Investigation Of Leishmania Parasites From Clinical Samples Using Polymerase Chain Reaction Technique In Cukurova Region, Turkey

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Türkiye'nin Çukurova Bölgesinde Kliniksel Materyalde Bulunan *Leishmania* Parazitlerinin Polimeraz Zincir Reaksiyonu Ile Araştırılması

Özet

Giriş: *Leishmania* cinsi parazitlerinin sebep olduğu Leishmaniazis, oldukça önemli bir sağlık problemi olduğu düşünülür. Klinik materyalde *Leishmania* parazitlerinin belirlenmesi ve tür ayırımında polimeraz zincir reaksiyonu-restriksiyon fragman uzunluğu polimorfizm metodunu (PCR-RFLP) kullanmayı ve mikroskobik inceleme sonuçlarıyla karşılaştırmayı amaçladık.

Metotlar: Kırk beş deri lezyonu, 9 kemik iliği ve 4 referans türünden oluşan toplam 58 örnek, bu çalışmaya alındı. Örnekler hem DNA elde edilmesi ve hem de preparat hazırlanmasında kullanıldı. DNA'lar, *Leishmania* alt-cinsinin belirlenmesi için PCR yöntemi ile çoğaltıldı ve PCR ürünü, tür ayırımı için *Hae*III ile kesildi.

Sonuçlar: Visseral *Leishmania* parazitleri *Leishmania infantum* olarak belirlendi. Deri leişmanyazın 45 hastanın, çoğunda *Leishmania tropica* belirlenirken, diğerleri *Leishmania donovani*'ye benzer bulundu. Aynı zamanda PCR'ın pozitiflik oranı (%100), mikroskobik inceleme (%67) sonuçlarından daha fazla olduğu saptandı.

Çıkarım: PCR-RFLP tekniği, *Leishmania* türlerinin tespit ve ayırımında çok hassas olduğu gözlendi.

Anahtar kelimeler: Tipleme, Leishmaniazis, PCR, RFLP

Abstract

Background: Leishmaniasis, caused by parasites of the genus *Leishmania* is still considered an important health problem. We aimed to appraise a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method for the detection and species differentiation of *Leishmania* parasites in clinical samples; and to compare with the results of microscopic examination.

Methods: Totally 58 samples were taken from 45 skin lesion, nine bone marrow aspirates, and four reference strains. The samples were used for both DNA and smear-slide preparations. The DNAs were amplified by PCR for the detection of *Leishmania* subgenus and PCR products were restricted with *Hae*III for the species differentiation.

Results: The visceral *Leishmania* parasites (VL) were genotyped as *Leishmania infantum*. Of 45 patients with CL, most of them were analyzed as *Leishmania tropica*, but the others were characterized similar to *Leishmania donovani* reference. It was also determined positivity rate in PCR (100%) was higher than microscopic examination (about 67%).

Conclusions: PCR-RFLP technique appears to be most sensitive for the detection and the differentiation of *Leishmania* species.

Key words: Identification, Leishmaniasis, PCR, RFLP.

Introduction

In some parts of World, cutaneous leishmaniasis (CL) is usually caused by *Leishmania major* (*L. major*), *Leishmania tropica* (*L. tropica*), or *Leishmania aethiopica* (*L. aethiopica*); mucocutenous leishmaniasis (MCL) by *L. aethiopica*; and visceral leishmaniasis (VL) by *Leishmania donovani donovani* (*L. donovani*) or *Leishmania d*onovani *infantum* (*L. d. infantum*). CL and VL has been an important health problem in Cukurova region as well as in the world (1).

Molecular techniques, such as the RFLP, have shown the heterogeneity in cutaneous *Leishmania* parasites in our region (2). *L. tropica* and *L. infantum* or *L. donovani* have been seen in both the skin lesion and the bone marrow in various countries (3-7). It is also important to make a difference the species of *Leishmania* for both clinical and epidemiological reasons (7, 8).

