Iranian J Publ Health, Vol. 37, No.1, 2008, pp.54-60

Original Article

Diagnosis and Characterization of *Leishmania* Species in Giemsa-Stained slides by PCR-RFLP

*E Kazemi-Rad[1], *M Mohebali[1], H Hajjaran[1], S Rezaei[1], S.Mamishi[2]

[1]*Dept. of Medical Parasitology and Mycology, School of Public Health, Medical Sciences/University of Tehran, Iran*

[2]*Dept. of Infectious Diseases, Pediatric Center, School of Medicine, Medical Sciences/University of Tehran, Iran*

(Received 25 Jun 2007; accepted 16 Dec 2007)

Abstract

Background: Direct identification of *Leishmania* species in Giemsa-stained slides without parasite culturing in the areas where multiple species exist, is very helpful. This study was designed to isolate *Leishmani* spp. from Giemsa-stained smears and to characterize them by PCR technique.

Methods: A total of 48 Giemsa-Stained slides from confirmed cases of leishmaniasis were examined under a light microscope at×1000 and classified based on grading of *Leishmania* parasites. DNA from each slide was extracted separately and subjected to PCR. The ribosomal internal transcribed spacer 1 (ITS1) was amplified with specific primers and the PCR products were digested with a restriction enzyme (HaeIII).

Results: Of the 48 microscopy-positive slides, 43(89.6%) were positive by PCR-RFLP and *Leishmania* species were identified. A statistically significant difference was observed between the both methods ($P< 0.05$) and also a concordance was found between microscopy and PCR-RFLP (k= 0.55).

Conclusion: PCR-RFLP seems to be an effective method to identify *Leishmania* species from Giemsa-stained smears which have been collected from both infected humans and animal reservoir hosts in Iran.

Keywords: *Leishmania* species, *Giemsa-stained smear, PCR-RFLP*

Introduction

Leishmaniasis is one of the 8 priority targets of the WHO and almost 1500000 new cases of the disease occur annually (1).

Cutaneous leishmaniasis (CL) caused by *Leishmania major, L. tropica* and visceral leishmaniasis (VL) caused by *L. infantum*, are major health problems in Iran (2). The majority of VL cases are reported from northwest, northeast and south of Iran and CL is also endemic in different parts of Iran (3). As the leishmanial signs and symptoms can be varied, characterization of *Leishmania* species is crucial for correct diagnosis and prognosis of the disease as well as for making decisions regarding treatment and control measures (4, 5).

Traditionally, *Leishmania* parasites are directly detected by microscopic examination of clinical specimens. However all *Leishmania* species are very similar and their species identification is not possible morphologically (5). Currently isoenzym analysis is a gold standard for differentiation of *Leishmania* species but this technique is demanding, laborious and usually requires prior cultivation in vitro (5,6). Frequently *Leishmania* species are identified based on their geographical distribution and on clinical manifestations of the resulting disease. However, geographical origin is an inadequate criterion in non-endemic areas, as well as endemic regions where multiple species of *Leishmania* may co-exist (7, 8).

Identification of the infected *Leishmania* species based on clinical signs and symptoms can be problematic because several species cause both visceral and cutaneous involvement (9). With the advent of the PCR technology, several PCR

***Corresponding author:** Tel: +98 21 88951400, Fax: +98 21 66462267, E-mail: mmohebali@hotmail.com

based assays such as the ssu rRNA gene (10), repetitive sequences (11), the gp63 gene locus (12), kinetoplast minicircle sequences (13-15), mini-exon gene sequences (8) for *Leishmania* species differentiation, were developed.

While all these different approaches provide a multitude of valid taxonomic characters for differentiation, most of the time it is necessary to multiply parasite in culture before using them (16). Culture techniques require a sophisticated laboratory set up, are time-consuming and increase risk of contamination (15) thus, in this study; we aimed to optimize a PCR method for the direct identification of *Leishmania* species on Giemsa-stained slides in Iran without need for their cultivation.

