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Original Article

Gene Cloning of Iranian *Leishmania major* Mannose-1-Phosphate Guanyltransferase

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Abstract

Background: *Leishmania* is an obligatory intracellular protozoan parasite, which infects human beings when infected sand fly vector takes a blood meal. Most efforts are towards designing an effective vaccine to prevent leishmaniasis. In this way, development of candidate antigen for vaccine has special important. In this study, we cloned mannose-1-phosphate guanyltransferase gene of Iranian *L .major* in pET32a expression vector.

Methods: Primers based on *L. major* mannose-1-phosphate guanyltransferase sequence gene was designed and synthesized. DNA of *Leishmania* promastigotes was extracted and PCR reaction was done. PCR product was cloned into pTZ57R and sub cloned into pET32a expression vector.

Results: Recombinant plasmid containing 1140 bp as *L. major* mannose-1-phosphate guanyltransferase gene was extracted and confirmed by restriction analysis. PCR product was sequenced and deposited to GenBank. There were some differences in amino acid sequences between Iranian *L. major* mannose-1-phosphate guanyltransferase and others previously accepted in GenBank

Conclusion: We amplified and cloned Iranian *L. major* mannose-1-phosphate guanyltransferase successfully.

Keywords: Leishmania major*, Mannose 1 phosphate guanyltransferas, Cloning*

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Introduction

Leishmania is an obligatory intracellular protozoan parasite, which infects human beings when infected sand fly vector takes a blood meal (1). Various species of *Leishmania* cause different clinical forms such as cutaneous; disseminated cutaneous; mucocutaneous; and visceral leishmaniasis (2).

Most efforts are towards designing an effective vaccine to prevent leishmaniasis (3, 4). In this way, recognition of candidate antigen for vaccine has vital important (5). The mannose-1-phosphate guanyltransferase as an antigen is one of the vaccine candidate. The gene encoding for the enzyme has 1140 bp and arranged on chromosome 23 (www.genedb.org). Mannose has a key role in glycosylation process in eukaryotes; glycocojugates are involved in correct protein folding, and solubility and sorting protein in the cell (Fig. 1). In the absence of the enzyme there are unable to synthesize man-containing glycoconjugates such as protein and lipid (6).

Many studies were done on the gene cloning of GDP-mannose pyrophosphorylase of *L.mexicana*, which is homologue to mannose-1-phosphate guanyltransferase of *L. major* and is important in *Leishmania* pathogenecity (6).

The aim of this study was cloning of mannose-1-phosphate guanyltransferase gene of Iranian *L .major* for further investigations.

