Tropical Biomedicine 33(2): 260–267 (2016)

Comparison of convensional and molecular methods used to determine *Leishmania* species

Soyal, A.[1], Gokmen, T.G.[2*], Kayar, B.[3] and Koksal, F.[1]
1Department of Medical Microbiology, Medical Faculty, Cukurova University, 01330, Adana, Turkey
[2]Department of Microbiology, Ceyhan Veterinary Faculty, Cukurova University, 01330, Adana, Turkey
[3]Tropical Disease Researh and Application Center, Cukurova University, 01330, Adana, Turkey
*Corresponding author e-mail: tulinguven01@hotmail.com
Received 2 May 2015; accepted in revised form 21 December 2105; accepted 23 December 2015

Abstract. Leishmaniasis is a multisystem disease, and have a broad spectrum ranging from skin lesions to systemic disease. Therefore diagnosis must be supported with laboratory results. We analyzed 153 smears, aspiration, blood and bone narrow samples collected from patients suspected with cutaneous and visceral leishmaniasis. The specificity and sensitivity of the four methods (culture, smear, miniexon-PCR-RFLP and ITS1-PCR-RFLP) were detected and *Leishmania* species were determined. The ITS1-PCR-RFLP method was found that the highest sensitivity and specifity. *L. infantum* and *L. tropica* were identified by molecular methods from samples. As a result, ITS-1-PCR has a high sensitivity and specificity and easily applicable method. However, it requires the miniexon-PCR or ITS1 sequencing the discrimination of the *L. donovani* complex. *L. infantum* is a agent both visceral and cutaneous leishmaniasis in our region.

INTRODUCTION

Leishmaniasis is an infectious disease, especially seen in tropical and subtropical areas (Croft & Coombs, 2003). According to World Health Organization data, approximately 12 million leishmaniasis cases are available in all over the world, 1.5-2 million cutaneous and 500,000 visceral *leishmaniasis* cases are added each year, and every year 70,000 people die due to this disease (WHO, 2012). Disease is endemic, especially the Mediterranean, Aegean and in Southeast Anatolia regions (Ateş *et al.*, 2011). Clinically, leishmaniasis can imitate many diseases, therefore, the diagnosis should be confirmed by a laboratory diagnosis.

Culture method in diagnosis of leishmaniasis is still considered as the gold standard, but it has low sensitivity. Microscopic diagnosis needs experienced staff and has low sensitivity and specificity. Last years, the molecular methods are prefered due from high specifity and sensitivity. PCR method, values at the level 100% specificity and sensitivity of 92-98%, shows a performance close to the gold standard. Besides, It is detected *Leishmania* spp. within 24 hours and identified level of species and sub-species (Andresen *et al.*, 1997; Gunes, 2006).

A well-established PCR method, targeting the internal transcribed spacer 1 (ITS1) region between the SSU and 5.8S rRNA genes, is useful for the direct diagnosis of different forms of leishmaniasis. The method is highly specific and sensitive, able to detect approximately 0.2 parasites per sample and identify all medically relevant *Leishmania* species which are distinguished by DNA sequencing or RFLP of the PCR product (Dávila & Momen, 2000, Schönian *et al.*, 2000, Schönian *et al.*, 2001 and Schönian *et al.*, 2003).

Miniexon PCR is used preliminary discrimination between the major complexes (Old World *Leishmania*, New World *Leishmania*, and New World *Viannia* complexes). After restriction enzyme digestion of the amplicon, a characteristic RFLP pattern is produced that depends on size variations in the polymorphic spacer regions as well as mutations in the recognition sites of a chosen restriction enzyme (Serin, 2005).

In our study, we aimed to determine the sensitivity and specificity of culture, microscopy, mini-exon PCR-RFLP and ITS1-PCR-RFLP methods, indicate their superiority to eachother and detect *Leishmania* species.

MATERIALS AND METHODS

In our study, we were evaluated samples of 153 patient admitted Cukurova University in Tropical Diseases and Application research center with suspected cutaneous and visceral leishmaniasis from May 2010 to September 2012. Smear and aspiration samples from 143 cutaneous lesions and 10 blood and bone marrow samples from patients suspected visceral leishmaniasis were taken.