Materials and Methods

Specimens Altogether, 48 Giemsa-stained smears from confirmed cases of leishmaniasis, were used. The smears were either referred to the Dept. of Medical Parasitology, Tehran University of Medical Sciences, Tehran, Iran, or from different studies which had been conducted for the epidemiological studies of cutaneous and visceral leishmaniasis in endemic areas of Iran. The smears were prepared form 33 human skin lesions, 4 rodent ear scrapings, 7 dog spleen biopsies, and 4 human bone marrow aspirations.

Cutaneous and visceral samples were prepared based on Evans protocol (6) and the samples were then smeared on a microscope slide, air dried, fixed with absolute ethanol and stained by Giemsa stain (17).

All the collected slides were examined under light microscope with high magnification (1000x). The slides had been prepared either less than one year (2006-2007) or more than 4 yr ago (2002-2003) and the positive smears were scored for amastigote numbers (17). Moreover, 12 smears from other infectious diseases including 6 mycotic and 6 bacterial diseases were included as control.

DNA extraction All the slides were washed with absolute ethanol and covered with 250 µl lysis buffer (50mM Nacl, 50 mM Tris, 10mM EDTA, pH 7.4, 1% v/v Triton x-100 and 100 µg of proteinase k per ml).

After a short time the smears removed completely and transferred to a 1.5 ml reaction tube (5). Cell lysis was accomplished after incubation for at least 3 h or over night at 56 °C (5). The lysate was extracted by phenol-chloroform followed by ethanol precipitation (18). The DNA was resuspended in 50 µl double distilled water (DDW) and stored at 4 °C (4).

PCR amplification A PCR was used to amplify the ribosomal internal transcribed spacer 1 (ITS1) region, which separates the genes coding for the ssu rRNA and 5.8S rRNA using the primers LITSR(5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCAC-TT-3') (5, 7,19).

Amplification reaction was performed in volume of 50µl. Five µl of isolated DNA were added to a PCR Master Mix, containing 2.0 mM $Mgcl_2$, 200 µM dNTP's, 20 pmol of each primers and 2U of Taq polymerase (Roche Biotech) in the PCR buffer (5). Reaction were over laid with 50µl of mineral oil and amplified in a thermocycler (Techne USA) as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 30s, 48 °C for 30s, and 72 °C for 1 min. Fifteen µl of PCR product were run along with a 50bp ladder on a 1.2% agarose gel containing ethidium bromide for 2 h at 70V. The gel was observed on a UV transilluminator and then, digital photographs were prepared (4). Also, PCR was evaluated with three *Leishmania* standard species including *L. major* (MHOM/ IR/75/ER). *L. infantum* (MCAN/IR/97/LON49) and *L. tropica* (MHOM/IR/99).

RFLP analysis of amplified ITS1 PCR products (20µl) were digested with 2µl of HaeIII at 37 °C for 4 h without prior purification using conditions recommended by the supplier (Fermentas Life Sciences, Germany). The restriction fragments were subjected to electrophoresis in 3% agarose gel containing ethidium bromide (0.5 µg/ml) for 3 h at 65v and visualized on a UV transilluminator (5).

DNA sequencing DNA for sequencing was prepared by the ITS1-PCR. The products of 3

different standard species of *Leishmania* were sequenced at Kawsar Biotechnology Center, Tehran, Iran. Nucleotide sequence data reported in this article have been submitted to the GenBank database with accession numbers, *L. tropica* EF 653267, *L. infantum* EF653268, and *L. major* EF653269.

Results

In this study, 48 *Leishmania* positive Giemsa-stained smears were used. Thirty one (64.6%) of the smears had been stored for more than 4 yr ago and 17 (35.4%) smears were prepared less than 1 yr. Twenty-six (60.5%) and 17 (39.5%) of PCR- positive slides, were prepared old (more than 4 yr ago) and freshly (less than one yr), respectively. No statistically difference was observed between old and freshly prepared smears ($P< 0.05$) (Table 1).

Table 2 shows that demonstration of DNA bands of *Leishmania* amastigotes is directly related to score of *Leishmania* numbers on the prepared smears.

Of the 48 positive slides tested with ITS1-PCR, 43 were positive (89.6%) for leishmanial DNA (The 300-350 bp ITS1 amplicon) (Fig.1).

