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Complete conservation of an immunogenic gene (*lcr1*) in *Leishmania infantum* and *Leishmania chagasi* isolated from Iran, Spain and Brazil

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Abstract

Background & objectives: Kala-azar is the visceral and most severe form of leishmaniasis that leads to death if untreated. The causative agents of visceral leishmaniasis (VL) are members of *Leishmania (L.) donovani* complex which includes *L. chagasi* and *L. infantum*. Genome sequences have raised the question whether *L. chagasi* and *L. infantum* are synonymous or different. This question has important implications for clinical and epidemiological studies, evaluation of vaccines and drugs, and disease control. LCR1 is an immunogenic molecule discovered from *L. chagasi* with potential as a component of a *Leishmania* subunit vaccine. If this protein has potentials for being used in a vaccine or diagnostic testing, there should be little variability in this molecule between *L. infantum* isolates from diverse geographic regions. The aim of this study was to determine whether *lcr1* of an Iranian strain of *L. infantum* was identical to *lcr1* of both *L. infantum* strain from a different geographic region (Spain) and that of an *L. chagasi* isolate from Brazil.

Methods: L. infantum isolated from an Iranian kala-azar patient was studied. *Lcr1* from this isolate was PCR amplified, cloned, and studied by restriction digest analysis and sequencing.

Results: The sequences of *lcr1* of the Iranian *L. infantum* were completely identical at nucleotide level to *lcr1* sequences of both the Spanish *L. infantum* and the Brazilian *L. chagasi* strains.

Conclusion: Complete conservation of the DNA sequence encoding for LCR1 molecule between geographically distinct *Leishmania* species adds credibility to the potential for LCR1 as a component of a subunit vaccine and diagnostic test for kala-azar.

Key words Internal transcribed spacer; kala-azar; *lcr1*; *Leishmania chagasi, Leishmania infantum*; vaccine

Introduction

The leishmaniasis is a spectrum of diseases of humans and other mammals. The disease is caused by kinetoplastid flagellates of genus *Leishmania (L.)*. Kala-azar is the visceral and most severe form of these diseases that leads to death if untreated. The yearly incidence of visceral leishmaniasis (VL) is 0.5 million cases[1,2]. VL is currently sporadic in all 30 provinces of Iran and endemic in at least threee provinces of the country[3,4]. In a year prospective survey in north-west Iran, the average incidence rate of infection was 2.8% per year with all ages equally at risk. One in 13 infections in children led to VL, and this ratio decreased significantly with age[5]. The causative agents of VL are members of *L. donovani* complex, classified into four species: *L. archibaldi*, *L. chagasi*, *L. donovani* and *L. infantum*, distinguished by the vectors, reservoir host and in pathology[6]. *Leishmania* strains isolated from kala-azar patients in Iran have been identified as *L. infantum*[3,7] which is the principal agent of the disease in animal reservoir hosts

in different parts of Iran[8]. *L. chagasi* is the usual causative agent of kala-azar in the Americas[9,10]. The New World species *Leishmania chagasi* is now widely accepted to be a synonym of *L. infantum*; however, in recent work Latin American authors still consider these species to be distinct[11]. Definitive proof that *L. chagasi* and *L. infantum* are synonymous is not yet established[12,13].

There is need for development of effective tools for detection, prevention and treatment of kala-azar[14]. An important feature of a vaccine candidate, or component of a subunit vaccine against leishmaniasis is conservation of the molecule throughout different species and strains of the parasites. A molecule used as a diagnostic criterion either by serology or PCR, would have the same requirement. Thus, the question of whether *L. infantum* and *L. chagasi* are identical or distinct, and the degree of conservation of antigenic proteins across species and strains, has important implications for development of a new diagnostic test or vaccine. An important implication would be use of the results obtained from studies on one species to the other, if these two *Leishmania* species are identical. An example would be the possible use of LCR1 of *L. chagasi* as part of a protective vaccine against both *L. chagasi* and *L. infantum*. LCR1 was discovered from *L. chagasi* and has been shown to confer partial protection against *L. chagasi* in a mouse model[15]. An *lcr1* homologue sequence has been reported from one strain of *L. infantum* (MCAN/ES/98/LLM-877), (GenBank Accession number: AM502245.1) from Spain. Whether LCR1 molecule is conserved in other strains of *L. infantum* remains to be studied. The aim of our study was to compare the sequence of *lcr1* between an Iranian isolate of *L. infantum* and *L. chagasi*. For this purpose we determined *lcr1* sequence in an Iranian isolate of *L. infantum* and compared it with homologous sequences from *L. chagasi* and *L. infantum* reported in GenBank.

