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Identification of Causative Species in Cutaneous Leishmaniasis Patients Using PCR-RFLP

Eroglu F[1]*, Koltas IS[1] and Genc A[2]

[1]Cukurova University, Faculty of Medicine, Department of Parasitology, Balcali, Adana, Turkey
[2]Adıyaman University, Vocational School of Health Services, Altinsehir, Adiyaman, Turkey

Abstract

Cutaneous leishmaniasis (CL) in Cukurova located in the southern part of Turkey is a public health problem. We assessed the efficiency of a PCR method in establishing the diagnosis of CL. We used two different targets, kinetoplast DNA (kDNA) for diagnosis and a mini-exon gene for species typing. 64 smear samples were taken from clinically CL suspected cases. The DNA was amplified kinetoplast DNA (kDNA) by using the polymerase chain reaction (PCR) with universal primers 13A-13B, specific for the *Leishmania* genus and DNA was amplified in the mini-exon region by PCR with Fme-Rme primers, specific for *Leishmania* species. We compared the sensitivity and specificity of conventional microscopy with kDNA and mini-exon PCR. kDNA PCR was found to have a specificty of 58.8% and a sensitivity of 100%, respectively. Furthermore, we performed a restriction fragment length polymorphism (RFLP) analysis on the PCR products of mini-exon for the genotyping of *Leishmania* species. Interestingly, the PCR-RFLP result showed that 31.5% of the isolates had *Leishmania infantum* (*L.infantum*) in CL cases without visceral leishmaniasis (VL) history.

Keywords: Cutaneous leishmaniasis; PCR; RFLP; *L.infantum*

Introduction

Cutaneous leishmaniasis (CL) is a skin disease caused by the *Leishmania* species [7,20]. CL is caused by *Leishmania major* (*L.major*), *Leishmania aethiopica* (*L.aethiopica*), *Leishmania tropica* (*L.tropica*), *Leishmania infantum* (*L.infantum*) and *Leishmania donovani* (*L.donovani*), in the Old World [24]. CL is endemic diseases in southeastern Turkey [10]. The causative agent of CL in this region is mainly *L.tropica* [2]. However, recently researches have reported that *L.infantum* is isolated from CL patients [30,32] CL is most frequently diagnosed by clinical evaluation and its typical acute lesions can be confused with other dermatological problems, such as sporotrichosis [1,25]. Therefore, a clinical evaluation is not enough for a conclusive diagnosis of CL, so the laboratory diagnosis is important for CL diagnosis [27,28]. The traditional diagnostic methods of CL consist of the microscopic examination of smears after Giemsa staining, culture and serological techniques [29]. These techniques are adequate for the diagnosis of CL, but these techniques are not adequate for the discrimination of species due to the morphological similarity of different *Leishmania* species [11,12].

Recently, several molecular methods, especially those based on PCR, have been used for the diagnosis and identification of *Leishmania* species [16,26]. The targets for amplification with PCR serve either nuclear DNA, such as the SSUrRNA gene, mini-exon regions, the gp63 gene locus, microsatellite DNA, the internal transcribed spacer 1 (ITS1) region or extrachromosomal DNA, such as the repetitive kDNA minicircles [5,17]. The ITS 1 region of the rRNA genes (40 to 200 copies) and ITS 1 region have previously been expoited for Old World species discrimination using restriction fragment length polymorphisms [31]. KDNA minicircles (10000 to 20000 copies) are used as ideal molecular targets for the diagnosis of the *Leishmania* genus [23]. The mini-exon regions of the rRNA genes (40 to 200 copies) have been used to identify *Leishmania* species, if the mini-exon amplicon is digested with restriction enzymes [17]. The PCR-RFLP method has reported sensitivity from 90% to 100% for identification of *Leishmania* sp [13], so we used PCR-RFLP method for identification of *Leishmania* sp in our study.