All the PCR-RFLP negative slides were prepared 4 yr ago and kept without cover slip, and also observed scarce amastigotes in the whole prepared slide during microscopy observation (Table 1, 2). By comparing the results obtained by ITS1-PCR with microscopy, a statistically significant difference was observed between the both methods ($P< 0.05$) and also a concordance was found between two the methods (k= 0.55).

In addition, a total of 12 leishmanial negative smears with other infectious diseases including 6 mycotic and 6 bacterial diseases were examined by PCR. No PCR band was observed in these samples.

Moreover, the ITS1-PCR products of three different standard species of *Leishmania* were sequenced. GenBank was searched for similar sequences with the BLAST program and a significant homology was detected with other *Leishmania* sequences. ITS1 region varies between the *Leishmania* species in nucleotide sequence thus; ITS1-PCR amplicons were digested by HaeIII, for the *Leishmania* characterization.

The fragments of 220 and 140 bp for *L. major*, and the fragments of 200, 80 and 60 bp for *L. infantum* were diagnostic. A banding pattern including two fragments of 200 and 60 bp were observed for *L. tropica*. Regarding to restriction map of the used enzyme, four fragments with approximate size of 200, 64, 55, and 20 bp should be observed in this case. However, due to small size of last fragment (20 bp) and overlapping of two fragments with near molecular weight (64 and 55) the above mentioned fragments (200, 60) were diagnostic for *L. tropica*. So by comparing the pattern of unidentified *Leishmania* isolates with those of ref. strains, *Leishmania* species were identified (Fig.2).

Table 3 shows the distribution of *Leishmania* species, isolated from clinical samples of human or animal reservoirs and their identification by PCR-RFLP in the prepared Giemsa-stained smears. The results showed that 17 (39.5%), 17(39.5%) and 9 (20.9%) of the isolates were similar to *L. tropica*, *L. major* and *L. infantum* reference strain, respectively. Nine isolates that were determined by PCR-RFLP as *L. infantum* were causative of visceral *Leishmania* infection in infected human and animal reservoir hosts. Also all *Leishmania* had been isolated from skin lesions, were the same of L. *tropica* and *L. major* of reference strain.

Table 1: Comparison of microscopy and PCR-RFLP results based on old and freshly prepared Giemsa-stained smears.

Slide preparation time	Microscopy-Positive		PCR-RFLP Positive	
	No.	%	No.	%
< 1 yr (2006-2007)	17	35.4	17	39.5
> 4 yr (2002-2003)	31	64.6	26	60.5
Total	48	100	43	100

Table 2: Relation between PCR-RFLP demonstration of *Leishmania* DNA with grading* of *Leishmania* amastigote numbers on Giemsa-stained smears

PCR-RFLP results	Grading of Leishmania Parasites				Total
	1+	2+	3+	4+	
Positive	3	8	15	17	43
Negative	5	0	0	0	5
Total	8	8	15	17	48

* Grading of *Leishmania* Parasites was obtained by average parasite density using x10 eyepiece and x100 oil-immersion lens as follows:

4+ 1-10 parasites/fields
3+ 1-10 parasites/10 fields
2+ 1-10 parasites/100 fields
1+ 1-10 parasites/1000 fields

Table 3: The results of microscopy examination and PCR-RFLP method on Giemsa-stained smears, prepared from human and animal reservoirs based on clinical features and *Leishmania* species identification.

Material	Kind of lesion/ sign and symptom	microscopy-Positive		PCR-RFLP Positive		Species identification
		n	%	n	%	
Human skin	Skin lesion(dry)	19	39.6	17	39.5	*L. tropica*
Human skin	Skin lesion(wet)	12	25	12	27.9	*L. major*
Human skin	Skin lesion(impetigo)	2	4.2	2	4.7	*L. major*
Smears from gerbil[a] ear	Papule	2	4.2	1	2.3	*L. major*
Smears from Balb/c[b]	Skin lesion (wet)	2	4.2	2	4.7	*L. major*
Dog spleen	Skin lesion, weigh less,	7	14.6	7	16.2	*L. infantum*
Human bone marrow	Lymphadenopathy	4	8.3	2	4.7	*L. infantum*
Total	Skin lesion, Fever, Anemia, Hepatosplenomegaly	48	100	43	100	*L. infantum/ L. major/ L. tropica*

a - Gerbils included on *Rhombymos opimus*

b- In the first, *Leishmania sp*. was detected from *P. papatasi* and then inoculated to the tail of Balb/c, the parasites were detected from skin lesion of inoculated Balb/c 3 months post inoculation.

