Protective Efficacy of Live recombinant Leishmania tarentolae Expressing KMP-11 – NTGP96-GFP fusion Combined with Naloxone as a vaccine candidate against visceral Leishmaniasis.

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4 AUTHORS, INCLUDING:

Vahid Nasiri
Razi Vaccine and Serum Research Institute

Abdolhossein Dalimi
Tarbiat Modares University

Fatemeh Ghaffarifar
Tarbiat Modares University

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Complete List of Authors:	Nasiri, Vahid; Razi Vaccine and Serum Research Institute, Department of Parasitology Dalimi, Abdolhosein; Tarbiat Modares University, Parasitology Ghaffarifar, Fatemeh; Faculty of Medical Sciences, Tarbiat Modares University, Department of Parasitology Bolhassani, Azam; Pasteur Institute of Iran, Department of Hepatitis & AIDS

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Protective Efficacy of Live *Leishmania tarentolae* Expressing KMP11-NTGP96-GFP fusion as a vaccine candidate against visceral Leishmaniasis

Vahid Nasiri [2], Abdolhossein Dalimi [1], Fatemeh Ghaffarifar [1], Azam Bolhassani [3]

[1] Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

[2] Department of Parasitology, Razi Vaccine and Serum Research Institute, Alborz, Iran

[3] Department of Hepatitis & AIDS, Pasteur Institute of Iran, Tehran, Iran

Correspondance: Abdolhossein Dalimi, Department of Parasitology and Entomology, Faculty of Medical Sciences, Tarbiat Modares University, P.O.Box: 14115-111, Tehran, Iran (e-mail: dalimi_a@modares.ac.ir).

Short Running: recombinant *Leishmania tarentolae* -KMP11-NTGP96-GFP vaccine

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Keywords: KMP-11, NT-GP96, *Leishmania tarentolae, Leishmania infantum*, visceral leishmaniasis,vaccine.

SUMMARY

Leishmaniases are neglected tropical diseases that cause human infections varying from self-healing cutaneous lesions to mucosal diffuse cutaneous and visceral forms. It has been reported that KMP-11 is fully protective antigen and induces very high cellular immune response and NT-GP96 acts as a strong biologic immunologic adjuvant. The use of the non-pathogenic *Leishmania tarentolae* as a live vaccine vector to deliver specific *Leishmania* antigens is a recent approach. KMP-11 and NT-GP96 genes cloned into the pJET1.2/blunt cloning vector and then into pEGFP-N1 expression vector. The KMP-11, NT-GP96 and GFP fused in pEGFP-N1 and subcloned into *Leishmania*n pLEXSY-neo vector. Finally this construct transferred to *Leishmania tarentolae* by electroporation. Tranfection confirmed by SDS-PAGE, WESTERN blot, flowcytometry and RT-PCR. Both humoral and cellular immune responses were assessed before and at 4 weeks after challenge with wild type *Leishmania infantum*. Results indicated that the strong protective efficacy was observed with live recombinant parasites.

INTRODUCTION

Leishmaniases include a wide variety of diseases that range in severity of self-healing cutaneous leishmaniasis to fatal disseminated visceral leishmaniasis that affect about 12 million people worldwide(1). As per WHO report, nearly 200,000 to 400,000 new cases of VL occur annually with 20,000 to 30,000 deaths per year (2). Already, there is no any effective vaccine for human leishmaniases and challenge with the disease is restricted only to chemotherapy of some resistance parasites with limited number of toxic drugs(3,4). Several antigens such as KMP-11, LeIF, GP63 ,p36/LACK, CP A-B, LD1, PSA-2, TSA/LmSTI1, PFR2, GP46, HASPB1, LCR1, and A2 have been tested as vaccine candidates(2), but they have not given any completely satisfied results.

Among various Leishmania molecules that have been identified as potential candidate antigens for second-generation vaccines, KMP-11 has attracted much attention because of its highly antigenicity for murine, canine and human T cells(5–8). Some findings suggest that the KMP-11 protein may be involved with mobility in both the parasite and in binding to the host cell. So, it could be considered as a candidate for vaccine production (9) .GP96 is a member of the HSP90 family and plays important roles in innate and adaptive immune responses, besides protein folding and assembly(10,11). Previously, it was demonstrated that the GP96 N-terminal domain has potent adjuvant activity toward hepatitis B surface antigen(12,13).

Live attenuated vaccines are the gold standard for protection against intracellular pathogens(14). Since 1986, it has been shown that *Leishmania* protozoan could be used to express foreign genes(15,16). Among Trypanosomatidae family, *Leishmania tarentolae* is a nonpathogenic parasite of the lizard *gecko Tarentolae annularis* and has been developed as a new potential eukaryotic expression system, as proven in the expression of erythropoietin and tissue plasminogen activator .This parasite can

differentiate into amastigote, but is not able to persist long enough within mammalian macrophages and thus can be used as a vaccine vector to deliver specific *Leishmania* antigens(17–19).

