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Original Article

The Accuracy of Diagnosis and Genotyping of *Leishmania* Species Based on Spliced Leader Mini-Exon Gene by Nuclear Magnetic Resonance and Sequencing Assays

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Abstract

Background: We aimed to evaluate the accuracy of genotyping of *Leishmania* species by the spliced leader mini-exon gene.

Methods: Suspected leishmaniasis patients, referred to Masieh Daneshvary Hospital, Tehran, Iran were included from May 2017 to September 2021. The *Leishmania* species were genotyped by PCR-RFLP based on the SL mini-exon gene and the *ITS1* region of *SSU-rRNA* gene and compared with the sequencing results. The expressed metabolites of metacyclic promastigotes were evaluated by Proton nuclear magnetic resonance (^{1}H-NMR).

Results: Out of 66 suspected cases, 36 (54.4%) were positive for *Leishmania* species based on the PCR assays. In 21 (31.8%) cases, promastigotes grew on culture tubes. Based on the RFLP of SL RNA profile, 13 (19.7%) *L. tropica*, 9 (13.6%) *L. major*, 3 (4.5%) *L. infantum*, and 8 (12.1%) *C. fasciculata* isolates, isolated from culture media, were identified; however, 3 (4.5%) cases were unidentifiable due to the low number of parasites. Seventeen metabolites were expressed by the metacyclic forms of *L. major*, *L. tropica* and *C. fasciculata* isolates. The top differential metabolites expressed more in *C. fasciculata* were FAD, p-Methoxybenzyl alcohol and S-b-G-5, 5-G-b-S (A = CH2) (P<0.005) whereas Veratryl glycerols and D-(+)-Mannose were significantly increased in *L. major* and Betulin, L-Tyrosine in *L. tropica* (P<0.01).

Conclusion: The invaluable techniques such as sequencing and ^{1}H-NMR confirmed the results of genotyping of *Leishmania* species based on the SL mini-exon gene. SL mini exon gene can be used as a diagnostic tool to differentiate various *Leishmania* genotypes and detect contamination of culture media with *C. fasciculata*.

Introduction

Leishmaniasis is a life-threatening vector-borne disease, which is endemic in Iran (1). Based on worldwide reports cutaneous Leishmaniasis is endemic in Iran (2, 3). The sensitivity of conventional diagnostic methods, such as microscopic examination and parasite culture from clinical specimens, is limited for the detection of this disease.

Nowadays, proteomics, genomics and metabolomics approaches have gained much attention. The metabolomics is enabling to detect metabolic changes in a short time (4). In the genomic field different target genes have been applied in PCR methods. One of the most common genetic markers used to detect *Leishmania* species in the Old World is the *ITS1 region of SSU-rRNA* gene (5). The SL mini-exon gene has been used as a more recent approach to identify *Leishmania* species (6). Accurate diagnosis of *Leishmania* species is essential to determine the clinical prognosis and selection of therapeutic approach; contrary wrong diagnosis may lead to mistreatment (7).

The first aim of the present study was to investigate the sensitivity and specificity of PCR methods using SL mini-exon gene and to make comparisons with *kDNA* gene and *ITS1 region of SSU-rRNA* gene for detecting and genotyping *Leishmania* species isolated from different samples.

There are multiple changes in biochemical and metabolite pathways in the different species of Trypanosomatidae, including *L. tropica, L. major* and *C. fasciculata* (8, 9). The biochemical dissimilarities in the different parasites can play an important role not only in the prevention and treatment of leishmaniasis but also in the identification of different species. The second aim of the study was to find the best tool for identifying and genotyping of *Leishmania* species. Accordingly, to confirm the result of genotyping methods, the analytical platform of proton nuclear magnetic resonance (^{1}H-NMR) was applied to identify differentially expressed metabolites in metacyclic promastigote of three different species isolated from the NNN medium.

Materials and Methods

During May 2017 to September 2021, suspected cases with Leishmaniasis referred to Masieh Daneshvary Hospital, Tehran, Iran were introduced to the study. For diagnosis of the disease, tissue specimens from skin sores, from bone marrow was stained and seen under a light microscope, was inoculated in Novy-Macneal-Nicolle (NNN) medium, and was diagnosed by various PCR methods and genotyped by RFLP assay. As well, paraffin embedded biopsies (for patients with a history of Leishmaniasis) was introduced to the project for identifying the *Leishmania* spp. A number of PCR products were selected and sequenced to confirm the genotyping results. The sequencing method was not used as the gold standard method because of limited budget. Finally, the expressed metabolites of different species grew on NNN medium were evaluated by ^{1}H-NMR.