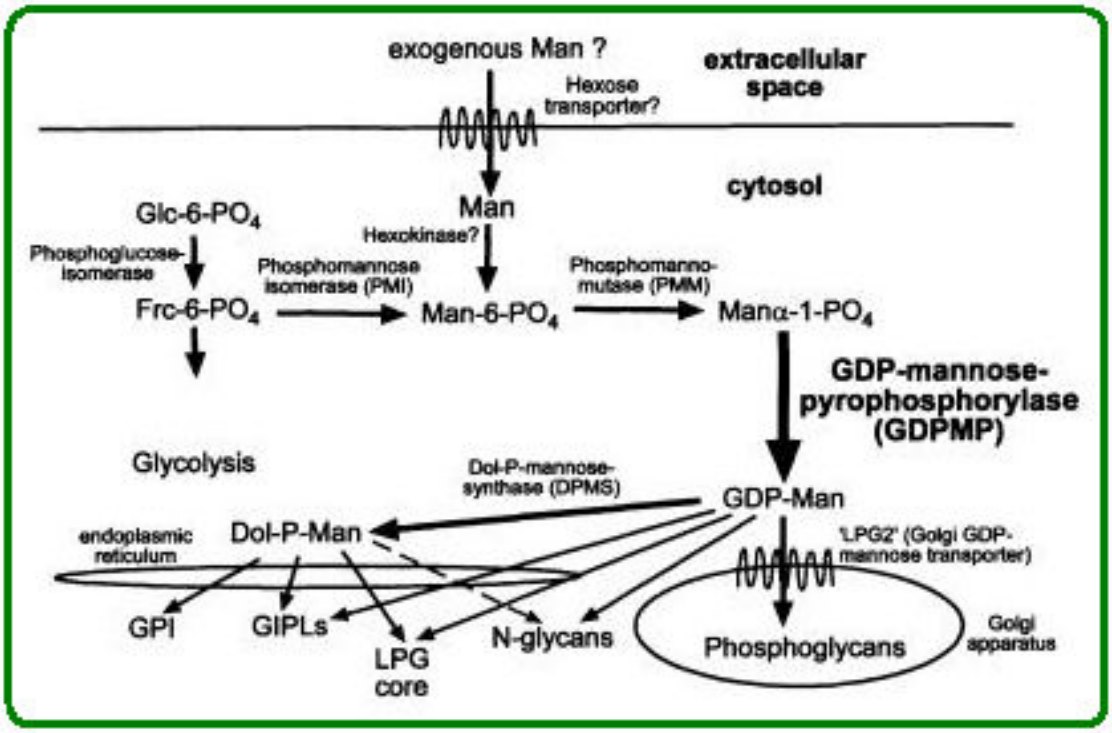

Fig. 1: Mannose activation pathways and glycoconjugates biosynthesis in *L. mexicana*. (Courtesy of Garami and Ilg 2001, ref 6)

Materials and Methods

This experimental work was done at Shahid Beheshti University, M.C., and Esfahan University of Medical Sciences, Iran.

Parasite and DNA extraction

L.major (MRHO/IR/75/ER) promastigotes were grown in N.N.N medium and mass cultured in $RPMI_{1640}$ supplemented with 10% heat inactivated Fetal Calf Serum (FCS). Promastigotes at stationary phase were collected by centrifugation at 8000 rpm for 5 min and subjected to DNA extraction as previously described (7).

Primers and PCR reaction

Primers for nested PCR were designed based on *Leishmania major* mannose-1-phosphate guanyltransferase sequence gene (Accession No. XM_001683281). Nest I primers: Man F1 5'-TGC ATC TCT CCG AGT GTG TG-3' and Man RI 5'-GAT CAC ACA CAC CGG CGA AA-3'. Nest II primers: Man F2 5'-GGA TCC GCC ATG TCT TCA TCC GAT GGC C-3' and Man R2 5'-GAA TTC CTA CAT GAT GAT RCC AGG CTC G-3'. The BamHI and EcoRI restriction sites were synthesized at the 5' ends of forward and reverse of nested II primers (Man F2 and Man R2) respectively. The Man F2 and Man R2 primers were amplified 1140 Pb fragment as mannose-1-phosphate guanyltransferase gene. Because there is no intron in *leishmania* genes (8), we used genomic DNA for PCR reaction. Each PCR reactions contained 1 µg DNA, 10 pmol each of forward and reverse primers, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1X PCR buffer, 1.25 unite of Taq DNA polymerase and dH_2O up to 25µL. PCR amplification was carried out within 94ºC for 5 min, followed by 30 cycles containing, denaturation at 94ºC for 60 s, annealing at 52ºC for 60 s (48 ^{O}C for nested II PCR reaction) and extension at 72 ºC for 90 s and post extension at 72 ºC for 5 min (9).

Gene cloning

PCR product was submitted to electrophoresis using 1% agarose gel and stained by ethidium bromide. The DNA band was visualized under UV Tran illuminator at 320 nm (10). PCR product was electrophoresed on LMP agarose gel and DNA band was sliced under 320 nm UV and recovered by phenol- chloroform extraction method (11). Recovered DNA was ligated in to pTZ57R cloning vector via T/A cloning method using T4 DNA ligase (12). The ligation reaction was transformed in *E.coli* Top10 strain competent cell (13) and dispensed on LB (Luria-Bertani) agar plate containing ampicillin (50 µg/mL), X-gal (20mM) and IPTG (200 mg/ml). White colonies containing recombinant plasmids were selected (14) and extracted by alkaline minipreparation method (15). Recombinant plasmid was digested by BamHI and EcoRI restriction enzymes, released DNA band was purified and sub cloned in BamHI and EcoRI digested pET32a expression vector. The reaction was transformed into *E.coli* BL21 strain. The resultant colonies containing recombinant plasmids were screened by colony PCR method (16) and mass cultured on LB medium. Recombinant plasmids were confirmed by restriction analysis using HindIII restriction enzyme. PCR product was purified and subjected to sequencing by di deoxy chain termination method (17).

