Diagnosis and identification of *Leishmania* spp. from Giemsa-stained slides, by real-time PCR and melting curve analysis in south-west of Iran

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Background: The aim of present study was describing a real-time PCR assay for the diagnosis and direct identification of *Leishmania* species on Giemsa-stained slides in south-west of Iran.

Materials and methods: Altogether, 102 Giemsa-stained slides were collected from different part of south-west of Iran between 2008 and 2011. All the Giemsa-stained slides were examined under light microscope. After DNA extraction, real-time PCR amplification and detection were conducted with fluorescent SYBR Green I. For identification, PCR products were analysed with melting curve analysis.

Results: One hundred and two archived slides from suspected lesion examined by microscopy and real-time PCR. The sensitivity of the real-time PCR on Giemsa-stained slide was 98% (96/102). The melting curve analysis (T_m) were $88.3 \pm 0.2^\circ\text{C}$ for *L. tropica* (MHOM/IR/02/Mash10), $86.5 \pm 0.2^\circ\text{C}$ for *L. major* (MHOM/IR/75/ER) and $89.4 \pm 0.3^\circ\text{C}$ for *L. infantum* (MCAN/IR/97/LON 49), respectively.

Conclusion: This study is first report in use of real-time PCR for diagnosis and identification of *Leishmania* spp. in Iran. Up to now, in Iran, the majority of identification of *Leishmania* species is restriction fragment length polymorphism (PCR-RFLP) of ITS1 and kinetoplast DNA. Our data showed that Giemsa-stained slides that were stored more than 3 years, can be used for *Leishmania* DNA extraction and amplification by real-time PCR. Compared to conventional PCR-based methods, the real-time PCR is extremely rapid with results and more samples can be processed at one time.

INTRODUCTION

Leishmaniasis is endemic diseases in 88 countries, 1.5–2 million new cases per year and 350 million at risk (Desjeux, 2001). The variety of clinical manifestations range from

self-curing cutaneous lesions to fatal visceral form of the disease.

About 21 *Leishmania* species have been reported to cause human infection (Croft and Coombs, 2003; Desjeux, 2004). Diagnosis and identification of the species level is important for prognostic, epidemiological and therapeutic reasons (Schriefer *et al.*, 2008).

Because of intrinsic difference in species sensitivity and parasite resistance to pentavalent antimonials, variations in the clinical

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response to drugs are a persistent problem in the treatment of leishmaniasis (Croft *et al.*, 2006; do Monte-Neto *et al.*, 2011).

Direct microscopy examination requires some expertise and they do not provide any species-specific diagnosis. Culture is labour-intensive, and the result is not known for weeks (Rioux *et al.*, 1990; Mondal *et al.*, 2011). The isoenzyme analysis, is slow, and needs to the cultured *Leishmania* (Brewster *et al.*, 1998). Numerous DNA-based assays, e.g. PCR plus sequencing, and restriction fragment length polymorphism (RFLP) analysis, have been developed for this purpose (Bezold *et al.*, 2001; Mauricio *et al.*, 2004; Chargui *et al.*, 2005; Oliveira *et al.*, 2005; Schönián *et al.*, 2008). However, conventional PCR assays provide results in one or two working days (Bretagne, 2003; Foulet *et al.*, 2007).

The relatively new method real-time PCR can process a sample in less than an hour, has been reported to rapidly diagnosis and differentiate single nucleotide mutations within a target DNA sequence (Wortmann *et al.*, 2005; Castilho *et al.*, 2008; Talmi-Frank *et al.*, 2010; Ben Abda *et al.*, 2011). This assay has the potential to provide the treating clinician with a rapid identification of the infecting *Leishmania* species, which subsequently offers the potential for targeted treatment strategies (Wortmann *et al.*, 2005).

South-west of Iran (Khuzestan Province with 3 700 000 populations and 60 000 km² area) is one of the important endemic areas of cutaneous leishmaniasis (CL) in Iran (Javadian and Mesghali, 1974), so that in some points, the incidence rate is 75 per 100 000 populations.

