Original Article *J Res Health Sci, Vol. 9, No. 2, 2009, pp.48-51*

Identification of *Leishmania* Species Isolated from Human Cutaneous Leishmaniasis Using PCR Method

Rahbarian N. PhD, **Mesgarian A. PhD, *Mahmoudi Rad M. PhD, ***Hajaran H. PhD ** Shahbazi F. PhD, * Mesgarian Z. BSc ** Taghipour N. MsC**

**Cellular and Molecular Biology Research Center, Shahid Behesthi University, M.C., Tehran, Iran*
***Dept. of Medical Parasitology and Mycology, Medicine Faculty, Shahid Behesthi University, M.C., Tehran, Iran*
****Dept. of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
*****Gonbad-e Qabus Health Center, Golestan, Iran*

(Received 2 Jul 2009; accepted 3 Sep 2009)

Abstract

Background: To determine the epidemiological status of cutaneous leishmaniasis outbreak, isolation and identification of the agent parasite, *Leishmania*, using PCR method in Gonbad-e Qabus County, north Iran, during 2006-2007.

Methods: Data were collected on the prevalence of scars and ulcers over a period of 3 months among 6990 inhabitants of five villages around Gonbad-e Qabus County, north Iran, during 2006-2007. Cultured promastigotes were identified using PCR technique. *Its1* and *its2* of Non Coding Transcribed region at ribosomal DNA of 46 *Leishmania* isolates were amplified and the PCR products were separated by electrophoresis in 1.5% agarose gel (200 mA, 140 V), visualized by staining with ethidium bromide, and photographed.

Results: Among 6990 inhabitants of 5 villages, 62.9% were identified as scars and 1.5% as active lesions. Individuals 11 to 20 years were the most highly infected age group. All the parasite isolates were *Leishmania major*.

Conclusion: Cutaneous leishmaniasis due to *L. major* is endemic in Gonbad-e Qabus County, north Iran.

Keywords: *Cutaneous leishmaniasis, ITS-PCR, Epidemiology, Iran*

Introduction

Leishmaniasis, a vector born disease, caused by obligate intramacrophage protozoa, is characterized by diversity and complexity. This parasite can cause a species dependent spectrum of diseases, ranging from self-healing cutaneous leishmaniasis to visceral infections that are fatal. Human cutaneous and visceral leishmaniasis both occur in some parts of Iran. In Iran, *L. major* is causative agent of zoonotic cutaneous leishmaniasis (ZCL). *L. tropica* causes anthroponetic cutaneous leishmaniasis (ACL) considered as old endemic disease in many parts of Iran (1, 2).

This study was done to inspect cutaneous leishmaniasis (CL) in Gonbad-e Qabus border villages using ITS-PCR method during 2006-2007.

Materials and Methods

To determine the CL species we did a descriptive research study in studied area. A total of 6990 inhabitants of five villages around Gonbad-e Qabus County, north Iran, were examined for CL and 124 patients, who had ulcers, were tested in public health center of this county, during 2006-2007. Cultivated specimens were transported to Cellular and Molecular Biology Research Center in Shahid Beheshti University of Medical Sciences, Iran. Diagnosis was done by direct detection of parasites by microscopic exami-

Corresponding author: Nourina Rahbarian, E-mail: nrahbarian@yahoo.com

nation of clinical specimens and by cultivation. The patients consented to their participation in the study and the study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences. For each case having ulcers or scars, a form was completed to record the necessary information such as name, address, age, sex, number of ulcers or scars, site of ulcer(s) or scar(s), date and place of acquiring the disease, etc.

Samples were taken from 46 patients (who did not have secondary infection) and cultured in NNN media, sloppy Evans' medium and Schneider's medium with 10% (v/v) FBS, incubated at 22-25 °C and monitored every 3 d, from day 4 for growth. Cultures were examined for 6 wk before being considered having no growth. All positive cultures were subcultured in RPMI1640 media every 15 d (3).

For DNA extraction, the parasites were added in 500µL of lysis buffer (10mM Tris-HCl [pH 8.0], 0.32M sucrose, 5mM MgCl2 and 1% SDS), digested at 37 ℃ for 24 h, and then 500 µL phenol was added and sedimented at 8000 rpm for 5 min. The supernatants were diluted in 500µL chloroform and were sedimented at 8000rpm. DNA precipitation was done in ethanol with 3 M sodium acetate at -20 °C for 1h. The remaining pellet was dried at 37 °C and dissolved in 30 µL of TE buffer. The DNA concentration was determined by measuring optical absorbance at 260 nm (4).