Leishmania parasites

Promastigotes of different *Leishmania* species were harvested from the Novy-MacNeal-Nicolle (NNN) medium. DNA was extracted by heat killing at 95 °C for 20 min. DNA of promastigotes from one standard *L. tropica* strain (MHOM/IR/02/MHOM/R) was used as a positive control in the study. DNA was extracted from the biopsies, lesions, and paraffin-embedded samples using a mini kit (Qiagen, Germany), based on the manufacturer's instructions. All extracted DNA samples were stored at -20 °C until further use.

PCR assay

Different primer pairs were used to detect *Leishmania* species, amplifying different target genes, including the *ITS1* region of *SSU-*

rRNA gene (10), SL mini-exon gene (11), *SSU-rRNA* gene (12), and mini circle *kDNA* gene (13), as described previously.

All PCR methods were performed at a final volume of 30 µL, containing 10 pmol of each primer, 15 µL of 2X Emerald Master Mix (Takara, Japan), 12% DMSO, and 10 µL of DNA template. To avoid contamination, pre-PCR and post-PCR preparation steps were performed in separate rooms using barrier tips. The expected amplicons of PCR products were visualized via electrophoresis on 1.5% agarose gel mixed with Safe Stain (Yekta Tajhiz, Iran).

To genotype *Leishmania* species, the amplicons were digested at a final volume of 30 µL, containing 5 µL of the PCR product, 2 µL of 10X buffer, and 1 µL of *HaeIII* (*BsuRI*) enzyme (Fermentase, Germany), and then were electrophoresed on 1.5% agarose gel (Fig. 1). The genotypes of *Leishmania* species were evaluated based on their product size (Table 1).

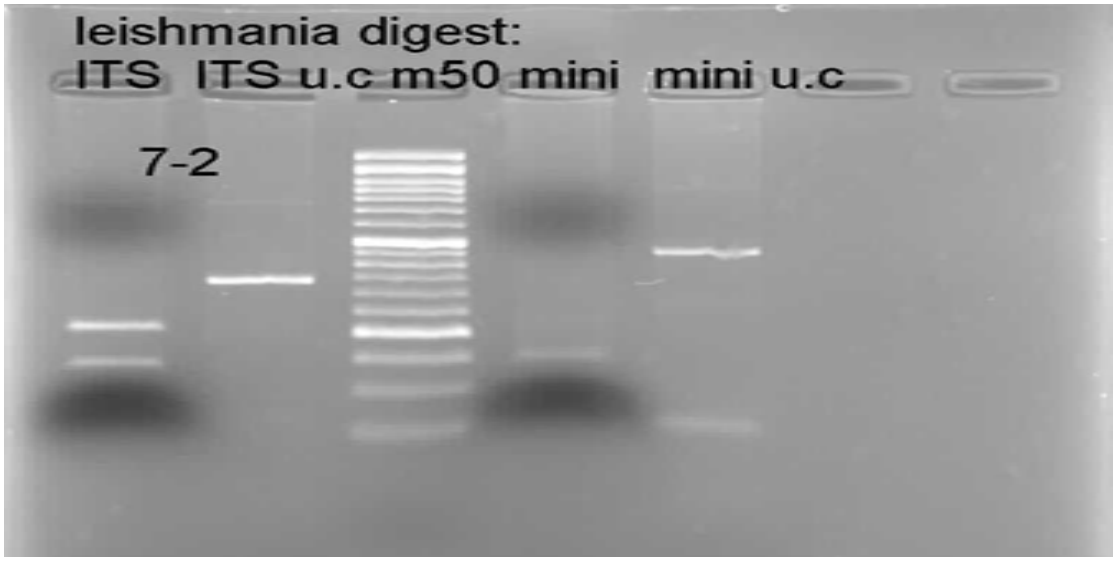

Fig. 1: Results of the digestion patterns of ITS1-rDNA and SL-mini exon amplicons. Lane 1: *Leishmania major* (ITS1 rDNA); lane 2: undigested product; Lane 3: *marker 50 bp; Lane 4: Leishmania major* (SL-mini exon gene); Lane 5: undigested product