The diagnosis of leishmaniasis, is performed by direct visualization of amastigotes using microscopic examination of stained material, by isolation of the parasite in culture, and by detection parasite using serological methods. Microscopic examination of stained-Giemsa slides, though rapid and low-cost, has limited sensitivity, particularly in chronic lesions (9-11). In vitro culture techniques, while more sensitive, are susceptible to microbiological contamination, and are hampered by the particular growth

requirements of different strains (11, 12). PCR-based methods are highly sensitive and specific, compared with standard methods and are considered valuable for diagnosis (11, 13, 14).
No single molecular test could serve as a "gold standard" against to the different assays could be evaluated. The PCR-RFLP-based genotyping assay has proven to be a reliable test for the detection of *Leishmania* in a wide range of clinical samples. The objective of our study is to contribute to the identification and differentiation of *Leishmania* species responsible from CL and VL in this region.

Material And Methods

Of 58 samples, 45 were collected from patients with skin lesions (mean ages 42±12) enrolled at the Cukurova University Tropical Diseases Research Center in Adana; nine was aspirated from VL patients (mean ages 3±2) who were enrolled at the Infection Unit of Pediatrics, Hospital of Faculty, in Adana between 1999 and 2000. However, four reference strains were used in this study (provided by J. Alvar, Spain). The lesion exudates and bone marrow aspirates were stained with Giemsa on slides for microscopic examination. In addition, the exudates (5-10µl) and the bone marrow (50µl) were used for DNA extraction. Furthermore, the reference strains were cultured in the liquid medium (Table 1). The samples with parasites were centrifuged at 13000×*g* for 5 min, washed twice with PBS (pH, 7.2), and the cells were lysed in 350µl-NET buffer (50mM NaCl, 50 mM EDTA, 50mM Tris-HCl, pH 7.4), including 0.5% SDS and 100µg/ml Proteinase K. DNAs were extracted with Chloroform/isoamyl alcohol (24/1) and precipitated with ethanol. Finally, DNAs were solved in appropriate distilled water, and two-µl of DNA template was subjected to the PCR analysis.

Table 1. Distrubition of PCR-RFLP profiles of *Leishmania* subgenus and species (13).

***Leishmania* groups**	**PCR product (bp)**	**Digested PCR product with *Hae* III (bp)**
L.infantum strains (type A sequence)	250	250
L.infantum strains (type C sequence)	500	250, 180, 70
L.donovani strains	250	180, 70
L.major	500	215, 155, 95, 35
L.tropica	250	215, 35
L.aethiopica	250	215, 35

The used reference strains*

L.donovani (MHOM/IN/80/DD-8)
L.tropica (MHOM/SU/74/K-27)
L.infantum (MHOM/FR//78/LEM-75)
L.infantum (MHOM/ES/90/LEM-2205)

*The reference strains were provided by Dr. J. Alvar (Spain).

The PCR was performed according to a modified procedure (13). PCR amplification carried out in a 10-µl reaction volume containing 1xPCR buffer, 2 mM $MgCl_2$, 2 µl DNA, 0.2 mM each deoxynucleotide triphosphote (dNTPs, Fermentas), 0.4 unit of Taq DNA Polymerase (Fermentas), and 0.2 µM of each primers (5'-CGGCTTCGCACCATGCGGTG-3' and 5'-ACATCCCTGCCCACATACGC-3') selected on repetitive DNA for detection of *Leishmania* subgenus. The PCR profile was as follows: initial denaturation at 94°C for 3 min, the followed by 40 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 20 s, with final extension at 72°C for 5 min.
PCR product (5µl) was digested with five Units of *Hae* III (Fermentas) restriction endonuclease overnight at 37°C in a 20-µl of reaction volume for *Leishmania* species differentiation. The digested PCR product was then separated on 3% agarose gel and analyzed for presence of DNA ladder (100-1500bp, Bio Basic Inc, Canada) with Alpha Imager System (Alpha Innotech, San Leandro, California USA).
We compared DNA fragments on gel according to previously published procedure.[13] Digested *L. tropica* PCR product yielded fragments of a 215 bp and 35 bp, *L. major* yielded fragments of 215, 155, 95 and 35 bp, and *L. donovani* yielded fragments of 180 and 70 bp (Table 1).