The aim of present study was evaluation of the Protective Efficacy of Live recombinant *Leishmania tarentolae* expressing KMP11-NTGP96-GFP fusion as candidates for live engineered recombinant vaccine against visceral leishmaniasis in BALB/c mice.

MATERIALS AND METHODS

DNA and Plasmids constructs

Genomic DNA of Iranian strain of *Leishmania infantum* (MCAN/IR/07/Moheb-gh.) promastigotes was extracted by commercial DNA Extraction kit (Bioneer, Korea) .A pBluescript-GP96 plasmid containing the Xenopus GP96 DNA (accession number AY187545, 2552 bp) was kindly provided by Dr. Jacques Robert (University of Rochester Medical Center, USA).*Escherichia coli* strains TOP10 (Novagene Co.), pJET1.2/blunt cloning vector (Clone JET™ PCR Cloning Kit ,Fermentas), pEGFP-N1 (Invitrogen Co) and pLEXSY-neo(Jena Bioscience, GmbH) expression vectors were used in this study.

Amplification of KMP-11

Oligonucleotide primers were designed based on the KMP- 11 gene sequences (accession number KF150697, 279 base pair) as follows:

Forward primer: 5'- AGA TCT ACC ATG GCC ACC ACG TAC GAG GAG-3'that ACC ATG: Kozak sequence and AGA TCT: *Bgl II* cut site.

Reverse primer: 5'- GAA TTC CTT GGA TGG GTA CTG CGC AGC-3' that GAA TTC: *EcoRI* cut site and primers without stop codon.

The PCR amplification with Pfu DNA polymerase (Vivantis) and DNA of *leishmania infantum* was done according to: 95ºc for 3 min as initial denaturation, 35 cycles at 95ºc for 30s, 60ºc for 30s, 72ºc for 30s and then 72ºc for 10 min as final extension.

Amplification of NT-GP96

The forward and reverse primers for amplifying the NT-GP96 of Xenopus GP96 DNA (accession number AY187545, 1014 base pair) were designed as following:

Forward primer: 5'- CGG GAA TTC GAA GAT GAC GTT GAA -3' that GAA TTC: *EcoRI* cut site.

Reverse primer: 5'- AT GGT ACC TTT GTA GAA GGC TTT GTA-3' that GGT ACC: *KpnI* cut site.

The following program was used for PCR amplification of NT-GP96 with using pBluescript-GP96 plasmid and Pfu DNA polymerase (Vivantis): 95ºc for 5 min as initial denaturation , 30 cycles at 95ºc for 1min, 62 ºC for 2min and 72 ºC for 1.5 min and then 72ºc for 20 min as final extension. Correct insertion confirmed by PCR, restriction enzymes digestion and sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing.

Design of the pLEXSY- KMP 11 -NTGP96-GFP construct

Cloning of KMP 11 -NTGP96 in pEGFP-N1 expression vector

First the KMP-11and NT-GP96 PCR products were ligated into pJET1.2/blunt cloning vector and transformed into *E.coli* TOP10 strain .Then, The plasmids pJET-KMP-11 and pEGFP-N1 were digested by *Bgl II* and EcoRI restriction enzymes. The digested bands of KMP-11 (279 bp) and pEGFP-N1 were purified by gel purification kit (Vivantis Co.) and KMP-11 fragment ligated into digested pEGFP-N1 expression vector and transformed into *E.coli* TOP10 strain .The

recombinant plasmids pEGFP- KMP 11 and pJET-NT-GP96 were digested by *EcoRI* and *KpnI* restriction enzymes and the digested bands of NT-GP96 fragment (1014 bp) ligated into digested pEGFP-N1- KMP 11 and transformed into *E.coli* TOP10 .PCR amplifications were performed on the recombinant colonies using forward of KMP-11and reverse of NT-GP96 genes primers. Colonies containing the recombinant plasmid were selected and recombinant plasmids were extracted by Vivantis plasmid extraction kit and digested by *Bgl II* / *EcoRI* (for KMP-11), *EcoRI* / *KpnI* (for NT-GP96) and *NheI* / *KpnI* (for KMP 11 -NTGP96 fusion) restriction enzymes (Fermentas Co.) for digestion confirmation.

Subcloning of KMP 11 -NTGP96 Fusion in pJET1.2/blunt cloning vector

The forward primer of KMP-11 and reverse primer of NTGP96 for amplifying the KMP 11 -NTGP96 Fusion (1293 base pair) were designed as following:

Forward primer: 5'- GGA TCC ACC ATG GCC ACC ACG TAC GAG GAG-3'that ACC ATG: Kozak sequence and GGA TCC: *BamHI* cut site.

