Cloning and production of an LPS-free immunogenic protein (LCR1) from an Iranian clinical isolate of *Leishmania infantum* and its recognition by sera from Iranian patients with visceral leishmaniasis

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Abstract. *Leishmania (L.) infantum* and *L. chagasi* are the causative agents of visceral leishmaniasis (VL). LCR1 is an immunogenic protein in a mouse model, discovered in *L. chagasi*. There is little data available concerning the human immune response to LCR1. The aim of our study was to clone lcr1 from an Iranian clinical isolate of *L. infantum*, produce its recombinant protein and study the human immune response to this protein. PCR using lcr1 specific primers was performed for amplification of the lcr1 sequence which was then cloned into a pRSETA plasmid. *E. coli* BL21(DE3)plysS was then transformed by the lcr1-containing plasmid for expression of the recombinant LCR1 protein. Expression of the recombinant LCR1 was confirmed by SDS-PAGE and immunoblotting using an anti His-tag antibody. Conditions for optimized expression of the recombinant protein were determined. The recombinant LCR1 protein was purified using an Ni²⁺ precharged resin. Lipopolysaccharide (LPS) was depleted from LCR1 recombinant protein by the technique known as “Triton X-114 phase separation”. Sera from Iranian VL patients recognized the recombinant LCR1 protein and also that there is an antibody response against this protein in Iranian VL patients.

Keywords: *Leishmania infantum*, *Leishmania chagasi*, lipopolysaccharide, recombinant protein

INTRODUCTION

Leishmaniasis is endemic in 88 countries with an estimated 12–15 million individuals infected and an annual incidence of around 2 million (WHO, 2010). Three major clinico-pathological categories are recognized: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis and visceral leishmaniasis (VL). Kala-azar is the visceral and most severe form of these diseases that leads to death if untreated. The incidence of fatal VL is rising, largely due to urbanization and the human immunodeficiency virus (HIV) pandemic (Roberts, 2006). The causative agents of VL are members of *Leishmania* (*L.) *donovani* complex which includes *L. chagasi* and *L. infantum* (Lainson and Shaw, 1987). Leishmania strains isolated from kala-azar patients in Iran have been identified as *L. infantum* (Edrissian et al., 1998; Kazemi-Rad et al., 2008; Mahmoudzadeh-Niknam et al., 2010). There is need for development of effective tools for detection, prevention and treatment of Kala-azar (Roberts, 2006). LCR1 is an immunogenic molecule which was discovered in *L. chagasi* and has been shown to confer partial protection against *L. chagasi* in a mouse model (Streit et al., 2000). The LCR1 epitope is part of a protein with molecular weight of more than 200 kD in *L. chagasi* (Wilson et al., 1995). The lcr1 is 100% identical, at the DNA level, with part of a gene in chromosome 27 of *L. infantum* which codes for a hypothetical "calpain-like cysteine peptidase" (GenBank Accession Number: AM502245.1). Calpains participate in a variety of cellular processes including remodeling of cytoskeletal/membrane attachments, different signal transduction pathways, and apoptosis (Goll et al., 2003). Calpain inhibitors have been shown to have anti-Leishmanial activity (d’Avila-Levy et al., 2006). An LCR1 recombinant protein has been produced (Wilson et al., 1995). However, this reported production procedure for LCR1 does not include all details and also does not address the issue of LPS contamination of the recombinant protein. In addition the immune response to LCR1 has mainly been studied in experimental animal models (Streit et al., 2000) and little data is available about the human immune response to LCR1. Therefore production of the LCR1 recombinant protein has justification from a technological point of view as well as potential for practical application. The aims of our study were to produce LPS-free LCR1 recombinant protein and to study the reactivity of sera from VL patients against it.

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**MATERIALS AND METHODS**

**Parasite**  *L. infantum* MHOM/04/IR/IPI-UN10 was isolated from a 1.5 year old boy from Iran in 2004. The details of parasite preparation have been reported (Mahmoudzadeh-Niknam et al., 2010).