Results

Leishmania promastigotes were grown in N.N.N medium and mass cultured in $RPMI_{1640}$ supplemented with 10% heat inactivated fetal calf serum (FCS) and subjected to DNA extraction by boiling method. Genomic DNA was electrophoresed on 0.8% agarose gel (Fig. 2 A).

Because there is no intron in *Leishmania* genes, PCR reaction was done using ge-

nomic DNA. Fig. 2 B shows an 1140 bp DNA fragment as PCR product of *L. major* mannose-1-phosphate guanyltransferase.

PCR product was purified and cloned in pTZ57R cloning vector and transformed in *E.coli*. Colonies containing recombinant plasmid was screened and mass cultured; plasmid was extracted and electrophoresed on 0.8% agarose gel.

Recombinant plasmid was digested by BamHI and EcoRI restriction enzymes and the 1140bp released fragment DNA band was recovered through electrophoresis on LMP agarose gel.

Mannose -1- phosphate guanyltransferase gene was sub cloned in BamHI, EcoRI digested pET32a expression vector, reaction was transformed into *E.coli* BL21, and colonies containing recombinant plasmid were screened by colony PCR method. Recombinant plasmid was confirmed by HindIII restriction enzyme, again, which has a restriction site on position 231 of mannose-1-phosphate guanyltransferase gene and position 173 on pET32a plasmid. Digested recombinant plasmid was yielded a 900 bp DNA fragment on gel electrophoresis (Fig. 2 C).

The PCR product (mannose-1-phosphate guanyltransferase gene) was purified and sequenced by di deoxy chain termination method (Sanger method) and deposited in GenBank at Accession No. FJ150423. There was 92% homology with sequence of gene related to mannose-1-phosphate guanyltransferase from *L .major* (Fig. 3). There are some amino acid replacements in Iranian *L. major* mannose-1-phosphate guanyltransferase relation to other trypanosomatide organism (alignment was done with online SIFT software: www.sift.jcvi.org) (Fig. 4).

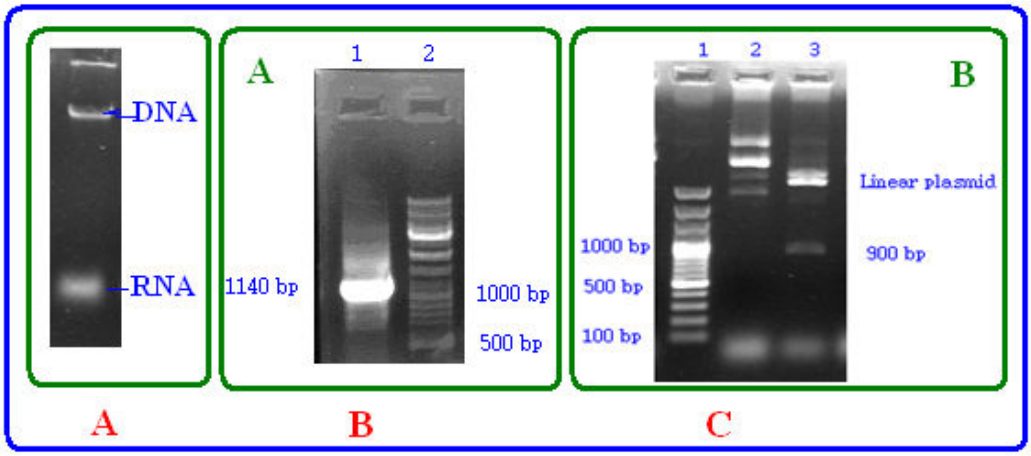

Fig.2: Agarose gel electrophoresis
A: Extracted genomic DNA
B: Lane 1: An 1140bp PCR product as *Leishmania major* mannose -1-phosphate guanyltransferase gene. Lane 2: 100 bp DNA Ladder Marker
C: Lane 1: 100 bp DNA ladder marker. Lane 2: Hind III digested pET32a. Lane 3: Hind III digested recombinant pET32a.