Ethics

The institutional review board approved the study and written informed consent obtained from all patients. The study complied with the Helsinki Declaration.

Results

The samples of CL and VL were analyzed by using the PCR-RFLP assay and microscopic examination. PCR-RFLP profiles produced the expected DNA bands. We only determined a single band (250-bp) in nine patients with VL, and these samples were genotyped as *L. infantum* strain (type A sequence) (Figure 2), when compared with previously published data and band patterns of reference strains, but not type C sequence. Total 45 cutaneous samples, of these, 39 was typed as *L. tropica* (215-bp and 35-bp) (Figure 2), six samples were detected as similar to *L. donovani* (about 180-bp and 70-bp) (Figure 3), but no *L. major* strains. Thirty-five-bp DNA was could not seen on the gel because of small DNA size. Amastigotes were detected in 30 of 45 skin exudates (67%) and six of nine bone marrow samples (68%) on stained-Giemsa slides (Figure 1, Table 2). PCR amplification of skin exudates resulted in 54 of the 54 samples, for an overall sensitivity of 100%. Microscopy was resulted as positive (about 67.5%) in 36 of the 54 samples and found to be less sensitive than PCR (100%).

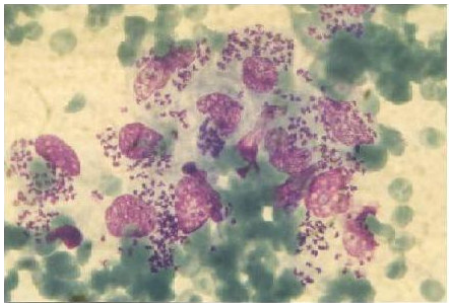

Figure 1. The images of amastigotes in human macrophages (100X, Giemsa stain)

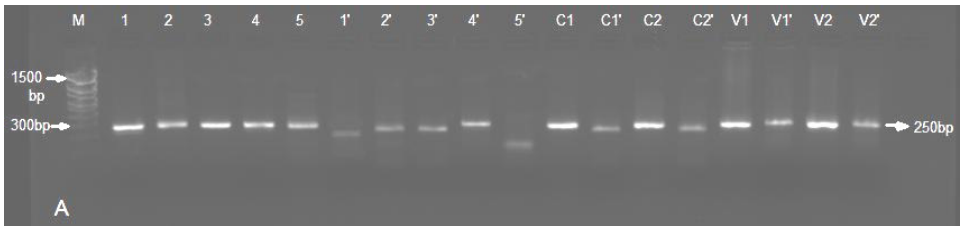

Figure 2. The PCR-RFLP profiles of CL and VL. Lane M: DNA marker (100-1500bp); lanes 1-5: undigested reference strains (*L. donovani* (DD-8), *L. tropica* (K-27) (two), *L. infantum* (LEM-75), and *L. infantum* (LEM-2205), respectively. Lanes 1'-5': digested reference strains, lanes C1-C2: undigested CL, lanes C1'-C1': digested CL samples, lanes V1-V2: undigested VL, and lanes V1'-V2': digested VL samples (35-bp fragment is not visible on the gel).