Table 1: The RFLP pattern of different genes in *Leishmania* spp. by HaeIII enzyme

Variable	*ITS1 region of the SSU-rRNA (ITS1)*	*Spliced leader Mini-exon (SL RNA)*	*Small Sub Unit of RNA (SSU)*	*Kinetoplast minicircles (kDNA)*
L. tropica	189/78/57/26	225/178	282/211/70/24/11/4	63/53
L. major	205/135	140/61/67/59/39/21/16/8	282/211/70/24/11/4	63/53
L. infantum	186/74/55	102/ 85/61/59/46/29/19	282/211/70/24/11/4	63/53
C. fasiculata	132/77	318/88	306/296	Undigested

DNA sequencing

Bidirectional DNA sequencing was performed using identical primers for amplification of SL mini-exon gene by Genomin Company, I.R.of Iran, in an ABI 3730 genetic analyzer (Bioneer Co., Daejeon, South Korea). Nucleotide sequences were also analyzed using Bioedit version 7.0.1. DNA sequences were then compared with sequences deposited

in the GenBank, using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov).

Preparation of Metacyclic Promastigotes

Approximately 2×10^7 cells/mL of the metacyclic promastigotes grown on the NNN medium at 24 to 25 °C with flagellum/ body length ratio was ≥2, were collected by the standard agglutination assay with peanut agglutinin (PNA) (Sigma, CA, USA) (14). Briefly, the PNA^- metacyclic promastigotes were washed with phosphate-buffered saline (PBS). The non-agglutinated metacyclic promastigotes and the culture medium compounds were separated from the PNA^+ cells (procyclic promastigotes) and harvested from the supernatants by centrifugation at 200g for 5min.

Cell Extraction

Cell disruption and metabolite extraction were performed on 6×10^7 cell/mL obtained from each vial (15). Briefly, the Child 1.8 M perchloric acid (Merck, Germany) was added to the cell suspension. After vortexing, sonication and adjusting the pH of supernatant at 6.8, the precipitated supernatant was taken for ^{1}H-NMR spectroscopy.

Proton Nuclear Magnetic

D2O (20%) with trimethylsilyl propionate (TSP, 0.25 mM) (Sigma, CA, USA), was used as the ^{1}H-NMR chemical shift reference. All the samples were analyzed on Bruker AVANCE 400 MHz (Bruker, Germany), equipped with a 5-mm probe at 298 K. The Carr-Purcell Meiboom-Gill (CPMG) spin-echo pulse sequencing was used to remove macromolecule signals. ^{1}H-NMR spectra were acquired in 150 scans per sample, with an acquisition time of 3.0 seconds and a spectral width of 8389.26 Hz.

Data Processing and Statistical Analysis

The ^{1}H-NMR spectra were processed using MestReNova (version 2.9). The data and bins of nine samples of *L. tropica*, seven samples of *L. major* and four samples of *C. fasciculata*, was studied. Combined offset less than 0.001 was applied to identify the most significant relevant metabolites. Metabolic pathways relating to these separated metabolites were studied using Biological Magnetic Resonance data Bank (BMRB) and Kyoto Encyclopedia of Genes and Genomes (KEEG) database.

Statistical analysis

Sensitivities, specificities, positive predictive value and negative predictive value were calculated using IBM SPSS Statistical Software version 22 (IBM Corp., Armonk, NY, USA). Kappa test was used for comparison of genotyping results.

Ethical consideration

This study was approved by the Ethics Committee of the Medical faculty of the Shahid Beheshti University of Medical Sciences. Ethical code: IR.SBMU.NRITLD.REC.1395.223.

Results

Out of 66 suspected patients with wet or dry lesions and bone marrow, 36 (54.4%) were positive for *Leishmania* species based on the PCR assay. In 21 (31.8%) cases, promastigotes grew on culture tubes after incubation at 25 °C. Based on the RFLP assay of SL mini-exon profiles, 13 (19.7%) *L. tropica*, 9 (13.6%) *L. major*, 3 (4.5%) *L. infantum,* and 8 (12.1%) *C. fasciculata* samples were identified, while 3 (4.5%) samples could not be identified by this assay because of the low number of parasites. On the other hand, based on the RFLP assay of *ITS1* region of *SSU-rRNA* gene, 10 (15.2%) *L. tropica*, 11 (16.7%) *L. major*, 2 (5.5%) *L. infantum*, and 8 (12.1%) undetermined samples were identified.

