Experimental Parasitology 123 (2009) 218–225

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Phlebotomus perfiliewi transcaucasicus is circulating both *Leishmania donovani* and *L. infantum* in northwest Iran

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ARTICLE INFO

Article history:
Received 21 January 2009
Received in revised form 8 July 2009
Accepted 14 July 2009
Available online 22 July 2009

Keywords:
Leishmania donovani complex
Phlebotomus perfiliewi
Vector
CPB
Iran
Mediterranean Basin

ABSTRACT

Leishmania infantum is the causative agent of infantile visceral leishmaniasis (IVL) in the Mediterranean Basin and, based on isoenzyme typing of the parasite isolated from dogs; this parasite was considered to predominate in the all foci of IVL in Iran. However, based on PCR detection and sequencing of parasite Cysteine Protease B (CPB), only one out of seven sandfly infections in *Phlebotomus perfiliewi transcaucasicus* was found to be *L. infantum* in the current investigation. The six other infections were haplotypes of *Leishmania donovani*, the causative agent of anthroponotic visceral leishmaniasis (AVL) in West Africa and India. The deduced amino acid of the *L. donovani* haplotype was found to be novel and the shortest CPB protein reported within the *Leishmania* spp. Circulation of both *L. donovani* and *L. infantum* by *P. perfiliewi transcaucasicus*, in addition to previous data indicating its ability to circulate *L. tropica,* suggests that this species, like other vectors of VL, is a permissive vector. Finding *L. donovani* infecting *P. perfiliewi transcaucasicus* in the area demands extensive and intensive typing of natural *Leishmania* infections in epidemiological investigations in Iran and the Mediterranean Basin in general.

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1. Introduction

Much of northwest Iran to the east of Turkey has a Mediterranean climate and, like the Mediterranean Basin, some parts are endemic for infantile visceral leishmaniasis (IVL) caused by *Leishmania infantum* (Nadim et al., 1978). The prevalence of human disease is relatively high in two districts of northwest Iran, the Meshkin-Shahr district of Ardabil province (Mohebali et al., 2005; Nadim et al., 1992) and, to the west, the Kaleybar district of East Azerbaijan province (Davies and Mazloumi-Gavgani, 1999; Gavgani et al., 2002a,b). Both districts appear to have a transmission cycle typical of the Mediterranean Basin, with domestic dogs incriminated as the reservoir hosts of *L. infantum* zymodeme MON-1 in rural villages and farms (Gavgani et al., 2002a,b; Mohebali et al., 2005). The Germi region, a part of Meshkin-Shahr district, was chosen as a study site to incriminate the regional sand fly vectors of *L. infantum*. The Germi region is located between two low mountain ranges enjoying a warm climate in summers and a moderate weather in winter.

Recently, different PCR or PCR-RFLP assays have been developed for discrimination of *Leishmania donovani/L. infantum*. These assays are mainly focused on the cysteine protease B (cpb) genes (Hide and Bañuls, 2006; Oshaghi et al., 2009). The loci involve in host–parasite interaction and have got two specific copies of cpbE for *L. infantum* and cpbF for *L. donovani* (Hide and Bañuls, 2006). In the current study we used the CPB PCR to detect *Leishmania* infection in sandflies and established that *L. infantum* is not the only species of the *L. donovani* complex circulating in the region. Our detection of *L. donovani* in sandflies from five out of six Germi villages raises the possibility that, this parasite is also established in the area.

2. Materials and methods

2.1. Reference strains of Leishmania

DNA of reference strains of four Old World species of the subgenus *Leishmania* was used: *L. major* MHOM/IR/75/ER, *L. tropica* MHOM/IR/03/Mash-878, *L. infantum* MHOM/FR/87/LEM1098, and

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doi:10.1016/j.exppara.2009.07.004

L. donovani MHOM/ET/00/HUSSEN. These strains were used as positive or negative controls in different PCR amplifications.

2.2. Sandfly collections

Six villages of (1) Kalansora, (2) Shah-Tapeh-si, (3) Hamzeh-Khanlo (4) Hasi-Kandy, (5) Sarv-Aghaji, and (6) Ghasem-Kandy with high incidence of VL have been selected for entomological surveys. Sandflies were sampled from the villages during May to late September 2006. The villages were systematically sampled by placing 100 sticky papers (A4 white paper soaked in castor oil) inside and outside of houses, domestic animal shelters, burrows around villages, crakes, and under bridges per village every two weeks for two day periods.

Specimens caught on sticky papers were removed with needles or fine brushes dipped in 70% ethanol. All sandflies were then stored in 80% analytical grade ethanol first at 4 °C (in Meshkinshahr research center) and later at −20 °C (in Tehran). They were washed in three changes of PCR water (Sigma) before dissection. Morphological identifications were based on characters of the head and abdominal terminalia slide mounted in Berlese fluid (Lewis, 1982; Nadim and Javadian, 1976), following dissection with sterilized forceps and micro-needles (Parvizi et al., 2005).

2.3. Extraction of DNA from sand flies

Total DNA was extracted from the dissected thorax and attached anterior abdomen of individual females using the method of Ish–Horowicz with minor modifications (Ready et al., 1991). Each sample contained the midgut, the location of most *Leishmania* promastigotes, and was homogenized in lysis buffer a 1.5 ml microfuge tube using a disposable plastic tip of a micropipette. Following ethanol precipitation, the DNA was dissolved in 15 µl 1 × TE (10 mM Tris–HCl, 1 mM EDTA pH 8.0), to give a concentration of 5–10 ng/µl, and stored at −20 °C.

