Vaccine Research
ISSN: 2423-4923
eISSN: 2383-2819

Phylogenetic Analysis, Cloning and Expression of Delta Amastin Encoding Gene from Leishmania major as a Vaccine Candidate

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ARTICLE INFO

Research Article

VacRes, 2022
Vol. 9, No.1, 63- 72
Received: October 21, 2022
Accepted: November 17, 2022
Pasteur Institute of Iran

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KEYWORDS: *Leishmania*, Amastin, Recombinant, Vaccine

ABSTRACT

Introduction: Amastin is a surface glycoprotein in *Leishmania* species and one of the most important vaccine candidates due to its involvement in pathogenesis and being an essential virulence factor for parasite replication within the mammalian host cells. There are more than 60 copies of Amastin gene per genome of the parasite. **Methods:** Following phylogenetic analysis, a selected *Amastin* sequence was optimized and cloned with signal peptide in *Escherichia coli*. The recombinant protein was evaluated by SDG-PAGE and a specific antibody by Western blotting. **Results:** Among the *Amastin* sequences within different chromosomes of *Leishmania major*, the main type known as delta Amastin (primary distributed on chromosome 34) could be expressed in *E. coli* host and was confirmed by SDG-PAGE and Western blotting. **Conclusion:** Due to its copy number and evolutionary conservation and its role in pathogenesis, δ-Amastin is considered as an important vaccine candidate against leishmaniasis which could be expressed as a recombinant protein in *E. coli*.

INTRODUCTION

Leishmaniasis is a tropical disease induced by a vector borne parasite of Leishmania genus and is an endemic disease within 102 countries and five continents. Approximately, 350 million people are at risk of the infection while 15 million of them are known as infected cases reported worldwide with 1.5 to 2 million new cases and 70,000 per year [1]. Cutaneous Leishmaniasis (CL) is endemic in tropical and subtropical countries and approximately 90% of the cutaneous forms of the disease have been reported in the Middle East [2]. Leishmania parasite exhibits an intracellular amastigote form inside macrophages of the vertebrate host and is adapted to resist anti-parasitic activities such as acidic conditions, such as nitric oxide and reactive oxygen species and can also persist against the adapted immune responses [3].

Amastins are a vast family of transmembrane glycoproteins conserved among the *Trypanosomatids* which is mainly expressed during the amastigote form of the parasite [4-6]. Amastin of Leishmania amastigotes contains 11 amino acids at positions of 52-62 which are highly conserved and unique to *Trypanosomatid* protoza [7]. It has been shown that Amastin proteins contain 4 hydrophobic transmembrane domains with two serine- and threonine-rich extracellular domains at N and C termini facing the cytosol. Comparative sequence analyses of *Amastin* genes have shown that the hydrophilic extracellular domain contains a conserved Amastin signature (i.e., C-[IVLYF]-[LF]-[WF]-G-X-[KRQ]-X-[DENT]-C) [8, 9].

Amastins are divided into 4 subfamilies of α, β, Y and δ [5]. It is believed that δ-Amastin subfamily is specific to *Leishmania* and none of these sequences were identified in other Typanosomatids, neither by phylogenetic analysis nor by similarity-based protein clustering approach. These genes were developed from ancestral proto-δ-*Amastin* genes [10]. The Amastin family of proteins may be involved in transport of ions and protons across the membranes along with the regulation of cytoplasmic pH [11, 12]. Being a surface glycoprotein, Amastin can induce the immune system toward a Th1-like immune response [13]. Stober et al. have revealed that Amastin proteins are very immunogenic in mice at their signature sequence (i.e., CITLWGLKTDC) [14].

Approximately, 63 copies of δ-Amastin have been found in the genome of *L. major*. The phylogenetic analysis of the Amastin sequence subfamily will reveal the main type of Amastin in the protozoa which involves in the adaptation of the parasite at the intracellular niche of the vertebrate host. It is envisaged that the selection and codon optimization of δ-*Amastin* sequence may lead to efficient expression of this immunogenic protein in *E. coli*. This protein can be further investigated as a vaccine candidate for eliciting effective immune response and protection against Leishmaniasis.

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[DOI: 10.52547/vacres.8.2.117]

MATERIALS AND METHODS

Phylogenetic Tree Reconstruction

The nucleotide sequences of Amastin gene were searched from GenBank, and were used based on LmjF.34.1960 *L. major* Friedlin strain putative Amastin-like surface protein partial mRNA 1-579 nucleotides, the aligned sequences were selected and used for phylogenic tree construction. The phylogenetic relationships among the isolates were achieved from the phylogenetic tree reconstruction by the Neighbor Joining (NJ) method using MEGA 7.0 with assumption setting and the sure of the internal branches was tested with 1,000 bootstrap replications.

Sequences Data of Amastin Gene of Kinetoplastida and *L. major* species.

The sequence of Amastin in *L. major* Friedlin strain LmajF.34.1960 (XM.001686241) was utilized for blast search and the sequences of *Amastin* gene sequences in order of *Kinetoplastida* including *Trypanosoma cruzi*, *Crithidia*, *Leishmania donovani*, *Leishmania infantum*, *Leishmania mexicana*, *Leishmania braziliensis* strains were used for tracing the phylogenetic tree construction. The phylogenetic association were analyzed by using the following sequences submitted to the GenBank, Leishmania donovani: MHOM/IN/1983/AG83 (ACCESSION CP019536), *Leishmania donovani* (ACCESSION CP022645.1), *Leishmania donovani*: LdBPK.080710 (ACCESSION FR799595.2), *Leishmania donovani*: LDHU3.24.1550 (ACCESSION LR812644.1), *Leishmania infantum*: LinJ.08.0680 (ACCESSION NC.009392.2), Leishmania infantum: LinJ.30.0930 (ACCESSION XM.001466901.1), *Leishmania infantum*: LinJ.24.1300 (ACCESSION FR796456.1), *Leishmania infantum*: LinJ.28.1510 (ACCESSION FR796460.1), *Leishmania mexicana*: LmxM.29.0850 (ACCESSION FR799582.1), Leishmania mexicana: LmxM.24.1270 (ACCESSION FR799577.1), *Leishmania mexicana*: LmxM.28.1400 (ACCESSION FR799581.1), *Leishmania mexicana*: LmxM MHOM/GT/2001/U1103 (ACCESSION XM.003886514.1), *Trypanosoma cruzi*: Tc00.1047053509051.20 (ACCESSION XM_801067.1), *Trypanosoma cruzi*: Tc00.1047053507485.150 (ACCESSION XM_807298.1), *Leishmania braziliensis*: LBRM.08.0680 (ACCESSION NC_009300.2), *Leishmania braziliensis*: LbrM.24.1280 (ACCESSION NC_009316.2), *Leishmania braziliensis*: LbrM.28.1550 (ACCESSION XM_003723148.1), *Leishmania braziliensis*: LbrM.30.0980 (ACCESSION XM_001566681.1), a-amastin [Crithidia sp. ATCC 30255]: CDFL9H15.10 (ACCESSION ACS87875.1), b-amastin [Crithidia sp. ATCC 30255]: CDFL7M16.03 (ACCESSION ACS87856.1), d-amastin, partial [*Crithidia* sp. ATCC 30255]: CDFL2E16.01(ACCESSION ACS87802.1), g-amastin [*Crithidia* sp. ATCC 30255]: CDFL6B24.01(ACCESSION GQ153665.1)