Firstly, normal saline were injected into the lesion patients with cutenous leishmaniasis and then aspirated. Aspiration samples was inoculated to NNN (Novy-Nicolle-McNeal) agar, spread onto a slide and were stored at -20ºC for DNA isolation. After that, smear was taken from lesion and stained 10% Giemsa stain.

The blood and bone marrow samples of patients suspected visceral leishmaniasis was inoculated to NNN (Novy-Nicolle-McNeal) agar, spread onto a slide and were stored at -20ºC for DNA isolation.

PCR and PCR-RFLP Applications

Collected 153 samples from patients with suspected leishmaniasis, was evaluated by PCR and PCR-RFLP method for detection of ITS-1 and miniexon specific sequences. In addition, 52 cutaneous leishmaniasis aspiration samples were sent in TE buffer from the Syrian Arab Republic Ministry of Health, Infectious and Heavy Diseases Department, Leishmaniasis Control Center. DNA isolation was performed with tissue extraction kit (QIA-GEN tissue extraction kit- Lot No: 11872534, Cat No: 51306) in these samples.

We were used the genomic DNAs of reference strains *L. infantum* (NYC/CN/80/RD_{13}, CAN/TR/02/EP77) and *L. tropica* (IROS/NA/87/8688, HOM/KE/81/NLB030B) provided by Prof. Kwang-Poo Chang (Department of Microbiology/Immunology, Chicago Medical School, North Chicago, IL) in a previous study of our department (Serin, 2005).

PCR Amplification of the Miniexon Region

For amplification of the regions located at the leishmania species, Fme 5'-ACAGAAA CTGATACTTATATAGCG-3' and Rme 5'-A-TATTGGTATGCGAAACTTCCG-3' primers were used (Serin *et al.*, 2005). Total of 50 µl PCR mixture for use in amplification was prepared as follows; PCR Buffer containing 10 mM NH_4CH_3COO (pH:8,3), 5 µl DMSO, 200 µM from each dNTP, 50 pmol from each primer, 2,5U Taq polymerase, 50 ng/5 µl template DNA. Amplification was performed; first denaturation at 94ºC for 5 min, 40 cycles of denaturation 94ºC for 1 min, annealing 54ºC for 1 min, extension 72ºC for 1 min and followed by last extension 72ºC for 7 min. Amplicons was separated on 1.5% agarose gel.

PCR Amplification of the ITS-1 Region

In this study, LITSR: 5'-CTGGATCATTTT CCGATG-3' and L5.8S: 5'-TGATACCAC TTATCGCACTT-3' primers were used (Schonian *et al.*, 2003). Total of 50 µl PCR mixture for use in amplification was prepared as follows; PCR Buffer containing 10 mM KCl (pH:8,3), 200 µM from each dNTP, 50 pmol from each primer, 2,5U Taq polymerase, 50 ng/5 µl template DNA. Amplification was performed as follows; first denaturation at 95ºC for 2 min, 34 cycles of denaturation 95ºC for 20 s, annealing 53ºC for 30 s, extension 72ºC for 1 min and followed by last extension 72ºC for 7 min. Amplicons was runned on 1.5% agarose gel.

RFLP Assay

PCR products were restricted by enzymes, EaeI (Fermentas, ER0161) and HaeIII (Fermentas, ER0151) to detection *Leishmania* spp. (Dweik *et al.*, 2007; Serin *et al.*, 2005). The total DNA amount was calculated to be 1 µg in a total volume. RFLP products were separated on 4% agarose gel.

Sequencing Assay

After amplification and RFLP assay, sequence analysis was performed with the same primers on ITS-1 and miniexon PCR products for *L. infantum*. The sequence analysis was done using the dye terminator cycle sequencing method and an ABI Prism Big Dye Terminator kit (Applied Biosystems, Foster City, USA). The assay was carried out according to the standard protocol. Data were collected on an ABI 3100 automated fluorescence sequencer (Applied Biosystems). The types of miniexon and ITS-1 genes were identified by comparing the sequences of the database in GenBank. Using Basic Local Alignment System Tool (BLAST) at www.ncbi.nlm.nih.gov/genbank.

The sensitivity and specificity of each assay were determined, and 95% confidence intervals (CI) were calculated using the standard normal-distribution formula for proportions.

RESULTS

Culture and Smear Assay

The *Leishmania* promastigotes was observed at 80 patients on NNN agar culture. In giemsa-stained smear method; *Leishmania* amastigotes was determined on 116 patients by microscopic examination.