2.4. Detection of Leishmania infections in sandflies

2.4.1. Preliminary screening

Preliminary screening for infections of sand flies to *Leishmania* species was performed using PCR against the mini-circle kDNA and ITS rDNA on female sandflies using the protocols described by Aransay et al. (2000) and Cupolillo et al. (1995), respectively. PCR positive specimens were positive against both kDNA and ITS rDNA loci were selected for further analysis by PCR against the CPB gene.

2.4.2. CPBE/F PCR

This PCR is species-specific for the *L. donovani* complex and has been developed by Hide and Bañuls (2006). The optimal conditions for CPBE/F amplification in 30 µl were 6 pmol of each primer (forward: 5′-CGTGACGCCGGTGAAGAAT-3′; reverse: 5′-CGTGCACTCG GCCGTCTT-3′), 4.5 nmol dNTPs, 1 U *Taq* polymerase, 3 µl Buffer 10× and 1 µl of DNA extracted from individual sand flies. Thirty cycles were necessary for amplification (denaturation 30 s at 94 °C, annealing 1 min at 62 °C and elongation 1 min at 72 °C), followed by 10 min at 72 °C. All of the amplification reactions were analyzed by 1–1.5% agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light. Standard DNA fragments (100 bp ladder, Fermentas) were used to permit sizing.

2.4.3. PCR direct sequencing and phylogenetic analysis

PCR products of the ITS rDNA and CPBE/F were directly sequenced to identify *Leishmania* haplotypes infecting individual female sand flies. About 538 bp of ITS2 region and 702/741 bp of the CPB gene were sequenced. Also some entries of the *L. donovani* complex (Kuhls et al., 2005) were retrieved from Genbank and used for data analysis.

Sequences were aligned using the multiple alignment program CLUSTAL V and manually adjusted. All haplotypes were identified to species level by sequence comparison with the Genbank entries and by the phylogenetic analysis using the neighbor-joining method embedded in the CLUSTAL V online program. Also the DNA sequences were translated to amino acids using the online program (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) and were compared with the published data available in Genbank.

Phylogenetic analysis was performed on a combination of the data obtained from this study plus representatives of different ITS or CPB haplotypes of the *L. donovani* complex available from the Genbank database. Representatives of eight (A, B, C, D, E, F, G, and H) and three intermediate (B/A, E/F, and D/E) ITS sequence types of the *L. donovani* complex (Kuhls et al., 2005) including *L. infantum*, *L. chagasi*, *L. donovani* and *L. archibaldi* from different zymodemes and geographical origin were selected for ITS sequence analysis (Table 1).

3. Results

3.1. Sandflies collected

In this study carried out during the seasonal activity of sand flies, we collected 3447 sand flies in six villages. In total, 1569 female sandflies belonging to 10 species (12 species if considering male specimens of three species of *Adlerius* group) were captured and screened for *Leishmania* infections (Table 2). Females of *Adlerius* subgenus group could not be separated morphologically but, based on accompanying males; they were *P. halepensis, P. brevis,* and *P. longiductus*. Except for two villages of Sarv-Aghaji and Ghasem-Kandy which the *Adlerius* group were dominant, the most dominant species in the area study was *Phlebotomus perfiliewi transcaucasicus* (see Table 3).

3.2. Leishmania infections identified in sandflies

3.2.1. kDNA and ITS rDNA PCR

Fourteen out of 1569 samples were positive in the semi-nested PCR against mini-circle kDNA molecules and out of theses, 11 specimens were positive for ITS rDNA. Nine were detected in *P. perfiliewi transcaucasicus*. 538 bp of the ITS2 region of 10 specimens were successfully sequenced and submitted to Genbank (Accession Nos. EU637914–EU637924). Sequence analysis revealed four parasite ITS2 haplotypes including three *L. donovani* complex and one *L. adleri-like* (76% homology), a causative agent of lizard leishmaniasis.

Three polymorphic sites were detected in the *L. donovani* complex haplotypes. Remarkably, all three polymorphisms were located within microsatellites and variations were due to two single nucleotide insertions/deletions (indels) and one variable repeats of a poly (G) microsatellite. Eight out of 10 sequences were identical and differed from the other two (AS327 and MG36) by just two and three nucleotides, respectively. AS327 and MG36 were different in just one nucleotide (Table 1; Fig. 1).

The haplotype of AS327 and MG36, respectively were found to be identical or differed by only one nucleotide to that of isolates of *L. infantum* from Spain (Genbank Accession Nos. AM502245 and AJ634352), Italy (Genbank Accession Nos. AJ634353 and AJ634354), China (Genbank Accession No. AJ000303), Tunisia (Genbank Accession No. AJ000289), and isolates of *L. chagasi* from Brazil (Genbank Accession Nos. AJ000304 and AJ000306). These isolates are characteristic for *L. infantum* from the European and Mediterranean regions and China, and for *L. chagasi* the synonymous species from the New World.

In the phylogenetic analysis using neighbor-joining method (Fig. 1), three distinct clades were found. The AS327 and MG36

Table 1
Designation and characteristics of *Leishmania* strains used for ITS2 analysis. The sequences of Iranian specimens were obtained in this study whereas other strains are derived from the Genbank database.