Reverse primer: 5'- AT GGT ACC TTT GTA GAA GGC TTT GTA-3' that GGT ACC: *KpnI* cut site.

The PCR amplification with Pfu DNA polymerase (Vivantis) and pEGFP-N1 - KMP 11 -NTGP96 Fusion plasmid and the following amplification program was done: 95ºc for 5 min as initial denaturation, 30 cycles at 95ºc for 1min, 60 ºC for 2min and 72 ºC for 1.5 min and then 72ºc for 20 min as final extension.

The KMP 11 -NTGP96 Fusion PCR product band was ligated into Pjet1.2 cloning vector and transformed into *E.coli* TOP10 strain. Correct insertion confirmed by PCR, restriction enzymes digestion and sending to the GenFanavaran ® Company (Iran, Tehran) for sequencing.

Sub cloning of KMP 11 -NTGP96 Fusion in pLEXSY-neo *Leishmania* expression vector

The recombinant plasmid pJET- KMP 11-NTGP96 Fusion was digested by *BamHI / KpnI* and pLEXSY-neo was digested by *BglII/KpnI* restriction enzymes and the KMP 11 -NTGP96 Fusion band ligated into digested pLEXSY-neo and ligation transformed into *E.coli* TOP10 strain competent cells and dispersed onto LB agar plates containing 100 µg/ml of ampicillin at 30˚c for overnight. After overnight incubation at 30ºc, colonies that appeared on the agar plate were detected and for confirmation, PCR amplifications were performed on these colonies using forward of KMP-11and reverse of NT-GP96 genes primers and also by using both KMP-11 and NT-GP96 genes forward and reverse primers at a single PCR reaction as a multiplex PCR. Correct insertion confirmed by PCR, restriction enzymes digestion and sending to the GenFanaVaran ® Company (Iran, Tehran) for sequencing.

Sub cloning of Green Florescent Protein (GFP) gene in pLEXSY- KMP 11 -NTGP96

The plasmids pEGFP-N1and pLEXSY- KMP 11 -NTGP96 were digested by *NotI* and *KpnI* restriction enzymes and the digested band of GFP fragment (741 bp) ligated into digested pLEXSY- KMP 11 -NTGP96 and transformed into *E.coli* TOP10.pLEXSY-KMP11-NTGP96-GFP correct insertion confirmed by PCR, restriction enzymes digestion and sending to the GenFanaVaran ® Company (Iran, Tehran) for sequencing.

Design the pLEXSY- KMP 11 -GFP construct

The plasmids pEGFP- KMP-11 and pLEXSY were digested by *BglII* and *NotI* restriction enzymes and the digested band of KMP-11-GFP fragment (1020 bp) ligated into digested pLEXSY and transformed into *E.coli* TOP10.pLEXSY-

KMP11 -GFP correct insertion confirmed by PCR, restriction enzymes digestion and sending to the GenFanaVaran ® Company (Iran, Tehran) for sequencing.

Cultivation and transfection of *Leishmania tarentolae*

The *Leishmania tarentolae* Tar II (ATCC 30143) strain was cultivated in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), at 26 ◦C. For transfection, 3.5×10^7 log-phase parasites were washed and re-suspended in 350 µl of electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose; pH 7.5) and mixed with 50 µl H_2O containing 10 µg of linearized pLEXSY-KMP11-NTGP96-GFP with *SwaI* restriction enzymes(Fermentas, USA), stored on ice for 10 min, and electroporated (Bio-Rad Gene Pulser Ecell, Germany) at 450 V and 500 mF as described previously(20). Then, the electroporated promastigotes were added to in 5ml of RPMI-20% FCS medium without any selective drug and incubated for 24 h at 26ºC.After this period recombinant parasites selected by adding 50 µg/ml of Geniticine (G418)(Sigma, USA) to overnight growth culture of transected parasites. The growth of cells highly resistant to Neomycin was observed after 7–10 days.

Screening of recombinant *Leishmania tarentolae* colonies and confirmation of the KMP11-NTGP96-GFP Fusion Gene Expression

PCR confirmation

To confirm the integration of the KMP11-NTGP96-GFP Fusion into the *ssu* locus of *Leishmania genome*, PCR was performed by using genomic DNA of transgenic strains as a template and *ssu* forward primer F3001 (5'-GATCTGGTTGATTCTGCCAGTAG-3') and reverse primer A1715 hybridizing within the 5'UTR of the target gene (5'- TATTCGTTGTCAGATGGCGCAC-3') with annealing temperature 60ºC according to the LEXSY Kit protocol (Jena bioscience,

Germany). One of the primers hybridizes within the expression cassette and other hybridizes to the *ssu* sequence not present in the plasmid. Integration of the expression cassette into the *ssu* locus yielded a 1 kb fragment that was not obtained in the control reactions with the genomic DNA of *Leishmania tarentolae* wild type.