PCR and RFLP Assay

Aspiration, blood and bone marrow samples taken from patients were extracted by the kit (QIAGEN DNA Extraction kit Lot No.: 11872534, Cat No: 51306). Extracted DNA samples were amplified by specific primers, targeting miniexon and ITS-1 gene region.

In miniexon-PCR method were obtained 400-435 bp in length fragments. The 119 of 153 samples were found positive for *Leishmania* spp. In 28 of 52 samples coming from Syrian Arab Republic were detected. Amplicons were restricted by *EaeI* restriction enzyme and band patterns were evaluated according to a previous study and RFLP pattern of reference strains *L. infantum* and *L. tropica* (Serin, 2007; Marfurt, 2003). As a result; 75 (64.2%) of 119 samples were found to be compatible with the RFLP pattern of *L. tropica* (227bp, 178bp) and the RFLP pattern of 42 samples (35.8%) is similar to *L. infantum* (326bp, 108bp). Two samples could not be typed because of weak DNA bands. In Syria samples identified as positive; 7 *L. infantum* and 21 *L. tropica* have been identified.

In the evaluation with ITS-1-PCR method, with specific primers targeted the operon between 18S and 5.8S rRNA genes in the genome of *Leishmania*, 350 bp in length fragments were obtained. By ITS-1 PCR; detected the Leishmania positivity in 125 of 153 patients samples. In 32 of 52 samples coming from Syrian Arab Republic were detected. PCR products were cut by *HaeIII* restriction enzyme. RFLP pattern were evaluated according to a previous study and RFLP pattern of reference strains *L. infantum* and *L. tropica* (Schönian, 2003). Eighty one of 125 samples were found to be compatible with the RFLP pattern of *L. tropica* (200bp, 60bp) and the RFLP pattern of 44 samples is similar to *L. donovani complex* (200 bp, 80bp and 50bp). In Syria samples identified as positive; 8 *L. donovani complex* and 24 *L. tropica* have been identified (Fig. 1).

Miniexon and ITS1 gene regions of *L. infantum* characterizated by the were performed sequencing analysis and comfirmed the results.

In our study, samples which was determined positive with a single method was considered positive. Sensitivity and specificity were calculated on the basis of total 127 positive and 26 negative samples (Table 1).

In compared four methods; ITS1-PCR was highest sensitivity with 98.4%, culture method was lowest sensitivity with 62.9%. The specificity of all methods was determined as 100% (Table 2).