Taxa[a]	WHO-code	Origin	Zymodeme	Pathology[b]	ITS seq. type	Accession number EMBL
L.infantum[a]	IPER/IR/2007/AS327	Iran	n.d.	CL(?)	A	EU637915
–[a]	ICHI/IR/2007/MG36	Iran	n.d.	CL(?)	A	EU637924
	MHOM/TN/80/IPT1	Tunisia	MON-1	VL	A	AJ000289
	MHOM/FR/78/LEM75	France	MON-1	VL	A	AJ634339
	MHOM/ES/93/PM1	Spain	MON-1	VL	A	AJ634341
	MHOM/PT/00/IMT260	Portugal	MON-1	CL	A	AJ634344
	MHOM/CN/54/Peking	China	MON-1	VL	A	AJ634345
	MHOM/FR/62/LRC-L47	France	n.d.	VL	B	AJ000288
	MHOM/MT/85/BUCK	Malta	MON-78	CL	A	AJ634350
	MCAN/ES/86/LEM935	Spain	MON-77	CanVL	B/A	AJ634355
	MHOM/IT/94/ISS1036	Italy	MON-228	VL	A	AJ634353
	MHOM/SD/97/LEM3472	Sudan	MON-267	PKDL	F	AJ634370
	MHOM/SD/62/3S	Sudan	MON-81	VL	E	AJ634361
L.donovani[a]	IPER/IR/2007/AC1	Iran	n.d.	CL(?)	I	EU637919
–[a]	IPER/IR/2007/HK2	Iran	n.d.	CL(?)	I	EU637923
–[a]	IPER/IR/2007/HZ15	Iran	n.d.	CL(?)	I	EU637922
–[a]	IPER/IR/2007/HZ6	Iran	n.d.	CL(?)	I	EU637921
–[a]	IPER/IR/2007/AS259	Iran	n.d.	CL(?)	I	EU637920
–[a]	IPER/IR/2007/HS10	Iran	n.d.	CL(?)	I	EU637918
–[a]	IPER/IR/2007/AS110	Iran	n.d.	CL(?)	I	EU637917
–[a]	IPER/IR/2007/AK11	Iran	n.d.	CL(?)	I	EU637916
	MHOM/CN/00/Wangjie1	China	MON-35	VL	C	AJ000294
	MHOM/ET/00/HUSSEN	Ethiopia	MON-31	VL	D/E	AJ634360
	MHOM/ET/67/HU3	Ethiopia	MON-18	VL	F	AJ634373
	MHOM/SD/93/338	Sudan	MON-18	PKDL	E/F	AJ634368
	MCAN/SD/00/LEM3946	Sudan	MON-274	CanVL	D	AJ634356
	MHOM/KE/83/NLB189	Kenya	MON-37	PKDL	G	AJ634374
	MHOM/IN/00/DEVI	India	MON-2	VL	H	AJ634376
	MHOM/IN/71/LRC-L51a	India	n.d.	VL	G	AJ000290
L.archibaldi	MHOM/ET/72/GEBRE1	Ethiopia	MON-82	VL	E	AJ634367

[a] Strain identification according to sequence analysis.
[b] VL, Visceral leishmaniasis; CL, Cutaneous leishmaniasis; PKDL, Post kala-azar dermal leishmaniasis; CanVL, Canine VL.; n.d., not done; CL(?): based on epidemiological data.

haplotypes from Iran and the other European haplotypes formed a clade distinct from the other clades. They comprised A, B, and B/A ITS sequence types of the *L. donovani* complex. Second clade contained different haplotypes of *L. donovani* from Ethiopia, Sudan, Kenya, China, and India (C, D, E, G, H, D/E, and E/F ITS sequence types), two haplotypes of *L. infantum* s.l. from Sudan (E and F ITS sequence types), a haplotype of *L. archibaldi* from Ethiopia (E ITS sequence type). Third clade includes other eight Iranian sequences which formed a clade diverse from the rest of the *L. donovani* complex; we call them ITS sequence type "I". The Iranian clade, however, were closer to the first clade containing European haplotypes than to the second clade including African haplotypes.

3.2.2. CPBE/F PCR

Species specific PCR of CPBE/F gene against the 10 positive ITS rDNA specimens revealed presence of the *L. donovani* complex in seven sandfly specimens. Interestingly all seven specimens were *P. perfiliewi transcaucasicus*. The PCR of the remaining three specimens (two *P. perfiliewi transcaucasicus* and one *Adlerius* group) were not positive against the CPBE/F species-specific primers of the *L. donovani* complex. The CPB region of the seven specimens was successfully sequenced and submitted to Genbank (Accession Nos. EU637907–EU637913). Overall, *L. donovani* (six samples) and *L. infantum* (one sample) haplotypes were identified from the specimens. Interestingly, the haplotypes of *L. donovani* were highly

Table 2
Details of female sand flies captured in the study area and tested for *Leishmania* infection by (1) mini-circle kinetoplast DNA (kDNA), (2) internal transcribed spacer (ITS) rDNA, and (3) Cysteine Protease B (CPB). Genus name, *P*, *Phlebotomus*; *S*, *Sergentomyia*. FS, Feeding status of infected specimens; E, empty; F, fully blood fed; G, gravid; SG, semi-gravid; Village code: ST, Shah-Tapeh-si; HL, Hamzeh-Khanlo; HK, Hasi-Kandy; GK, Ghasem-Kandy; KS, Kalansora; SA, Sarv-Aghaji.