RNA Extraction and Reverse-Transcription PCR

Total RNA extracted from promastigote of *Leishmania tarentolae* with the RNX Plus kit (Cinnagene ®) according to the manufacturer's instructions. The RNA concentration and quality was assessed by both UV absorbance and electrophoresis on the 2% agarose gel. The total RNA reverse was transcribed to cDNA (with using RevertAid™ H Minus Reverse Transcriptase, Fermentas®) and this cDNA was used as template DNA for RT-PCR amplification. To detect the KMP11, NT-GP96 and Fusion, PCR reactions were carried out using specific primer pairs to amplify each gene separately. The RT-PCR product was analyzed by electrophoresis on a 1.2% agarose gel.

Flow Cytometry and Fluorescence Microscopy analysis

Analysis promastigote forms of *L.tarentolae*-A2-CPA-CPB-CTE-GFP were examined for GFP expression by Epifluorescence microscopy. Promastigotes were centrifuged in 3000 rpm for 10 min and after washing once with PBS, cells were re-suspended in PBS and mounted on microscope slides. Expression of EGFP protein was evaluated by Epifluorescent microscopy. For flow cytometric measurement, parasites at two different growth phases (logarithmic and stationary phases) were centrifuged at 3000 rpm for 10 min, washed with PBS and then resuspended at 10^{6} cell/ml in PBS and stored on ice. Cells were analyzed on a FACS caliber flow cytometer (BD: Becton Dickinson, Franklin Lakes, NJ) equipped with a 15 mV, 488 nm, air-cooled argon ion

laser. 50,000 events were recorded and EGFP expression in transgenic *Leishmania tarentolae* was measured in comparison with wild type (WT) parasites.

SDS–PAGE and Western Blot Analysis

Promastigote forms of the transformed *L. tarentolae*-A2-CPA-CPB-CTE-GFP and the wild type parasites were harvested by centrifugation at 3000 rpm for 15 min and washed in PBS and the pellets were lysed in 5X SDS-PAGE sample and then boiled for 5 min. Samples were then loaded on a 12.5% SDS-PAGE. The gels were transferred onto a nitrocellulose membrane and Western blotting was performed according to the standard procedure(21).

Mice, Immunization Schedules and challenge infection

Female inbred BALB/c mice, 7 week-old were acquired from the Animal Breeding Facility Centre of Razi Vaccine and Serum Research Institute, Karaj, Iran. They were housed in clean cages and fed ad libitum. The immunization experiments were carried out in four groups of mice (n= 15 at each group) and all tests were done in triplicate. The first group received PBS only and Group 2 immunized with 2×107 Wild *Leishmania tarentolae* as the control; group 3 vaccinated with 2×10^7 recombinant *L. tarentolae*-KMP11-GFP; group 4 vaccinated with 2×10^7 recombinant *L.tarentolae*-KMP11-NTGP96-GFP. All groups were immunized subcutaneously via footpad. Four weeks after the last immunization, all animals were challenged with 10^7 stationary phase of *Leishmania infantum* strain JPCM5 (MCAN/ES/98/LLM-877) virulent promastigotes by intra peritoneal injection.

Determination of Parasite Burden

Five mice from each group were sacrificed at 4 weeks after challenge and parasite burden in the spleens was quantitatively determined by serial dilutions method. Briefly, A piece of spleen was excised, weighed and then homogenized with a tissue

grinder in 2 ml of RPMI-1640 medium (Sigma, USA) supplemented with 20% heat-inactivated fetal calf serum. Under sterile conditions, serial dilutions ranging from 1 to 10^{-20} were prepared in wells of 96 well microtitration plates. After 3, 7 and 14 days after incubation at 26ºC, plates were examined with an inverted microscope at a magnification of 40×. The presence or absence of mobile promastigotes was recorded in each well. The final titer was the last dilution for which the well contained at least one motile parasite. The number of parasites per gram was calculated in the following way: parasite burden = $-\log_{10}$ (parasite dilution/tissue weight)(22,23).

Determination of Antibody response

Before challenge and 4 week after challenge, all groups of mice were bled retro-orbitally and the levels of anti-soluble Leishmania antigen(SLA) IgG1 and IgG2a Abs were evaluated using ELISA method according to the manufacturer's instruction (Mouse IgG2a&1 detection kit, ebioscience, USA).