Material & Methods

Parasite: Leishmania infantum MHOM/04/IR/IPI-UN10 was isolated from a 1.5 yr old boy from Imam Khomeini Hospital in Tehran, Iran in 2004. Diagnosis of kala-azar was confirmed in this child by isolation of the parasite from bone marrow culture in NNN media. The isolate was stored in liquid nitrogen soon after isolation. The parasite was recovered from liquid nitrogen by thawing and culturing it in NNN media[16]. Logarithmically growing parasites were harvested and washed by centrifugation in phosphate buffer saline (PBS) twice and were stored in aliquots of 100×10^8 parasites (for DNA extraction) and 1×10^9parasites (for isoenzyme electrophoresis) in –70°C. The following reference strains were used in isoenzyme electrophoresis studies: *L. major* strain MRHO/IR/75/ER, *L. tropica* strain MHOM/SU/74/K27, and *L. infantum* strain MHOM/TN/80/IPT1.

Isoenzyme electrophoresis: Discontinuous vertical polyacrylamide gel electrophoresis (PAGE) and cellulose acetate were used for isoenzyme analysis of the isolate. About 1×10^9 parasites were mixed with equal volumes of a hypotonic aqueous solution of enzyme stabilizer (1 mM EDTA, 1 mM ε-aminoncaproic acid, 1 mM dithiothreitol), frozen and thawed thrice. Soluble extract of lysed promastigotes was prepared by centrifugation at 30,000× g at 4°C for 30 min, and stored at –70°C until use. Four enzymes were used for analysis of isolates: malate dehydrogenase (MDH), malic enzyme (ME), glucose phosphate isomerase (GPI), superoxide dismutase (SOD)[17,18].

ITS1 sequencing: Genomic DNA was extracted by LiCl extraction[19]. The internal transcribed spacer 1 (ITS1) region was amplified using DNA extracted from parasite and ITS1 specific primers LITSR and L5.8S and analyzed by restriction fragment length polymorphism (RFLP) analysis using the restriction endonuclease *Hae*III[20]. PCR amplification of genomic DNA of *L. infantum* by ITS1 specific primers resulted in a sharp single band on agarose gel electrophoresis. The PCR product was directly sent for sequencing (Macrogene Company, Korea). Each PCR product was sequenced at least twice by ITS1 specific primers: once by forward primer (LITSR)

and once by reverse primer (L5.8S). Nucleotide in each position was considered correct if two sequencing results (which were sequenced in opposite directions) confirmed each other.

Amplification of lcr1: Polymerase chain reaction (PCR) was performed in 200 µl thin wall tubes. Each reaction consists of 3 µl genomic DNA (containing 6–8 ng DNA), 0.4 µl Taq DNA polymerase (5 units) (GenetBio Co., Korea), 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM) and 2.5 µl 10 × PCR buffer (GenetBio Co., Korea), 0.125 µl of each forward and reverse primers (100 pmol/µl). The primers flanked the reported antigenic fragment of *lcr1*[21].

The sequences and names of forward and reverse primers were: 5'-TAGGGATCCCATGAGCTG GCCAAA-3' (named LCR1-F1) and 5'-GTCGACAAGCTTACTCGAGGTCCT CGATGG-3' (named LCR1-R1), respectively. The primers included restriction sites (underlined) for *Bam*HI (in *lcr1*-F1) and *Hind*III (in *lcr1*-R1). PCR was performed in thermocycler (Mastercycler gradient, Eppendorf Co., Hamburg, Germany) under the following program: 95ºC for 2 min, 35 cycles of 95ºC for 20 sec, 65.1ºC for 30 sec, and 72ºC for 1 min, and 72ºC for 25 min. Great cares were taken to prevent contamination including using disposable tips and tubes and performance of the procedure under laminar air flow. Template negative tube was included in all runs and results were disregarded if template negative control showed any band (even faint) in agarose gel electrophoresis. All the PCR products were electrophoresed in 1% agarose gel.

Restriction fragment length polymorphism (RFLP): PCR product obtained by using primers specific for *lcr1* was digested by restriction enzyme *Hae*III (*Bsu*RI) (Fermentas Co., Ontario, Canada) at concentration of 10 unit/µl for 4 h at 37ºC. The reaction was terminated on dry plate at 80ºC for 20 min. The digested and undigested PCR products were electrophoresed in 1% agarose gel and their molecular weight were approximately determined using 1 Kb molecular weight marker (Fermentas Co., Ontario, Canada).