**DNA extraction** Genomic DNA was extracted by an LiCl extraction method (Medina-Acosta and Cross 1993) with some modifications. Parasites were lysed by addition of 150 μl TELT lysis buffer (50mM TrisCl (pH 8.0), 62.5mM EDTA, 2.5M LiCl, 4% (v/v) TritonX100) to a tube containing 10×10^7 parasites. The tube was vigorously shaken and incubated for 5 minutes at room temperature. Equal volume of Phenol: Chloroform: Isoamyl Alcohol (ratio 25:24:1, respectively) (phenol from Cinnagen, Iran; Chloroform and Isoamyl Alcohol from Sigma, USA) was then added to the tube which was mixed vigorously. The tube was centrifuged at 12,000 rpm (17000×g) at room temperature for 3 minutes. The aqueous phase (top layer) was carefully transferred to a new tube. The volume was increased to 200μl by addition of ddH₂O. An equal volume of chloroform was added to the tube and centrifuged at 12,000 rpm (17000×g) at room temperature for 3 minutes. The supernatant was transferred to a new tube and 5M NaCl (1/10 of supernatant volume) and Isopropyl alcohol (Merck, Germany) (equal to supernatant volume) were then added to the tube, which was mixed well by repeated inversion. DNA was precipitated by centrifugation at 12,000 rpm (17000×g) for 15 minutes at room temperature. The precipitate was washed two times in 400μl of 70% ethanol at room temperature. The supernatant was discarded and the residual ethanol was dried out by incubation of the tube for 10-15 minutes at room temperature. DNA was resuspended in 100 μl of TE buffer (10 mM TrisCl, pH 8.0, 1 mM EDTA, pH 8.0). DNA quantity was assessed through determination of absorbance at 260 and 280 nm using a spectrophotometer (Biophotometer, Eppendorf, Germany). DNA quality was assessed through electrophoresis in 1% agarose gel.

**Bacterial strains and plasmid** *Escherichia coli* Top10F’ and *E. coli* BL21(DE3)plysS (Invitrogen, USA) were used for cloning and expression, respectively. Plasmid vector, pRSETA (Invitrogen, USA) was used for cloning and expression.

**Cloning and sequencing of lcr1** The lcr1 gene from *L. infantum* genomic DNA was amplified by polymerase chain reaction (PCR), before being cloned and sequenced. The details have been reported elsewhere (Mahmoudzadeh-Niknam et al., 2010).

**Expression and identification of the recombinant protein** *Escherichia coli* strain BL21(DE3)plysS (Invitrogen, USA) was transformed using the recombinant pRSETA-lcr1 plasmids, as mentioned previously (Mahmoudzadeh-Niknam et al., 2010). The transformed bacteria were grown in Luria Bertani (LB) medium supplemented with 100 μg/ml ampicillin and 170 μg/ml chloramphenicol at 37°C overnight. LB medium containing antibiotics was inoculated by overnight culture to obtain OD₆₀₀ = 0.1 units. The cultures were grown at 37°C with vigorous shaking until the OD₆₀₀ reached 0.4 – 0.6 and induced by isopropylthio- β-D-galactoside (IPTG) (Roche, Germany) at concentrations of 1, 5, and 10 mM for 4 hours. The bacteria from 50 ml culture media were precipitated by centrifugation (17000×g, 10 minutes, 4°C) and the supernatant was discarded. The pellet was stored frozen at -20°C prior to use. The cell pellet was re-suspended in PBS and passed through five cycles of freeze and thaw (liquid nitrogen and 37°C respectively), then centrifuged at 16000×g for 10 minutes at 4°C. The supernatant and the pellet were kept separately for further study.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)** The molecular weight of the recombinant LCR1 was determined by separation of whole cell lysates by SDS-PAGE (Gallagher SR. 1999). The discontinuous SDS system was used with using a 10% concentration of acrylamide (Cinnagen, Tehran, Iran) in the resolving gel. Samples were prepared by adding the sample buffer and boiling for 5 minutes before loading onto an SDS-PAGE gel.

The running buffer consisted of 1g SDS (Cinnagen, Tehran, Iran), 3.03g Tris base (Sigma, Saint Louis, USA, T1503) and 14.04g Glycin (Applichem, Darmstadt, Germany) in 1000 ml ddH₂O. The samples were subjected to electrophoresis at 10 mA and 15 mA in stacking and resolving gel, respectively. Finally, the gel was stained with coomassie brilliant blue R 250 (Acros Organics, New Jersey, US).