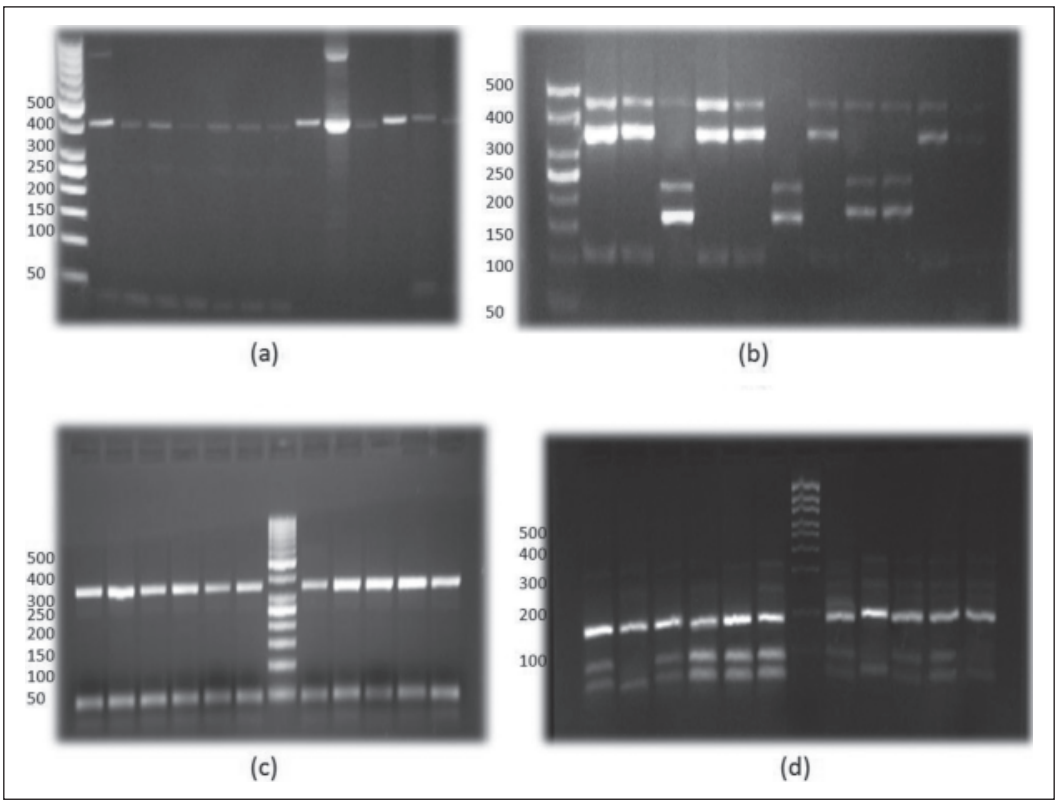

Figure 1. (a) Lane 1: 50 bp DNA ladder; Lane 2-12: Miniexon-PCR products (400-435 bp). (b) Lane 1: 50 bp DNA ladder; Lane 2: RFLP with EaeI for reference *L. infantum* (326 bp and 108 bp); Lane 3: RFLP with EaeI for reference *L. tropica* (227 bp and 178 bp); Lane 4-5-7-10: RFLP with EaeI for *L. infantum* miniexon gene region (326 bp and 108 bp); Lane 6-8-9: RFLP with EaeI for *L. tropica* miniexon gene region (227 bp and 178 bp). (c) Lane 7: 50 bp DNA ladder; Lane 1-11: ITS1-PCR products (350 bp). (d) Lane 7: 100 bp DNA ladder; Lane 1: RFLP with HaeIII for reference *L. infantum* (200bp, 80 bp and 50 bp); Lane 3: RFLP with HaeIII for reference *L. tropica* (200bp and 60 bp); Lane 3-4-5-6-8-10-11-12: RFLP with HaeIII for *L. infantum* miniexon gene region (200bp, 80 bp and 50 bp); Lane 9: RFLP with HaeIII for *L. tropica* miniexon gene region (200 bp and 60 bp).

Table 1. Positive and negative values of the samples according to methods

POSITIVE		NUMBER
Culture(+)Smear(+)	ITS(+) MNX(+)	80
Culture(-)Smear(+)	ITS(+) MNX(+)	34
Culture(-)Smear(-)	ITS(+) MNX(+)	5
Culture(-)Smear(+)	ITS(-) MNX (-)	2
Culture(-)Smear(-)	ITS(+) MNX (-)	6
Total		127
NEGATIVE		
For 4 metods (-)		26

Table 2. Sensitivity and spesifity values of all the methods used in our study

	True Positive	False Positive	True Negative	False Negative	Sensitivity (%)	Sensitivity 95% CI	Spesifity (%)	Specificity 95% CI	PPV (%)	NPV (%)
Culture	80	0	26	47	62,9	53.98%–71.39%	100	86.65%–100%	100	35,6
Smear	116	0	26	11	91,3	85.03%–95.59%	100	86.65%–100%	100	70,2
Miniexon PCR	119	0	26	8	93,7	87.96%–97.23%	100	86.65%–100%	100	76,5
ITS-1 PCR	125	0	26	2	98,4	94.41%–99.76%	100	86.65%–100%	100	92,8

DISCUSSION

In the literature, molecular techniques have proved to be more sensitive and powerful tools than traditional culture methods and smear for detecting Leishmania directly in clinical samples. While the sensitivity of traditional culture methods is ranged between 40-75%, microscopic examination methods is ranged between 74-90% (Bensoussan *et al.*, 2006; Graça *et al.*, 2012; Bart *et al.*, 2013; Tomas-Perez *et al.*, 2013). When combined two methods, the ratio rises to 85% (Culha *et al.*, 2006; Boggild *et al.*, 2007; Chouihi *et al.*, 2009; Saki *et al.*, 2009).