Sequencing of lcr1 through T/A cloning: Amplification of the *lcr1* gene from *L. infantum* genomic DNA by *lcr1* specific primers resulted in a major band of the expected molecular size (785 bp). The band was extracted from agarose gel using Silica Bead DNA Gel Extraction kit (Fermentas Co., Ontario, Canada). The gel extracted DNA fragments were cloned using the InsTA cloning kit (Fermentas Co., Ontario, Canada) according to the manufacturer's instructions. Briefly, gel extracted DNA fragment was ligated into pTZ57R plasmid and transformed into DH5α strain of *Escherichia coli*. Plasmids were prepared using the Accu Prep Plasmid Mini Extraction plasmid purification kit (Bioneer Co., Korea) according to the manufacturer's instructions. The DNA inserts were sequenced (Macrogene Company, Korea) at least twice using the forward and reverse plasmid specific primers. Nucleotide in each position was considered correct if the two sequencing results confirmed each other.

Cloning and sequencing of lcr1 by Pfu DNA polymerase: To determine whether the sequence variants were created during PCR due to Taq polymerase error, *lcr1* was amplified from the parasite genomic DNA by *Pfu* DNA polymerase (Fermentas Co., Ontario, Canada) which exhibits 3'→5' exonuclease (proof reading) activity according to manufacturer's instructions. PCR product of *lcr1* was extracted from gel as mentioned above for T/A cloning. The PCR product was inserted into pRSET A plasmid (Invitrogen Co., Carlsbad, USA) by double digestion of both *lcr1* PCR product and the plasmid, extraction of digested products from gel, and ligation of *lcr1* into the plasmid by ligase (Fermentas Co., Ontario, Canada) according to manufacturer's instructions. The insert containing plasmid was transformed into TOP10F' *E. coli* (Invitrogen Co. Carlsbad, USA). The plasmid was purified from transformed bacteria, screened by colony PCR method[22] followed by digestion by *Hae*III (Fermentas Co., Ontario, Canada) for finding plasmids with correct insert. Two plasmids containing *lcr1* were sequenced by plasmid specific primers (Macrogen Company, Korea).

Results

Species identification by isoenzyme electrophoresis: The *Leishmania* isolate studied in the present report (MHOM/04/IR/IPI-UN10) was identified as *L. infantum* by isoenzyme electrophoresis. The isoenzyme profiles of this isolate were consistent with the profile of *L. infantum* strain MHOM/TN/80/IPT1. This conclusion was based on the isoenzyme profiles of four enzymes: malate dehydrogenase (MDH) (Fig. 1), malic enzyme (ME), glucose phosphate isomerase (GPI), and superoxide dismutase (SOD).

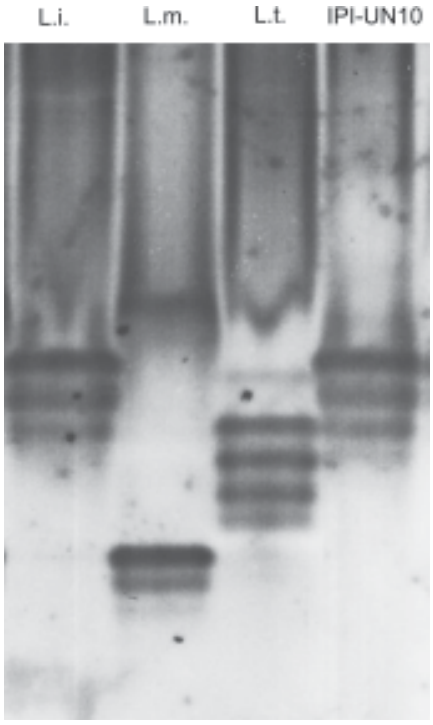

Fig. 1: The isoenzyme results show that the Iranian isolate of the present study is *L. infantum*. The malate dehydrogenase (MDH) isoenzyme profile of the isolate is shown and compared with those of reference strains. L.i.—*Leishmania infantum* reference strain MHOM/TN/80/IPT1; L.m.—*Leishmania major* reference strain MRHO/IR/75/ER; L.t.—*L. tropica* reference strain MHOM/SU/74/K2; and IPI-UN10—*L. infantum* strain MHOM/IR/04/IPI-UN10.

Species identification by ITS1 sequencing: The ITS1 of the *Leishmania* isolate studied in the present report was sequenced and submitted to GenBank (accession number GQ444144) (Fig. 2). The species of this isolate was confirmed to be *L. infantum* as its ITS1 is completely identical to ITS1 sequence located in chromosome 27 of *L. infantum* strain MCAN/ES/98/LLM-877 reported in GenBank (Accession No. AM502245.1). The ITS1 sequence of *L. infantum* MHOM/04/IR/IPI-UN10 (studied in the present study) was completely identical to ITS1 sequence of many *L. chagasi* strains, e.g. strain MHOM/BR/85/M9702 (Accession No. AJ000306).