**Immunoblot analysis** The reactivity of the histidine tag of the LCR1 recombinant protein with anti HisG-HRP antibody (Invitrogen) was studied through immunoblotting according to the established methods (Gallagher et al., 2004). The Anti-HisG antibody recognizes the sequence -His-His-His-His-His-Gly (6xHis-Gly epitope). Briefly, proteins were transferred from unstained SDS-PAGE gel to a nitrocellulose membrane (Sigma, Saint Louis, USA) by Semi-dry apparatus (Pharmacia, Sweden) at 13.1 volts for 60 minutes. The transfer was confirmed by Ponceau S (Sigma, Saint Louis, USA) staining. The membrane was incubated in 50 ml of TTBS blocking buffer (Tris HCl 100 mM pH 7.5, NaCl 150 mM, and 1%Tween 20) at 4°C overnight. Ten milliliters of anti-HisG-HRP antibody (diluted at 1/5000 in TTBS blocking buffer) were then added and incubated with continuous shaking at 4°C overnight. The membrane was then washed three times in 200 ml of washing buffer (Tris HCl 100 mM pH=7.5, NaCl 150 mM, 1%Tween 20) for 5 minutes in a shaker. Finally, the DAB (3,3‘-Diaminobenzidine) (Sigma, Saint Louis, USA) solution (30 ml of TTBS, 20 mg of DAB powder, 30 μl of H₂O₂) was added to the membrane, and after signal development the membrane was washed with ddH₂O and results were recorded.

Reactivity of sera from VL patient with recombinant LCR1 was examined as above except for the following differ-
ences in reagents: the blocking buffer was 3% BSA (Merck, Darmstadt, Germany) in TBS; the patient sera was used for primary antibody; anti human IgG HRP-conjugated (Bethyl, Montgomery, USA) was used as secondary antibody, and soluble leishmania antigen (SLA) was used as a positive control. The optimum dilutions of primary and secondary antibodies were determined empirically.

**LCR1 protein purification and concentration** The lcr1-containing transformed bacteria were cultured in conditions as mentioned above in a volume of one liter (in aliquots of 50 to 100 ml in Erlenmeyers, whose volume were at least 5 times the volume of the culture). The culture was then harvested and centrifuged (1700 x g, 10 minutes, 4°C), and LCR1 recombinant protein was purified from the bacterial pellet using ProBond (Invitrogen, USA) according to the manufacturer's instructions. Briefly, a bacterial pellet from 50 ml bacterial culture was re-suspended in 8 ml native binding buffer (50 mM NaH2PO4, 0.5 M NaCl, pH=8.0) and subjected to 5 cycles of freeze and thaw (as mentioned above), centrifuged (1700g, 10 minutes, 4°C), and the supernatant was passed over ProBond resin (precharged with Ni2+, average bead size: 45-165 microns). The resin was washed 3 times using native washing buffer (native binding buffer with 20 mM Imidazole, pH=8.0) and eluted using 8 ml of native elution buffer (native binding buffer with 250 mM Imidazole, pH=8.0). The extracted recombinant protein was concentrated through ultrafiltration using Amicon Ultra-15 (Millipore Ireland, County Cork, Ireland). The elution buffer present in the protein solution was washed away through addition of 15 ml PBS to the protein solution before concentrating it to a volume of 1.5 ml. The wash process was repeated 5 times. The recombinant protein was stored at -80°C prior to use.

**LPS depletion** Lipopolysaccharide (LPS) was depleted from LCR1 recombinant protein by the technique known as “Triton X-114 phase separation” (Liu et al., 1997). Briefly, Triton X-114 was added to the protein preparation to a final concentration of 1%. The mixture was incubated at 4°C for 30 minutes with constant stirring to ensure a homogenous solution. The sample was then transferred to a 37°C water bath, incubated for 10 minutes, and centrifuged (16,000g, 10 minutes) at 25°C. The upper aqueous phase containing the protein was carefully removed and subjected to Triton X-114 phase separation for five more cycles. Quantitation of endotoxin was carried out by the Quality Control Department of Pasteur Institute of Iran using Limulus Amebocyte Lysate QCL-1000 (Lonza, MD, USA) according to the manufacturer's instructions.

**Patients** Patients were selected from the North Khorasan province in the North East of Iran. Seven patients, 6 female and 1 male, with an age of 3.8 ± 1.9 years were studied. The VL was confirmed in all patients by isolation of parasite from bone marrow or a positive DAT (Direct Agglutination Test) of ≥ 1/102400. They were recovered from the disease 2-10 months before sampling for the present study. Parents of all patients gave written informed consent, and the study protocol was approved by the ethical committee of the Pasteur Institute of Iran. Peripheral blood was withdrawn from patients, sera was separated after clotting, and stored at -70°C. Sera were pooled and used in immunoblot analysis.

**RESULTS**

**Quality of extracted DNA** The ratios of optical density at 260 to 280 nm for the extracted DNA were 1.80-2 as measured by a spectrophotometer. This ratio showed sufficient purity of the DNA to be used as a template for PCR. The parasite-extracted DNA samples showed a compact band without any smear on an agarose gel which indicated no major DNA degradation has taken place (Data not shown).

