Original article

A molecular study on cutaneous leishmaniasis lesions in Khuzestan province (South west of Iran)

Jasem Saki, PhD
Shahram Khademvatan, PhD

Department of Medical Parasitology and Mycology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Address for correspondence:
Dr. Shahram Khademvatan, Department of Medical Parasitology and Mycology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
Tel: +98611 3201147
Fax: +98611 3337370
Email: Khademvatan@yahoo.com

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Abstract

Introduction and objective: One of the most likely factors influencing on the clinical manifestations of cutaneous leishmaniasis, is genetic variability of *Leishmania* parasites that is currently controversial. The aim of this study was to identify the possible correlation between clinical cutaneous lesions and genetic of the causative *Leishmania* agents in Khuzestan, south west of Iran.

Materials and methods: Fourteen samples from patients with different size of cutaneous leishmaniasis (CL) lesions referred to health centers and medical diagnosis laboratories of Ahvaz, Dashteazadegan, Shush, Hendijan and Ramhormoz were collected and studied by mini-exonePCR-RFLP and sequencing methods. The diameter of lesions was measured by a blinded caliper and flexible ruler.

Results: Enzyme electrophoretic analysis of different isolates of *L. major* and *L. tropica* separated from lesions with different sizes have revealed no heterogeneity in this species, whereas alignment of the mini-exonesequencing isolates revealed 97-99% identity and 2-8 nucleotide substitution. Identity was 99% in isolates obtained from Lesions with near Sizes.

Conclusion: This study revealed nucleotides substitutions among sequences of causative agents for different size of lesions. In order to find any correlation between genetic and clinical manifestation in CL, a comprehensive study with more samples from more geographical area is needed.

Significance and impact of the study. Information about relationship between the parasite's genetic variability and the clinical form of CL may help us to understand the pathway and a molecular target for devising effective treatment.

Keywords: *Cutaneous leishmaniasis*; RFLP; Sequencing

Introduction

Leishmaniasis is a disease caused by protozoan parasites that belong to the genus *Leishmania*. It is a serious public health problem and affects over 12 million people in many parts of the world [1]. Leishmaniasis has a variety of clinical manifestations ranging from self-curing cutaneous lesions to fatal visceral form. Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis.

The prevalence of infection has been reported from 1.8% to 37.9% in different provinces of Iran and *Leishmania major* and *L. tropica* are the etiological agents of zoonotic cutaneous leishmaniasis (ZCL) and anthroponotic cutaneous leishmaniasis [2,3]. Khuzestan province is located in the southwest of Iran, with a population of 4,300,000 in 2007 residing in an estimated area 63.238 km² is known to be an endemic area for CL [4,5]. In the Iran-Iraq War lasting from September 1980 to August 1988, Thousands of ZCL cases appeared among soldiers and paramilitary men who were sent to the war front in the south-west in the first two years [6].

The different clinical sing of CL is dependent on a number of factors, such as the type and duration of clinical lesion, strain of organism and geographical location [7,8]. One of the most likely factors influencing the clinical manifestations, is genetic variability of parasites that is currently controversial [9,10]. Different feature of CL lesions referred to leishmaniasis clinic of Ahvaz Jundishapur University of Medical Sciences were observed. Although CL in Khuzestan province has previously been studied, but genetic variability of the causative agents and morphological analysis of lesions were not emphasized. In the present study, restriction fragment length polymorphism (RFLP) analysis and sequencing of the amplified mini - exon gene were used to investigate the genetic variations among *Leishmania* isolates and correlated the findings with the clinical manifestations of CL.

Materials and methods

Fourteen persons in 12-15 years old, who provided informed consent, ultimately participated in the study between August 2007 and May 2008. The study was approved by Ahvaz Jundishapur University of Medical Sciences Ethics Committee, and all subjects granted informed consent to participate.

Inclusion criteria

Individuals suspected for CL, who were referred to the leishmaniasis clinic, had clinical indication for skin scraping were included.

Exclusion criteria

Patients with bacterial and fungal contaminated ulcers and those undergone active treatment for cutaneous leishmaniasis were excluded.

Sampling

Sampling was conducted in five regions of the Khuzestan province; North (Shush), South (Hendijan), West (Dashteazadegan, Susangerd, Hovizeh, Bostan), East (Ramhormoz) and Center (Ahvaz). Eight samples from Ahvaz and Dashteazadegan and two samples from each other regions. In addition, *L. major*, *L. infantum* and *L. tropica* (Iranian reference strains) were used as controls. A questionnaire including gender, age, patient location and lesion type information was filled for each patient. All of the lesions were measured by flexible ruler and caliper. Three samples from each patient were taken by scraping the internal border of skin lesions by a surgical blade.