In this study, we used kDNA for the diagnosis and the mini-exon region for the species typing of CL smear samples. We calculated the sensitivities and specificities of kDNA and the mini-exon PCR assay using a microscopic examination as the gold standard of CL diagnosis. However, *Leishmania* isolates have identified genotypes using the mini-exon region by PCR-RFLP.

Materials and Methods

Cases, microscopic examination and DNA isolation

The smear samples were taken from 64 cases, which were suspected for CL. The participants were living in the locality were informed about CL disease, a questionnaire was applied to people suspected of having CL. Then, these lesions were cleaned with 70% alcohol. Using a sterile lancet, 2-3 mm long superficial incisions were made on the margins of the lesion. A smear of dermal tissue scrapings was collected from each patient. The smear samples were stained with Giemsa and then examined with (oil X100 objective) for the presence of the amastigote

kDNA PCR	Microscopic Examination		Mini-exon PCR
+	22	13	+
-	0	0	
+	8	1	-
-	0	20	
Total	30	34	

Table 1: Results of microscopic examination, kDNA PCR and Mini-exon PCR.

***Corresponding author:** Eroglu F, Cukurova University, Faculty of Medicine, Department of Parasitology, Balcali, Adana, Turkey, E-mail: eroglufadime@hotmail.com

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form of the parasite. The amastigotes were quantitated according to the method by [6] and the density of the amastigotes was 10 to 100 amastigotes per high power field (hpf).

The genomic DNA was extracted from all of the smear samples using the Agencourt Genfind v2 DNA isolation kit (Beckman Coulter, Beverly, USA) according to the manufacturer's protocol.

PCR amplification and restriction digestion

The kDNA PCR using the primers 13A (5'-GTG GGG GAG GGG CGT TCT-3') and 13B (5'-ATT TTC CAC CAA CCC CCA GTT-3') was carried out essentially [21]. On the other hand, mini-exon PCR using the primers Fme (5'-ACA GAA ACT GAT ACT TAT ATA GCG-3') and Rme (5'-TAT TGG TAT GCG AAA CTT CCG-3') was used to identify *Leishmania* species [17]. In addition, it was used to compare PCR methods (kDNA and mini-exon) and microscopic examinations.

All PCR reaction mixtures consisted of PCR buffer 1X (75 mM Tris-HCl, P^H 8.3 and 20 mM $(NH4)_2SO_4$, 1.5 mM $MgCl_2$,1 U Taq polymerase (Fermantas SB83), 0.2 mM dNTPs (Fermantas RO 191), 12% DMSO (Fluka Chemika, Germany), 0.5 pmol of each primer, and 5 µL of the DNA sample. DNA amplification was performed by a first cycle at 94°C for 5 mins, followed by 40 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min and 30 sec, with a final extension cycle at 72°C for 10 mins. The PCR products were analysed on 2% agarose gels by electrophoresis at 100 V in 1 X Tris-Boric-EDTA buffer (0.04 mM Tris-boric and 1mM EDTA P^H 8) and visualized by UV light after being stained with ethidium bromide.

Mini-exon PCR products (10-20 µL) were digested with the EaeI restriction enzyme (Takara, 1123A) according to the manufacturer's instructions, and the restriction fragments were analysed by 3% agarose gel electrophoresis. Fragment sizes were determined with bands of a DNA length Standard (50-1000 / 100-1000 bp ladder, Bio-Basic GM345/GM346).

Results

Specimens from 64 suspected CL patients infected in the Cukurova region, in the southern part of Turkey, were examined by microscopic examination, kDNA and mini-exon PCR (Table 1). The microscopic examination results were positive in 30 (46.9 %) and negative in 34 (53.1%) cases (Table 2).

44 (68.75 %) of the 64 smear samples were positive and 20 (31.25%) smear samples were negative, according to kDNA PCR. When we compared the sensitivity and specificity of the kDNA PCR and the microscopic examination, a sensitivity of 58.8% and 100% were found, respectively (Table 2). 35 (54.7%) of these samples were found to be positive and 29 (45.3%) smear samples were found to be negative by mini-exon PCR. We calculated the specificity and sensitivity of the mini-exon PCR according to the microscopic examination; the specificity was 85.2% and the sensitivity was 100% (Table 3).