Species	No. (%)	No. of infected			*Leishmania* species (No.)	FS(No.)	Village code (No.)
		kDNA	ITS	CPB			
P. perfiliewi transcaucasicus	954(60.8)	9	9	7	CPBF *L. donovani* (6)	E(5); G(1)	ST(4), HL(1), KS(1)
					CPBE *L. infantum* (1)	G(1)	ST(1)
					L. tropica/L.donovani complex(2)	E(2)	HL(1), KS(1),
Adlerius group	131(8.3)	1	1	0	*L. tropica/L.donovani complecx*(1)	E(1)	GK(1)
P. papatasi	47(3.0)	1	0	0	*L. sp* (1)	E(1)	HK(1)
P. kandelakii	45(3.0)	1	0	0	*L. sp* (1)	E(1)	GK(1)
P. sergenti	32(2.0)	0	0	0		-	-
P. alexandri	1(0.06)	0	0	0		-	-
P. mongolensis	1(0.06)	0	0	0		-	-
S. dentata	313(19.9)	2	1	0	*L. adleri*-like	F(2)	HK(1), HL(1)
S. sintoni	44(2.8)	0	0	0		-	
S. powlowsky	1(0.06)	0	0	0		-	
Total	78(5.0)	14	11	7		E(10); G(2); F(2)	ST(4), HL(3), HK(3), GK(2) KS(2)

Table 3
Leishmania strains used for CPB sequence analysis.

International code	Species	Origin	Zymodeme	Genbank Accession Nos.
IPER/IR/2007/HS10	*L. donovani*	Iran	n.d	cpbF EU637913
IPER/IR/2007/AS110	*L. donovani*	Iran	n.d	cpbF EU637912
IPER/IR/2007/HZ6	*L. donovani*	Iran	n.d	cpbF EU637911
IPER/IR/2007/AS259	*L. donovani*	Iran	n.d	cpbF EU637910
IPER/IR/2007/AK11	*L. donovani*	Iran	n.d	cpbF EU637909
IPER/IR/2007/AC1	*L. donovani*	Iran	n.d	cpbF EU637908
MHOM/ET/67/HU3	*L. donovani*	Ethiopia	MON-18	cpbF AY896783
MHOM/ET/00/HUSSEN	*L. donovani*	Ethiopia	LON-42	cpbF AY896785
MHOM/KE/67/MRC(L)3	*L. donovani*	Kenya	LON-44 or 46	cpbF AY896786
MHOM/SD/82/GILANI	*L. donovani*	Sudan	MON-30	cpbF AY896784
n.d	*L. donovani*	n.d	n.d	cpbF AF309627
IPER/IR/2007/AS327	*L. infantum*	Iran	n.d	cpbE EU637907
n.d. (JPCM5 strain)	*L. infantum*	Spain	n.d	cpbE AJ628943
MHOM/FR/78/LEM75	*L. infantum*	France	MON-1	cpbE AY896781
MHOM/FR/82/LEM356	*L. infantum*	France	MON-33	cpbE AY896779
MHOM/FR/87/LEM1098	*L. infantum*	France	MON-1	cpbE AY896776
MHOM/FR/85/LEM716	*L. infantum*	France	MON-1	cpbE AY896777
MHOM/FR/85/LEM663	*L. infantum*	France	MON-1	cpbE AY896780
MHOM/ES/81/BCN1	*L. infantum*	Spain	MON-29	cpbE AY896778
MHOM/MA/67/ITMAP263	*L. infantum*	Malta	MON-1	cpbE AY896782
n.d	*L. chagasi*	n.d	n.d	cpbE AF217087