A direct smear for microscopical examination (Fig. 1), one was cultured in Novy-MacNeal-Nicolle medium and the last one in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 200U/ml penicillin G. For mass production, primary

Leishmania isolates were subcultured in RPMI 1640 medium with 10% FBS at 26°C. The cultures were checked for promastigotes every three days for four weeks.

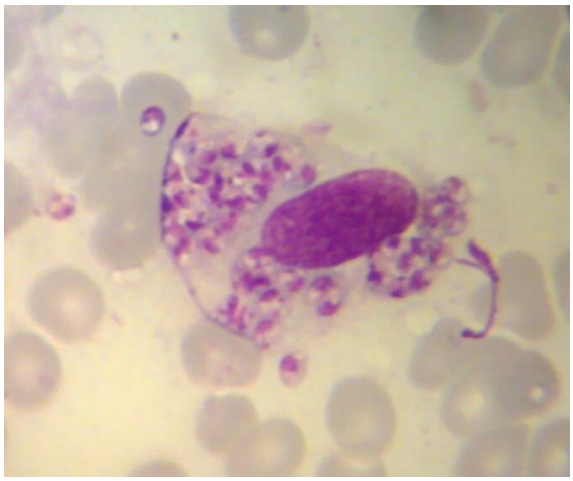

Fig. 1: Amastigotes of *Leishmania* sp. in skin lesion smear (100X, Giemsa stain)

Measurement methods

The diameter of lesions was measured by a blinded caliper using the ballpoint-pen technique [11]. In order to improve the accuracy, the measurement process was repeated. The flexible ruler was used to reproduce the usual conditions of testing.

DNA extraction

The DNA of mass cultured promastigotes was extracted by QIA DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified DNA was eluted in 100µl of elution buffer and stored in -20°C until use. PCR control DNA preparations were also extracted from *L. major* MHOM/IR/75/ER, *L. tropica* MHOM/IR/02/Mash 10 and *L. infantum* MCAN/IR/97/LON 49 (Iranian standard strains).

PCR amplification

Amplification of the mini-exonegene was performed as a single PCR with forward (5'-TATTGGTATGCGAAACTTCCG-3') and reverse (5'-ACAGAAACTGATACTTAT-AT AGCG-3') primers as described before [12]. Amplification reaction was performed in volume of 20µl. Two µl of each isolated DNA were added to a PCR Master Mix (Bioneer Korea), and amplified in a thermocycler (Eppendorf AG 22331, Hamburg, Germany) as follows: initial denaturation at 94°C for 5mins followed by 35 cycles of 94°C for 30s, 51.5°C for 30s, 72°C for 45s and a final elongation at 72°C for 10mins. For each sample one positive control and one negative control were included. 15µl of PCR product was run along with a 50bp ladder on a 1.5% agarose gel containing ethidium bromide for 2h at 70V and visualized by staining with ethidium bromide.

RFLP and sequencing

For digestion, 1.5µl of each Eae I (Fermentase life science, Germany) and Hae III (Takara Bio Inc, Japan) was added to 15µl of the PCR products and incubated at 37°C for 2h [12]. Digestion products were separated by gel electrophoresis in 2.5% agarose and visualized with ethidium bromide staining. Three Iranian reference strains and nine samples from five different parts of the province were selected based on the lesion diameter variability. The PCR products of 17 samples were purified using an Accuprep Gel Purification kit (Bioneer, Deajeon, Korea) then sequenced (MWG-Biotech, Ebersberg, Germany) by the primers employed in the PCR. Sequencing alignments were constructed using the program CLUSTAL W version 1.83 (http://www.ddbj.nig.ac.jp/search/clust-alw-e.html).

Results

Twelve of 14 positive samples detected as *L. major* and two as *L. tropica* by PCR- RFLP (Figs. 2 and 3). The two *L. tropica* isolates were detected in the samples collected from Ahvaz and Susangerd. Comparison of the mini-exonesequences of *Leishmania* isolates revealed 97-99% identity and 2-8 nucleotide substitution. Enzyme electrophoretic analysis of different isolates of *L. major* and

two *L. tropica* have revealed no heterogeneity in this species. Morphological data analysis of CL lesions showed two separate sizes in different geographical areas. Size of skin lesions (in 3-7cm) of infected people in Ramhormoze and Hendijan, East and South regions respectively, were significantly ($p<0.05$) smaller than others (with 1-11 cm).

