Original Article

Accurate Identification of *Leishmania* Parasites in Sand Flies by Polymorphism Analysis of Cytochrome Oxidase Subunit 2 Gene Using Polymerase Chain Reaction and Quantitative PCR-High Resolution Melting Techniques in Iranian Border with Iraq

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Abstract

Background: Firmly identification of *Leishmania* in *Phlebotomus papatasi* and understanding of natural transmission cycles of parasites in sand flies are important for treatment and local control.

Methods: Modified and developed method of High Resolution Melting (HRM) as a preferable technique was employed to accurate identification of *Leishmania* in sand flies from Iranian border with Iraq, by targeting cytochrome oxidase II (COII) gene and designing suitable primers. PCR products cloned into pTG19-T vector, then purified plasmid concentration was measured at 260 and 280nm wavelength. The melting curve plots were generated and DNA sequences were analyzed using Sequencher 3.1.1, CLC Main Workbench 5.5, MEGA 6, DnaSP5.10.01 and MedCalc® version 13.3.3 soft wares.

Results: Among about 3000 collected sand flies, 89 female *Ph. papatasi* were identified and two with *L. major*. In amplified fragment of COII gene among 611bp, 452bp had no genetic variations with low polymorphic sites (P= 0.001) and high synonymous (79.8%) as compare to non-synonymous sites (20.2%). *Leishmania major* was discriminated in *Ph. papatasi* with 0.84 °C melting temperature (T_m) and unique curve based on thermodynamic differences was an important criterion using HRM technique.

Conclusion: Subsequent war in Iraq made a high risk habitat for parasites transmission. It is important to discover accurate diagnostic procedures for leishmaniasis control.

Keywords: Cloning; Cytochrome oxidase II; qPCR-HRM

Introduction

Phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) are the only proven natural vectors of *Leishmania* species, causative agents of a neglected tropical disease, leishmaniasis (1-3). *Leishmania* is a digenetic parasite with the extracellular stage within an invertebrate vector which called promastigote and the intracellular stage within a vertebrate hosts and reservoirs which called amastigote (4). Incrimination and identification of *Leishmania* parasite, has been developed and performed using different molecular methodologies and analyses for more than 30 years (2, 5-7). Genetic analysis of mitochondrial and nuclear genes has commonly been employed for *Leishmania* species identification considering their sensitivity, simplicity, reliability and specificity in epidemiological studies (2, 8).

High Resolution Melting (HRM) method is an efficient and cost-effective method that eliminates the risk of laboratory contaminations. In recent years, quantitative PCR using SYBR Green or TaqMan chemistries have been developed and evaluated for detection, quantification, identification and *Leishmania* species differentiation in human samples, a few in res-

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ervoir hosts and few in sand flies (9-13).

Suitable methodology helps to distinguish among *Leishmania* species especially in tiny insect of female sand flies as natural vectors of leishmaniasis. This is crucial and essential in epidemiological studies to determine appropriate procedures (14). In this research for typing at intra-species level of *Leishmania* species, new discriminative molecular markers have been applied (2, 8). qPCR-HRM, may answer the epidemiological questions of ZCL in the region of border between Iran and Iraq such as transmission cycles of animal reservoirs and transmitting of the vectors, infection's origin, modeling of spreading in this border and disease distinction of imported cases. The objectives of this investigation were to determine *Leishmania* species and genotyping of *Leishmania* parasites in sand flies of Ilam Province.

Materials and Methods

Locations, sand flies trapping and morphological identification

Sand flies were captured from gerbil burrows and nearby domestic animal shelters in two locations of Ilam Province, border with Iraq where *Ph. papatasi* is proven vector of Zoonotic Cutaneous Leishmaniasis (ZCL). Accordingly, Mehran and Dehloran were considered (31°, 58' to 34°, 15' N and 45°, 24' to 48°, 10' E) in West of Iran, with altitude of 2740 meters above sea level (masl) (Fig. 1 and Table 1). The sticky papers, CDC miniature light traps (with the white light bulb 1–2m above ground level) which were set overnight to sample sand flies in domestic animal shelters and inside houses in the morning and manual aspirators were applied for sand flies sampling by expert field collectors. Besides, funnel traps were used to sample from rodent borrows. The collected specimens were put in microtubes without ethanol and then were frozen. All female sand flies were identified by morphological characters of the head and terminal genitalia, following a dissection of fed females with sterilized forceps and microneedles and mounted in Berlese fluid (15)(Fig. 2).

DNA extraction, PCR and sequencing

DNA from thorax and abdomen of sand flies were extracted using ISH-Horovize method with minor modifications and also using Genet Bio kits (Genet Bio, Daejeon, Korea) (5, 15).

Total DNA was extracted from the dissected thorax and attached anterior abdomen of individual females of *Ph. papatasi*. Each sample contained the midgut, the location of most *L. major* promastigotes, and was homogenized in a 1.5ml microfuge tube using a disposable plastic tip of a micropipette. Following ethanol precipitation, the DNA was dissolved in 15µl 1×TE (10mM Tris-HCL, 1mM EDTA pH 8.0), to give a concentration of 5–10ng/µl, and stored at −20 °C (5, 15). Cytochrome oxidase subunit II (COII) as an enzymatic gene was amplified to detect any *Leishmania* infection among sand flies (2, 8). New sensitive and specific primers were designed and employed to amplify COII gene to detect *Leishmania* parasite in sand flies.