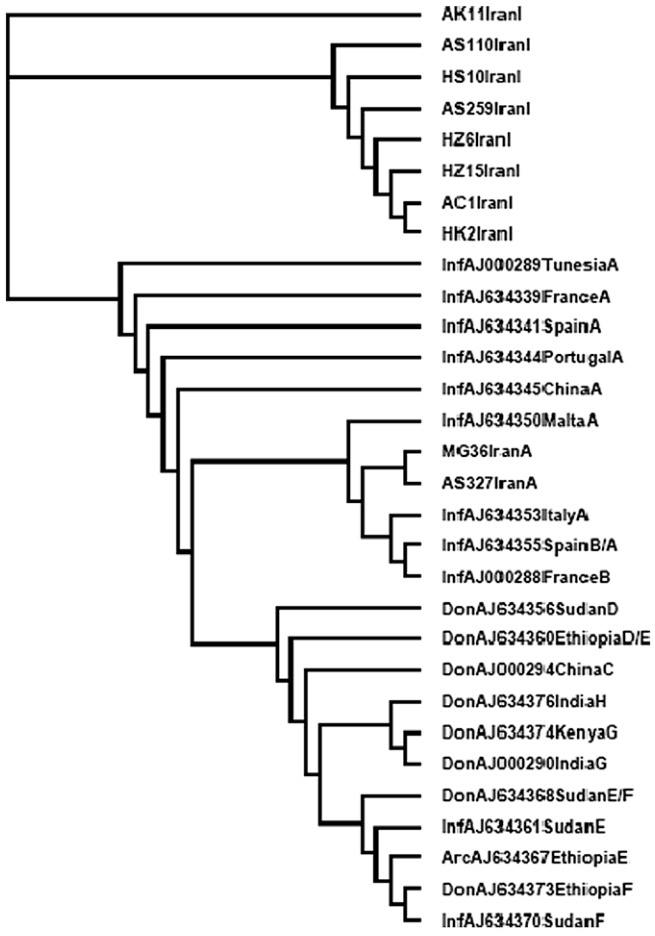

Fig. 1. The neighbor-joining tree for ITS rDNA sequences of *Leishmania* species. The species, origin and accession number of the data retrieved from Genbank is shown on the branches. The capital letter at the end of branches refers to the ITS haplotypes designated by Kuhls et al. (2005). ITS haplotype of the Iranian samples which are differ from the Kuhls ones are shown as I haplotype (IranI). Inf, Arc, and Don refer to *L. infantum*, *L. archibaldi*, and *L. donovani*, respectively.

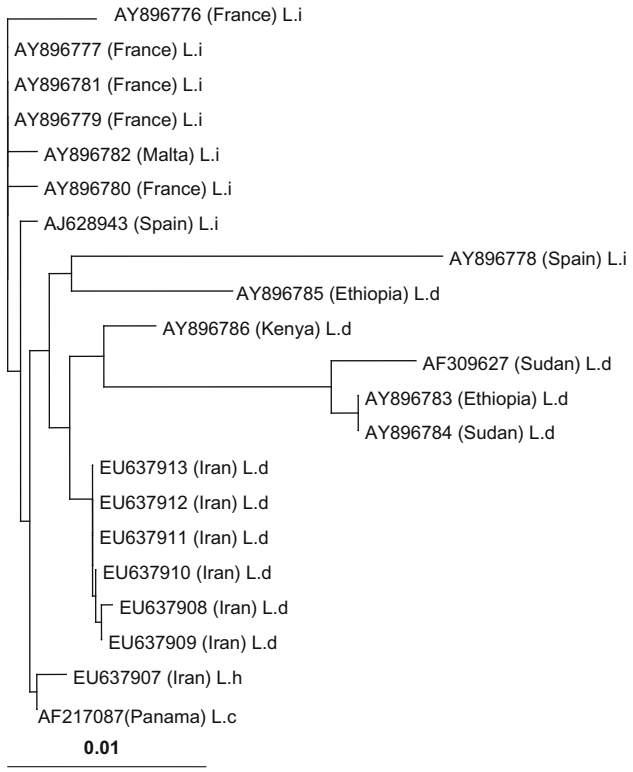

Fig. 2. The neighbor-joining tree for CPB sequences of the *Leishmania donovani* complex. The species, origin and accession number of the data retrieved from Genbank is shown on the branches. L.i, L.d, and L.c refer to *L. infantum*, *L. donovani*, and *L. archibaldi*, respectively.

similar to the African haplotypes. They differed pairwise by only one nucleotide position from a haplotype of *L. donovani* from Kenya (Genbank Accession Nos. AY896786), and by two to three nucleotide positions from haplotypes of Kenya (Genbank Accession No. AY896788), Ethiopia (Genbank Accession Nos. AY896785 and AY896783), Sudan (Genbank Accession No. AY896784). Also they were found to be 98% similar to a haplotype of *L. donovani* from China (Genbank Accession No. AY896787).

The haplotype of *L. infantum* were found to be identical to that of isolates of *L. infantum* from Spain (Genbank Accession Nos. AJ628943 and XM001463394), France (Genbank Accession Nos. AY896781, AY896779, AY896777) and Croatia (Genbank Accession No.

EU145976) and differed pairwise by only one nucleotide position from a haplotype of *L. infantum* from Malta (Genbank Accession No. AY896782) and France (Genbank Accession No. AY896780) but due to the 39 bp indel (Fig. 3) was very different in many nucleotide positions from all haplotypes of *L. donovani*. Also it was identical to that of isolates of *L. chagasi* from New World (Genbank Accession No. AF217087). In the phylogenetic analysis using neighbor joining method (Fig. 2), the Iranian haplotypes of *L. infantum* was a sister branch of *L. chagasi*, and both were associated with other *L. infantum* haplotypes. The Iranian *L. donovani* haplotype formed a separate clade but close to the *L. donovani* African haplotypes including two haplotypes from Sudan (Genbank Accession Nos. AF309627 and AY896784), and two haplotypes of *L. donovani* from Ethiopia and Kenya (Genbank Accession Nos. AY896783, and AY896786, respectively).

3.3. CPBE/F deduced amino acid

Comparison of the deduced amino acid sequences of CPB sequences of the Iranian *L. donovani* and *L. infantum* haplotypes with other translated CPB sequences showed that the Iranian *L. infantum* haplotype is identical to published *L. infantum* haplotypes (e.g. Genbank Accession Nos. AY896776, AY896780, AY896782, and AF217087).