The aim of present study was describing a real-time PCR assay for the diagnosis and direct identification of *Leishmania* species on Giemsa-stained slides without cultivation in south-west of Iran.

MATERIALS AND METHODS

Leishmania Reference Strain Culture

Leishmania major (MRHO/IR/75/ER), *Leishmania tropica* (MHOM/IR/02/Mash10)

and *Leishmania infantum* (MCAN/IR/96/LON49) were kindly provided by Dr Mohebbi (Tehran University of Medical Sciences, Tehran, Iran). Briefly, 5×10^5 cells/ml were cultured in the RPMI 1640 media (pH 7.2, containing 25 mM HEPES) (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% heat inactivated fetal bovine serum and antibiotics at 24°C for 96 hours and stationary-phase promastigotes were harvested at cell densities of 2×10^5 cells/ml. Cells were washed with PBS and pellet obtained by centrifugation (4000 rev/min for 5 minutes) and stored at -20°C.

Patient Samples and Microscopic Examination

Altogether, Giemsa-stained slides were collected from 102 confirmed cases of CL who presented in different part of south-west of Iran (Khuzestan) including North (Shush), South (Hendián), West (dasht-e azadegan), East (Ramhormoz) and Centre (Ahvaz) between 2008 and 2011. All the Giemsa-stained slides were examined under light microscope with magnification ($\times 1000$) and the positive smears were graded for amastigote numbers based on WHO laboratory Methods (WHO, 1991). Briefly, *Leishmania* amastigote grading was acquired by average parasite using $\times 1000$ magnification as follows: 1–10 parasites/fields means +4, 1–10 parasites/10 fields means +3, 1–10 parasites/100 fields means +2 and 1–10 parasites/1000 fields means +1.

DNA Extraction

Briefly, all the slides were cleaned with xylol, covered with 250 μ l lysis buffer and smears scraped completely with scalpel (Schönián *et al.*, 2003). DNA extraction was performed by QIA DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Extracted DNA was eluted in 100 μ l of elution buffer and stored in -20°C until use. Real-time PCR control DNA were also

extracted from mass cultured promastigotes of Iranian standard strains *L. major*, *L. tropica* and *L. infantum*.

Real-Time PCR and Melt Curve Analysis

PCR primers JW13 (5'-ACTGGGGGTT-GGTGTAAAATAGG-3') and JW14 (5'-TTTCGCAGAACGCCCTACCC-3'), are designed on the conserved region of *Leishmania* kDNA minicircle, which amplify the DNA from a wide range of *Leishmania* spp., including *L. infantum*, *L. major* and *L. tropica* (Nicolas *et al.*, 2002). Each sample has been subjected to real-time PCR assay for the detection of *Leishmania* DNA as previously described (de Monbrison *et al.*, 2007).

Real-time PCR amplification and detection were conducted with fluorescent SYBR Green I in a reaction volume of 20 µl, including (5 µl) extracted DNA, 15 µl of reaction mixture containing 4 mM of MgCl₂, 0.5 µM primers and 2 µl of LightCycler FastStart DNA Master SybrGreen I buffer (Roche Applied Science, Meylan, France).

DNA was amplified using thermal cycler (ABI StepOne Plus™) under the following conditions: 4 minutes at 95°C, followed by 35 cycles of 10 seconds at 95°C, 10 seconds at 62°C and 10 seconds at 72°C with watching of fluorescence at the end of elongation step. PCR product identity was confirmed with melting curve analysis (Nicolas *et al.*, 2002).

Briefly, a melting curve program was obtained by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 67°C, keeping it at 67°C for 30 seconds and then slowly heating at 0.1°C/s to 95°C. The transition rates were 20°C/s except for the extension step and the final step, which have a temperature transition rate at 1°C/s and 0.1°C, respectively. Fluorescence was measured through the slow heating phase.

DNA Sequencing

kDNA minicircle was prepared for sequencing. The amplicons of samples and three of

Iranian reference strains including: *L. major*: MHOM/IR/75/ER, *L. infantum*: MCAN/IR/97/LON 49 and *L. tropica*: MHOM/IR/02/Mash10 were subjected to sequencing by MWG (Germany) by the primers employed in the real-time PCR.