Cytokine Assays

To determine the levels of IFN- γ and IL-4, in each group of experiment five mice were sacrificed before and also 4 weeks after challenge and spleen of them were removed and homogenized in PBS. After erythrocytes lysis using ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2-EDTA), splenocytes were washed with PBS and resuspended in RPMI-10% FCS. Cells were then seeded at a density of 3.5×10^6 cells/ml in the presence of *Leishmania tarentolae*-KMP11-NTGP96-GFP Freeze/Thawed (25 mg/ml). Concanavalin A (Con A; 5 mg/ml) and medium alone were used as the positive and the negative control respectively. Plates were incubated for 72h at 37ºC in 5% CO2 humidified atmosphere for IFN- γ and IL-4 measurement. The IFN- γ and IL-4 production in supernatants of splenocytes cultures was measured

by ELISA kits (U-CyTech, Netherlands), according to the manufacturer's instructions. All experiments were run in triplicates.

Ethics Statement

This research was carried out accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Tarbiat Modares university and all animals experiments including maintenance, handling and blood collection were approved by Institutional Animal Care and Research Advisory Committee of Tarbiat Modares university based on the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education of Iran.

Statistical Analysis

Statistics were performed using SPSS version 18 and one way ANOVA (Multiple-comparison Tukey post Hoc test) and Student's t-test was employed to assess the significance of the differences between the mean values of control and experimental groups. Differences were considered statistically significant when $p < 0.05$. Data shown represent the mean values ± standard error of the mean (SEM) of three independent experiments

RESULTS

Constructing molecular structures

Following PCR amplification, a 279 bp DNA fragment for KMP-11 and 1014 bp for NT-GP96 were identified by agarose gel electrophoresis. The PCR products were successfully ligated into a pJET1.2/blunt cloning vector and subsequently, KMP-11 fragment was ligated into pEGFP-N1 expression vectors and then recombinant pEGFP-N1- KMP 11 -NTGP96, pJET- KMP 11 -NTGP96, pLEXSY- KMP11-NTGP96 and pLEXSY- KMP11-NTGP96-GFP plasmids were constructed

successfully. Following PCR amplifications using pLEXSY- KMP11-NTGP96-GFP plasmids as the template and forward and reverse primers specific for KMP-11 and NT-GP96 genes all three DNA fragment (279 bp for KMP-11, 1014 bp for NT-GP96 and 1293 bp for KMP11-NTGP96 fusion) was identified by agarose gel electrophoresis of the PCR products (Fig. 1).Accurate presence of all constructs confirmed by enzyme digestion, PCR amplification and sequencing.

Confirmation of construction of Recombinant *Leishmania tarentolae* expressing the KMP11-NTGP96-GFP Fusion

Recombinant *Leishmania tarentolae* stably expressing the KMP11-NTGP96-GFP Fusion gene were generated by introducing the linearized pLEXSY- KMP11-NTGP96-GFP vector into the 18S rRNA *ssu* locus of *Leishmania tarentolae*. Specific targeting of the expression cassette into the *ssu* locus was confirmed both by genomic PCR with gene specific primers (Fig.2.A) and vector specific primers (Fig.3) . Amplification of all expected genes in a RT-PCR product, confirmed the expression of KMP11-NTGP96-GFP by *Leishmania tarentolae* at the mRNA level (Fig. 2.B). Expression of EGFP (marker for expression of KMP11-NTGP96-GFP) in recombinant *Leishmania tarentolae* parasites was confirmed by fluorescence microscopy (Fig. 4.B) and also by fluorescence-activated cell sorting (FACS) analysis (Fig. 4.C). The KMP11-NTGP96-GFP expression was also assessed by western blot analysis. As shown in Figure 4.A, immunoreactive bands were detected in recombinant *Leishmania tarentolae* parasites using polyclonal antibody.

Immunization with recombinant *Leishmania tarentolae* and determination of Parasite burden

Four groups of mice were considered for immunization with two subsequent repeats as described in Materials and Methods. The results are shown, obtained from these

two independent experiments. The degree of protection against infection was determined by measurement of the parasite burden in the spleen at 4weeks after challenge with *Leishmania infantum*. As shown in Figure 5, immunization using a *Leishmania tarentolae*-KMP11-NTGP96-GFP reduced the infection in the spleen at 4 weeks after challenge in contrast to the control groups (PBS and wild *Leishmania tarentolae*).

Evaluation of IFN- γ /IL-4 ratio after immunization with Live Recombinant parasites

The levels of IFN-γ and IL-4 production were analyzed before and 4 weeks after challenge in the supernatant of the spleen cells culture of all five groups following stimulation with Freeze/Thawed *Leishmania tarentolae*-KMP11-NTGP96-GFP. As shown in Figure 6-I-A, stimulation of isolated splenocytes from vaccinated group with *Leishmania tarentolae*-KMP11-NTGP96-GFP prior and 4 weeks after challenge elicited a significantly higher IFN-γ production than other groups ($p < 0.05$).