The sequence of *Amastin* in *L. major* Friedlin strain LmajF.34.1960 (XM.001686241) was used to find the homologous sequences in genome of L. major Friedlin strain. The phylogenetic relationship were analyzed by using the following sequences (LmajF.08.0670) ACCESSION XM.001681044.1 (, LmajF.08.0680) ACCESSION XM.001681045.1 (, LmajF.08.0690) ACCESSION XM.001681046.1 (, LmajF.08.0700) ACCESSION XM.001681047.1 (, LmajF.08.0710) ACCESSION XM.001681048.1 (, LmajF.08.0720) ACCESSION XM.001681049.1 (, LmajF.08.0730) ACCESSION XM.001681050.1 (, LmajF.08.0740) ACCESSION XM.001681051.1 (, LmajF.08.0750) ACCESSION XM.001681052.1 (, LmajF.08 0760) ACCESSION XP.001681105 (, LmajF.08.0770) ACCESSION XM.001681054.1 (, LmajF.08.0800) ACCESSION XM.001681057.1 (, LmajF.08.0810) ACCESSION XM.001681058.1 (, LmajF.08.0820) ACCESSION XM.001681059.1 (, LmajF.08.0830) ACCESSION XM.001681060.1 (, LmajF.08.0840) ACCESSION XM.001681061.1 (, LmajF.08.0850) ACCESSION XM.001681062.1 (, LmajF.08.0640) ACCESSION XM.001681041.1 (, LmajF.10.0220) ACCESSION XM.001681302.1 (, LmajF.10.0230) ACCESSION XM.001681303.1 (, LmajF.16.0490) ACCESSION XM.001682075.1 (, LmajF.24.1250) ACCESSION XP.001683641 (, LmajF.24.1260) ACCESSION XM.001683590.1 (, LmajF.24.1270) ACCESSION XP.001683643 (, LmajF.24.1280) ACCESSION XM.001683592.1 (, LmajF.27.0540) ACCESSION XM.003721812.1 (, LmajF.28.1120) ACCESSION XM.001684342.1 (, LmajF.28.1130) ACCESSION XM.001684343.1 (, LmajF.28.1390) ACCESSION XM.001684369.1 (, LmajF.28.1400) ACCESSION XP.001684422 (, LmajF.30.0850) ACCESSION XP.001684680.1 (, LmajF.30.0860) ACCESSION XP.001684681 (, LmajF.30.0870) ACCESSION XP.001684682 (, LmajF.31.0450) ACCESSION XP.001685030 (, LmajF.34.0500) ACCESSION XP.001686141 (, LmajF.34.0960) ACCESSION XM.001686137 (, LmajF.34.0970) ACCESSION XM.001686138.1 (, LmajF.34.0980) ACCESSION XM.001686139.1 (, LmajF.34.1080) ACCESSION XM.001686149.1 (, LmajF.34.1560) ACCESSION XM.001686201.1 (, LmajF.34.1580) ACCESSION XM.001686203.1 (, LmajF.34.1600) ACCESSION XM.001686205.1 (, LmajF.34.1620) ACCESSION XM.001686207.1 (, LmajF.34.1640) ACCESSION XM.001686209.1 (, LmajF.34.1660) ACCESSION XM.001686211.1 (, LmajF.34.1680) ACCESSION XM.001686213.1 (, LmajF.34 1700) ACCESSION XM.001686215.1 (, LmajF.34.1720) ACCESSION XM.001686217.1 (, LmajF.34.1740) ACCESSION XM.001686219.1 (, LmajF.34.1760) ACCESSION XM.001686219.1 (, LmajF.34.1780) ACCESSION XM.001686223.1 (, LmajF.34.1800) ACCESSION XM.001686225.1 (, LmajF.34.1820) ACCESSION XM.001686227.1 (, LmajF.34.1840) ACCESSION XM.001686229 (, LmajF.34.1860) ACCESSION XM.001686231.1 (, LmajF.34.1880) ACCESSION XM.001686233.1 (, LmajF.34.1900) ACCESSION XM.001686235.1 (, LmajF.34.1920) ACCESSION XM.001686237.1 (, LmajF.34.1940) ACCESSION XM.001686239.1 (, LmajF.34.1960) ACCESSION XM.001686241 (, LmajF.34.1980) ACCESSION XM.001686243.1 (, LmajF.36.1270) ACCESSION XM.001686647.1 (, LmajF.36.4140) ACCESSION XM.001686945.1.

Analysis of Amino Acid Sequences of Amastin

The amino acid sequences of Amastin, Amastin-like and putative Amastin proteins of *L. major* Friedlin strain were

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[DOI: 10.52547/vacres.8.2.117]

aligned using MEGA7 software (Molecular Evolutionary Genetics Analysis, version 7.0 for bigger datasets) [15]. The conserved amino acid sequence parts similar to Amastin of LmjF.34.1960 with 192 amino acids were determined by default setting.