However, the Iranian *L. donovani* haplotype was found to be a novel deduced amino acid. Due to an insertion of a C nucleotide within the open reading frame of the gene (Fig. 3) a reading frame shift occurred and translation of the gene were halted seven amino acids further down the insertion, resulting in a 76 amino acid polypeptide. It is the shortest CPB protein that has been reported in *Leishmania* species so far. Comparison of the deduced amino acid sequence with the translated CPB sequences from some representatives of other species of the *L. donovani* complex is shown in Fig. 4.

4. Discussion

In this study, for the first time, we showed that not only *L. infantum* but also *L. donovani* is circulating in the region by *P. perfiliewi transcaucasicus*. Finding *L. donovani* is important in epidemiology and ecology of VL in the region since *L. donovani* is largely anthroponotic and produces mainly the visceral form whereas *L. infantum* is anthropozoonotic with a dog reservoir and can produce visceral and cutaneous forms in humans. Previous studies have shown that domestic dogs are the main reservoir of *L. infantum* in the VL focus of Ardabil province (Hajjaran et al., 2007) and elsewhere in Iran (Motazedian et al., 2002). However, for the *L. donovani* haplotype in the region, extensive research is needed to distinguish its clinical

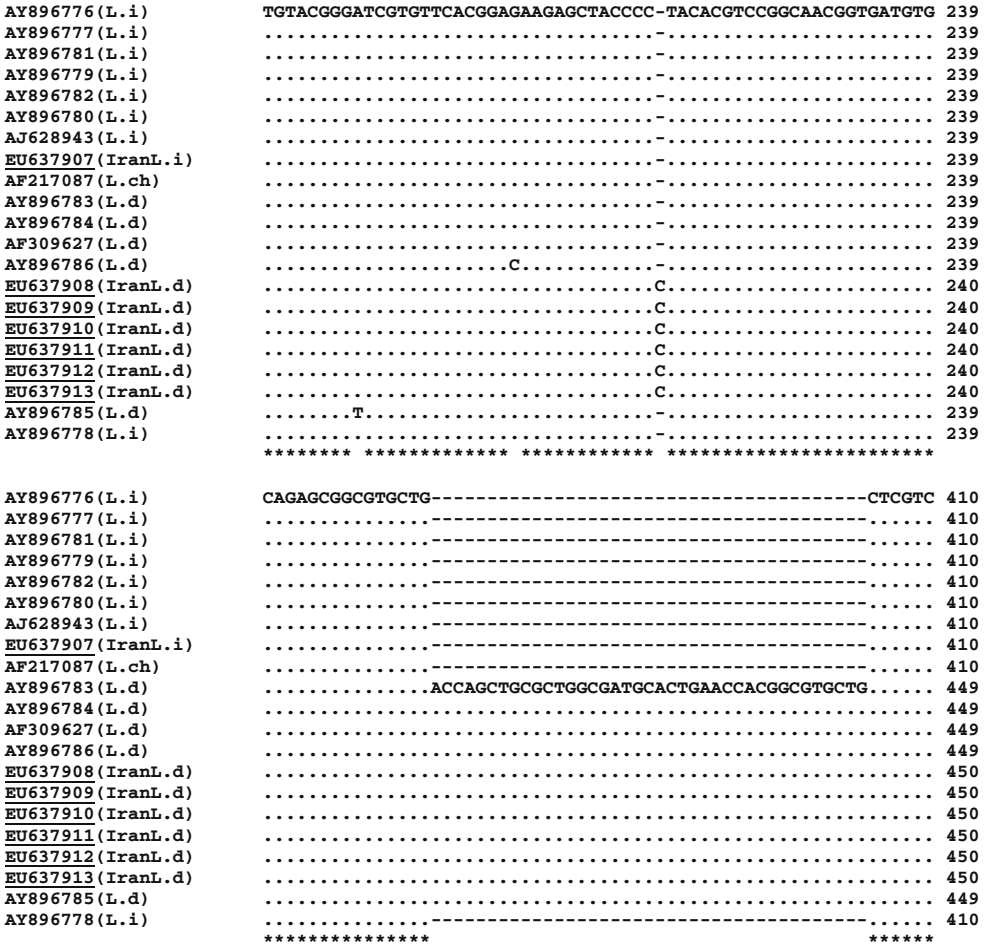

Fig. 3. Sequence alignment of two portions (181–240 and 350–410) of CPB gene of the Iranian *L. infantum* haplotype (EU637907L.i) and *L. donovani* haplotype (EU637908–EU637912L.d), with some reference strains obtained from Genbank. These representatives included *L. infantum* (AY896776-82L.i and AJ628943L.i), *L. chagasi* (AF217087L.ch), and *L. donovani* (AY896783-86L.d and AF309627L.d). The 39 bp gap (indel in the position 366–405) is shown as a distinct DNA marker for *L.donovani* and *L.infantum* identification. Insertion of the "c" nucleotide in the position 236 in the Iranian *L.donovani* haplotype caused a reading frame shift producing the shortest CPB protein reported in *Leishmania* species yet (Fig. 4).