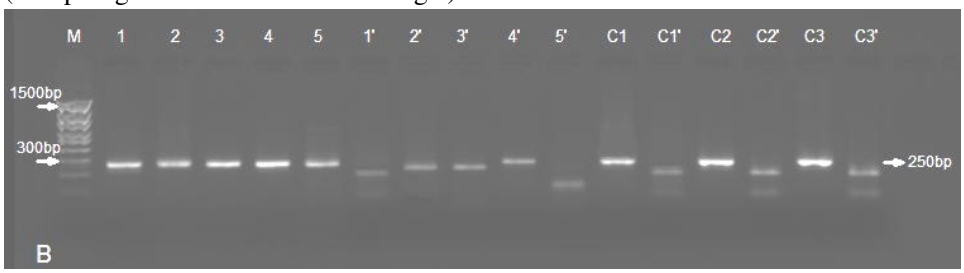

Figure 3. The comparison of PCR-RFLP profiles belong to skin lesions. Lane M: DNA marker (100-1500bp), lanes 1-5: undigested reference strains (*L. donovani* (DD-8), *L. tropica* (K-27) (two), *L. infantum* (LEM-75) and *L. infantum* (LEM-2205), respectively. Lanes 1'-5': digested reference strains. Lanes C1-C3: undigested CL samples, Lanes C1'-C3': digested CL samples (35-bp fragment is not visible on the gel).

Table 2. Positivity of diagnostic procedures in patients with CL and VL attended in Cukurova

Diagnostic test	No of positives		Positivity (%)	
	Cutaneous	Visceral	Cutaneous	Visceral
Direct visualization of amastigotes	30/45	6/9	67	68
PCR-RFLP	45/45	9/9	100	100

Discussion

Frequently, *Leishmania* species are identified based on their geographical distribution and 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 (13).

To identify some parasites in clinical materials can be problematic due to cause both cutaneous and visceral disease (8). Characterization of *Leishmania* species in clinical infections is important, as different species may require distinct treatment regimens (11, 14). PCR methods using either genomic or kinetoplast DNA (kDNA) are now frequently cast in this role. When the amplicon is digested with restriction enzymes, it is possible to identify almost all pathogenic *Leishmania* species by RFLP, allowing direct, rapid characterization and identification of the infecting parasite (7, 8, 14-16).

VL is rare seen in Silifke area of the west of Cukurova region. We used the PCR-RFLP method for the detection and the differentiation species of CL and VL in this region, which is near to Syria. The recent study showed that one human Syrian isolate was identified as *L. major* (17). Our PCR results showed that there is *L.tropica* in 39 of 45 patients with skin lesions. In addition, among the CL patients obtained from our region, six samples were characterized as similar to *L. donovani* reference strain (DD-8). It was important to be seen similarity to visceral Leishmaniasis parasites in patients with skin lesions. CL is endemic in Cukurova region, whereas VL is sporadic. In Turkey, is confined to the Southern and South-Eastern Anatolia where cutaneous leishmaniasis caused by *L. tropica* and visceral leishmaniasis by *L. infantum* (18). On the other hand, our data provided an evidence for the previous studies, which reported that leishmaniasis. Elamin *et al*. showed that *L. donovani* was isolated from patients with CL in Sudan (6). Serin *et al.* and Chargui *et al*. indicated that *L. infantum* was obtained in skin lesions in Turkey, and Tunisia (7, 11). In this study, PCR results showed that the *L. infantum* is a visceral agent in the west of Cukurova region (Silifke). In addition, we did not found viscerotropic agents in these areas. We determined that there was not any *L. major* in CL in our region. These findings are important to treat the cutaneous and visceral Leishmaniasis. Our results reveal that *L. donovani* and *L. infantum* could be playing a possible role in the generation of CL in our region. In present study, parasites in the skin lesions were similar to *L. donovani*, whereas the parasites in the study of Serin *et al*. in the same region were *L. infantum* (7). It is require designing the advanced studies on this issue.

We also examined the smear of 45 exudates and 9 bone marrow aspirates using microscope and showed *Leishmania* amastigotes in 30 patients with skin lesion and 6 patients with VL. PCR resulted in a higher sensitivity (100%) than microscopy, detecting an additional 67-68% of the positive cases and identified the species. This finding is consistent with previous reports that have found the PCR to be more sensitive than conventional methods for parasitological detection in clinical specimens. Culha *et al*. reported 100% and 68% sensitivities in PCR and microscopic examination, respectively (10). Chargui *et al*. pointed that PCR was 99.3% sensitive and microscopy technique resulted in sensitivity of 80.3% (11).

As a conclusion, this study pointed out a diagnostic PCR method for leishmaniasis that combines high sensitivity with species differentiation and more reliable, more economical and less time consuming than microscopic examination.

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