**lcr1 sequence** Restriction mapping as well as sequencing confirmed that lcr1 from L. infantum MHOM/04/IR/UN-10 (GenBank Accession Number GQ850521.1) was identical to the reported lcr1 sequence of L. chagasi (Mahmoudzadeh-Niknam et al., 2010).

**Expression of LCR1 protein** The expression of recombinant LCR1 protein was studied by induction of the *Escherichia coli* strain BL21(DE3)pLyS containing the lcr1 recombinant pRSETA plasmid under the following conditions: 1 mM IPTG, 37°C, vigorous shaking (more than 150 rpm) overnight. The same bacteria transformed with a pRSETA plasmid without the insert were used as negative control. The results showed that the LCR1 protein is expressed in bacterial culture for 5 hours after IPTG induction but is not stable and cannot be detected after overnight culture (Figure 1). No recombinant protein was detected in the bacteria transformed by pRSETA plasmid lacking lcr1 insert (Figure 1).

**Verification of LCR1 protein** The expression of recombinant LCR1 protein was confirmed by immunoblot analysis using anti-HisG-HRP antibody (Figure 2). The molecular weight of LCR1 was about 40 kD, as expected.

**Optimization of LCR1 expression** The optimized conditions for LCR1 expression in *E. coli* BL21(DE3)pLyS were determined as follows: culture at 37°C with shaking at 250 rpm, 1 mM IPTG final concentration, start of induction at OD600 = 0.4-0.6, and an induction period of 2 hours (Figure 3).

**Recombinant LCR1 is soluble** To study the solubility of the LCR1 recombinant protein, the bacterial pellet was subjected to 5 cycles of freeze-thaw, centrifuged, and the supernatant and pellet were studied separately by SDS-PAGE for the presence of the recombinant protein. The LCR1 recombinant protein was present only in the supernatant.
Production of recombinant Leishmania protein

Figure 1. LCR1 recombinant protein is expressed by the recombinant E. coli. This figure shows SDS-PAGE of E. coli strain BL21(DE3)pLysS containing lcr1 recombinant pRSETA plasmid cultured in the presence of 1 mM IPTG, with vigorous shaking, at 37°C, for 5 hours (5h) or overnight (24h). The same E. coli strain containing pRSETA plasmid lacking lcr1 insert was used as negative control (Neg.). The apparent molecular weight of LCR1 (shown by arrow) is about 40 kD according to the molecular weight markers (MW) shown. The recombinant protein was not detected in the overnight culture.

No recombinant protein was detected in the pellet of the recombinant bacteria or in bacteria transformed by pRSETA plasmid lacking lcr1 insert (Figure 3).

Purification of LCR1 protein The bacteria containing the recombinant plasmid were harvested, lysed and the LCR1 recombinant protein was purified by ProBond. The purified LCR1 recombinant protein was shown to have no extra protein presence, as judged by the SDS-PAGE bands (Figure 4).

LPS depletion from recombinant LCR1 protein Recombinant LCR1 protein eluted from the Ni⁺ precharged resin had an LPS contamination of >200 IU/ml. LPS was depleted from the purified LCR1 protein through the “Triton X-114 phase separation” method. This method resulted in complete removal of LPS from the protein preparation (<0.1 Endotoxin Units/ml). The efficiency of protein recovery after LPS the depletion process was about 50%.

Sera from VL patients recognize the recombinant LCR1 protein A series of experiments were carried out with different dilutions of patient sera and anti human IgG HRP-conjugated, and the optimal dilution for primary and secondary antibody were determined to be 1:500 and 1:8,000, respectively. The absence of primary antibody resulted in no visible band being present on the immunoblot, confirming the specificity of the secondary antibody. As is shown in Figure 5, a 40 kD band corresponding to LCR1 is present on the immunoblot, verifying the recognition of LCR1 by VL patient sera. SLA, used as a positive control, resulted in multiple bands being present.

Figure 2. The presence of LCR1 was verified by immunoblot. The expression of recombinant LCR1 protein was confirmed by immunoblot analysis using anti HisG-HRP antibody recognizing the histidine tag of the recombinant protein. A positive band is seen for lcr1-containing bacteria cultured for 5 hours (5h), while no band is detected for the same bacteria cultured for 24 hours (24h) or lcr1-lacking bacteria cultured for 5 hours (Neg.). The molecular weight of LCR1 (shown by arrow) was about 40 kD as expected according to the molecular weight markers (MW) shown.