PCR was performed on every female *Ph. papatasi* using COII specific new designed forward primer, COII F (5′-ATGGCTTTTATA TTATCATTTTG-3′) and reverse primer, COII R (5′- GGCATAAATCCATGTAAGAC-3′).

The amplification reaction was carried out in a total of 20µl containing 1× Taq polymerase buffer (Promega), 1.5mM MgCl2, 60µM of each dNTP, 1µM both forward and reverse primers, 1µM primer unit Taq polymerase (Promega) and 1.5µl of DNA (5–10ng/µl) extracted from individual wild caught sand flies. The mixture was incubated in a PE GeneAmp ®PCR Thermocycler 9700 (0.2ml block) at 94 °C for 3min followed by 37 cycles, each consisting of 30s at 94 °C, 30s at 58 °C and 90s at 72 °C. After the last cycle, the extension was continued for a further 10min then held at 4 °C (16).

PCR products were subjected to electropho-

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resis on 1.5% agarose gel and *Leishmania* positive PCR products were used for sequencing by ABI PRISMTM 310 automated sequencer (Applied Biosystems, Thermo Fisher Scientific, Foster City, USA) in order to accurate identification of species and haplotypes (2, 8). Sequences of COII gene was compared in different *Leishmania* species to determine conserved areas in sand flies of Ilam. The amplified sequences with those from GenBank, were compared and analyzed in aspect of their phylogenetic and polymorphism.

Cloning

In this study, in order to prepare standards for qPCR-HRM and besides for re-sequencing of ambiguous sites, PCR products were cloned directly into pTG19-T vector using SinaClon PCR TA Cloning kit (SinaClon Bioscience, Tehran, Iran). To confirm cloning results, colony PCR with vector specific primers was performed (17). Plasmid was extracted from transformed bacterias, using SinaClon Plasmid Isolation Kit (SinaClon Bioscience, Tehran, Iran). Purified plasmid concentration was measured at 260 and 280nm wavelength using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). In accordance with the following formula DNA copy number was calculated. Then, seven serial dilutions of plasmids were prepared in the ratio of one to ten (Fig. 3).

DNA (copy number)= [6.02× 10^{23} (copy/mol)× DNA amount (g)]/ DNA length (dp)× 660 (g/mol/dp)

Quantitative PCR-High Resolution Melting

High Resolution Melting (HRM) analysis were carried out by Corbett Rotor-Gene 6000 HRM Real Time PCR instrument (Corbett Life Science, Sydney, Australia). A part of COII gene was amplified using designed CO.A. 470.2 forward and reverse primers in the presence of Evagreen dye (Fig. 4a). In this method, the observed differences in the melting temperature were noticed and the use of HRM method was considered for separation of different species.

HRM procedure was designed and performed in 20µl containing 2µl of DNA or plasmid (10 ng/reaction), 0.7µl of each primer (10pmol/µl or 10µmol/l) and 4µl of 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Bio Byne, Tartu, Estonia). Then, 12.6µl of PCR-grade H_2O was added to the volume. The initial denaturation for 1 cycle was 15 minutes at 95 °C then it was followed by 45 cycles, 15 seconds in each cycle for denaturation; 30 seconds at 55 °C for annealing, 30 seconds at 72 °C for extension and final extension was 5 minutes at 72 °C (1 cycle) and then stored for 10 minutes at 4 °C. The qPCR-HRM products were followed by a conventional melting step: melting curve was performed from 65 to 92 °C, with an increasing slope of 0.1 °C each step, with 2 seconds rest at each step afterwards. Finally, to determine the average T_m for each *Leishmania* spp., the melting curve plots were generated and analyzed using HRM software (Corbett Life Science, Sydney, Australia).

Statistical analysis

DNA sequences were edited and aligned using Sequencher 3.1.1 and CLC Main Workbench 5.5 softwares. Polymorphism and phylogenetic analyses of our sequences with standard sequences from GenBank were carried out using MEGA 6 and DnaSP5.10.01 softwares (18). In order to compare the median and T_m, the MedCalc® version 13.3.3 software and Mann-Whitney U test were used. The calculation of diversity between sequences and neutrality were performed by DnaSP 5.10.01 software. The ratio of non-synonymous substitution (dN) to synonymous substitution (dS) was assessed using DnaSP 5.10.01 software (19).

Results

Sand flies sampling and preparation

Out of more than 3000 collected sand flies, 234 sand flies were dissected, mounted and five

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species were identified (Table 1). More than collected samples were male sand flies and more of the sand flies were not *Ph. papatasi* so 234 were female *Ph. papatasi*. Eighty nine out of 234 were fed which were screened in order to detect *Leishmania* infection. *Leishmania major* was found at least in two *Ph. papatasi* out of 89 female sand flies from two locations (Mehran and Dehloran) (Table 1).