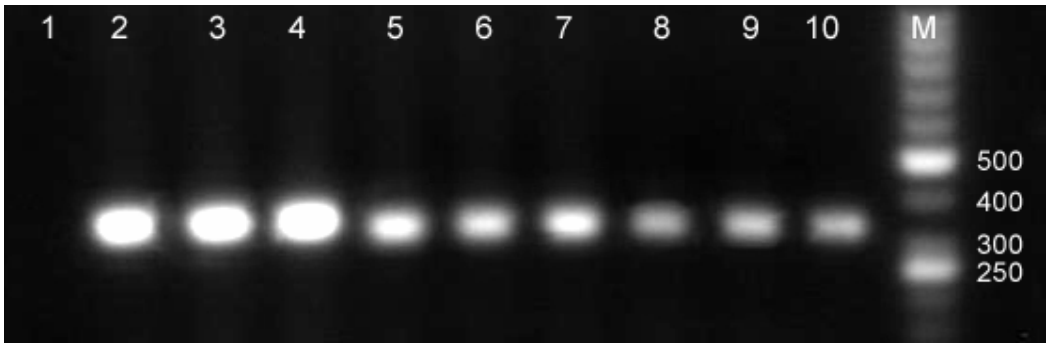

Fig. 1: Electerophoresis results of ITS1-RCR from *Leishmania* stocks and Giemsa-stained smears. Lane 1, Negative control. Lanes 2, 3 and 4 represent *L. major*, *L. tropica* and *L. infantum* reference stocks respectively. Lanes 5, 6, 7, 8, 9 and 10 are isolates from Giemsa-stained smears. M: 50 bp size marker (Fermentas).

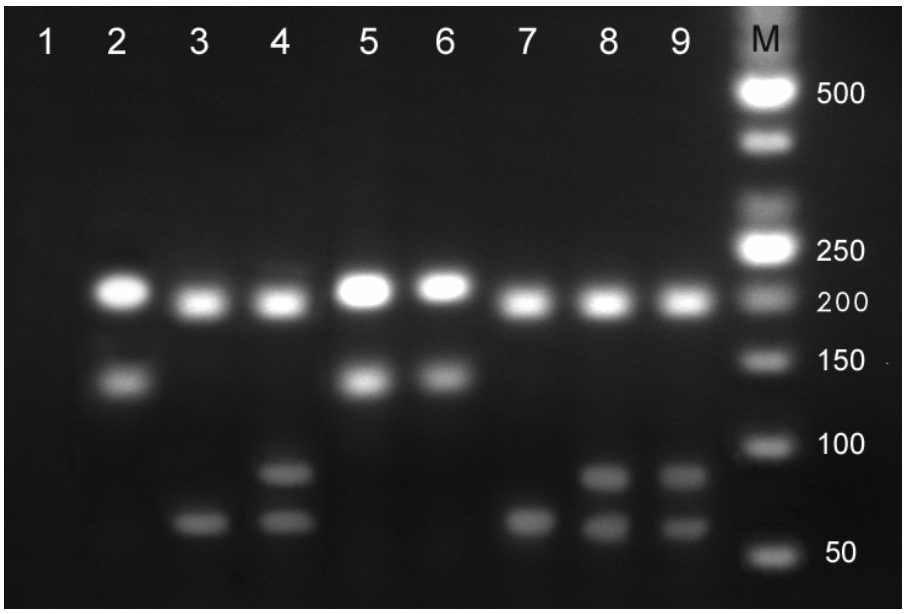

Fig. 2: Restriction fragment length polymorphism (RFLP) patterns obtained from *Leishmania* stocks and Giemsa-stained smears. Lane 1, Negative control. Lanes 2, 3 and 4 represent *L. major, L. tropical* and *L. infantum* reference stocks respectively. Lanes 5 and 6 are *L. major*. Lane 7 is *L. tropica*. Lanes 8 and 9 are *L. infantum.* M: 50 bp size marker (Fermentas).