DISCUSSION

The lcr1 sequence was successfully cloned and sequenced in this study, using E. coli bacteria which are often used as the expression organism in the production of recombinant proteins. Lipopolysaccharide (LPS) (also known as endotoxin), an integral component of Gram negative bacteria, is a potent stimulator of the immune system (Erridge et al., 2002), so one inherent problem with using bacterial expression systems is the inevitable contamination of protein samples with LPS. LPS from the bacterial cell wall is present in protein preparations purified from the bacteria and may influence results in cell culture assays where the re-
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Figure 3. LCR1 recombinant protein is soluble. E. coli strain BL21(DE3)PlysS containing lcr1-recombinant pRSETA plasmid were induced for LCR1 expression and harvested after 2, 4, and 6 hours of culture. The harvested bacterial pellet was subjected to 5 cycles of freeze-thaw, centrifuged, and supernatants and pellets (S2, S4, S6 for supernatants and P2, P4, P6 for pellets harvested at 2, 4, and 6 hours cultures, respectively) were studied by SDS-PAGE. The LCR1 recombinant protein (shown by arrow) was present only in the supernatants and no recombinant protein was detected in the pellets. The maximum expression level of LCR1 is detected at 2 hours culture.

Combinant proteins are used as antigens to measure antigen-specific stimulation of T cells (Jensen et al., 2008). A body of evidence has suggested that some of the effects previously attributed to some recombinant proteins may be the direct result of low amounts of endotoxin contained within the recombinant protein used experimentally to investigate the roles in vitro (Gao and Tsan 2003a; Gao and Tsan 2003b; Bausinger et al., 2002). It is therefore crucial to remove or at least significantly reduce LPS levels from recombinant proteins before analyzing antigen specific responses (Velickovic et al., 2007). Many recombinant proteins are constructed with a (His)6-tag for easy purification from bacteria. This complicates the removal of LPS from the proteins since LPS has an affinity for histidine (Matsumae et al., 1990). In our study the LCR1 recombinant protein was expressed in E. coli and purified by resin precharged with Ni2+. The eluted LCR1 protein was heavily contaminated with LPS (> 200 Endotoxin Units/ml). However, the LPS was successfully depleted from the LCR1 (to < 0.1 Endotoxin Units/ml) in our study. This is lower than the maximum permissible endotoxin level of 0.25-0.5 Endotoxin Units/ml approved by FDA for sterile water. Our results show that LPS depletion from recombinant proteins is quite feasible, at least for LCR1 and probably for other recombinant proteins.

Our results show that Iranian patients with VL produce antibody against the LCR1 protein. Similar results have been reported for Brazilian VL patients (Wilson et al.,

Figure 4. The purified LCR1 recombinant protein shows to have no extra protein bands. The LCR1 expressing bacterial pellet was re-suspended in native binding buffer, lysed by freeze-thaw, centrifuged and the supernatant passed over ProBond resin. The figure shows SDS-PAGE of flow through of the first wash (1), second wash (2), and eluate (LCR1) that contains LCR1 recombinant protein (shown by arrow). Molecular weight markers are shown on the right.

Figure 5. Sera of VL patients recognize the recombinant LCR1 protein. LCR1 and SLA were run on SDS-PAGE, blotted on nitrocellulose membrane and reacted with patient sera followed by anti-human IgG HRP conjugated antibody, and finally the chromogenic substrate DAB. A clear band of about 40 kDa is seen for the LCR1 sample. SLA, acting as positive control for the test, resulted in multiple bands. Abbreviation used: LCR1 - recombinant LCR1 protein; SLA - soluble leishmania antigen.
1995). These findings show the potential of LCR1 as a vaccine or diagnostic candidate; this requires further study.

In summary, we successfully cloned and expressed LCR1 recombinant protein from an Iranian clinical isolate of *L. infantum*, and achieved purification of the protein to ensure it was free from any bacterial proteins as well as LPS contamination. We also showed that LCR1 induces an antibody response in Iranian VL patients, as was the case in Brazilian patients previously studied. The purified LCR1 is now ready to be used for further “in vitro” human studies that will reveal more details of the human immune response against this protein and will show to what extent this protein has potential as part of a diagnostic test for or for the production of a subunit vaccine against Leishmaniasis.

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**REFERENCES**


Velickovic, T.C., Thunberg, S., Polovic, N., Neimert-Anderson, T., Grönlund, H., van Hage, M. and Gafvelin, G. 2007. Low levels of endotoxin enhance allergen stimulated proliferation and reduce the threshold for activation in human peripheral blood cells. *International Archives of Allergy and Immunology* 146: 1-10.