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AY896783L.d      VTPVKNQGACGSCWAFSAVGNIESQWARAGHGLVSLSEQQLVSCDDKDNGCNGGLMLQAF 60
AY896784L.d      ............................................................ 60
AY896780L.i      ..................S......................................... 60
EU637907L.i      -------..................................................... 53
AF217087L.ch     ............................................................ 60
AY896782L.i      ............................................................ 60
AY896776L.i      ............................................................ 60
AY896786L.d      ............................................................ 60
EU637912L.d      --------.................................................... 52
                         **********.****************************************

AY896783L.d      EWLLRHMYGIVFTEKSYPYTSGNGDVAECLNSSKLVPGARIDGYVMIPSNETVMAAWLAE 120
AY896784L.d      ............................................................ 120
AY896780L.i      ....................................Q....................... 120
EU637907L.i      ....................................Q....................... 113
AF217087L.ch     ....................................Q....................... 120
AY896782L.i      ....................................Q....................... 120
AY896776L.i      ....................................Q....................... 120
AY896786L.d      ............D.......................Q....................... 120
EU637912L.d      ..................LHVRQR---------------------------------- 76
                 ************:****    :

AY896783L.d      NGPIAIGVDASSFMSYQSGVLTSCAGDALNHGVLLVGYNKTGGVPYWVIKNSWGEDWGEK 180
AY896784L.d      ............................................................ 180
AY896780L.i      ......A..........-------------.............................. 167
EU637907L.i      ......A..........-------------.............................. 160
AF217087L.ch     ......A..........-------------.............................. 167
AY896782L.i      ......A..........-------------.............................. 167
AY896776L.i      ......A..........-------------.............................. 167
AY896786L.d      ......A..................................................... 180
EU637912L.d      ------------------------------------------------------------

AY896783L.d      GYVRVAMGLNACLLSEYPVSAHVPQSLTPASTASGNFCEACWTVMLHRILSVLKTNGWLL 240
AY896784L.d      ............................................................ 240
AY896780L.i      ..........................G......S..............P.P..... 227
EU637907L.i      ..........................G......S..............P.P..... 220
AF217087L.ch     ..........................G......S..............P.P..... 227
AY896782L.i      ..........................G...G..S..............P.P..... 227
AY896776L.i      ..........................G......S...S.....R.....P.P..... 227
AY896786L.d      ..........................G......S.......................... 240
EU637912L.d      ------------------------------------------------------------

AY896783L.d      GRRPSAR 247
AY896784L.d      ....... 247
AY896780L.i      ....... 234
EU637907L.i      ....... 227
AF217087L.ch     ....... 234
AY896782L.i      ....... 234
AY896776L.i      ....... 234
AY896786L.d      ....... 247
EU637912L.d      -------
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Fig. 4. Alignment of deduced amino acid sequence of the Iranian *L.infantum* haplotype (EU637907L.i) and *L.donovani* haplotype (EU637912L.d), with deduced amino acid sequences obtained from Genbank. These representatives included *L.infantum* (AY896780L.i, AY896782L.i, and AY896776L.i), *L.chagasi* (AF217087L.ch), and *L.donovani* (AY896783L.d, AY896784L.d, and AY896786L.d).

outcome and to establish its transmission cycle. Recently, some researchers indicated the possibility of humans being a reservoir and source of transmission of VL agent in south of Iran (Alborzi et al., 2008a). However, more humans and dogs must be screened before concluding whether or not they are important reservoir hosts of *L. donovani* in northwest Iran. Other possible reservoirs of *L. donovani* in the region include rodents, in which this species has once been isolated and typed from *Meryones persicus* in northwest of Iran (Mohebali et al., 2004). Having found *L. donovani* in Iran is also important in association with different clinical outcomes as one of the most important distinctions between *L. donovani* and *L. infantum* is their virulence in humans and the expression of the disease, for example the presence of PKDL in *L. donovani* infections. Interestingly, there are some reports on some PKDL cases from the VL foci in Iran (Baghestani et al., 1998; Alborzi et al., 2008b) which might be due to *L. donovani*.

Leishmania donovani has been limited to Africa, India, and China, however, it seems that its geographical distribution is gradually expanding since there are some reports on the presence of this species in the Mediterranean region and Middle East countries such as Turkey, Iraq, Yemen, Saudi-Arabia, and Pakistan (WHO, 1990; Ozbel et al., 2000), all neighbors of Iran. One implication of circulating *L. donovani* in Iran is that more extensive and intensive typing of natural *Leishmania* infections should become a feature of epidemiological investigations in Iran and neighboring countries.

Phlebotomine vectors of leishmaniasis in some instances display striking species restricted competency for the parasite strains that they transmit in nature, i.e. certain sand flies are able to transmit only certain species of *Leishmania* (Killick-Kendrick, 1985). There is, for example, no evidence that *P. papatasi* is involved in the natural transmission of any species other than *L. major*, despite the fact that this sand fly has a wide distribution in regions endemic for other species of *Leishmania*. Similarly, *Phlebotomus sergenti* is a proven vector of only *L. tropica*, again despite the fact that it is found in biotopes containing other *Leishmania* species. On the other hand, the natural vectors of the *L. donovani* complex appear to be broadly permissive to diverse *Leishmania* species. In this study we show that *P. perfiliewi transcaucasicus*, could transmit both *L. donovani* and *L. infantum*. Recently, a study by Parvizi et al. (2008) showed that *P. perfiliewi transcaucasicus* is able to

transmit *L. tropica. This, in addition to the* results of present investigation, shows that *P. perfiliewi transcaucasicus*, like other vectors of the *L. donovani* complex, is broadly permissive to multiple *Leishmania* species including *L. donovani, L. infantum,* and *L. tropica. Lutzomyia longiplapis*, for example, which is the natural vector of *L. chagasi* in the New World, has been used to study the complete development of *L. amazonensis* and *L. major* (Molyneux et al., 1975; Walters et al., 1993). *P. argentipes*, which is the natural vector of *L. donovani* transmission in India, was also susceptible to the full development of *L. major, L. tropica* and *L. amazonensis* (Pimenta et al., 1994; Kamhawi et al., 2000).