Discussion

In endemic areas where more than one *Leishmania* species is present, diagnostic tools are required for the detection of parasites directly in samples and distinguish all relevant *Leishmania* species (5).

Characterization of *Leishmania* species is important, because different species may require distinct treatment regimens (1). Furthermore, such information is also valuable in epidemiologic studies where the distribution of *Leishmania* species in human and animal hosts, as well as in insect vectors, is a prerequisite for designing appropriate control measures (5, 7).

Based on previous experiments (5, 20, 21), we used PCR-RFLP methods for diagnosis and characterization of *Leishmania* species on Giemsa-stained slides without the need for cultivation them. Giemsa-stained slides are appropriate for field condition as such samples can be easily stored and sent to the diagnostic laboratory (5, 22). This study showed that *Leishmania* DNA could be efficiently extracted and amplified even from old Giemsa-stained microscopic slides that were stored more than 4 yr and if these were protected by a cover slip, DNA extraction could have been more efficient.

This method can be helpful when re-evaluating the diagnosis of controversial cases or in retrospective epidemiologic studies (5).

In this study, the Giemsa-stained slides were examined by both microscopy and ITS1-PCR. Most of the slides that were high scored amastigote numbers as microscopy- positive were also positive by PCR-RFLP.

This study also showed that Giemsa-stained slides used for the diagnosis with microscopy, could readily be used as samples for identifying of *Leishmania* species with PCR-RFLP.

Although the costs for PCR-RFLP diagnosis are higher and its concordance is lower than microscopic examination but this method can identify *Leishmania* species without need for cultivation them (5, 20). In a study in Iran, *Leishmania* sp. was detected from archived, Giemsa-stained slides, for the diagnosis of cutaneous leishmaniasis by PCR, but species of *Leishmania* had not been determined (22).

The advantage of PCR-RFLP is sufficient to distinguish almost all medically relevant *Leishmania* species including *L. major*, *L. tropica* and *L. infantum* that predominate in Iran. A drawback to the present study is the relatively small number of healthy controls because it is very important to evaluate validity of the PCR-RFLP method in low leishmanial prevalence populations, such as patients from non-endemic areas. In conclusion, the PCR-RFLP procedure seems to be applied as a suitable tool for direct diagnosis and characterization of *Leishmania* species from Giemsa-stained slides.

Acknowledgments

This study was financially supported by Vice-Chancellors for Education and Research of School of Public Health, Med Sciences/University of Tehran. We thank Prof MR Yaghoobi-Ershadi, Mr AA Akhavan, Mr MR Abaei, Mr Z Zarei, Mrs B Akhoundi, Mrs S Charehdar, Dr E Zibafar, Miss F Mesgarian, Mrs Z Talaei, Mr MH Falaki, Mrs A Assare and Mrs Sh Jafarian for valuable helps. The authors declare that they have no Conflict of Interests.