Bacterial strains, Plasmids, and Chemicals

E. coli DH5α was used as the cloning host and *E. coli* BL21 (DE3) strain as the expression host. For cultivation of the cloning and the expression hosts, ampicillin (100 µg/ml) and kanamycin (33 µg/ml) antibiotics were added to the culture medium, respectively. Plasmid pUC19 was used as the cloning and pET28 was utilized as the expression vectors. When required, for transformation of the cloning vector in competent cells, *E. coli* DH5α strain competent was prepared by a 2:100 overnight culture transfer in fresh LB broth (37 ° C, at 180 rpm, 3 h). The culture medium was transferred on ice for 1 h. After centrifuging 1.5 ml of the culture medium (5 min, 5000 rpm, 10 min, 4 ° C), the pellet was resuspended in 0.5 ml of 100 mM $CaCl_2$ and put on ice for 45 min. The cells were washed again and were resuspended in 100 µl of 100mM $CaCl_2$. For transformation of cells, approximately 0.5 µg of plasmid was mixed with 50 µl suspension of competent cells and incubated on ice for 45 min. The cells were transferred on hot plate at 42° C for 90 s, and then were put on ice. After adding 500 µl LB broth to the pellet and incubation at 37 ° C for 45 min, 150 µl samples with and without the construct were cultured on LB agar plate containing the antibiotic and incubated 37° C for 16-20 h. The transformed clones were selected and cultured in 4 ml LB medium containing the antibiotic and incubated for 16-24 h at 37 °C. The plasmid was purified with Favorgen kit (Yektatajhiz Azma, Iran) according to the manufacturer's instruction and analyzed by restriction map preparation.

Construction of the Expression Vector with a Signal Peptide of *E. coli*

The pUC57 cloning vector containing nucleotide optimized sequence (Pishgam Biotech Company, Iran) had a *δ-Amastin* sequence containing the *Hind* III restriction sequences at both ends. After transformation and plasmid purification, the vector was digested with *Hind* III and the digested sequence was extracted from agarose gel by GeneAll kit (GeneAll Biotechnology, South Korea).

The type-1 fimbrial subunit of *E. coli* is encoded by *fimA* gene. To design primers for amplification of *fimA* gene signal peptide, type 1 fimbriae major subunit of *E. coli* strain K-12, substrain MG1655 was selected (NCBI GeneID: 948838; 549 bp). Using Gene Runner software, the primers were designed for the signal peptide part of this gene, and NdeI restriction site was placed in the forward primer (GTTTTTTGAAAGGAAAGC**CATATG**), and *Hind* III restriction site was placed in the reverse primer (AAA**AAGCTT**GGTCCCACCATTAAC). The restriction sites are shown in bold in the sequence. The amplification reaction for the signal peptide was conducted using the designed primers and the genomic DNA of *E. coli* which was purified and used as the template. The reaction began with an initial denaturation step at 96 °C for 3 min and 30 cycles of 45 s at 96 °C, 45 s at 56 °C, and 60 s at 72 °C with a final extension at 72 °C for 5 min. After digestion and purification of the amplicon, it was ligated with a pET28 plasmid digested with the same restriction enzymes. In order to transform of *E. coli* DH5α competent cells, 5 µl of the ligation mix were used. The transformed clones were confirmed by plasmid purification and restriction digestion. The clones containing fimA sequence were used for the next step.

Both the purified pET28 plasmid (with the signal peptide sequence) from the previous step and pUC57 vector containing *δ-Amastin* sequence were digested with *Hind* III enzyme. Both ends of the amplified DNA fragment cleavage site and the ends of the vector used in this study were sticky. A molar ratio of 1:2 vector: insert gel purified DNA was used to prepare the ligation mixture (4 °C, 16 h) with T4 DNA Ligase (Thermo Fisher Scientific Inc.,). The product was added to *E. coli* DH5α competent cells. The transformed colonies were identified by purified plasmid size comparison and enzyme-digestion using *Hind* III and *Nde* I enzymes. The selected transformants of the cloning host were used for plasmid purification and the purified product was transformed to the expression host BL21 DE3. The resulting clones were examined for the expression of *δ-Amastin*.

Recombinant δ-Amastin Protein Expression

A clone of the transformed plasmid in the BL21 DE3 expression host was cultured in LB medium containing Kanamycin 33 µg/ml and incubated for 16-24 hours at 37 °C. Subculture was then performed in the fresh medium and shacked for 4 hours at 37 °C at 180 rpm. After reaching the optical absorption of the culture medium to 0.6 at a wavelength of 600 nm, equal volume of fresh medium with kanamycin 33 µg/ml was added to culture medium. To the final volume of the culture, IPTG was tested at different final concentrations (0.2, 0.4, 0.6 and 0.8 mM). The medium was incubated at 25 °C or 37 °C and shacked at 180 rpm for 20 h. The cells were harvested by centrifugation at 5000 rpm for 5 minutes.

Confirmation of *δ-Amastin* Protein Expression by SDS-PAGE, Dot Blot and Western Blotting

A 500 µl culture medium of cells harboring the construct were harvested and lysed in 40 µl of 50 mM Tris-HCl (pH 6.8) by vortexing. After adding the sample buffer, the mixture was boiled and was incubated on ice for 10 min. The sample was centrifuged at 6000 rpm (4°C, 10 min) and loaded on 12% polyacrylamide gel. The gel was stained with 0.05% Coomassie brilliant blue for 40 min and then destained (5% acetic acid, 25% methanol v/v).

To investigate the expression of the target protein in the cell lysate, the dot blot method was performed. Fifty µl of sample pellet was dissolved in 50 mM Tris solution (pH 6.8) and vortexed. The cell lysate was centrifuged, poured on the nitrocellulose membrane and dried. The nitrocellulose membrane was incubated in the blocking solution (2% skimmed milk powder without fat in TBST, NaCl 250 mM, Tris Base 10 mM, and pH 7.5) for 1 h at room temperature on the shaker. The membrane was washed 3 times with Washing Buffer (TBST; every time for 10 min on shaker at room temperature). The membrane was placed in the primary antibody solution (anti-His Tag Human-Mouse hybrid monoclonal diluted 1:750 in TBST; Sina Biotch Company, Iran), for 1 h on shaker at room temperature and washed again. In the next step, the membrane was soaked in a secondary antibody solution (Sheep anti-human IgG HRP conjugated polyclonal antibody diluted 1:1000 in TBST; Sina Biotch Company, Iran), for 1 h at room temperature on the shaker. The antibody was washed off 3 times with TBST. Finally, 10 mg of DAB, 20 ml of TBST and 1 µl of H2O2 30% were mixed and poured on the nitrocellulose membrane and kept in the dark for 15 to 30 min. After the dot appeared, the membrane was washed with distilled water.