Table 1: Amino acid replacement in Iranian *Leishmania major* mannose-1-phosphate guanyltransferase

Amino acid position	Replaced aa	Amino acid position	Replaced aa
Threonine 147	Serine	Glycine 153	Serine
Valine 155	Isileucine	Proline 159	Methionine
Serine 173	Arginine	Cysteine 197	Proline
Glutamic acid 202	Alanine	Serine 208	Alanine
Alanine 121	Threonine	Isoleucine 227	Valine
Prolone 230	Arginine	Leucine 235	Phenylalanine
Tyrosine 240	Phenylalanine	Phenylalanine 243	Serine
Valine 245	Alanine	Lysine 235	Histidine
Lysine 260	Glutamic acid	Isoleucine 270	Valine
Glycine 319	Aspartic acid	Serine 320	Threonine
Valine 322	Methionine		

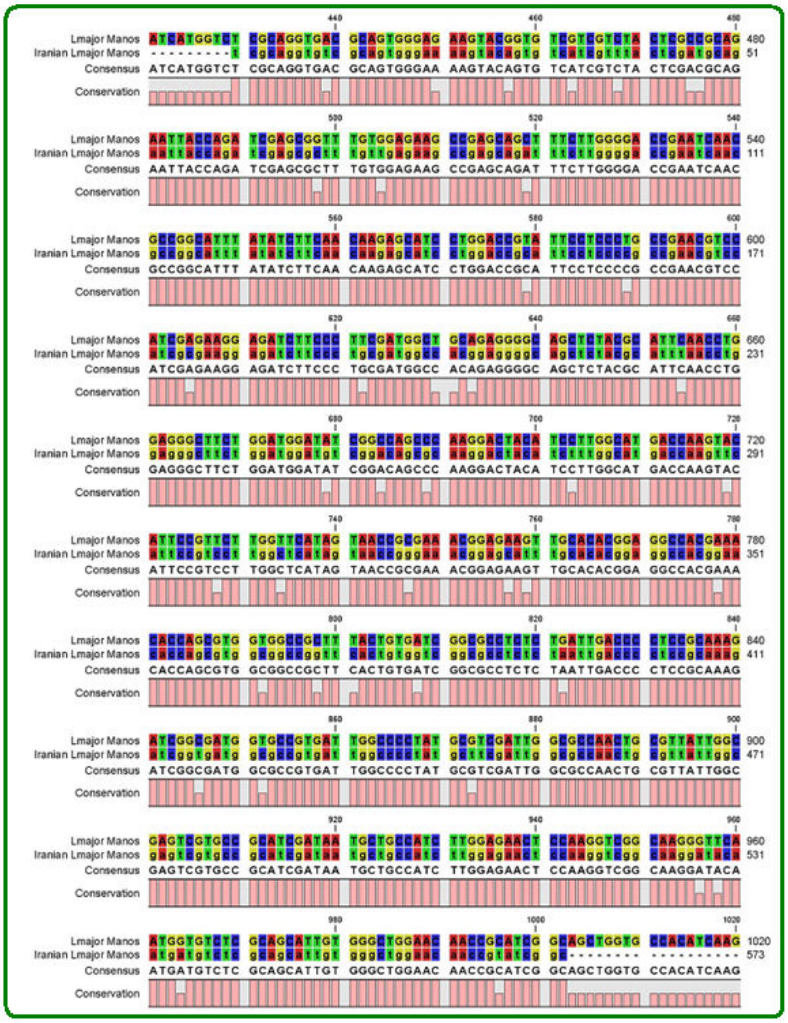

Fig. 3: Comparison of nucleic acids sequence of *leshmania major* mannose-1-phosphate guanyltransferase gene (Accession No. XM_001683281) with sequence of Iranian *Leishmania major* mannose-1-phosphate guanyltransferase gene (Accession No. FJ150423). As illustrated, there are some different nucleic acids in two sequences