There were some conflicting results in the genotyping of eight samples using two different assays. Seven samples that were genotyped as *L. major* and one sample that was genotyped

as *L. tropica* based on the PCR of *SSU-rRNA* ITS1 region were identified as *C. fasciculata* based on the PCR-RFLP of SL mini-exon gene. The sequencing results confirmed the contamination of cultures with *C. fasciculata*. According to the results, genotyping using the PCR-RFLP assay of *SSU-rRNA* ITS1 region could not detect contamination with *C. fasciculata* in the culture media. The sensitivity, specificity, positive predictive value, and negative predictive value of the PCR-RFLP assay of *SSU-rRNA* ITS1 region were compared to the SL mini-exon assay (Table 2).

Table 2: Genotyping of *Leishmania* spp. based on two different target genes

Variable		***Genotyping based on spliced leader mini-exon***					
		Negative	*L .tropica*	*L. major*	*L. infantum*	*C. fasciculata*	Total
Genotyping based on *ITS1* region of *SSU-rRNA*	Negative	30	2	1	0	0	33
	L. tropica	0	10	0	1	1	12
	L. major	0	1	8	0	7	16
	L. infantum	0	0	0	2	0	2
	C. fasciculata	0	0	0	0	0	0
	Unidentified	0	0	0	0	0	0
	Total	30	13	9	3	8	66

Sensitivity of ITS1 for detection of *L. tropica*: 10/13=76.92%
Sensitivity of ITS1 for detection of *L. major*: 8/9=88.9%
Sensitivity of ITS1 for detection of *L. infantum*: 2/3=66.7%
Sensitivity of ITS1 for detection of *C. fasciculata*: 0/8=0%
Sensitivity: 30/33=90.91%
Specificity: 33/33=100%
Positive Predictive value: 30/30=100%
Negative predictive value: 33/36=91.6%

Metabolite Identification of three kinetoplastidea Species and Pathway Analysis

The expressed metabolite of nine isolates of *L. tropica,* seven isolates of *L. major*, and four isolates of *C. fasciculata* contaminated the culture tubes were evaluated by ^{1}H-NMR. The highest importance spectral bins of the metacyclic promastigotes of the Iranian isolates of *L. major, L. tropica*, and *C. fasciculata* were determined according to combined offset values. Twenty-seven significantly expressed metabolites were identified ($P \leq 0.01$) which 4 of them were common in three different species, three were common between *L. tropica* and *L. major*, three between *L. major* and *C. fasciculata* (Table 3).

Table 3: The metabolites of the metacyclic promastigote extracts of *Leishmania major*, *Leishmania tropica* and *Crithidia fasciculate*

	BMRB ID	*Metabolite**	*Matching shifts*	*Parasites species*
1	bmse000344	4-Guanidinobutyric acid	H:3.18	*L. major/L. tropica/C. fasciculata*
2	bmse000780	Tetramethylammonium	H:3.18	*L. major/L. tropica/C. fasciculata*
3	bmse010341	lignin_cw_compounds	H:3.18	*L. major/L. tropica/C. fasciculata*
4	bmse001303	Pseudoyohimbine	H:1.2498	*L. major/L. tropica/C. fasciculata*
5	bmse001308	Neohesperidin	H:3.2002	*L. tropica/L .major*
6	bmse001133	N-(4-aminobutyl) acetamide	H:3.1997	*L. tropica/L. major*
7	bmse000148	Diethyl oxalacetate	H:1.25	*L. tropica/L. major*
8	bmse010173	S-b(t)-G-b(e)-S5	H:1.88	*L. major/C. fasciculat*
9	bmse001319	(-)-dehydrocostuslactone	H:1.8795	*L. major/C. fasciculat*
10	bmse001273	Simvastatin	H:1.2999	*L. major/C. fasciculat*
11	bmse010091	Veratryl glycerol	H:3.62	*L. major*
12	bmse000018	D-(+)-Mannose	H:3.56	*L. major*
13	bmse001204	2-METHYL-BICYCLO(2.2.1)HEPT-5-ENE-2-CARBOXYLIC ACID	H:1.8804	*L. major*
14	bmse000829	IPTG	H:1.3	*L. major*
15	bmse001102	(Z)-octadec-11-enoic acid	H:1.3	*L. major*
16	bmse000051	L-Tyrosine	H:3.2	*L. tropica*
17	bmse001296	Betulin	H:3.19	*L. tropica*
18	bmse000973	Ethyl 4-aminobutyrate	H:1.25	*L. tropica*
19	bmse000226	FAD	H:4.51	*C. fasciculata*
20	bmse010025	p-Methoxybenzyl alcohol	H:4.51	*C. fasciculata*
21	bmse010151	S-b-G-5,5-G-b-S (A = CH2)	H:4.51	*C. fasciculata*
22	bmse000394	6-Aminohexanoic acid	H:2.19	*C. fasciculata*
23	bmse010011	2,3-Diacetoxypropioveratrone	H:2.19	*C. fasciculata*
24	bmse010178	H-b-H5e	H:2.19	*C. fasciculata*
25	bmse010176	S-a-S-b-S	H:2.18	*C. fasciculata*
26	bmse010183	G-5-O-4-G diacetate	H:2.18	*C. fasciculata*
27	bmse000727	trans-2,3-dimethylacrylic acid	H:1.71	*C. fasciculata*