The sand fly species infected with the *L. donovani* complex in the region was predominantly *P. perfiliewi transcaucasicus* (confirmed by kDNA, ITS, and CPB loci), though one specimen belonging to the *Adlerius* subgenus group was also infected (confirmed by kDNA and ITS loci). Most Mediterranean vectors of this parasite belong to the same subgenus as *P. perfiliewi transcaucasicus*, namely *Larroussius*, and members of *Adlerius* subgenus. There were leptomonad infection confirmed only by kDNA in one *P. kandelakii*, one *P. papatasi*, and two *S. dentata*. However, non-vectorial sandflies may feed on an infected host and support some parasite growth but not metacyclogenesis (the production of infective forms) (Killick-Kendrick, 1990). Such infections only indicate which parasites are present in a focus. Additional criteria must be met to incriminate a vector (Killick-Kendrick and Ward, 1981). However, previously in northwest Iran, non-specific *Leishmania* infections were found in putative vectors of IVL: *P. kandelakii* (Nadim et al., 1992; Rassi et al., 2005), *P. major* (Azizi et al., 2008), *P. alexandri* (Azizi et al., 2006), and *P. perfiliewi* (Nadim et al., 1992).

Analysis of ITS haplotype found in *S. dentata* revealed 76% identity with those of *L. (sauroleishmania) adleri* of Genbank (Accession No. EU637914). However, further studies are needed to establish the species identity of the leptomonad. This reptile related *sauroleishmania* parasites lacks LPG required for entrance to human phagocytes, and hence are not human pathogens. However, the GlycoInositoPhosphoLipid (GIPL) molecules of this parasite reacts with sera VL patients and may cause false positive scores in seroepidemiological surveys for the disease. It is shown that some *Sergentomyia* species such as *S. garnhami* and *S. clydei* are also man-biting species and are able to transmit *sauroleishmania* parasites to human (Lewis and Ward, 1987; Lane, 1993).

Comparison of the deduced amino acid of CPB sequences showed that the Iranian haplotype of *L. donovani* was diverse from other *L. donovani* haplotypes. After translation of the DNA to amino acids, due to an insertion occurring in the middle of reading frame of the CPB gene, a stop codon occurred and translation halted resulting in a short peptide. In comparison with other CPB peptides, it is the shortest among all *Leishmania* species. It is shown that CPBs have key roles in infection, expression of disease and in effective autophagy (catabolic system, whereby eukaryotic cells can degrade and recycle proteins and organelles) (Hide and Bañuls, 2008). This short protein lacks the COOH terminal extension (CTE) domain. This domain is important as a target of immune response in canine leishmaniasis (Nakhaee et al., 2004), and there is some evidence to suggest that it has a role in immune evasion (Rafati et al., 2003). CTE appears highly immunogenic, as assessed by the presence of antibodies and the presence of CTE reactive peripheral blood mononuclear cells, and may play a role in diversion of the host immune response (Hide and Bañuls, 2008). Therefore the implication of this short protease in host–parasite interactions of the haplotype should be further investigated. If it causes milder disease, it is unlikely that AVL has been significantly underreported in the region.

To understand phylogenetic relationships of the Iranian haplotypes within the *L. donovani* complex, we have analyzed sequences of ITS2 and CPB loci. Results of both analyses showed that the Iranian *L. infantum* haplotypes were associated with the European *L. infantum* haplotypes or *L. chagasi*, the synonymous species of *L. infantum* from the New World. On the other hand, the Iranian *L. donovani* haplotype formed a phylogenetic group well separated from other *L. donovai* complex haplotypes but their relationships were quite different in ITS2 and CPB trees. In the ITS2 tree, they branched early from the European *L. infantum* haplotypes and were very distinct from *L. donovani* haplotypes whereas in the CPB tree they were closely related to the African *L. donovani* haplotypes. The function of the ITS2 and CPB gene is pretty diverse and the later has important implication in host–parasite interactions (Mottram et al., 1997; Hide and Bañuls, 2008). In contrast, ITS2 is a none coding region and has no functional role in the life cycle of the parasite. Therefore we should place the emphasis on the CPB tree for correct taxonomy and phylogenetic relationship within the *Leishmania* spp. Study of Kuhls et al. (2005) on the ITS sequences of the *L. donovani* complex showed that sequence variation in the region was very low within the *L. donovani* complex, 0–2.9% for ITS1, and 0–2.3% for ITS2 and in phylogenetic analysis some of the *L. infantum* haplotypes did not resolve from *L. donovani* haplotyps.

Acknowledgments

We are grateful to staff of Germi Center of Disease Control, Ardebil Province, Iran for their contributions and helps in field sand fly collection. We also thank Dr. Alimohammadian and Dr. Piazak from Iran Pasteur Institute for preparing different species of *Leishmania*. This work was financially supported partly by the Tehran University of Medical Sciences and partly by the EMRO WHO (TDR 6/36, T5/72/6, SGS06/77).

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