Polymorphism analysis

From 89 female *Ph. papatasi* only two were infected with *Leishmania* which identified and sequenced. The monomorphic and polymorphic positions of COII were compared using Chi-square test. Of 611bp nucleotides, 452bp had no genetic variations. Also, the number of polymorphic sites were significantly lower than that of monomorphic sites sequences (P= 0.001). For informative and non-informative sequences in the evaluated gene, genetic variation occurred in 46 nucleotide positions. Bioinformatics analyses were performed by DnaSP5.10.01 software and number of synonymous sites was significantly greater than non-synonymous sites (P= 0.00). In the expression gene (COII), among 26% segregating sites in nucleic acid variation areas, 20.2% sites were non-synonymous mutations also, more mutations might have occurred in the negative selection side. Based on the findings by DnaSP5.10.01 software, dS/dN ratio for COII had the low amino acid changes (dS: Synonymous mutations in silent sites, dN: Non-synonymous mutations in replacement sites). According to Tajima's D Index, dN/dS ratio was calculated to be 0.14 (in the positive direction) so COII gene has produced through evolutionary process (Table 2) (20).

A natural evolution was reported, based on the comparison of mean nucleotide diversity (π= 0.075) and expected number of mutations (Θ= 41.51) in each sequence of gene. The study of different COII sites of *Leishmania* species showed no significant difference in nucleotide diversity (π) or expected number of mutations (Θ) (P= 0.86). The comparison of synonymous mutations in silent sites (dS) and non-synonymous mutations in replacement sites (dN) in COII expression gene showed a significant difference between two *Leishmania* species (P= 0.001). The number of dS (79.8%) was greater than dN (20.2%).

The greatest dissimilarities in dS site were detected in the comparison of *L. major* and *L. tropica* populations (P= 0.019). Among 12 sequences of *L. major*, 19 nucleotide positions (13 dS and 6 dN) were different in terms of gene expression. Also, among four sequences of *L. tropica* species, 27 nucleotide positions (7 dS and 20 dN) varied in terms of gene expression. Haplotype diversity was analyzed for *L. major* and *L. tropica* and was observed 81.35% and 28.38%, in *L. tropica* and *L. major* populations respectively. The average, standard deviation (SD), and coefficient variation (CV) showed low to high haplotype diversity (P= 0.000). To determine the extent of natural selection in *Leishmania* species, the average number of nucleotide differences (k) and the number of expected genetic differences in the whole sequence (Θ) were compared, using Tajima's D index. Results showed that evolution was positive in COII gene (Table 2).

Genetic evaluation and phylogenetic analysis

30 *Leishmania* sequences were employed. These species are included two *L. major* (two haplotype were found in two *Ph. papatasi* in Ilam) and 28 *Leishmania* species reference strains: 10 *L. major*, four *L. tropica*, three *L. donovani*, and three *L. infantum* from Old World and five from New World: two *L. mexicana* and two *L. tarentolae* and one *L. amazonensis*. They were employed to evaluate the sensitivity and specificity of COII gene for accurate identification of *Leishmania* species (Fig. 5 and Table 3).

To determine the extent of natural selection in *Leishmania* species, the average number of nucleotide differences (k) and the number of expected genetic differences in the whole

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sequence (Θ) were compared, using Tajima's D Index between *L. major* and *L. tropica* species. Based on the findings, COII gene belongs to *L. tropica* species has been produced through evolutionary processes (Tajima's D= 2.85, P< 0.01). This indicates that evolution was positive in COII gene.

Molecular phylogenetic analysis of COII gene is presented in Figure 5 based on the Maximum Liklihood (ML) method. It presents the concatenated tree of COII gene. Figure 5a was drawn, based on the nucleotide sequences. Kimura's two-parameter method was used for 30 nucleotide sequences. The topology of the tree revealed that this mitochondrial gene caused the separation of *Leishmania* species, whereas this was unable to separate subspecies or determine haplotype diversity. By applying the ML method, 1326 sites were analyzed. Also, under similar conditions, the phylogenetic tree of COII gene based on the mitochondrial genetic amino acid codes, showed a similar topology by ML model (Fig. 5b).

Quantitative PCR-High Resolution Melting

Different dilutions of DNA template were prepared for validation, accuracy and sensitivity of HRM assay (R^2= 0.94, efficiency= 4.49). High sensitivity of HRM could pull out minimum amount of DNA to draw appropriate melting curves. Serial dilutions of specimens were experienced on three consecutive days with similar reactions. Identical DNA concentrations were repeated with COII gene. Melting curves for serial dilutions were compared. Identical DNA were analyzed and melt patterns were appropriated for both species on different days. According to results, two common *Leishmania* species in Ilam (*L. major* and *L. tropica*) were discriminated with 0.84 °C difference in T_m, using HRM technique (mean T_m were 74.8 °C and 75.64 °C for *L. major* and *L. tropica* respectively (Fig. 6, 7 and Table 4).