BMRB: Biological Magnetic Resonance data Bank. KEEG: Kyoto Encyclopedia of Genes and Genomes; * variable with combined offset equal to 0 were considered as the mean and common metabolites of each species

The metabolite set enrichment analysis of biochemical pathways was performed using the Biological BMRB and KEEG database. In this analysis, the significantly common expressed metabolites between *L. tropica, L. major and C. fasciculata* were measured relative to the predefined biochemical pathway sets in the database. According to the result, the significantly altered biochemical pathways with a P value of less than 0.001 were 17 metabolic pathways. Most of the common metabolic pathways in the metacyclic promastigotes of the Iranian isolates of *L. major*, *L. tropica and C. fasciculata* are presented in table 3.

Seventeen significantly differentially expressed metabolites were detected between the metacyclic forms of *C. fasciculata* and standard strains of *L. major, L. tropica*. The top differential metabolites, which were expressed more in C. fasciculata, were: FAD, p-Methoxybenzyl alcohol, S-b-G-5, 5-G-b-S (A = CH2) and trans-2, 3-dimethylacrylic acid (P<0.005) whereas Veratryl glycerols and D-(+)-Mannose were significantly increased in *L. major* and Betulin, L-Tyrosine in *L. tropica* (P<0.01). The four metabolite, 4-Guanidinobutyric acid, Tetramethylammonium, lignin_cw_compounds and Pseudoyohimbine were commonly expressed in three different species.

The comparison of the metabolites models in three different species is depicted in Fig. 2. Those metabolites pathways were influenced by *Leishmania* species (P<0.01) (Fig. 3).