Specific oligonucleotide primer was designed based on GenBank accession no. AJ300485, LeishF (5′-CAA CAC GCC GCC TCC TCT CT-3′), LeishR (5′-AAA CAA AGG TTG TCN GGG -3′). PCR reaction was carried out based on the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles including denaturation at 94°C for 35 S, annealing at 60°C for 35 s, extension at 72°C for 45 S, and final extension at 72°C for 5 min. At the end, 5 µl of the reaction mix was analyzed by 2% agarose gel electrophoresis. The primer was evaluated with *Leishmania* standard species including *L. infantum* (MCAN/IR/97/LON490), *L. major* (MHOM/IR/75/ER), and *L. tropica* (MHOM/IR/o4/Mash10 (5).

Results

Electrophoresis patterns from each isolates were compared with reference strains of *L. tropica*, *L. major* and *L. infantum*. PCR products from *L. major*, *L. tropica* and *L. infantum* were 626, 485, and 565 bp, respectively. The results showed that from 46 isolates, all (100%) were *L. major* in comparison to reference strains (Fig. 1).

Forty six isolates determined by ITS-PCR method as *L. major,* could produce ulcer at the base tail of BALB/c mice, 4-12 wk after inoculation.

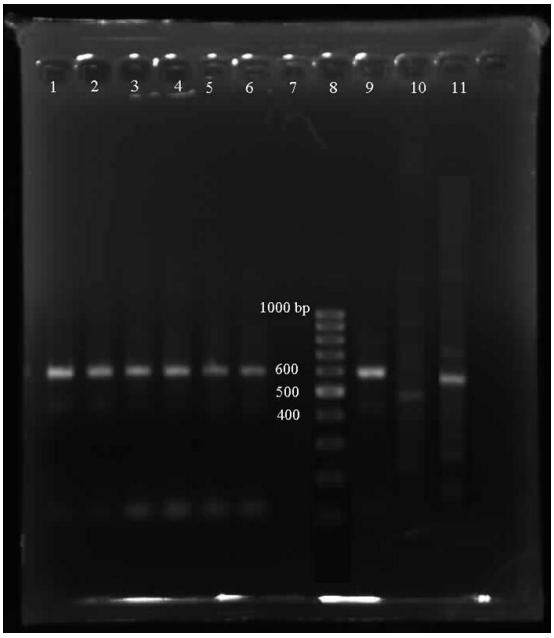

Fig. 1: Amplification of the ITS-1 and ITS-2 fragments in ribosomal DNA, using primers LeishF and LeishR. Six clinical isolates that were determined as *L. major* by ITS-PCR (lanes 1-6), negative control (lane 7), molecular weight marker (lane 8), reference strains of *L. major*, *L. tropica*, and *L. infantum* (lanes 9-11, respectively)

Discussion

Two species of *Leishmania* are involved in CL infections in Iran. *L. major* is causative agent of ZCL and *L. tropica* causes ACL. ZCL is found in many rural foci in the north, east and south of Iran (6).

The ability to distinguish among *Leishmania* is crucial when proscribing treatment, as well as in epidemiologic studies to determine possible control measures. The classic diagnostic techniques for cutaneous leishmaniasis include microscopic diagnosis, the Montenegro skin test, serological diagnosis, and parasite culture, all of which have important limitations (7).

In this study, we used ITS-PCR method to determine species of *Leishmania* isolates from human patients clinically diagnosed as having tegumentary leishmaniasis. The results indicated that *L. major* species was dominant in the studied areas of Gonbad-e Qabus County, north Iran, and ITS-PCR technique was a suitable tool for *Leishmania* characterization in epidemiological studies.

Bensossuan et al. (8) used restriction enzyme analysis of the ITS-PCR product and identified 74.6% positive samples, which included strains of *L. major* (50.9%), *L. tropica* (47.2%), and *L. braziliensis* complex (1.9%). Cupolillo (9) classified *Leishmania* species of new world using a ITS-PCR method. Jawabreh et al. (10) isolated 49 strains of *Leishmania* from the cutaneous lesions of Palestinians living in and around Jericho and applied ITS-PCR to their cultured promastigotes. Of 49 cultures, 28(57%) were *L. major* and 21(43%) *L. tropica*. Although CL has been present in Gonbad-e Qabus border villages in recent years, this is the first time that an endemic focus of zoonotic CL has been identified. There are many active colonies of rodents around some of the infected villages. The occurrence of ZCL in Gonbad-e Qabus border villages seems to be the result of construction of buildings in farms near the colonies of rodents, storage of waste materials around the villages, which are suitable for building nests by rodents.

In conclusion, cutaneous leishmaniasis due to *L. major* is endemic in Gonbad-e Qabus County, north Iran.

Acknowledgements

The present study was conducted with the support of Cellular and Molecular Biology Research Center in Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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