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[DOI: 10.52547/vacres.8.2.117]

For Western blotting, the lysate of the protein sample was prepared as above. The protein concentration was determined with spectrophotometry based on absorbance at 280 nm. The protein samples (1 to 2 mg) were gel-loaded and run by SDS-PAGE. The gel-separated proteins bands were transferred to a nitrocellulose membrane by electro-transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% v/v methanol; 1 h, 80 V). The membrane was washed with distilled water. The nitrocellulose membrane was incubated in the blocking buffer for 1 h and followed as described above for the dot blot procedure.

RESULTS

Phylogenic Relationship of Amastin Gene within Kinetoplastida and *L. major* Species

Amastins have homology in species and different strain of *Leishmania*. It has four subfamilies α, β, δ and γ. In this study, the similarity of LmjF.34.1960 sequence was searched with the selected *Amastin* sequences within the order of Kinetoplastida. According to the constructed three as shown in Fig. 1, the sequences of *L. donovani*: LDHU3.24.1550, *L. infantum*: LinJ.24.1300, g-amastin [Crithidia sp. ATCC 30255]: CDFL6B24.01, *L. mexicana*: LmxM.24.1270, *L. major*: LmjF.24.1270, *L. braziliensis*: LbrM.24.1280 and *L. major* and LmjF.24.1280 include in the subfamily γ. The subfamily α contains the Amastin sequences a-amastin [Crithidia sp. ATCC 30255]: CDFL9H15.10, *L. braziliensis*: LbrM.28.1550, *L. mexicana*: LmxM.28.1400, *L. major*: LmjF.28.1400, *L. infantum*: LinJ.28.1510 and *L. donovani*: MHOM/IN/1983/AG83.

In the clade of subfamily δ, *L. infantum*: LinJ.08.0680, *L. donovani*: LdBPK.08.0710, *L. mexicana*: LmxM.29.0850, *L. mexicana*: LmxM.33.1900.1, d-amastin, partial [*Crithidia* sp. ATCC 30255]: CDFL2E16.01, *T. cruzi*: Tc00.1047053507485.150, *L. major*: LmjF.08.0760, *L. major*: LmjF.08.0810, *L. major*: LmjF.31.0450, L. major: LmjF.34.1840, *L. major*: LmjF.34.1860 and LmjF.34.1960 in *L. major* Friedlin strain are seen. Finally, the sequences of *L. major*: LmjF.30.0860, *L. braziliensis*: LbrM.08.0680, *L. braziliensis*: LbrM.30.0980, *L. infantum*: LinJ.30.0930, *L. major*: LmjF.30.0870, b-amastin [Crithidia sp. ATCC 30255]: CDFL7M16_03, *T. cruzi*: Tc00.1047053509051.20, *L. donovani*: CP022645.1 are within the subfamily β. The result revealed that the LmjF.34.1960 in *L. major* Friedlin strain is in the subfamily δ. There are not any *Amastin* sequences of *T. cruzi* related to the α and γ subfamilies.

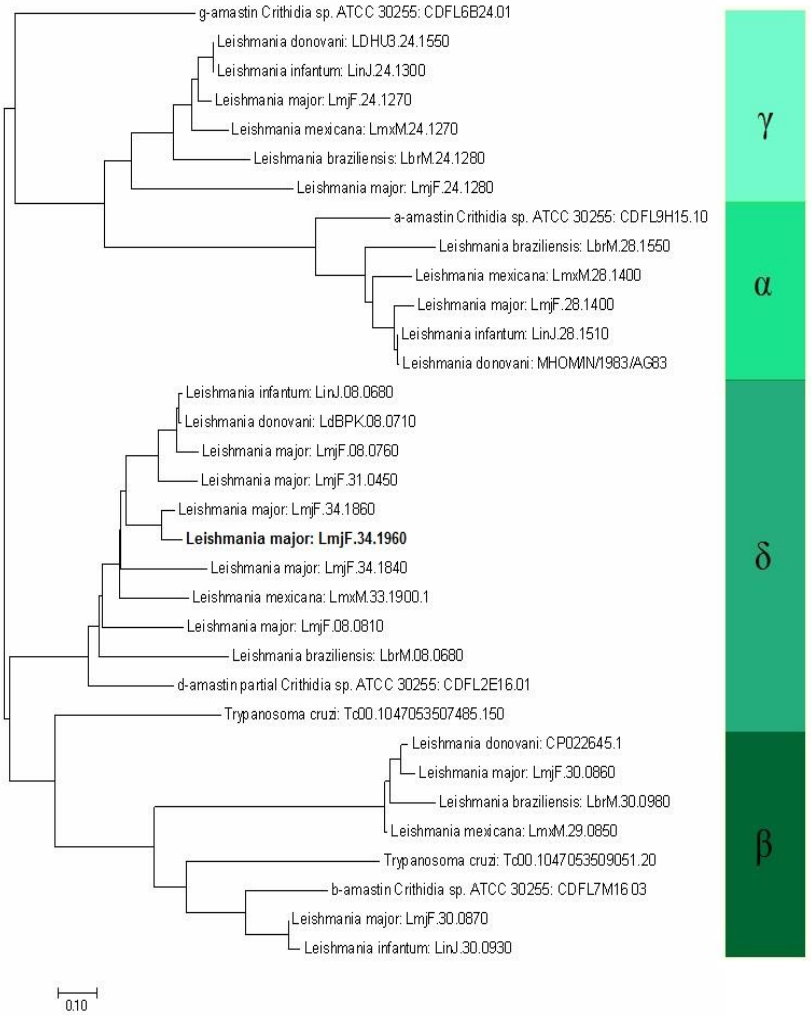

Fig. 1. Phylogenetic relationship of sequences α, β, γ and δ-Amastin subfamilies of Kinetoplastida (*T. cruzi*, Crithidia and *Leishmania* species) based on LmjF.34.1960 *L. major* Friedlin strain putative Amastin-like surface protein partial mRNA 1-579 nucleotides. *Amastin* sequences of different species are divided in four cades. The evolutionary relationship was prepared using the Neighbor-Joining method, using MEGA 7.0 by default setting and the reliability of the internal branches was tested by 1,000 bootstrap replications.