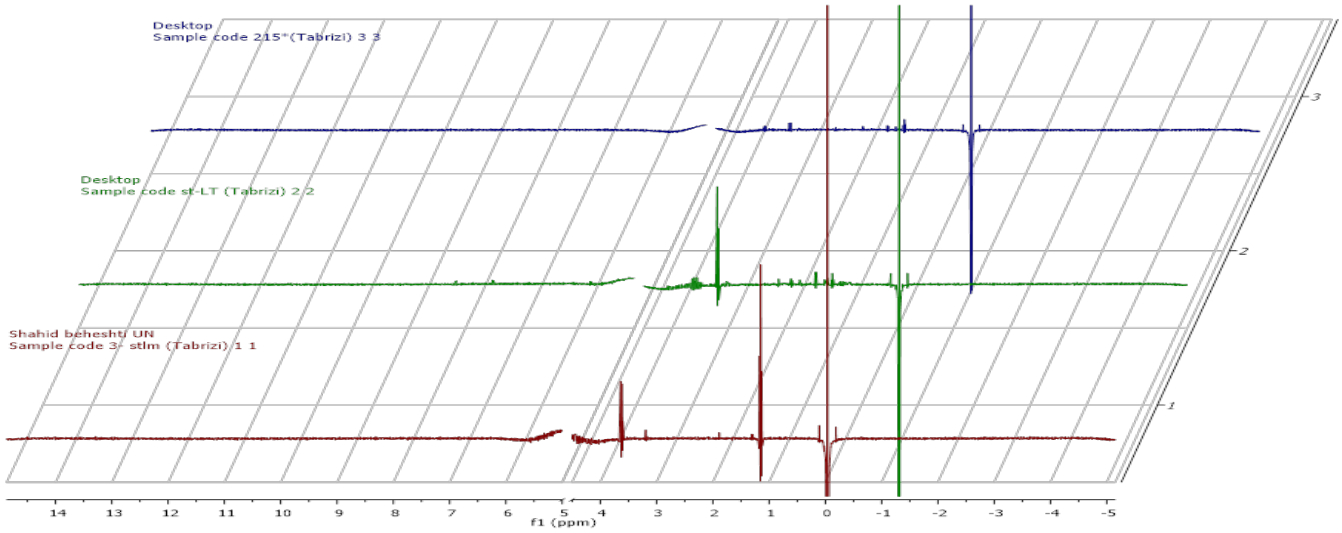

Fig. 2: Comparison of different metabolite pattern of *C. fasciculata* (sample code 215) with standard species of *L. tropica* (sample code st-Lt) and *L. major* (sample code stlm).

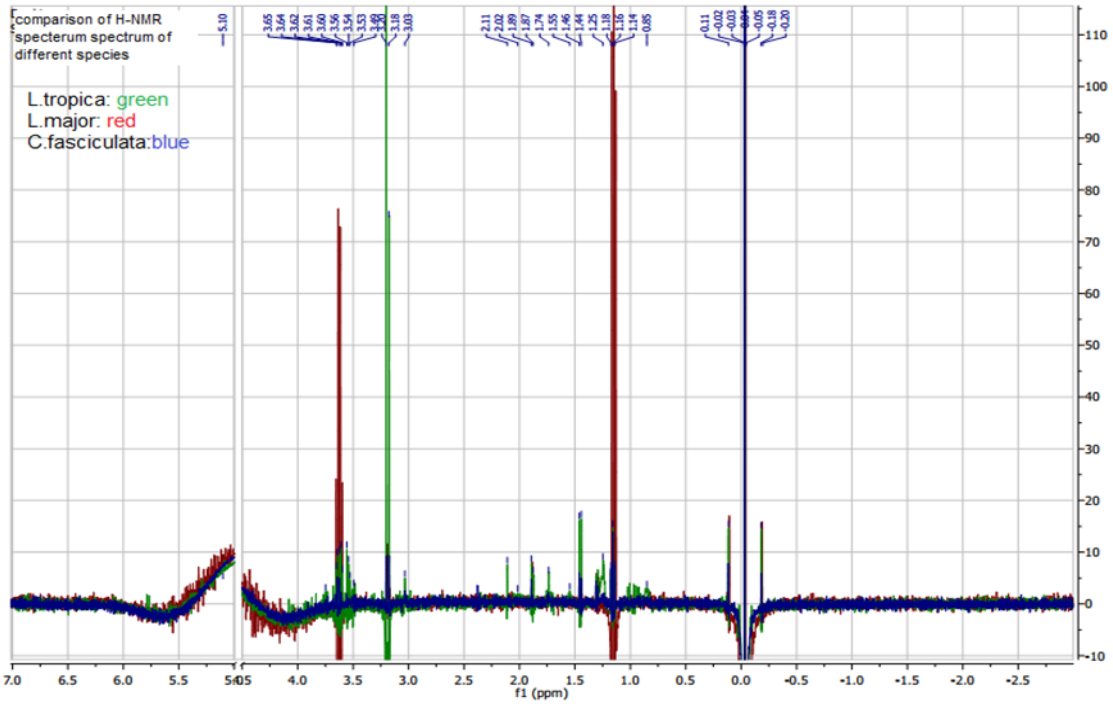

Fig. 3: Comparison of aligned ^{1}H-NMR spectrum of different species of kinetoplastidea

Discussion

Early and accurate identification of *Leishmania* species plays an important role in preventing severe clinical manifestations and mortality in patients. Different diagnostic methods are used to detect and identify *Leishmania* species. The traditional diagnosis approaches are not always reliable and can be time-consuming, costly, and difficult to perform (16).