Fig. 1. Geographical location of Ilam Province, and sampling sites

Table 1. Sampled sand flies collected in June to August 2016, from Ilam Province has shown base on gender, species and locations

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Species		*Sergentomia clydei*				*Sergentomia dentata*				*Sergentomia sintoni*				*Phlebotomus mongolensis*				*Phlebotomus papatasi*			
Gender		F			M	F			M	F			M	F			M	F			M
Location		In	RB	ASH		In	RB	ASH		In	RB	ASH		In	RB	ASH		In	RB	ASH	
Mehran	**Nirogah Bargh**	0	0	1	0	0	0	1	0	0	0	1	3	0	0	0	0	0	9*	2	38
	Imamzadeh Hasan	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	2	11	0	4	56
Dehlouran	**Dasht Akbar**	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	2	0	0	15	7
	Chehmeh gheer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	10
	Janbazan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	12
	Roosta Ali	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	3	6	0	33*	2
			1		1		1		0		6		4		0		7	89		125	
Total		234 (97F+137M)																			

In: Indoor, ASH: Animal shelter, RB: Rodent burrow, F: Female, M: Male, **Leishmania* positive

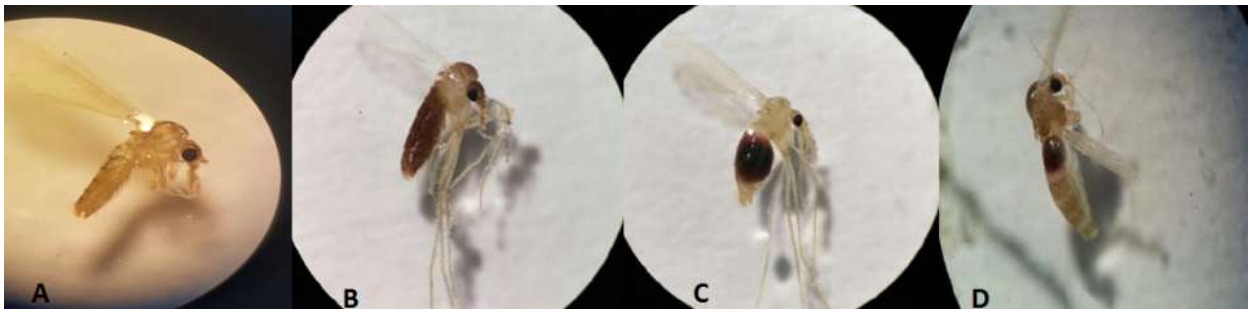

Fig. 2. *Phlebotomus papatasi* female, A: unfed, B: Fed, C: Gravid, D: Semi gravid

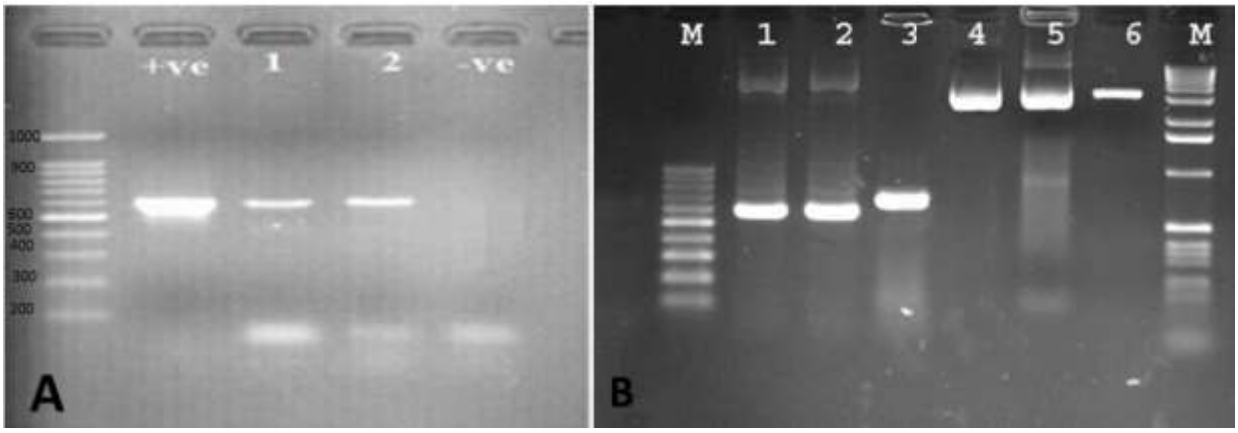

Fig. 3. A: Two *Leishmania* infection were detected by PCR. The sequences were identified as *L. major* based on sequencing. -ve: Negative control, +ve: Positive control, M: Markers (100bp left). B: COII gene was cloned in the pTG19-T vector. Plasmids were extracted and serial dilution of them were prepared. M: Markers (1000bp right, 100bp left). 1: PCR products with COII primers for plasmid including COII in *L. tropica* with 611bp, 2: PCR products with COII primers for plasmid including COII in *L. major* with 607bp, 3: PCR products with M13 primers for plasmid including COII in *L. major* with 800bp, 4: Plasmid extraction products contains COII gene in *L. major* with 3500bp, 5: Plasmid extraction products contains COII gene in *L. tropica* with 3500bp, 6: Plasmid without pTG19-T vector with 2880bp

Table 2. Polymorphism analysis of COII sequences from *Leishmania* species by DnaSP5.10.01 software

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A

Gene	No. Seq.	No. Nucleotide (bp)	V (%)	Singleton variable (%)				Parsimony variable (%)				Synonymous/replacement change			
				2 Variants	3 Variants	4 Variants	Total	2 Variants	3 Variants	4 Variants	Total	C.C	dS (%)	dN (%)	dN/dS ratio
COII	29	611		24	0	0	24	114	21	1	136	15	147 (87)	21 (12)	0.14