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Analysis of Amastin Amino Acid Sequences in L. major Friedlin strain and their Phylogenic Relationship

There are 63 sequences of Amastin genes located in 9 different chromosomes in *L. major* Friedlin strain. They are classified in four amastin subfamilies, α, β, δ and γ- amastin. There are 44 numbers δ-Amastin on chromosome 8, 31, 34, 36; 2 proto-δ (pδ) Amastin on chromosome 34; 2 α-Amastin genes on chromosome 28; 4 γ amastin on chromosome 24 and 11 β amastin on chromosome 8,10,16, 27, 28, 30, 36 in *L. major* Friedlin strain. The prominent type of amastin sequence in the genome of *L. major* Friedlin strain is δ -amastin which including more than 73 percent of amastin gene copies followed by β-amastin copies with more than 17 percent. The α and γ-amastin types form about 10 percent of gene copy numbers. (Table 1).

In *L. major* Friedlin strain, the conserved amino acids sequences of Amastins are 11 amino acids (C-[IVLYF]-[TS]-[LF]-[WF]-G-X-[KRQ]-X-[DENT]-C), 2 amino acid G [FY] in position 40-50 and 152-153 according to the LmjF.34.1960 sequence, respectively. The conserved 11 amino acids segment is absent in 8 sequences of 63 amastin protein copies in L. major as follow LmjF.10.0220, LmjF.10.0230, LmjF.16.0490, LmjF.27.0540, LmjF.08.0640, LmjF.28.1120, LmjF.28.1130 LmjF.36.4140. The conserved motif of G [FY] at position 152-153 is absent in three copies, LmjF.16.0490, LmjF.27.0540, LmjF.36.4140.

Table 1. Comparison of 63 sequences of amastin gene copies located on 9 different chromosomes in *L. major* Friedlin strain with their accession number, amastin type, conserved sequence domain and related chromosome. the conserved amino acids sequences are 11 amino acids (C-[IVLYF]-[TS]-[LF]-[WF]-G-X-[KRQ]-X-[DENT]-C), 2 amino acid G[FY] in position 40-50 and 152-153 of LmjF.34.1960, respectively.

Gene	Accession number	Conserve sequences of protein		Chromosome	Type
LmajF.08.0670	XM.001681044.1	CITLWGAKTDC	GF	8	δ
LmajF.08.0680	XM.001681045.1	CITLWGAKTDC	GF	8	δ
LmajF.08.0690	XM.001681046.1	CITLWGAKTDC	GF	8	δ
LmajF.08.0700	XM.001681047.1	CITLWGLKTDC	GF	8	δ
LmajF.08.0710	XM.001681048.1	CITLWGAKTDC	GF	8	δ
LmajF.08.0720	XM.001681049.1	CITLWGLKTDC	GF	8	δ
LmajF.08.0730	XM.001681050.1	CITLWGAKTDC	GF	8	δ
LmajF.08.0740	XM.001681051.1	CITLWGLKTDC	GF	8	δ
LmajF.08.0750	XM.001681052.1	CITLWGAKTDC	GF	8	δ
LmajF.08.0760	XP.001681105	CITLWGLKTDC	GF	8	δ
LmajF.08.0770	XM.001681054.1	CITLWGLKTDC	GF	8	δ
LmajF.08.0800	XM.001681057.1	CLTLWGGKEEC	GF	8	δ
LmajF.08.0810	XM.001681058.1	CLTLWGYKSEC	GF	8	δ
LmajF.08.0820	XM.001681059.1	CLTLWGGKEEC	GF	8	δ
LmajF.08.0830	XM.001681060.1	CLTLWGGKEEC	GF	8	δ
LmajF.08.0840	XM.001681061.1	CLTLWGGKEEC	GF	8	δ
LmajF.08.0850	XM.001681062.1	CLTLWGYKSEC	GF	8	δ
LmajF.08.0640	XM.001681041.1	_____	____	8	β
LmajF.10.0220	XM.001681302.1	_____	____	10	β
LmajF.10.0230	XM.001681303.1	_____	____	10	β
LmajF.16.0490	XM.001682075.1	_____	GF	16	β
LmajF.24.1250	XP.001683641	CYTMWGYRKF	GF	24	γ
LmajF.24.1260	XM.001683590.1	CYTFIGYKTEC	GF	24	γ
LmajF.24.1270	XP.001683643	CYTFWGMKSDC	GF	24	γ
LmajF.24.1280	XM.001683592.1	CYTMWGYRKFC	GF	24	γ
LmajF.27.0540	XM.003721812.1	_____	GF	27	β
LmajF.28.1120	XM.001684342.1	_____	GF	28	β
LmajF.2.1130	XM.001684343.1	_____	GF	28	β
LmajF.28.1390	XM.001684369.1	CYTFWGYRANC	GF	28	α