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            QUERY  XNTENT-TYP EAPPLICATI -N-CTET-ST REAMSQVSQW EKYS-
VIVYSM
              A4I048  XXXXXXXXXX XXXXXXXXXX XXXXXXXXTI MVSQVTQWEK
YGVVVYSPQN
              Q4QBG5  XXXXXXXXXX XXXXXXXXXX XXXXXXXXTI MVSQVTQWEK
YGVVVYSPQN
              B5TZ85  XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXSQVSQWEK
YSVIVYSMQN
              Q9BLW4  XXXXXXXXXX XXXXXXXXXX XXXXXXXXTI MVSQVTQWEK
YGVVVYSQQS
              A4HCM4  XXXXXXXXXX XXXXXXXXXX XXXXXXXXTI MVSQVEQWEK
YGVVVYSPQT
              Q581X7  XXXXXXXXXX XXXXXXXXXX XXXXXXXXTI AVTKVKDWRK
YGVVVHDEVT
              Q4CU94  XXXXXXXXXX XXXXXXXXXX XXXXXXXXTI AVTKVTDWQK
YGVVVFDEAT
              Q4CMK4  XXXXXXXXXX XXXXXXXXXX XXXXXXXXTI AVTKVTDWQK
YGVVVFDEAT

                      51
100
               QUERY  QNYQIERFVE KPSRFLGDRI NAGIYIFNKS ILDRIPPRRT
SIAKEIFPAM
              A4I048  YQIERFVEKP SRFLGDRINA GIYIFNKSIL DRIPPRRTSI
EKEIFPAMAA
              Q4QBG5  YQIERFVEKP SSFLGDRINA GIYIFNKSIL DRIPPCRTSI
EKEIFPSMAA
              B5TZ85  YQIERFVEKP SRFLGDRINA GIYIFNKSIL DRIPPRRTSI
AKEIFPAMAT
              Q9BLW4  YQIERFVEKP SSFLGDRVNA GIYIFNKSIL DRIPPCRTSI
EKEIFPAMAA
              A4HCM4  YQIERFVEKP KKFLGDRINA GIYIFNKSIL KRIPPCRASI
EKEIFPVMAS
              Q581X7  GAIKQFVEKP PEFVGDRINA GIYIFNKSIL NRIKLEKTSI
ERQVFPMMAS
              Q4CU94  GVIDQFVEKP KNFVGDRINA GIYVFNKSVL NRIKLEKTSI
ETQVFPQMAS
              Q4CMK4  GVIDQFVEKP KNFVGDRINA GIYVFNKSVL NRIKVEKTSI
ETQVFPQMAS

                      101
150
               QUERY  ATEGQLYAFN LEGFWMDVGQ RKDYIFGMTK FIPSLAHSNR
ETEHLHTEAT
              A4I048  EGQLYAFNLE GFWMDVGQPK DYILGMTKFI PSLVHGNRET
EQLHTEDMEH
              Q4QBG5  EGQLYAFNLE GFWMDIGQPK DYILGMTKYI PFLVHSNRET
EKLHTEATKH
              B5TZ85  EGQLYAFNLE GFWMDVGQRK DYIFGMTKFI PSLAHSNRET
EHLHTEATEH
              Q9BLW4  EGELYAFNLE GFWMDVGQPK DYILGMTKFI PSLLDGDRKT
EQLHTEATEH
              A4HCM4  EGQLYAFNVE GFWMDIGQPK DYILGMSKFI ESLVSSGCET
EQLRTEAKEH
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                Q581X7  DSQLSAFHLE GFWMDIGVPK DYIEGMGKYL DSLAGTSTEV
GFSCDERRVS
                Q4CU94  AKQLCAFILE GFWMDIGVPK DYIEGVGKYL RSL-NGTPKE
SQEVYGLGQA
                Q4CMK4  AKQLCAFILE GFWMDIGVPK DYIEGVGKYL RSL-NGTPKE
SQEVYGFGQA

                        151
200
                 QUERY  EHQRGGRFTV VGASLIDPSA KIGDGAVIGP YASIGANCVI
GESCRIDNAA
                A4I048  QRGGRFTVIG ASLIDPSAKI GDGAVIGPYA SIGANCVIGE
SCRIDNAAIL
                Q4QBG5  QRGGRFTVIG ASLIDPSAKI GDGAVIGPYA SIGANCVIGE
SCRIDNAAIL
                B5TZ85  QRGGRFTVVG ASLIDPSAKI GDGAVIGPYA SIGANCVIGE
SCRIDNAAIL
                Q9BLW4  QHGGRFTVVG ASLIDPSAKI GDGAVIGPCA SIGANCVIGE
SCRIDNAAIL
                A4HCM4  QNGSRFAVVG ASLIHPTAKI GDGAVIGPHA SIGANCVIGE
SCRINNAAIL
                Q581X7  DRS--YVLKG CVMIHPTAKI GEGSVIGPHV SIGPGCVIGP
CCRIQRTAIL
                Q4CU94  HKTDDFTVIG SVIIDPSAKI GKGCVIGPFA TIGPGCVIGP
TSRIRHSAIL
                Q4CMK4  HKTDDFTVIG SVIIDPSAKI GKGCVIGPFA TIGPGCVIGP
TSRIRNSAIL