On the other hand, Miniexon-PCR and ITS1-PCR are the most commonly used molecular techniques in the diagnosis leishmaniasis. Sensitivity of miniexon-PCR methods is ranged between 53,8-98% (Marfurt *et al.*, 2003; Bensoussan *et al.*, 2006; Eroglu *et al.*, 2011; Bart *et al.*, 2013). Studies showed differences from 63,5 to 91% in the sensitivity of ITS1-PCR (Bensoussan *et al.*, 2006; Azmi *et al.*, 2011; Graça *et al.*, 2012; Tomas-Perez *et al.*, 2013).

In the calculation of sensitivity and specificity, Culture positivity, smear positivity and at least one PCR positivity were made on the basis. Because there is not a method as the gold standard to calculating for the sensitivity and specificity.

In the most studies, kDNA-PCR considered as the gold standard method of in several studies (Reithinger *et al.*, 2007; Nasereddin *et al.*, 2008). On the other hand, Specific gold standard was undetermined and calculations were made by methods in the other studies analogous to us (Bensoussan *et al.*, 2006; Azmi *et al.*, 2011). kDNA-PCR did not accept as the gold standard, because of genus specific identification and low specificity (50-60%) (Bensoussan *et al.*, 2006; Reithinger *et al.*, 2007; Nasereddin *et al.*, 2008; Azmi *et al.*, 2011).

In our study, we determined NNN agar culture, smear, miniexon-PCR and ITS1-PCR sensitivity were 62.9%, 91,3%, 93,7% and 98,4%, respectively. Specificity of all methods were calculted as 100%.

The low sensitivity of the culture method may be connected to false-negative results. The high sensitivity of smear method may depend on the experienced personnel and examination of two smear samples (aspiration and skin scraping).

When we compared both methods, the ITS1-PCR method is more sensitive than the miniexon-PCR method were determined. In a study, sensitivity of ITS1-PCR methods was determined as 91% and miniexon-PCR as 53.8% (Bensoussan *et al.*, 2006). In a another study, DNA amplification with primers ITS1, at 10fg was observed the band formation, for amplification with miniexon modified primers at 50fg, and for conventional Miniexon primers at 1pg band are determined (Roelfsema *et al.*, 2011).

To evaluate RFLP pattern, Schonian *et al.* (ITS1-PCR-RFLP), Serin *et al.* (Miniexon-PCR-RFLP) studies and genomic DNA of reference strains were made on the basis. However, ITS-1-PCR-RFLP is insufficient to separate the *L. donovani* complex. This deficiency can be removed with the ITS1 Sequencing or Miniexon-PCR-RFLP methods.

Most cases of cutaneous leishmaniasis (CL) in the Mediterranean Basin are caused by two *Leishmania* species: *Leishmania major* and *Leishmania tropica*. The responsibility of *L. infantum* as an agent of CL has been confirmed in several countries of the Mediterranean Basin (Pascal del Giudice, 1998; Serin *et al*., 2005; Eroglu *et al*., 2011). In our study, *L. infantum* was found to be agent for cutaneous leishmaniasis.

Actually, the most important agent is *L. donovani* for visceral leishmaniasis in the old world. However, *L. infantum* was found to be agent in our study. In many studies, *L. infantum* was identified as a factor in the Mediterranean visceral leishmaniasis.

During our study, we had the the sample collection opportunity from Aleppo in Syria which is close to our city. We identified *L. infantum* and *L. tropica* in cutaneous leishmaniasis samples. Besides, some studies showed that after the civil war in Syria, in our country is an increase in the number of patients with the settlement of refugees (Salman *et al*., 2014). In the future, these strains can cause an increase in the incidence of leishmaniasis in our region.

One limitation of our study, kDNA-PCR can be used as a gold standard for determining sensitivity of our study methods. However, we can not be used due to low specificity and genus-level identification. Second limitation, discrimination of dermatotropic and visserotropic variants can be done by MLEE analysis for *L. infantum*. But there was no equipment required and it was difficult isolation with culture method.

As a result, ITS-1-PCR has a high sensitivity and specificity and easily applicable method, when it compared other methods in this study. However, it requires the miniexon-PCR or ITS1 sequencing the discrimination of the *L. donovani* complex. *L. infantum* is a agent both visceral and cutaneous leishmaniasis in our region. *Leishmania* spp. will continue to be a agent that will not lose its importance for our region due to Syria Civil War.

Acknowledgments. In this study, Dr. Lama Jalouk contributed with sending samples from Leishmaniasis Control Center, Aleppo, Syria.

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