B

Gene	No. Seq.	No. Nucleotide (bp)	S	Eta	K	π	Θ per site	Θ per seq.	Tajima's D	H	Hd
COII	29	611	149	183	45.97	0.075	0.067	41.51	0.42	27	1

A: V: Variable (polymorphic) nucleotide site, C·C: Total number of sites in other codons (complex codons), which were not analyzed because of their highly variable regions, dS: Synonymous mutations in silent sites, dN: Non-synonymous mutations in replacement sites

B: S: segregation site, Eta: Total number of mutations, K: Average number of nucleotide differences between pairs of sequences, π: Mean nucleotide diversity, Θ per site: Expected number of mutations per a site, Θ per seq: Expected number of mutations per a sequence, Tajima's D: the statistical test proposed by Tajima (1996), H: No. of haplotypes, HD: Haplotype diversity

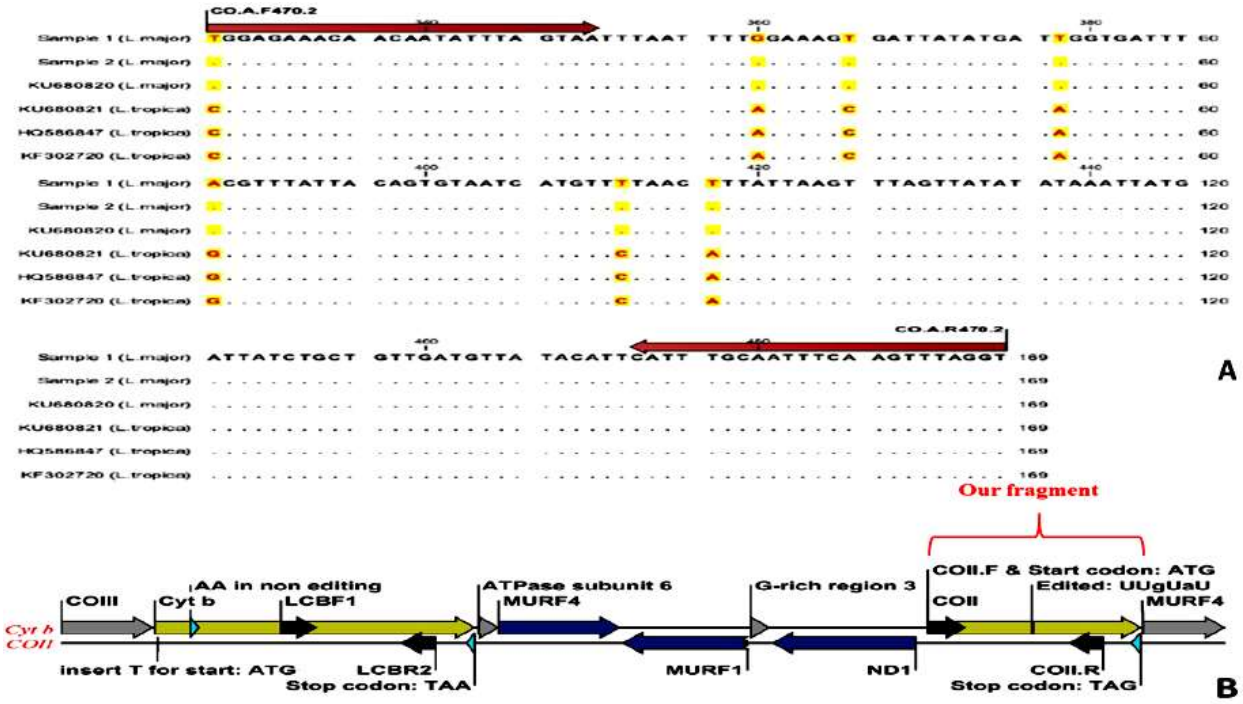

Fig. 4. A: Based on obtained sequences and polymorphism analysis results, forward and reverse primers were designed for qPCR of COII gene. CO.A.R470.2 (F: TGGAGAAACAACAATATTTAGTAA, R: CCTAAACTTGAAATT-GCAAATG) B: Schematic illustration of COII gene, amplified with specific primers (small black arrows) for *Leishmania* species using CLC bioinformatics software (8)

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Table 3. Details of COII sequences of *Leishmania* parasites from Ilam and GenBank sequences for constructing phylogenetic tree