                        201
250
                 QUERY  ILENSKVGKD TMMSRSIVGW N
                A4I048  ENSKVGKGTM VSRSIVGWNN R
                Q4QBG5  ENSKVGKGSM VSRSIVGWNN R
                B5TZ85  ENSKVGKDTM MSRSIVGWNN R
                Q9BLW4  ENSKVGKGTM VSRSIVGWNN R
                A4HCM4  DNTKVGKGTI VVCSIVGWNS R
                Q581X7  DNSTVGRGTL IESSIVGWNG R
                Q4CU94  DESTIGKGTL VDSSIIGWKS R
                Q4CMK4  DESTIGKGTL VDSSIIGWKS R
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Fig. 4: Alignment of amino acids sequence of *Leishmania major* mannose-1-phosphate guanyltransferase with online SIFT software

A4I048: *L. infantum* Mannose-1-phosphate guanyltransferase. Q4QBG5: *L. major* Mannose-1-phosphate guanyltransferase. B5TZ85: Mannose-1-phosphate guanyltransferase. Q9BLW4: *L. Mexicana* GDP-mannose pyrophosphorylase OS=*Leishmania*. A4HCM4: *L. braziliensis* Mannose-1-phosphate guanyltransferase. Q581X7: *T. brucei* Mannose-1-phosphate guanyltransferase. Q4CU94: *T. cruzi* Mannose-1-phosphate guanyltransferase. Q4CMK4: *T. cruzi* Mannose-1-phosphate guanyltransferase

Discussion

Coetaneous leishmaniasis is a main health problem in Iran (18-20). For some biological and ecological aspects, the sand fly vector control has had no considerable success (21-24). WHO researchers emphasize on development of *Leishmania* products, which can affect on *Leishmania* - host immune responses. In this way, *Leishmania* recombinant proteins, which are involved in activating of host immune system, are important. In addition, it is possible to manipulation gene encoding special protein, which is important in pathogenicity of *Leishmania* (25).

Mannose has a key role in glycosylation process in eukaryotes and *Leishmania* glycocojugates are essential for living and proliferation of parasite in the host cell (26, 27). The mannose-1-phosphate guanyltransferase as an antigen is one of the vaccine candidate. In this study, the gene encoding mannose-1-phosphate guanyltransferase from Iranian strain of *L .major* was cloned for the first time. Some investigator suggested that it is appropriate candidate for further investigation either for traditional vaccine or DNA vaccine. Few differences were observed among amino acid sequences of the mannose-1-phosphate guanyltransferase from other strains of *L. major*. Because cysteine amino acid 197 is replaced with proline and some arginin amino acid are inserted into protein strand, these replacement amino acids lead to changes in protein conformation and may be different from other related proteins on its function.

In conclusion, we amplified Iranian *L. major* mannose-1-phosphate guanyltransferase enzyme successfully. The gene was sequenced and cloned in expression vector. It is ready to protein expression.

Acknowledgements

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References

1. Bailey MS, Lockwood DNJ. Cutaneous leishmaniasis. Clinics in Dermatology. 2007; 25: 203–211.
2. Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis. 2004; 27: 305-318.
3. Chappuis F, Sundar S, Hailu A et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbol. 2007; S1- S10.
4. Handman M. Leishmaniasis: Current Status of Vaccine Development. Clin Microbiol Rev. 2001; 14(2): 229-243.
5. Brodskyn C, De Oliveira CI, Barral A, Barral-Netto M. Vaccines in leishmaniasis: advances in the last five years. Expert Rev Vaccines. 2003; 2(5): 705-717.
6. Garami A, Ilg T. Disruption of mannose activation in *Leishmania mexicana*: GDP mannose pyrophosphorylase is required for virulence, but not for viability. EMBO J. 2001; 20(14): 3657-3666.
7. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd edition. Cold Spring Harbor . N.Y. Cold. 2001.
8. Fong D, Lee B. Beta tubulin gene of the parasitic protozoan *Leishmania* Mexicana. Mol Biochem Parasitol. 1988; 31(1): 97- 106.
9. Pherson MC, Moller MJ. The Basics from Background to Bench. Understanding PCR 1th. Oxford, UK: Bios Scientific publishers. 2000; P.9-21.