The kDNA PCR products were considered positive when band of correct size kDNA of ~ 120 bp was observed for *Leishmania* isolates (Figure 1). The mini-exon PCR product sizes of cutaneous isolates were found to be approximately 403-435 bp for *Leishmania* sp (Figure 2). However, to be sure about the exact type of species, the PCR products were digested with EaeI for RFLP. 11 mini-exon PCR according to the microscopic examination; the specificity was 85.2% and the sensitivity was 100% (Table 4).

The kDNA PCR products were considered positive when a band of correct size kDNA of ~ 120 bp was observed for *Leishmania* isolates (Figure 1). The mini-exon PCR product sizes of cutaneous isolates were found to be approximately 403-435 bp for *Leishmania* sp (Figure 2). However, to be sure about the exact type of species, the PCR products were digested with EaeI for RFLP. 11 mini-exon PCR samples (31.5%) produced two bands approximately 327 and 108 bp after digestion with EaeI indicating *L.infantum* and the other 24 of ITS 1 PCR products (68.5%) produced two bands approximately 227 and 178 bp after digestion with EaeI indicating *L.tropica* (Figure 3). We did not find any other *Leishmania* species in this study.

Discussion

A diagnosis of CL can be made on the basis of clinical and epidemiological data, but it has to be confirmed by laboratory methods [9]. All conventional methods such as stained smear, culture, histopathology and immunologic methods, employed for the diagnosis of CL has modest to low rates of positivity [18]. Microscopic examination has been the gold Standard for CL diagnosis for the last 100 years [19,22]. However, it has reported sensitivity from 42% to 67% [26]. We found similar results in our study regarding the sensitivity of microscopic examination for CL diagnosis.

Assay	Positive	Negative	%of Positive	%of Negative	Sensitivity		Specificity	
					kDNA PCR	Mini-exon PCR	kDNA PCR	Mini-exon PCR
Microscopic Examination	30	34	46.9	53.1	68	85	100	100

Table 2: Results of microscopic examination compared with kDNA PCR and mini-exon PCR.

Assay	Positive	Negative	%of Positive	%of Negative	Sensitivity		Specificity	
					ME*	Mini-exon PCR	ME*	Mini-exonPCR
kDNA PCR	44	20	68.75	31.25	100	100	58.8	68.9

*ME: Microscopic examination

Table 3: Results of kDNA PCR compared with microscopic examination and mini-exon PCR.

Assay	Positive	Negative	%of Positive	%of Negative	Sensitivity		Specificity	
					ME*	kDNA PCR	ME*	kDNA PCR
Mini-exon PCR	35	29	54.7	45.3	100	79.5	85.2	100

*ME: Microscopic examination

Table 4: Results of mini-exon PCR compared with microscopic examination and kDNA PCR.

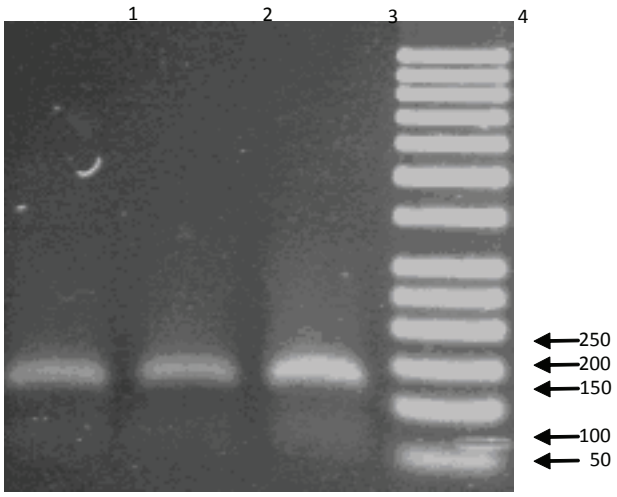

Figure 1: PCR products of *Leishmania* isolates. Lanes 1-3: *Leishmania* isolates. PCR products of cutaneous isolates with 13A-13B primer of kDNA PCR. Lane 4: 50-1000 bp DNA ladder.