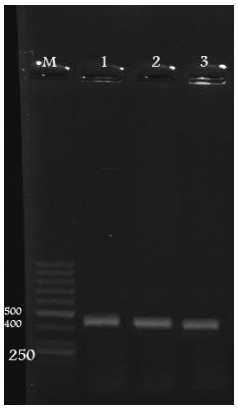

Fig. 2: PCR products of *Leishmania* isolates on 1.5% agarose gel. M: 50 bp molecular weight marker; lane 1-3: Clinical *Leishmania* isolates

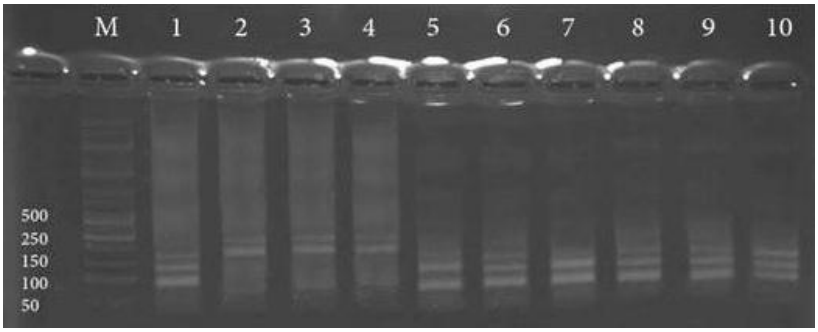

Fig. 3: RFLP patterns of *Leishmania* species after digestion with *Eae* I (for *L. major*) and *Hae* III (for *L. tropica*) on 2.5% agarose gel. M: 50 bp molecular weight marker; lane 1: *L. major* (reference HOM/IR/75/ER); lane 2: *L. tropica* (reference MHOM /IR/02/Mash10); lane 3,4: *L. tropica* isolates (center and west regions); lane 5-10 *L. major* isolates

Discussion

Cutaneous leishmaniasis is endemic in 88 countries in Africa, Asia, Europe and North and South America [13] and caused mainly by two species of *L. major and L. tropica* [14,15]. Ninety percent of all cases occur in six countries including Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria [16]. Although very intensive attempts are being conducted to control the infection but this disease is still a great health problem in Iran. The prevalence of infection has been reported as 1.8% to 37.9% in different provinces of Iran [2,3]. Khuzestan province is an endemic area for the disease [4].

The main molecular identification and genotyping of *Leishmania* species causing cutaneous leishmaniasis in Iran were based on KDNA (Kinetoplast DNA) and internal transcribed spacer (ITS) of small subunit ribosomal RNA (SSU rRNA) analysis [17,18]. This is the first study in Iran used Mini- exon gene for genotyping of *Leishmania* species. We evaluated the Mini-exonepolymorphisms in 14 *Leishmania* isolates from infected people in different geographical areas of Khuzestan province using PCR- RFLP and sequencing. DNA sequencing of mini-exon confirmed the results of RFLP analysis for genotypes.

We have demonstrated the predominance of *L. major* in the studied endemic areas of Khuzestan which agrees with previous study [19]. Alignment of the mini-exonesequences with recorded ones in Genbank and RFLP species-specific fragment patterns showed *Leishmania* at the species level. This study indicated that the isolates which cause the different clinical forms of CL, which had no variation in mini-exon RFLP patterns but there were nucleotides substitutions among obtained sequences. Mini-exon sequence of etiologic agents for highest diameter lesion (11cm) was different in eight nucleotides with sequence of etiologic for lowest diameter lesion (2cm).

Alignment of etiologic sequences for close diameter lesions indicated 99% of homology. However this correlation was not

general for all subjects. In four cases there was statistical difference in lesion size diameters but two nucleotides were substituted. In contrast with the present finding, considerable heterogeneity has been reported within the ITS–RFLP of *L. aethiopica* strains [10], and kDNA of *L. Mexicana* strains [9]. This non-agreement can be due to either conservation in the target gene, mini-exon, which exon and intron portions much conserve in different *Leishmania* species [12]. Whereas other target such as KDNA revealed extensive intraspecific polymorphism among strains of one species [20], or heterogeneity in the species which are related to different geographical areas of the world.

Conclusion

Our study revealed diversity in mini-exon gene of CL agents but the heterogeneity is not coordinated with the size of lesions. In order to find confirmed correlation between genetic and clinical manifestation in CL, comprehensive study with more samples from more geographical area is needed.

Acknowledgements

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Conflict of interest statement: There is no conflict of interest in this paper.

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