The production of IL-4 upon antigen stimulation before and 4 weeks after challenge was also higher in this vaccinated group (Fig. 6-I-B).We further calculated the IFN- γ to IL-4 ratio for each vaccinated group as an indicator of potential immunization (Fig. 6-I-C). The Leishmania specific IFN- γ /IL-4 ratio were higher in *Leishmania tarentolae*-KMP11-NTGP96-GFP vaccinated group compared to the others both at before and 4 weeks after challenge.

IgG antibody isotypes response to Immunization with Live Recombinant *Leishmania tarentolae*

To compare IgG isotypes in different groups, all sera were assayed by ELISA before and 4 weeks after challenge. As shown in Figures 6-II-A´ and 6-II-B´, IgG1 and IgG2a isotypes before challenge were higher in groups that vaccinated with

Leishmania tarentolae-KMP11-NTGP96-GFP and *Leishmania tarentolae*-KMP11-GFP in comparison to the control groups ($p<0.05$). Also, increased amount of IgG2a was seen in these groups at 4 weeks after challenge. Interestingly, in the group vaccinated with Live *Leishmania tarentolae*-KMP11-NTGP96-GFP, a decreased amount of specific IgG1 was detected in comparison to other vaccinated and control groups. The ratio of IgG2a/IgG1 was significant in *Leishmania tarentolae*-KMP11-NTGP96-GFP as compared with all other groups (Fig. 6-II-C´) ($p<0.05$).

DISCUSSION

Leishmania vaccine development has proven to be a difficult and challenging task, which is mostly hampered by inadequate knowledge of parasite pathogenesis and the complexity of immune responses needed for protection(24). If the patient with visceral leishmaniasis left without any treatment it can be lead to death in 90% of cases. The drugs which are currently used as treatment for leishmaniasis cannot be handled easily due to a number of problems including high toxicity and various side effects(25). Therefore, efforts to introduce new candidates for vaccine production are currently being considered. Despite advances in *Leishmania* genomics and proteomics (26), modern biotechnology for antigen expression, purification and delivery, and the large availability of murine models in the field of experimental immunology, *Leishmania* vaccinology still suffers from several bottlenecks that limit the progress towards effective and universal vaccines(27).

KMP-11 is found in all kinetoplastid protozoa (28) and is highly conserved (> 95% homology) in all Leishmania species, suggesting an essential role for this protein in the biology of the parasite(29). Studies on KMP-11 protein have shown that KMP-11 has clearly three immunological roles: B-cell immuno stimulatory, inducer lymphocyte proliferation and response cytotoxic and Immuno protective

in animal models (30,31). The ability of KMP-11 protein to induce proliferation of T lymphocytes was demonstrated (32,33). Ramirez showed that immunization of BALB/c mice with an attenuated strain of *Toxoplasma gondii* expressing the Leishmania KMP-11 protein , induces a specific immune response and immunoprotective in such animals (34). Researches indicated that KMP11 was found in two forms: one in all *Leishmani*a species with two gene copies and the four C-terminal amino acids YPSK, and one in all species except *L. major* with a single-copy gene and ending with NMCK.Besides the C-terminal variation, KMP11 is nearly completely conserved(35).These evidences, strongly indicate that this gene is very excellent target for immunization against Leishmaniasis and our results indicated that above prediction about immunological function of KMP11 is correct..

GP96 as one of the most abundant intracellular heat shock proteins possesses multiple functions. Among these functions, its ability to bridge the innate and adaptive immune systems has attracted extensive interest. Immunotherapies using HSPs to generate specific antitumor responses have been evaluated in clinical studies. GP96 and its N-terminal fragment, but not the C-terminal fragment showed adjuvant effects in enhancing the peptide-specific CTL response against HBV infection and HCC, and this response was peptide concentration dependent(36).The immune effect of GP96 is probably dependent on its ability to bind peptide epitopes. It was reported that both the N- and C-terminal fragments of GP96 are able to bind peptides, with the N-terminal fragment behaving at a similar capacity to the full-length GP96(37,38). Linkage of antigens to HSPs (Hsp70, calreticulin, Hsp60, GP96) represents a potential approach for increasing the potency of DNA vaccines. For example, vaccines containing full lengthHPV16E7 fused to Mycobacterium tuberculosis HSP70 increased the frequency of E7-specific CD8+ T cells by at least 30 fold relative to

vaccines containing the wild-type E7 gene(36). Recently a research indicated that the adjuvant activity of NT (gp96) was more efficient in the induction of immune responses when fused to the C-terminal end of the HCV DNA polytope and the NT (gp96) improved the efficacy of the DNA vaccine, and this immunomodulatory effect was dependent on the position of the fusion(39).All of these research confirms our results that indicated to positive immunological effects of NT-gp96 fusion with KMP11 to make a new effective fusion construct for better stimulation of protective immunity.