RFLP of lcr1 sequence: PCR amplification of *lcr1* fragment from *L. infantum* MHOM/04/IR/IPI-UN10 resulted in an amplicon with size of the expected 785 bp (Fig. 3). Digestion of *lcr1* with *Hae*III resulted in two bands of 631 and 142 bp long (and a 12 bp band that cannot be seen, due to its too short length in agarose gel) (Fig. 3). This restriction digestion pattern is consistent with the published *lcr1* sequence of *L. chagasi*[23].

Lcr1 sequence: Plasmids containing *lcr1* sequence obtained by T/A cloning method (PCR amplification was performed by *Taq* DNA polymerase) were extracted from three different clones of the recombi-

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   1  ctggatcatt  ttccgatgat  tacacccaaa  aaacatatac  aactcgggga  gacctatgta
  61  tatatatgta  ggcctttccc  acatacacag  caaagttttg  tactcaaaat  ttgcagtaaa
 121  aaaaaggccg  atcgacgtta  taacgcaccg  cctatacaaa  agcaaaaatg  tccgtttata
 181  caaaaaatat  acggcgtttc  ggtttttggc  ggggtgggtg  cgtgtgtgga  taacggctca
 241  cataacgtgt  cgcgatggat  gacttggctt  cctatttcgt  tgaagaacgc  agtaaagtgc
 301  gataagtggt  atca
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Fig. 2: ITS1 containing sequences in *L. infantum* isolate MHOM/IR/04/IPI-UN10 (GenBank Accession: GQ444144.1). Genomic DNA of *L. infantum* was amplified by ITS1 specific primers and the PCR product was directly sequenced. Each PCR product was sequenced at least twice (from opposite directions).

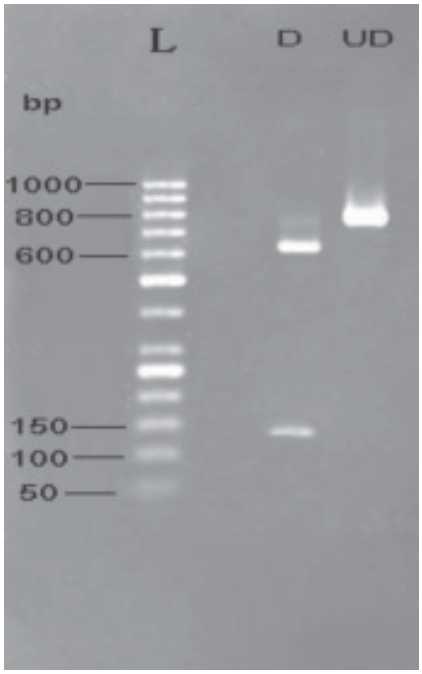

*Fig. 3: Hae*III restriction enzyme digestion pattern of *lcr1* fragment amplified from genomic DNA of *L. infantum* isolate MHOM/IR/04/IPI-UN10. The full amplified sequence is 785 bp, and restriction digestion resulted in two fragments of 631 and 142 bp. Lanes contain the molecular weight ladder (L), and PCR amplified *lcr1* products from the Iranian *L. infantum* isolate. Products were either digested using *Hae*III (D) or undigested (UD).

nant bacteria and sequenced using plasmid specific primers. These three *lcr1* sequences were compared with the published sequence from *lcr1* of *L. chagasi* (Fig. 4). Each of these three *lcr1* sequences had 1–2 different nucleotides in comparison to *lcr1* of *L. chagasi*. The difference in the nucleotides present in these clones was attributed to lack of proof reading of *Taq* DNA polymerase used for amplification. This conclusion is valid because each discordant nucleotide is only present in one clone and is absent in the other two clones. This is true for all nucleotide discrepancies observed between the three clones. In addition, sequences of two clones obtained by *Pfu* DNA polymerase confirmed the sequence concluded from the *Taq*-amplified sequences (Fig. 4). These data show that the sequence of *lcr1* of the Iranian *L. infantum* is completely identical to *lcr1* sequences of *L. chagasi.* The concluded sequence of *lcr1* from *L. infantum* MHOM/04/IR/UN-10 was submitted to GenBank (Accession No. GQ850521.1) (Fig. 5), and was compared with *lcr1* reported for *L. chagasi* and *L. infantum* in GenBank. *Lcr1* sequence of our Iranian isolate is identical to *lcr1* sequence of *L. chagasi* and *lcr1* sequence of *L. infantum* (Accession Nos. U23437.1 and AM502245.1 respectively).