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LmajF.28.1400	XP.001684422	CFTYWGYKDNC	GF	28	α
LmajF.30.0850	XP.001684680.1	CVTVWGLKNDC	GY	30	β
LmajF.30.0860	XP.001684681	CVTVWGLKNDC	GY	30	β
LmajF.30.0870	XP.001684682	CFTLWGLHSDC	GY	30	β
LmajF.31.0450	XP.001685030	CLTLWGEKLDC	GF	31	δ
LmajF.34.0500	XP.001686141	CITLWGWKSRC	GF	34	δ
LmajF.34.0960	XM.001686137	CVTLWGAKLGC	GF	34	δ
LmajF.34.0970	XM.001686138.1	CLTIWGFKDKC	GF	34	p_{δ}
LmajF.34.0980	XM.001686139.1	CVSLWGIRDRC	GF	34	p^{δ}
LmajF.34.1080	XM.001686149.1	CVTLWGAKLGC	GF	34	δ
LmajF.34.1560	XM.001686201.1	CITLWGEFTC	GF	34	δ
LmajF.34.1580	XM.001686203.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1600	XM.001686205.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1620	XM.001686207.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1640	XM.001686209.1	CITLWGLRLTC	GF	34	δ
LmajF.34.1660	XM.001686211.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1680	XM.001686213.1	CLTLFGLKFDC	GF	34	δ
LmajF.34.1700	XM.001686215.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1720	XM.001686217.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1740	XM.001686219.1	CITLWGERFTC	GF	34	δ
LmajF.34.1760	XM.001686219.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1780	XM.001686223.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1800	XM.001686225.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1820	XM.001686227.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1840	XM.001686229	CITLFGVKVDC	GF	34	δ
LmajF.34.1860	XM.001686231.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1880	XM.001686233.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1900	XM.001686235.1	CITLWGERFTC	GF	34	δ
LmajF.34.1920	XM.001686237.1	CITLWGDKLAC	GF	34	δ
LmajF.34.1940	XM.001686239.1	CLTLFGFKLDC	GF	34	δ
LmajF.34.1960	XM.001686241	CLTLFGFKLDC	GF	34	δ
LmajF.34.1980	XM.001686243.1	CLTLFGFKLDC	GF	34	δ
LmajF.36.1270	XM.001686647.1	CITLWGLRLTC	GF	36	δ
LmajF.36.4140	XM.001686945.1	_____	GY	36	δ

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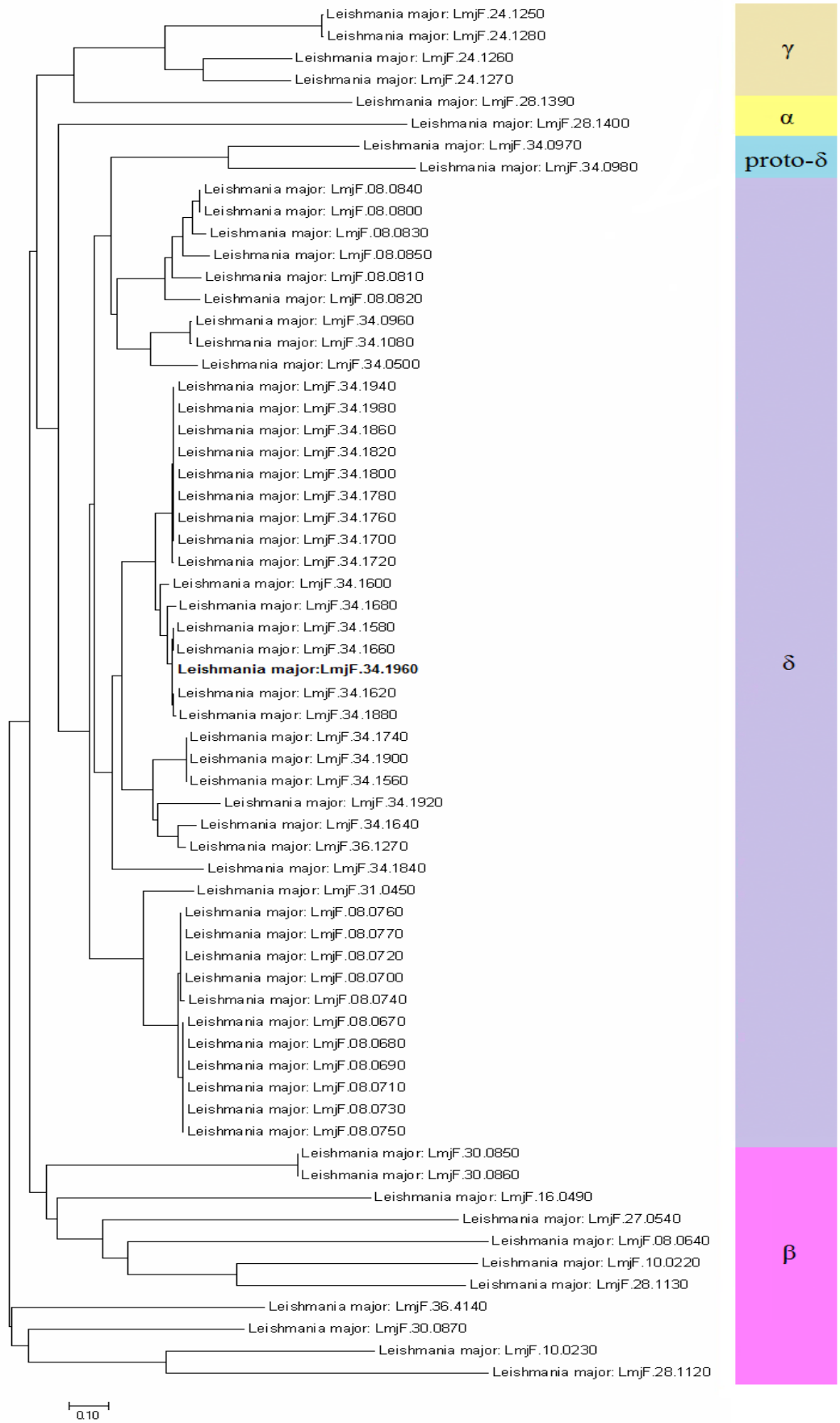

Fig. 2. Phylogenetic relationship of sequences *α, β, γ* and *δ-Amastin* subfamilies of of *L. major* Friedlin strain based on LmjF.34.1960 *L. major* Friedlin strain putative Amastin-like surface protein partial mRNA 1-579 nucleotides. *Amastin* sequences of different copies are divided mainly in the *δ- Amastin* clade, the minority, less than 30% of the copies form the other 3 clads, namely *α, β* and *γ-Amastin*. The evolutionary relationship was prepared using the Neighbor-Joining method, using MEGA 7.0 by default setting and the reliability of the internal branches was tested by 1,000 bootstrap replications.

