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\textsuperscript{1,2}. VL is currently sporadic in all 30 provinces of Iran and endemic in at least three provinces of the country\textsuperscript{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\textsuperscript{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\textsuperscript{6}. *Leishmania* strains isolated from kala-azar patients in Iran have been identified as *L. infantum*\textsuperscript{3,7} which is the principal agent of the disease in animal reservoir hosts.
in different parts of Iran. L. chagasi is the usual causative agent of kala-azar in the Americas. 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. Definitive proof that L. chagasi and L. infantum are synonymous is not yet established.

There is need for development of effective tools for detection, prevention and treatment of kala-azar. 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. 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. 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 1×10^9 parasites (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-aminocaproic 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).

ITS1 sequencing: Genomic DNA was extracted by LiCl extraction. 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 HaeIII. 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 MgCl₂ (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 lcr1²¹.

The sequences and names of forward and reverse primers were: 5’-TAGGGATCCCATGAGCTGCCAAA-3’ (named LCR1-F1) and 5’-GTCGACGTCCTCTGGAAGTGCATGGGC-3’ (named LCR1-R1), respectively. The primers included restriction sites (underlined) for BamHI (in lcr1-F1) and HindIII (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 for BamHI and HindIII. PCR was performed in 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 MgCl₂ (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 lcr1²¹.

Restriction fragment length polymorphism (RFLP): PCR product obtained by using primers specific for lcr1 was digested by restriction enzyme HaeIII (BsuRI) (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).

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 followed by digestion by HaeIII (Fermentas Co., Ontario, Canada) for finding plasmids with correct insert. Two plasmids containing lcr1 were sequenced by plasmid specific primers (Macrogen Company, Korea).
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).

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 recomb-
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. This implies that new diagnostic assays or vaccine candidates may exhibit identical effi-

<table>
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Fig. 3: HaeIII 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 HaeIII (D) or undigested (UD).

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.
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.\(^\text{12, 24–27}\)

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.

**References**

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