The name of species	Accession No.	Origin	Source	Reference
L. major	**KU680818		Ulcers of patients	This study
	**KU680819		Ulcers of patients	This study
	KU680820			
	AF287688	Soviet Union	Ulcers of patients	8
	EU140338	Iran	Not found	21
	EF633106	Italy	Ulcers of patients	22
	KF815208	Sudan	Not found	21
	KF815210	Japan	Clinical samples:HB,HBM,HLN*	23
	KF815211	Not found	" " "	23
	MH443402	Sudan	" " "	23
		" " "	" " "	Direct submission (Aghai Maybodi et al. 2018), Unpublished
	MH443403	" " "	Not found	
		Not found		Direct submission (Eslami et al. 2019), Unpublished
	MH443404		Not found	
		Not found		Direct submission (Aghai Maybodi et al. 2018), Unpublished
			Not found	
		Not found		
L. donovani	FJ416603	US	Cultivated parasites	Nebohacova et al. 2009
	KF815198	Sudan	Ulcers of patients	23
	AY660023	" " "	Clinical samples:HB,HBM,HLN	23
L. infantum	KF815207	" " "	" " "	23
	KF815206	" " "	" " "	23
	KF302727	Not found	Not found	Direct submission (Soares, 2013), Unpublished
L. tarentolae	KU680825	Germany	Ulcers of patients	8
	L07544	USA	Cultured parasites	25
L. amasonensis	HQ586836	China	" " "	26
L. mexicana	HQ586845	" " "	" " "	26
	KU680824	Brazil	Cultured parasites	8
L. tropica	KF302720	Brazil	Not found	Direct submission (Soares, 2013), Unpublished
	KU680821	Iran	Ulcers of patients	8
	HQ586846	Shandong, China	Cultured parasites	26
	HQ586847	Soviet union	Cultured parasites	26
				26
L. sp.	HQ586841	China	Cultivated parasites	26
	HQ586843	Jiangsu, China	" " "	26

HB: blood, HBM: bone marrow, HLN: lymph node, **: Identified *L. major* in this study, " " ": Same as above

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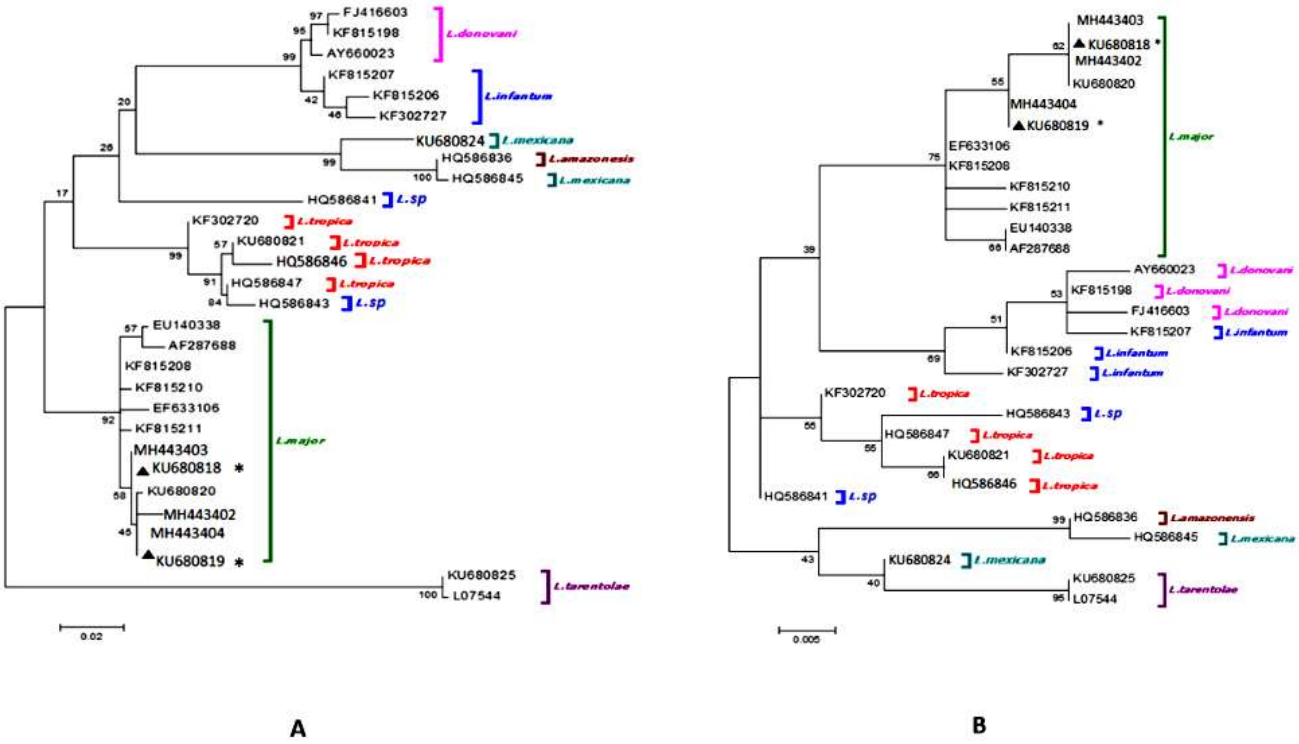

Fig. 5. Phylogenetic analyses discriminated *Leishmania* species well. A: Phylogenetic tree based on the mitochondrial nucleotides (COII gene) drawn by Maximum Likelihood method. B: Phylogenetic tree based on the mitochondrial genetic amino acid codes, drown by Maximum Likelihood method