Discussion

Sequence analysis of the *Leishmania* spp. genomes is leading to the conclusion that two *Leishmania* species causing visceral leishmaniasis, *L. chagasi* isolated from subjects in the New World and *L. infantum* derived from patient in the Old World, may be one and the same[13]. This implies that new diagnostic assays or vaccine candidates may exhibit identical effi-

Sequence origin	Enzyme used for amplification	Position 254	Position 312	Position 396	Position 691
Clone No. 4	*Taq* DNA polymerase	A	G	A	C
Clone No. 6	*Taq* DNA polymerase	G	A	A	T
Clone No. 3	*Taq* DNA polymerase	G	G	G	T
Clone No. 2	*Pfu* DNA polymerase	G	G	A	T
Clone No. 16	*Pfu* DNA polymerase	G	G	A	T
L. chagasi (GenBank Accession No. U23437.1)	–	G	G	A	T

Fig. 4: Lcr1 nucleotide differences between 5 clones obtained from the Iranian *L. infantum* and the reported *L. chagasi* in GenBank. PCR amplification was performed by *Taq* or *Pfu* DNA polymerases for the indicated clones. The rest of nucleotides in the 785 nucleotide span of *lcr1* were identical in all five clones of the Iranian *L. infantum* and *L. chagasi*.

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  1   cccatgagct ggccaaagtc gaactggcga aggaccgtgc cttcctcgac cctgagccgg
 61   agggcgtgcc actggcggac ctcccgctca gcgacgaccc ggagttcaac gtactggcga
121   agcagcgtca ggcgctgaag aacaccagga ggggccgcga ccccgaaatg aaggacctgg
181   aggagaggat gaacgaccgt gtccacgaca tcgcaaggga gttcctcagc aagcaccgcg
241   gctacctgaa cccggagccg cagaatgtac ccattgccga catccccctc aaccgcgacc
301   cgatcttccg cgaaatggag aacgagctgt tgaaggctat gaaggacccc cgcagcaatg
361   cgggcaagat tgcagagctg caggacgacc tcaacaaccg cgcagacgac ctcgcgaagg
421   acctacggcg caaggagctt gctaatcagg agcaggagcc tctcggcgtg ccgctggaag
481   agctgccact caactacgac ccgatcctca atccactgga acgcaagcgc cgcgacatca
541   agaaaaaccc gaagcggaat gccgatgtgc tgcgcaacct cgagcgggag atcgccgcgc
601   gcatcgatga catcgcgcgc gactttctgg cgaaggagcg tgctttcctg gaccaggaac
661   cggaggcggt gcaattggag cgcctgccgc tgtcagatga cagggagttt cacgaaatgg
721   agagggacct gcgcgcgctg aagaagcaac cagcaaagaa cagggacgcc atcgaggacc
781   tcgag
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Fig. 5: Lcr1 sequence of *L. infantum* isolate MHOM/IR/04/IPI-UN10 (GenBank Accession: GQ850521.1).

cacy for each of these species. Nonetheless, in advance of full agreement that these are indeed identical species, it is important to compare the nucleotide and protein sequences of genes and gene products chosen for clinical assay development. The purpose of this study was to assess the potential utility of the LCR1 protein for diagnosis of or immunization against visceral leishmaniasis caused by *L. infantum* in Iran. We, therefore, compared the sequence of *lcr1* fragment in an Iranian isolate of *L. infantum* with the homologous regions from a Brazilian *L. chagasi* isolate (strain MHOM/BR/00/1669) and a Spanish *L. infantum* (MCAN/ES/98/LLM-877).

The data presented in this paper are novel; the first report about *lcr1* from an Iranian *L. infantum* isolate. Our results showed that the ITS1 and the *lcr1* sequences were identical among all three genomes (Iranian *L. infantum*, Spanish *L. infantum*, and Brazilian *L. chagasi*). This finding is in agreement with reports that *L. infantum* and *L. chagasi* are synonymous[12, 24–27].

Complete identity of the antigenic region of the LCR1 protein in isolates of *L. infantum* from different regions of the Old World (Iran and Spain) and with that of *L. chagasi* from Brazil (New World) is an important step in evaluation of this molecule for potential clinical use. The complete conservation of LCR1 indicates that LCR1 could be evaluated as a potential component of a subunit vaccine against VL. The next step will be to study immune response to LCR1 protein in human individuals exposed to *L. infantum* or *L. chagasi* in different countries. We propose that similar sequence evaluation in strains from diverse geographic regions be performed prior to testing antigenic peptides for their potential as protective vaccines.

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