In our research, following immunization, a notable pattern was observed on the profile of cytokines and immunoglobulin expression. A significant production of IFN-γ cytokine was detected following immunization with *Leishmania tarentolae*-KMP11-NTGP96-GFP.We also detected considerable levels of IL-4 in immunized mice with this group. These results indicated that our vaccine strategy mediated protection was associated with a mixed Th1/Th2 that is typical of other successful experimental vaccines against visceral leishmaniasis(40). But, the important point is the matter that the level of IFN-γ cytokines were increased after challenge, but this pattern was decreasing about IL-4. Interestingly, the expression ratio of IFN-γ/IL-4 of mice immunized with *Leishmania tarentolae*-KMP11-NTGP96-GFP was significantly higher before and after challenge in comparison to control groups. Also, a significant difference in the expression ratio of IgG2a/IgG1 was observed in mice immunized with this vaccination group.

The important result is that this group also displayed a significant reduction in the parasite load in spleen four weeks after challenge, that suggesting this modulation in the immune response is capable to control infection. The significant parasite load reduction observed in the *Leishmania tarentolae*-KMP11-NTGP96-GFP immunized

group after challenge could be a result of the increased expression ratio of IFN-γ/IL-4 and IgG2a/IgG1 that indicated to activation of Th1cell mediated immune response that could have an impact on parasite establishment and disease development pattern. Vaccines based on combination of different antigen candidates have been shown to improve protection. In fact, a recombinant Q protein formed by genetic fusion of five parasite intracellular antigens has been successfully tested in dogs(41–43).

The data shown herein indicates that *Leishmania tarentolae*-KMP11-NTGP96-GFP group was able to confer a significant degree of protection against *L. infantum*.

For effective intervention measures to control VL in endemic areas, it is imperative to design a vaccine, which is the most economical way of controlling infectious diseases(2). For many years, many laboratories around the world have worked on the development of an effective vaccine against leishmaniasis(44). Different Vaccination studies in experimental murine models have shown that live attenuated forms of parasites gives better protection when compared to other recombinant antigens(45). *Leishmania tarentolae* is a parasite of the *gecko Tarentolae annularis* and because of its several unique features, including higher specific growth rate compared to mammalian cells, cultivation in low cost media, safety for humans, possibility to introduce several copies of a foreign gene into the parasite genome and production of recombinant proteins with an animal-like N-glycosylation pattern is a feasible eukaryotic expression system for high level production of active recombinant biopharmaceuticals (18,46). A couple of recombinant pharmaceutical and non-pharmaceutical glycoproteins such as human erythropoietin, tissue plasminogen activator and laminin- 332 have already been produced in this expression system and in all cases, the expressed proteins were biologically active(18,19,47). These advantages, in addition to feasibility for constitutive or regulative protein production,

make *Leishmania tarentolae* an attractive host for high level production of heterologous proteins(18,19,48,49) and as a ideal candidate for vaccine against leishmaniases.

In conclusion, this study shows that immunization with live recombinant *Leishmania tarentolae*-KMP11-NTGP96-GFP provides significant protection against visceral leishmaniasis in the BALB/c mice. According to the results and information presented in this study, it seems that using fusion strategy between KMP-11 and GP96 can lead us to making an effective construct for increasing the efficiency of a live recombinant vaccine against visceral leishmaniasis. Designing of effective live recombinant vaccines is attractive in terms of low cost, optimal safety, stability and potency when compared with other vaccination strategy. In addition, further investigation about its efficacy should be done in the susceptible hamster and dog model of visceral leishmaniasis and this strategy could have a broader impact on vaccine development efforts for visceral leishmaniasis worldwide.

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Conceived and designed the experiments: VN, AD, FG and AB. Performed the experiments: VN, AD, FG and AB. Analyzed the data: VN, AD, FG and AB. Contributed reagents/materials/analysis tools: VN, AD, FG and AB.Wrote the paper: VN, AD, FG and AB. The present work is part of PhD. thesis, supported financially only by Medical Sciences Faculty of Tarbiat Modares University (Grant No. 52.112071).

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Fig.2. Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes in PCR reaction with using: A. recombinant *Leishmania tarentolae* DNA as the template and genes specific primers that Lane1: 1kb DNA ladder; Lanes 2, 3, 4: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively) ;and **B.** recombinant parasite cDNA as the template and genes specific primers that Lanes 1, 2, 3: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively); lane 4: wild *leishmania tarentolae* as the negative control; Lane5: 1kb DNA ladder.