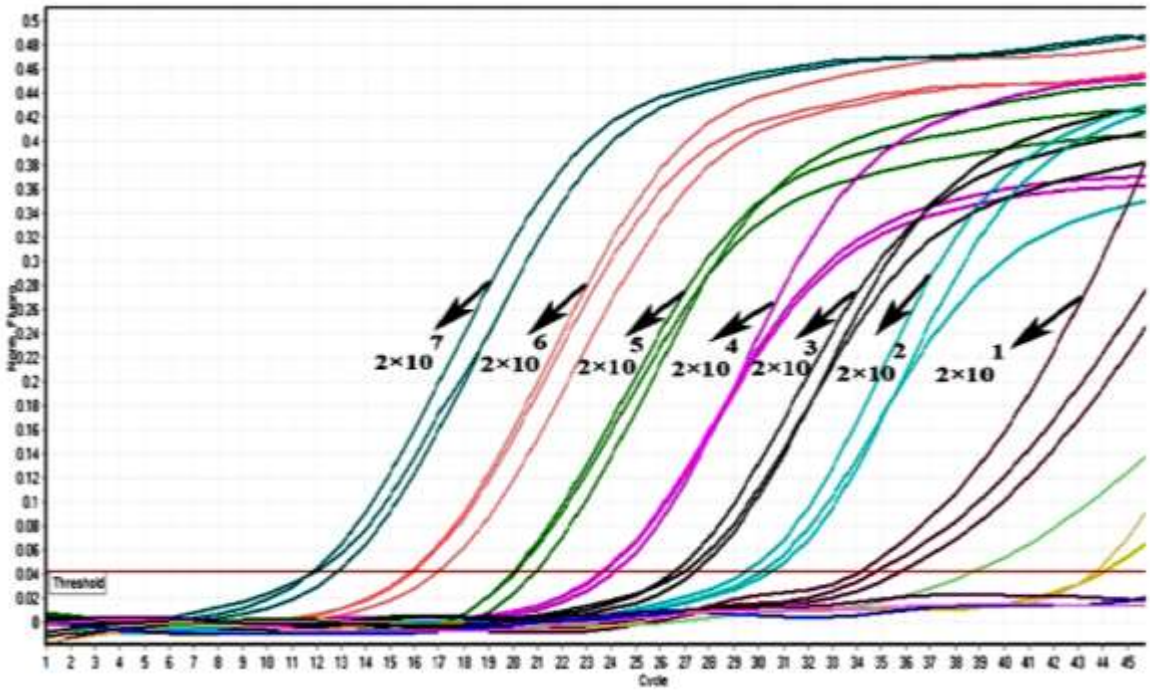

Fig. 6. Specifications performance of *Leishmania* detection by qPCR validation. Amplification plots derived from serial dilutions of cultured parasites (*L. major* and *L. tropica* standards with R and F CO.A.470.2 primers), ranging from 20–2× 10^7 copies / reaction plasmids by qPCR

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Table 4. Quantitative PCR-High Resolution Melting analysis of COII gene for *Leishmania major* and *L. tropica* in different plasmid copy number

Leishmania species	Plasmid copy number	qPCR		HRM	T_m mean
		Efficiency	R^2	T_m	
L. major	10^4	4.49	0.94	74.60	74.8
	10^4			74.83	
	10^5			74.65	
	10^5			74.58	
	10^6			74.55	
	10^6			74.63	
	10^7			74.63	
	10^7			74.85	
L. tropica	10^4	4.49	0.94	75.08	75.64
	10^4			75.22	
	10^5			75.00	
	10^5			74.98	
	10^6			75.50	
	10^6			75.42	
	10^7			74.25	
	10^7			74.22	