References

1. World Health Organization (updated 2002). TDR Strategic Direction: Leishmaniasis. available from: http://www.who.int/tdr/diseases/leish
2. Nadim A (2000). Leishmaniasis in: *Epidemiology and control of common disorders in Iran.* Ed, Azizi F, Janghorbani M, Hatam H, 2nd ed. Endocrine & Metabolism Research Center. Shaheed Beheshti University of Medical Sciences, pp.524-34.
3. Mohebali M, Edrissian Gh H, Nadim A, Hajjaran H, Akhoundi B, Hooshmand B, et al. (2006). Application of Direct Agglutination Test (DAT) for the diagnosis and seroepidemiological studies of visceral leishmaniasis in Iran. *Iranian J Parasitol*, 1(1):15-25.
4. Hajjaran H, Mohebali M, Razavi MR, Rezaei S, Kazemi b, Edrissian GhH, Mojtabavi J, Hooshmand B (2004). Identification of *Leishmania* species isolated from human cutaneous leishmaniasis, using Random Amplified Polymorphic DNA (RAPD-PCR). *Iranian J Publ Health*, 33(4):8-15.
5. Schönian G, Nasereddin A, Dinse N, Schweynoh C, Schalling HD, Presbe W, Jaffe C (2003). PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diag Microbiol Infect*, 47(1):349-58.
6. Evans D (1989). *Hand book on isolation. Characterization and Cryopreservation of Leishmania.* First ed. UNDP/ WORLD BANK/WHO.
7. El Tai NO, Osam OF, Elfari M, Presber W, Schönian G (2000). Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. *Trans R Trop Med Hyg*, 94(5):575-79.
8. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I (2003). Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the Miniexon sequence and subsequent Restriction Fragment Length Polymorphism analysis. *J Clin Microbiol*, 41(7):3147-53.
9. Marfurt J, Niederwieser I, Makia ND, Beck HP, Felger I (2003). Diagnostic genotyping of old and new world *Leishmania* species by PCR-RFLP. *Diagno Microbiol Infect*, 46(2):115-24.
10. Van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB (1992). Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. *Mol Biochem Parasitol*, 51(1):133-42.
11. Piarroux R, Fontes M, Perasso R, Gambarelli F, Joblet C, Dumon H, Quilici M (1995).

Phylogenic relationships between old World *Leishmania* strains revealed by analysis of repetitive DNA sequence. *Mol Biochem Parasitol,* 73(1):249-52.

12. Victor K, Banuls AL, Arevalo J, Llanos A, Hamers R, Noel S, et al. (1998). The gp63 gene locus, a target for genetic characterization of *Leishmania* belonging to subgenus. *Viannia Parasitol,* 117(1):1-13.
13. Rodgers M, Popper S, Wirth D (1990). Amplification of Kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. *Exp Parasitol,* 71(3):267-75.
14. Smyth AJ, Ghosh A, Hassan MQ, Basu D, De Bruijn MH, Adhya S, et al. (1992). Rapid and sensitive detection of *Leishmania* kinetoplast DNA from spleen and blood samples of kala-azar patients. *Parasitol,* 105(2):183-92.
15. Ravel S, Cuny G, Reynes J, Veas F (1995). A highly sensitive and rapid procedure for direct PCR detection of *Leishmania infantum* within human peripheral blood mononuclear cells. *Acta Trop,* 59(3):187-96.
16. Noyes HA, Reyburn H, Bailey JW, Smith D (1998). A nested PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. *J Clin Microbiol,* 36(10):2877-81.
17. Worth Health Organization (1991). *Basic laboratory Methods in Medical Parasitology*. First ed. WHO, Geneva.
18. Kelly JM (1993). Isolation of RNA & DNA from *Leishmania*. In: *Method* in *Molecular Biology.* Ed, Hyde JE. Human a Press Newjersey USA.
19. Daliva AM, Momen H (2002). Internal-Trascribed-Spacer (ITS) sequences used to explore polygenetic relationships within *Leishmania*. *Ann Trop Med Parasitol,* 94(6):651-4.
20. Al-Jawabreh A, Schönian G, Hamarsheh O, Presber W (2006). Clinical diagnosis of cutaneous Leishmaniasis: A comparison study between standardized graded direct microscopy and ITS1-PCR of Giemsa-stained smears. *Acta Trop,* 99(1):55-61.
21. Al-Jawabreh A, Schnur LF, Nasereddin A, Schwenkenbecher JM, Abdeen Z, Barghuthy F, et al.(2004). The recent emergence of *Leishmania tropica* in Jericho (A'riha) and its environs, a classical focus of L. major. *Trop Med Int Health,* 9(7):812-16.
22. Motazedian H, Karamian M, Noyes HA, Ardehali S (2002). DNA extraction and amplification of leishmania from archived, Giemsa-stained slides, for the diagnosis of cutaneous leishmaniasis by PCR. *Ann Trop Med Parasitol,* 96(1):31-34.