Vaccine Research

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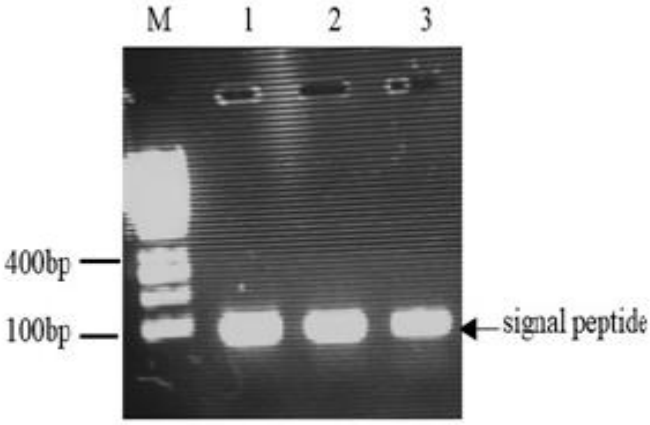

Fig. 3. The PCR product amplified with two specific primers for the *fimA* gene signal peptide in 1% agarose gel and 100 bp DNA Ladder (Vazyme). Lane M: DNA Ladder, lane 1 to 3: the PCR product amplification of the signal peptide of the fim A gene in the genomic DNA of *E. coli*.

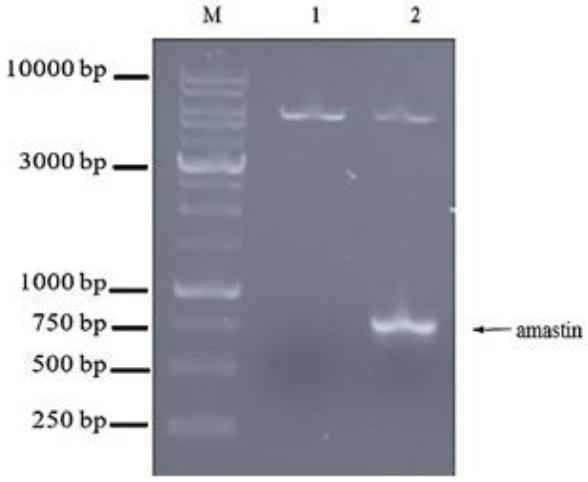

Fig. 4. The pET28 expression construct containing fla gene signal peptide and amastin coding sequence digestion with *Hind* III enzyme and without enzymatic cleavage. Lane M: 100 bp DNA Ladder (Vazyme), lane 1: The construct without enzymatic cleavage, lane 2: The construct with *Hind* III enzyme digestion.

Recombinant δ-Amastin Expression

The expression of recombinant δ-Amastin protein with an expected molecular weight of 37 kDa was examined at 0.2, 0.4, 0.6, and 0.8 mM IPTG at 25°C and 37 °C temperatures. The results indicated that the highest amastin expression was achieved with an IPTG concentration of 0.8 mM and at 25°C (Fig. 5A). The expression at 25°C was also assayed using pellets prepared from the culture medium with 0.8 mM IPTG protein expression at 12, 24, and 36 h after the induction (Fig. 5B).

Confirmation of δ-Amastin Expression by Dot Blot and Western Blotting

The recombinant expressed protein was confirmed using the host cell lysate (0.8 mM IPTG induction at 25 °C for 24 h) by a dot blot procedure. The reaction of recombinant δ-Amastin with a specific anti-His tag antibody was revealed by an intense colored dot compared to the negative control on the membrane (Fig. 6 A). For confirmation of the expressed protein molecular weight, the cell lysate was analyzed by Western blotting which detected an approximately 37 kDa band using a specific anti-His tag antibody (Fig. 6 B).

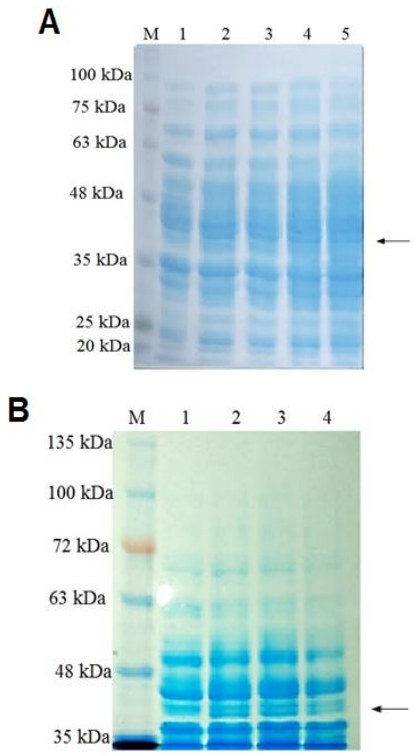

Fig. 5. (A) Expression of recombinant δ-Amastin with different IPTG concentrations (0.2 mM to 0.8 mM) at 25 °C . Lane M: Pre-stained Protein Molecular weight Marker (Sinaclon), Lane 1: unindiuced negative control, Lane 2: induced at 25 °C with 0.2 mM IPTG, Lane 3: induced at 25 °C with 0.4 mM IPTG, Lane 4: induced at 25 °C with 0.6 mM IPTG, Lane 5: induced at 25 °C with 0.8 mM IPTG. **(B)** Investigation of Amastin expression at different time intervals after 0.8 mM IPTG induction at 25 °C. Lane M: Pre-stained Protein Molecular weight Marker (Biolegend, USA), Lane 1: uninduced negative control, Lane 2: 12 h after the induction, Lane 3: 24 h after the induction, Lane 4: 36 h after the induction of protein expression.

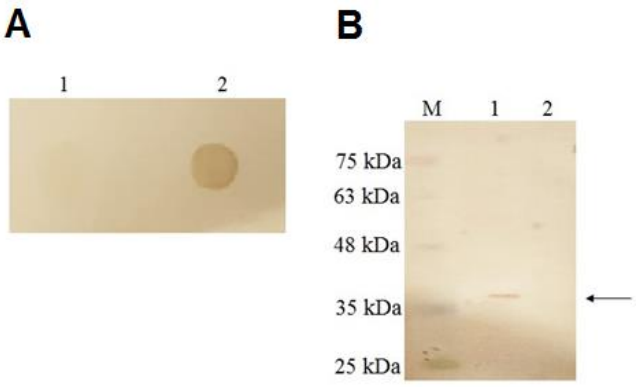

Fig. 6. Final Confirmation of recombinant δ-Amastin expression by dot blot and Western blotting methods. **A**) Dot blot: Lane 1: negative control, Lane 2: Recombinant δ-Amastin test sample. **B**) Western blotting procdure, Lane M: Pre-stained Protein Molecular Weight Marker (Sinaclon, Iran), Lane 1: Recombinant δ-Amastin, Lane 2: Negative control.