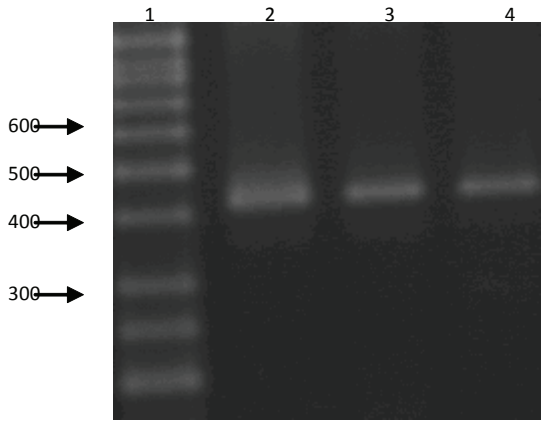

Figure 2: PCR products of cutaneous isolates with Fme-Rme primer of mini-exon PCR. Lane 1: 50-1000 bp DNA ladder, lane 2-4: *Leishmania* isolates.

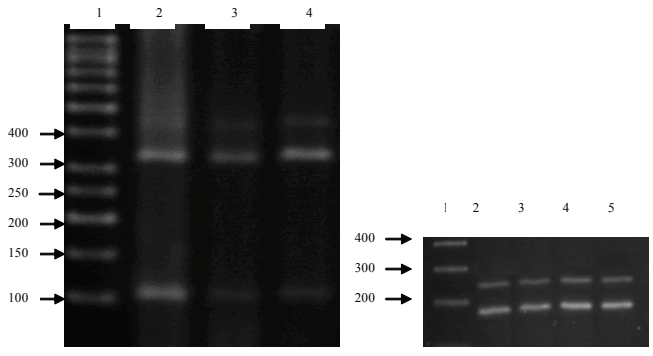

Figure 3: RFLP band patterns of cutaneous isolates after digestion with *EaeI*. A:Lane 1: 50-1000 bp DNA ladder, lane 2-4: *L. infantum*, B: Lane 1: 100-1000 bp DNA marker, lanes 2-4: *L. tropica*.

PCR offers certain advantages over conventional methods for the diagnosis and characterization of CL [22]. When approximately applied, PCR can be more specific, sensitive, versatile, and rapid than conventional methods; in addition, genetic information can be obtained in the process [4].

PCR has shown high sensitivities, varying from 80-100 %, as reported by various groups working on CL diagnosis [15,20]. The positivity rate of kDNA PCR was found to be 99.3% [8], 87% [1], and 100% [4]. Our study confirms these findings, showing that kDNA PCR has a specificty of 58.8% and sensitivity of 100%, while the specificity and sensitivity of mini-exon PCR were found to be 85.2% and 100%, respectively. kDNA PCR was found to be superior to parasitological methods for the diagnosis of CL in our study. However, the PCR-RFLP assay targeting mini-exon region in nuclear DNA was shown to be useful in the diagnosis and identification of speicies of *Leishmania* in this study.

CL in the Old World is mainly caused by *L.major*, *L.tropica*, *L.aethiopica* and occasionally *L.infantum* [14]. It has been reported that *L.infantum* strains may be causng CL in the Middle-East, Africa, Southern Europe, Iran and Tunisia [3,21]. Chargui et al. [8] Isolated *L.infantum* (1%) from leisons of the kin and they found that 99% were *L.major* in Tunisia by zymodeme analysis [8]. However, *L.infantum* in CL cases without VL history has been reported in our country [30,32]. In our study, 68.5% were probably caused by *L.tropica* and 31.5% by *L.infantum*. We found that *L.infantum* could cause CL but these findings are very important for prescribing the appropriate therapy as *L.infantum* strains may be enter the visceral. However, more studies are needed to confirm this issue.

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