To confirm leishmaniasis, isolation of parasites is critical. The sensitivity of parasite culturing and histopathological microscopic smears varies from 27% to 85%, depending on the clinical presentations, parasite species, technical expertise, and other factors (17). One of the most important disadvantages of culture is contamination with *C. fasciculata* which cannot be differentiating from other *Leishmania* species because of their similar morphology. Today, several molecular assays have been developed for diagnosis of leishmaniasis. In the current study, we tried to detect *Leishmania* species using several PCR assays and compared the sensitivity and specificity of these methods. Generally, the use of highly repetitive genomic loci or extrachromosomal *kDNA* sequences is the most common method, which is confined to the detection of parasites at the genus or complex levels (18). The present study showed that, while PCR based on minicircle *kDNA* sequences could correctly detect the positive samples, it is not a good tool to differentiate and identify the *Leishmania* isolates. Moreover, the SSU-rRNA region in *Leishmania* species could not also differentiate different species owing to similar digestion patterns in numerous *Leishmania* variants. Therefore, these two methods were not suitable for determining the genotype of *Leishmania* and were discarded.

To date, no gold standard has been established for the diagnosis of leishmaniasis. Several studies have indicated the high sensitivity of PCR based on the ITS1 region of SSU-rRNA gene due to the highly polymorphic region and high copy number of *ITS1* region of *SSU-rRNA* gene in *Leishmania* genomes (19,

20). ITS1 region as a target could not differentiate *C. fasciculata* from other species in the contaminated culture. In other words, PCR assay based on the *ITS1 region of SSU-rRNA* gene had 0% power for the detection and differentiation of *C. fasciculata* from *L. major* and *L. tropica* (Table 2).

There are 100-200 copies of SL mini-exon gene within the nuclear *Leishmania* genome, differ among *Leishmania* species, consisting of transcribed conserved and non-transcribed variable regions (21). The sensitivity of PCR assay of SL mini-exon gene was 87.5% for the detection of *L. siamensis* in clinical specimens (20). But the current study showed that it could accurately detect and differentiate *Leishmania* species in clinical samples; it may even detect culture contamination with *C. fasciculata*. Contamination of laboratory cultures with *C. fasciculata* is extremely important in the diagnostic process, because it is difficult to differentiate it morphologically from *Leishmania* parasites (22).

The ^{1}H-NMR results showed that the metabolic model of metacyclic promastigote of contaminated culture with *C. fasciculata* was significantly differed with *L. major* and *L. tropica* (Fig. 2 & 3). Indeed, the result of ^{1}H-NMR and metabolic analysis confirmed the result of PCR-RFLP based on SL mini-exon and genotyping based on sequencing. Our study confirmed that the metabolic models of different species of *Leishmania* were exclusive and species-specific (8, 23-28). The species-specific metabolic model can lead to different clinical manifestations and cause the resistance to treatment; therefore, it is very important to identify the species of parasite accurately. Our study also showed the different and unique metabolic pathways in *L. tropica, L. major* and *C. fasciculata* (Table 3). Although, either isolated from animals or cultured axenically can be more informative. The outcome provides clear evidence that there is a species-specific metabolic feature, and gives a way to conduct further studies exploring the host-parasite interactions.

The present study showed that the PCR-RFLP assay of SL mini-exon gene was 100% accurate in detecting culture contamination with *C. fasciculata*. Conversely, another study indicated the cross-species amplification of *C. fasciculata* in Iranian patients, using the PCR-RFLP assay of *ITS1* region of *SSU-rRNA* gene (29). In the current study, *C. fasciculata* could not be detected by PCR-RFLP assay of *ITS1* region of *SSU-rRNA* gene, while the SL mini-exon gene could differentiate it from *L. major* and *L. tropica* in 24.2% of positive samples. So the *SL RNA* gene can be recommended as a target for rapid characterization of cultured parasites, because accurate information on the type of parasites and culture contamination is necessary for selecting suitable therapeutic regimens and controlling the disease.

Conclusion

Genotyping of *Leishmania* species based on the SL mini-exon gene, confirmed by invaluable technique such as sequencing and ^{1}H-NMR, are more reliable and accurate than genotyping based on the *ITS1 region* of *SSU-rRNA* gene. This method can be used as an accurate and fast tool to diagnose and differentiate various *Leishmania* genotypes and detection of contamination of culture media with *C. fasciculata*. Our study also showed that the metabolic pathways of *L. major, L. tropica* and *C. fasciculata* were unique and highly species-specific.

Acknowledgments

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Conflict of Interest

There is no conflict of interest to declare.

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