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DISCUSSION

Leishmaniasis is a neglected vector-borne disease in tropical and subtropical areas with increased prevalence during the last decade. The infection is correlated to the socio-economic conditions in the endemic foci. At the present, the control measurements against leishmaniasis are mainly relied on controlling the vectors and the reservoirs of the parasite. As a result, the sensitive population who remain at the infected areas can increase the risk of infection in the subsequent years in those areas. Hence, studying the antigenic molecules as vaccine candidates which are capable of eliciting effective immune responses in human population and protection against leishmaniasis in the infected areas is a public health priority.

Amastin surface proteins are encoded by a broad family of genes known so far in 45 species of *Leishmania*. All of these genes have a common structure and have an extracellular domain with 11 highly conserved amino acids. Most of these genes are expressed in the amastigote stage of the parasite and are present upstream of 3'-UTR. A fragment of 450 nucleotides is conserved in the region that regulates the gene expression at the transcriptional level, and possibly other mechanisms may be involved in controlling or regulating their transcription in macrophages [4]. Another study has shown that a 450-nucleotide region responds to heat shock by initiating the transcription of Amastin mRNA in Leishmania. Upon entering the host cell, it has also been shown that another 100-nucleotide region in the 3'-end of Amastin mRNAs increase the translation of this protein in response to temperature. In addition, the acidic pH present in phagolysosomes and macrophages has been shown to produce large amounts of Amastin mRNA, which is independent of the 450-nucleotide and the 100-nucleotide fragments. Post-transcriptional regulation of Amastin mRNA in *Leishmania* is complex and various mechanisms are involved in its stability and translation [16]. The expression of this protein is induced at an acidic pH. Moreover, the fact that Amastin encoding gene is conserved in all species of Leishmania, is of particular importance. Due to its immunogenicity, Amastin has been used for both diagnosis and immunization[7]. For instance, in a study by Vale *et al*., Amastin of *L. infantum* was evaluated for disease diagnosis in dogs and humans. The results indicated high sensitivity of these tests for diagnosis of the disease when used simultaneously in dogs and humans [11].

In order to obtain an appropriate antigen for immunization against *L. major* infection in this study, the conserved and common form of *Amastin* gene was cloned into a pET expression vector and was expressed in *E. coli* and the results showed a proper expression of the antigen. By examining the suitable temperature conditions for the optimal expression of the recombinant protein, 25°C was found to be the best temperature for the expression. The likely causes of this are the slow metabolism and decreased secretion of toxic metabolites in the culture medium at this temperature. The IPTG concentrations in range of 0.5 and 1 mM IPTG indicated that 0.8 M concentration was the most appropriate IPTG concentration for the optimal expression of Amastin in *E. coli*. The expression of the recombinant protein at different times after the induction indicated that the expression began at approximately 9 hours after the induction and increased to up to approximately 24 hours with no increase in the amount of protein after this time point.

Previously, Zhang and colleagues have revealed multiepitope vaccine composed of KMP-11, Amastin and GP63 proteins is protective in BALB/c mice. Following two injections, the animals were challenged with Leishmania promastigotes. In the animal sera the antibodies specific to the epitopes and in spleen cells secreted cytokines CD3+, CD4+, CD8+ were detected by the ELISA technique. Moreover, the parasitic load was reduced in the GP63, KMP-11, and amastin treated groups to 89, 86, and 79 percent compared to the control group [22].

The amastin of *L. infantum* has been evaluated for immunogenicity and protection in BALB/c mice. It has been reported that at forty-five days after infection with L. infantum promastigotes, based on protein and parasite-specific IFN-γ, IL-12, GM-CSF, and the IgG2a isotype antibody, a Th1 immune response occurs in vaccinated mice [23].

The phylogenetic relationship of Amastin protein in *L. major* indicated that the majority of Amastin gene copies are in the main form of δ-Amastin which comprise more than 73 % of the copies. This form of Amastin is evolutionary one of the most conserved forms; therefore, has an important role in biology and virulence of the parasite, especially in the vertebrate host cells. The evaluation of the immunogenicity and efficacy of Amastin in animal models have revealed that it can elicits protective immune responses in animals against leishmaniasis; although, the type of the Amastins that were used in these studies are not well-specified [17]. The placement of an *E. coli* signal peptide upstream of the Amastin encoding gene transports the expressed protein into the bacterial cell membrane; thereby, it can reduce the concentration of Amastin within the cytoplasm during the induction of the expression by IPTG. This in turn, reduces the transcription and translation inhibition of the Amastin gene, resulting in continuous expression of the protein in the cell, in its natural form.

In this study, the total cellular protein amount was 50 µg/ml of the culture medium while the expression of Amastin was evaluated as 0.5 µg/ml of the culture medium. This indicated that the expression of the protein in the cell can increase up to approximately 1-2% of the total amount of the cellular protein at the best environmental conditions. Since in other studies, 5 to 50 µg of this protein has been used for immunization [13, 18], the obtained concentration of the recombinant Amastin by the described method seems to be sufficient for immunization in future animal studies. Therefore, the next step is to prepare the appropriate dose and formulation of this recombinant protein to assess the outcome of using it for the immunization assays.

In conclusion, Amastin is one of the most important vaccine candidates for protection of sensitive population in the endemic areas against leishmaniasis. The phylogenetic analysis of Amastin surface glycoprotein protein family encoding sequences in *L. major* revealed that the abundant type is *δ-amastin* which is widely distributed on chromosomes 34 and 8 of the parasite. The copy number and the evolutionary conservation of this prominent type emphasize its role in the pathogenesis and survival of the parasite in the vertebrate hosts. Due to the conserved nature of this virulence factor within the genus of *Leishmania* and *Trypanosomatidae* family, the cloning and expression of *δ-Amastin* encoding sequence described in this study appears to be a promising mean to prepare an effective antigen for induction of protective immune response in future animal studies.

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ACKNOWLEDGMENT

The authors would like to thank Departments of Mycobacteriology as well as Hepatitis and HIV of Pasteur Institute of Iran for providing certain laboratory facilities. The authors would like to appreciate Ms. Mariam Amin for her supervision and laboratory assistance.

CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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