RESULTS

Comparison of Real-Time PCR Assay Results with Standard Microscopy Method

One hundred and two archived slides examined by microscopy and real-time PCR. All slides were classified based on date of preparation: less than 1 year 2010–2011, 2 years ago 2009–2010 and 3 years ago 2008–2009.

Thirteen (12.74%) slides were prepared less than 1 year and 65 (63.72%) of the smears had been stored for more than 3 years ago (Table 1). All the real-time PCR negative smears were prepared 2 and 3 years ago. In addition, results of real-time PCR were directly related to grade of *Leishmania* amastigotes numbers on the Giemsa-stained smears (Table 2). Statistically significant difference was observed between the results of real-time PCR with microscopy methods ($P<0.05$). Results showed that real-time PCR was not enough sensitive on slides with low parasite grade.

Melting Curve Analysis of Real-Time PCR

Primers targeting kDNA minicircle were employed for identification of *Leishmania*

TABLE 1. The microscopy and real-time PCR results based on time of Giemsa-stained slides preparation

Smear preparation time	Microscopy-positive		Real-time PCR-positive	
	<i>n</i>	%	<i>n</i>	%
<1 year	13	12.74	13	13.54
2 years	24	23.52	22	22.9
3 years	65	63.72	61	63.54
Total	102	100	96	100

FIG. Melting curve analysis of three reference strains of *Leishmania* spp. and negative control. The melting temperatures (T_m) are $86.5 \pm 0.2^\circ\text{C}$ for *L. major* (MHOM/IR/75/ER), $88.3 \pm 0.2^\circ\text{C}$ for *L. tropica* (MHOM/IR/02/Mash10) and $89.4 \pm 0.3^\circ\text{C}$ for *L. infantum* (MHOM/IR/75/ER). Each point represents the means of three independent tests.

species based on a real-time PCR melting curve analysis. The melting temperatures (T_m) analyses of PCR product can differentiate *Leishmania* species. Three culture adapted strains of *Leishmania* include *L. tropica* (MHOM/IR/02/Mash10), *L. major* (MHOM/IR/75/ER) and *L. infantum* (MHOM/IR/75/ER) discriminate by post-amplification melting temperature (T_m). The melting curve analysis (T_m) were $88.3 \pm 0.2^\circ\text{C}$ for *L. tropica* (MHOM/IR/02/Mash10), $86.5 \pm 0.2^\circ\text{C}$ for *L. major* (MHOM/IR/75/ER) and $89.4 \pm 0.3^\circ\text{C}$ for *L. infantum* (MHOM/IR/75/ER) ($P < 0.02$), respectively (Fig.). The variability of three independent experiment runs did not exceed 0.2%. In addition, the melting

temperature (T_m) under 80°C in non-specific products of real-time PCR, like primer-dimers and lack of melting curve in non-infected samples, was seen.

To evaluate the effect of DNA concentration on melting temperature, five-fold dilutions of *Leishmania* standard strains, ranging from 1 to 100 pg DNA per reaction, were tested, which was not significantly different in results (data not shown). Among the 96 positive samples from south-west of Iran, 90 cases showed melting temperature (T_m) at $86.5 \pm 0.8^\circ\text{C}$ that belong to *L. major*. Five samples showed melting temperature at $88.3 \pm 0.2^\circ\text{C}$ that belong to *L. tropica*. One sample did not show melting temperature (T_m) related to *L. major* and *L. tropica*. Melting curve of recent case was $89 \pm 0.3^\circ\text{C}$ that it was related to *L. infantum* melting temperature (T_m). Analyses of the molecular sequences confirm that this case related to *L. infantum* strain.