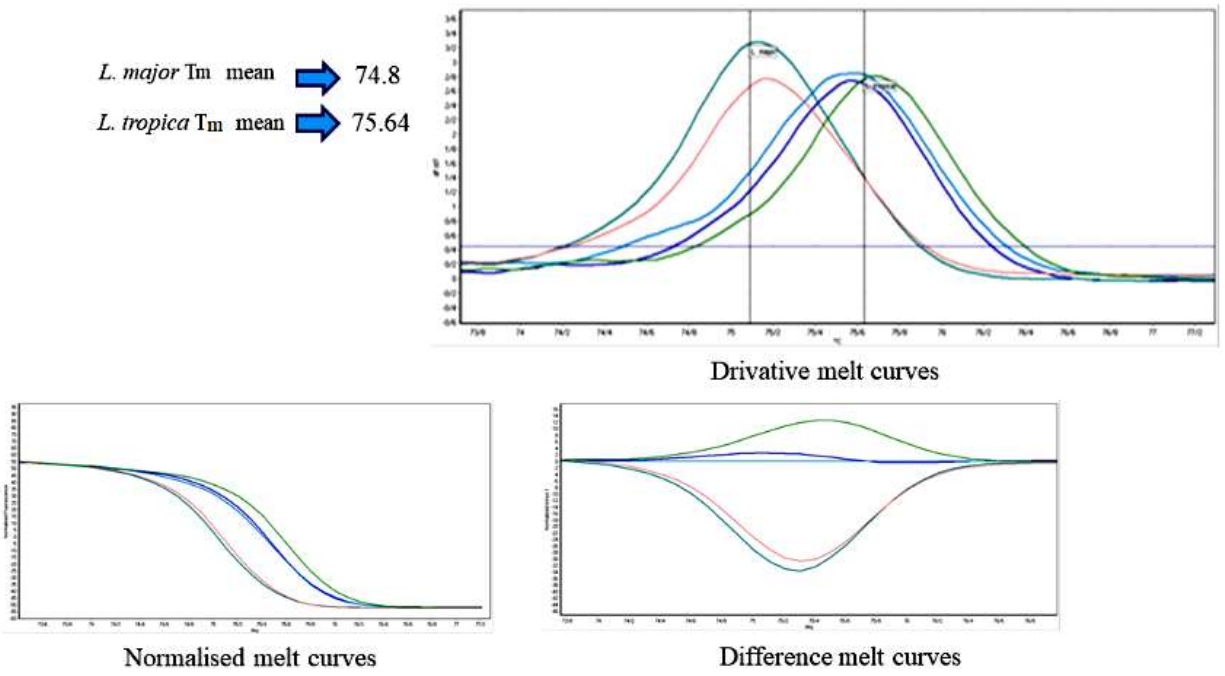

Fig. 7. Melting temperatures and normalized melting profiles obtained with the HRM assays for *L. major* and *L. tropica*. A: Derivative melt curves, B: Aligned and normalized melt curves, C: Differences melt curves

Discussion

For this investigation, blood fed female sand flies were screened for detecting *Leishmania* infections from two locations in Ilam Province (Mehran and Dehlouran) in Iranian border with

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Iraq where the endemic foci of ZCL places in these two countries. In these locations, *Ph. papatasi* is proven and main vector of *L. major* (27).

We were not surprised to find only two *Ph. papatasi* which carried *L. major* out of 89 sampled in our preliminary screen. The infection rates of *Ph. papatasi* can be low even in well studied in different ZCL foci in the world while infection rates can be high in human and reservoir hosts (5, 28-29). In about 20 past years, the sensitivity and specificity of molecular techniques was compared targeting different mitochondrial and nuclear genes for identifying *L. major* (2, 8). Now, we have tried to take an effective step with a new modified molecular method and genetic analysis for detection and identification of infection in native *Ph. papatasi* samples, in Ilam Province.

Rapid, sensitive and accurate diagnostic procedures are crucial for detecting and characterizing of *Leishmania* parasites in sand flies, in order to provide accurate treatment, precise prognosis and appropriate control measurements. For this research a new technique of HRM was employed for fast detecting of *Leishmania* species and mix infections targeting COII gene in sand flies.

COII gene is an expression gene. The silent site (dS) and replacement site (dN) of *L. major* and *L. tropica* were significantly different (P= 0.001). Nucleotides were 611 positions in the final dataset and 452 out of 611bp had no genetic variations. Evolutionary relationships of taxa for COII maxicircle mitochondrial were analyzed using Molecular Phylogenetic of Maximum Likelihood method.

The nucleotide's substitution in COII gene in *L. major* had 86% similarity with sequences available in the GenBank. COII is a mitochondrial gene and all mitochondrial genes have more mutations to compare with nuclear genes. When COII gene compared with other polymorphic genes in different *Leishmania* species has less diversity. Although Boite and colleagues (2012) mentioned that COII was less capable for discriminating and distinguishing different *Leishmania* species but this research and other investigations showed that the phylogenetic analyses and trees of this gene is able to identify *Leishmania* strains at the species level (8, 30). COII gene is conserve enough to discriminate *Leishmania* parasites and has polymorphism sites to discriminate some species, more over it has 20–50 copy numbers (8, 26). According to previous findings, COII is the most sensitive as compared with ITS-rDNA, HSP70, nagt and Cyt b genes that were tested (2, 8).

In this research new primers were designed to detect *Leishmania* species and strains. The prediction's results were analyzed using CLC software and followed by HRM method. The altered regions could make a desirable temperature difference in species of *Leishmania* parasites (31). According to polymorphism and phylogenetic analyses of COII gene, *L. major* was discriminated in *Ph. papatasi* from Ilam Province with 0.84 °C T_m using HRM technique.

Determining unique curves based on thermodynamic differences is the important criterion of HRM method. HRM can detect single nucleotide polymorphisms based on small differences in the nucleotide composition for any suitable mitochondrial and nuclear genes (6, 32). HRM to compare with conventional PCR is more sensitive, specific, simpler, less expensive and faster. DNA can be extracted directly from samples, blood and other tissues. HRM results can be obtained without additional post-PCR processing in less than 2.5 hours (33). So far, many effective and efficient studies have been conducted to isolate *Leishmania* parasite from sand flies (9, 33-35).

Because of eight years-imposed war between Iraq and Iran subsequently Kuwait then long time Iraq occupation by USA following ISIS, much transmission of *L. major* occur in locations in both sides of Iranian border with Iraq. These situations have provided refuges for many sand fly species as well as reservoir

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hosts to make a high risk habitat for transmission of parasites (5, 36).

Conclusions

The transmission cycles of *L. major* might depend on many criteria including diversity of *Leishmania* species and intraspecific strains, sand fly species, reservoir hosts, locations and migration of two countries people. Different parasite species interact to maintain *L. major* infections in reservoir hosts and people. Understanding the roles of *Ph. papatasi* as a proven vector which transmits *L. major* to the reservoir hosts and people, is important; also it is equally important to discover the complementary roles of rapid, sensitive and accurate diagnostic procedures for leishmaniasis in order to disease control and treatment in Iranian border with Iraq.

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Ethical considerations

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Conflict of interest statement

The authors declare there is no conflict of interests.

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