowest one was in the winter season with 4 cases. In patients with *L. major*, the highest frequency was in the summer season with 38 cases and the lowest frequency was in winter season with 1 case (Figure 3). It revealed a significant correlation between onset of lesions and the parasite species (*P*<0.05).

Figure 2. The pattern of PCR of kDNA gene with corresponding primers. Lanes 1 to 3: PCR product from patients infected with *L. major* species with molecular weight 600 bp; Line 4: PCR product from patients infected with *L. tropica* with molecular weight of 800 bp; Lane 5: 100 bp DNA marker; Lane 6: Standard *L. major*; Lane 7: Standard *L. tropica*; Lane 8: Negative control.

Figure 3. Time distribution of onset of lesions in patients with cutaneous leishmaniasis according to species.
3.5. Amount of parasites

In patients with L. tropica infection, severe slides with 19.6% had the highest percentage, while none of the patients had rare slides. Among slides of patients infected to L. major, a few slides with 31.3% had the highest percentage, while no severe slide was observed in this species (Table 4). The results illustrated a significant relationship between amount of parasites on the slides taken from patients and the parasite species (P<0.05).

Table 4

<table>
<thead>
<tr>
<th>Parasite amount</th>
<th>L. tropica</th>
<th>L. major</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percent</td>
<td>Number</td>
</tr>
<tr>
<td>Rare</td>
<td>0</td>
<td>0.0</td>
<td>8</td>
</tr>
<tr>
<td>Few</td>
<td>3</td>
<td>3.6</td>
<td>27</td>
</tr>
<tr>
<td>Moderate</td>
<td>11</td>
<td>13.0</td>
<td>19</td>
</tr>
<tr>
<td>Severe</td>
<td>17</td>
<td>19.6</td>
<td>0</td>
</tr>
<tr>
<td>Sum</td>
<td>32</td>
<td>37.2</td>
<td>53</td>
</tr>
</tbody>
</table>

3.6. Distribution of species according to districts

Nevertheless, the largest number of patients came from Hokm Abad town with 54 patients while the smallest number came from Rudab town with only 1 patient. Maximum number of L. tropica was for Sabzevar City with 12 cases and maximum number of L. major was for Hokm Abad town with 43 cases. There was a significant relationship between patient’s locations and the species (P<0.05).

4. Discussion

At the past, studies on detection and differentiation of parasite species are accompanied with many problems and need information about clinical symptoms, epidemiology of the disease, induced disease in animals and growth in culture medium. Identification of the Leishmania species seems to be necessary for especial treatment and also for programmers to design controlling strategies against Leishmania reservoirs[12,18]. Applying biochemical methods and using isoenzymes can partially overcome the problems of identification of Leishmania parasite species. Usage of the methods mentioned above required multiple enzyme systems and special skills, even though lack of parasite identification would reduce the value of those approaches[19]. Nowadays, with powerful, fast and sensitive tools such as molecular diagnostic methods, the conventional methods are eliminated. Different PCR techniques have been commonly used as potential tools for this purpose[20,21]. In this study we used a consensus sequence of kDNA gene for designing especial PCR for identification of different Leishmania species. The number of parasites among slides of the patient’s lesions varied according to the species. Among the slides taken from infected patients with L. tropica, “severe” with 17 cases had the highest frequency, while “rare” slides accounted for the smallest number. Besides, among the patients’ slides infected by L. major, the “few” slides accounted for most of slides, but no “severe” slide was observed in this species. Furthermore, statistical tests represented that there was a significant relationship between the parasite species and number of parasites in stained slides (P<0.05), so the slides with high parasites (severe) were observed in lesions of infected patients with L. tropica. However, slides with low parasites (few) were seen in lesions of infected patients with L. major species.

According to acquired information in the present study, Sabzevar cannot be presented as a dry core because it includes both focuses. The epidemiology variation could be due to immethodical construction in towns and villages, accumulation of construction debris, dumping waste near houses which cause the growth of L. major reservoirs and carriers of both species and agriculture in the habitat which provides the situation for the growth of parasite reservoirs. Outbreak time of the disease is different in each species, in leishmaniasis with L. tropica, the highest frequency was related to spring season with 12 cases, while in leishmaniasis with L. major, the highest frequency observed in summer season with 38 cases. Lesions size was different between two species, the lesions of L. major were larger than the lesions of L. tropica. Types of lesions also were different between them so that L. tropica–induced lesions were often generally dry, while L. major–induced lesions were wet and secreted. In conclusion, based on the findings of this study, Sabzevar can be divided into two focal points: one focal point where merely L. tropica is observed including Sabzevar City and Rudab; and the other where both species are seen including Hokm Abad, Quzalgharshy, Jaghatay, Rivadeh and Mohammad Abad. Our study proved that both parasites were seen in Sabzevar. Therefore, Sabzevar cannot be considered as the dry focal point. Characteristics of the collected Leishmania isolates from Sabzevar showed that regions in western and south eastern of Iran, L. major is a predominant parasite of cutaneous leishmaniasis, thus significant relationship reported among Leishmania species to time distribution, type of lesion, amount of parasites and infection districts[22,23].

Conflict of interest statement

We declare that we have no conflict of interest.
Acknowledgements

The authors would like to appreciate the kind collaboration of microbiology laboratory staffs of Mashhad University of Medical Sciences, and staffs of Health Centre of Sabzevar University of Medical Sciences. This research is supported in part by Baqiyatallah University of Medical Sciences under grant No. 91–1127.

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