TABLE 2. Giemsa-stained smears grading and diagnosis of *Leishmania* with real-time PCR

Real-time PCR	Grading of <i>Leishmania</i> amastigote				Total
	+1	+2	+3	+4	
Positive	20	12	38	26	96
Negative	5	1	0	0	6
Total	25	13	38	26	102

Leishmania kDNA Sequencing

The real-time PCR products of three different standard species of *Leishmania*

were sequenced. Sequence alignments were constructed using the program CLUSTAL W version 1.83 (<http://www.ddbj.nig.ac.jp/search/clustalwe.html>). All sequences submitted in Genbank are as follows: IranJWinf AB678348 for *L. infantum*, IranJWmaj AB678349 for *L. major* and IranJWtrop AB678350 for *L. tropica*. GenBank was searched for similar sequences with the BLAST program and a significant homology was detected with other *Leishmania* sequences.

DISCUSSION

In this report, we performed a real-time PCR assay for diagnosis and characterization of *Leishmania* in Giemsa-stained slides. Our data showed that Giemsa-stained slides that were stored more than 3 years, could be used for *Leishmania* DNA extraction and amplification by real-time PCR. The use of archived Giemsa-stained slides for molecular diagnosis and differentiation of causative agent of CL has been described in several studies (Motazedian *et al.*, 2002; Al-Jawabreh *et al.*, 2006; Kazemi-Rad *et al.*, 2008). Motazedin *et al.* indicate that the PCR-based checking of Giemsa-stained smears appears to be reasonably sensitive and specific in revealing the presence of *Leishmania* parasites (or, at least undegraded leishmanial DNA) in such chronic lesions (Motazedian *et al.*, 2002).

Up to now, in Iran, the majority of identification of *Leishmania* species are DNA base molecular methods include restriction fragment length polymorphism (PCR-RFLP) of ITS1, kDNA minicircle sequences and minicircle gene (Baghaei, 2005; Tashakori *et al.*, 2006; Saki *et al.*, 2010; Ghasemian *et al.*, 2011).

In *Leishmania* species, kDNA minicircles are a heterogeneous group in sequence and size (0.5–1.5 kb) (Brewster *et al.*, 1998). Although the SYBR green I dye used in this study binds to double-strand DNA, our results indicate that the fluorescence melting

curve analysis (T_m) of kDNA can be used for differentiation of three species strains of *L. major*, *L. infantum* and *L. tropica*, 86.5 ± 0.2 , 89.4 ± 0.3 and $88.3 \pm 0.2^\circ\text{C}$, respectively (Fig.). The sequencing of the real-time PCR products confirmed the outcome of the melting curve analysis. Also melting curve analysis allows discriminating non-specific products of amplification, like primer-dimers that melted at temperatures below 80°C (data not shown).

Results of our study showed that *L. major* is predominant strain (93.75%), according to previous results in south-west of Iran (Saki *et al.*, 2010).

In similar study, Nicolas *et al.* has evaluated real-time PCR for differentiation of Old World *Leishmania* species and indicated that melting curve analysis of PCR products offers a rapid alternative for identification of *L. major*, *L. donovani*, *L. tropica* and *L. infantum* species in diagnostic or epidemiological studies of leishmaniasis or asymptomatic parasitism (Nicolas *et al.*, 2002). Differential diagnosis of species or strains with melting curve analysis has been described in several pathogens including *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Campylobacter* spp. (Corless *et al.*, 2001; Logan *et al.*, 2001).

In a prospective study, skin lesions spotted on filter paper have been evaluated by real-time PCR for the *Leishmania* species identification in Algeria. Monbrison *et al.* showed that the melting curve analysis discriminate four culture adapted strains (*L. infantum* MHOM/TN/80/IPT1, *L. major* MHOM/SU/73/5-ASKH, *L. donovani* MHOM/IN/80/DD8 and *L. tropica* MHOM/SU/74/K27) and indicated that real-time PCR provides significant results with filter paper and represents a tool for the identification of old world CL (de Monbrison *et al.*, 2007). In another study, the melting curve analysis has been used for the identification of species and intraspecies groups within the *L. donovani* complex and because the capacity to type parasites directly from clinical samples has been suggested alternative for molecular

diagnosis and epidemiological studies of visceral leishmaniasis (Quispe-Tintaya *et al.*, 2005).

In conclusion, the real-time PCR procedure seems to be a suitable tool for direct diagnosis and characterization of *Leishmania* species from Giemsa-stained slides.

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