10. Boffey SA. Agarose gel electrophoresis of DNA. In methods in molecular biology vol.2: Nucleic acids. United states : Walker JM, Humana Press. 1984; p.43-50.
11. Gaastra W, Jorgensen PL. The extraction and isolation of DNA from gels. In molecular biology. Vol.2: Nucleic acids. London: Walker JM. 1984; p. 67-76.
12. Gaastra W, Hansen K. Ligation of DNA with T4 DNA ligase. In: methods in Molecular Biology, Vol.2 Nucleic Acids. London: Walker JM, Humana Press. 1984; p.225-230.
13. Hanahan D. Studies on transformation on *E. coli* with plasmids. J Mol Biol. 1983; 166(4): 557-580.
14. Bothwell AL, Yancopulos GD, Alt FW. Methods for cloning and analysis of eukaryotic genes. Spi edition. Boston: Jones and Bartlett publishers. 1990; p. 247-60.
15. Feliciecello I, Chinali G. A modified alkaline lysis method for the preparation of highly purified plasmid DNA from *E.coli*. Anal Biochem.1993; 212(2): 394-401.
16. Agrawal V, Roy N. Contaminating insert degradation by preincubation colony PCR: A method for avoiding false positives in transformant screening. Anal Biochem. 2008; 375(1): 159-161
17. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA. 1977; 74(12): 5463-5467.
18. Mohebali M, Hajjaran H, Hamzavi Y, Mobedi I, Arshi S, Zarei Z, et al. Epidemiological aspects of canine visceral leishmaniosis in the Islamic Republic of Iran. Vet Parasitol. 2005; 129(3-4): 243-251.
19. Rahim KM, Ashkan MM. Epidemiological, clinical and therapeutic features of pediatric kala-azar. Southeast Asian J Trop Med Public Health. 2007; 38(4): 626-630.
20. Yaghobi-Ershadi MR, Akhavan AA, Zahraei-Ramazani Av, et al. Epidemiological study in a new focus of cutaneous leishmaniasis in the Islamic Republic of Iran. East Mediterr Health J. 2003; 9(4): 816-826.
21. Emami MM, Yazdi M. Entomological survey of phlebotomine sand flies (Diptera: Psychodidae) in a focus of visceral leishmaniasis in central Iran. J Vector Borne Dis. 2008;45(1):38-43.
22. Moin-Vaziri V, Depaquit J, Yaghobi-Ershadi MR, Oshaghi MA, Derakhshandeh-Peykar P, Ferte H, Kaltenbach M, Bargues MD, Nadim A, Javadian E, Rassi Y, Jafari R. Geographical variation in populations of Phlebotomus (Paraphlebotomus) caucasicus (Diptera: Psychodidae) in Iran. Bull Soc Pathol Exot. 2007a; 100(4): 291-295.
23. Moin-Vaziri V, Depaquit J, Yaghobi-Ershadi MR et al. Intraspecific variation within Phlebotomus sergenti Parrot (1917) (Diptera: Psychodidae) based on mtDNA sequences in Islamic Republic of Iran. Acta Trop. 2007b; 102(1): 29-37.
24. Nadim A, Javadiyan E, Mohebali M. The experience of leishmaniazation in the Islamic Republic of Iran. Med J Islamic Rep Iran. 1997; 3(2): 284-289.
25. Khamesipour A, Rafati S, Davoudi N, Maboudi F, Modabber F. Leishmaniasis vaccine candidates for development: A global Overview. Indian J Med Res. 2006; 123: 423-438.
26. Descoteaux A, Turco SJ. Glycoconjugates in *Leishmania* infectivity. Biochim Biophys Acta. 1999 ;1455(2-3): 341-352.
27. Desjardins M, Descoteaux A. Survival strategies of *Leishmania donovani* in mammalian host macrophages. Res Immunol. 1998 ;149(7-8): 689-692.