Fig.3. Electrophoresis of the amplified fragments (1 Kb) of specific targeting of the expression cassette into the ssu locus in PCR reaction with using recombinant Leishmania tarentolae DNA as the template and vector specific primers on 1.2 % (w/v) agarose gel. Lane1: 1kb DNA ladder; Lanes 2: expanded bands of 1Kb.

Fig.4. Expression of the fusion gene by *Leishmania tarentolae*. (A) Western blot analysis for evaluating expression of the KMP11-NTGP96-GFP fusion protein. (B) Expression of EGFP by recombinant *Leishmania tarentolae*

promastigotes.(C)Percentage of the EGFP positive population in *Leishmania tarentolae* promastigotes transfected with either pLEXSY-KMP11-NTGP96-GFP (I) or pLEXSY-KMP11-GFP (II) that shown in green lines in comparison to wild type parasite that shown in red lines as determined by flowcytometry.

Fig.5. spleen parasite burden in all groups following immunization and infectious challenge with *Leishmania infantum*. The parasite number in spleen was evaluated at 4 weeks after challenge.

Figure.6. Analysis of the specific cellular and humoral response factors in vaccinated and control groups before and after challenge. (I) Cytokine production by splenocytes in vaccinated and control groups .A: IFN- γ production by splenocytes in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP ($p < 0.05$);**B:** IL-4- γ production by splenocytes in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP ($p < 0.05$);**C:** IFN- γ /IL-4 ratio in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP ($p < 0.05$). **(II)Analysis of the specific IgG isotypes in vaccinated and control groups. A´:** Specific IgG2a antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups ($p < 0.01$); **B´:** Specific IgG1 antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (*P < 0.01 compared to PBS); **C´:** IgG2a/IgG1 ratio in vaccinated and control groups before and 4 weeks after challenge (*P < 0.01 compared to PBS).

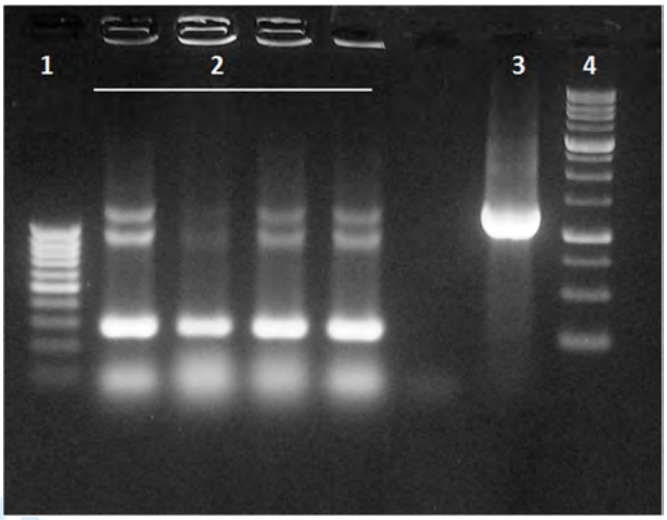


Fig.1

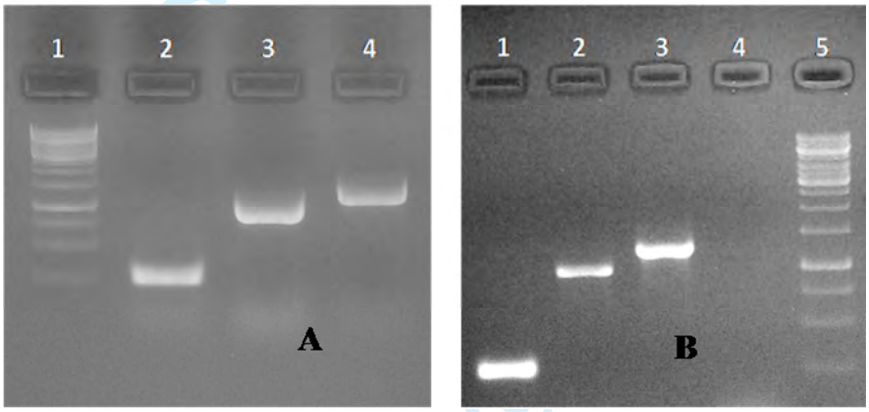


Fig.2

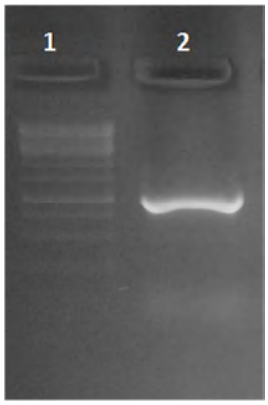


Fig.3

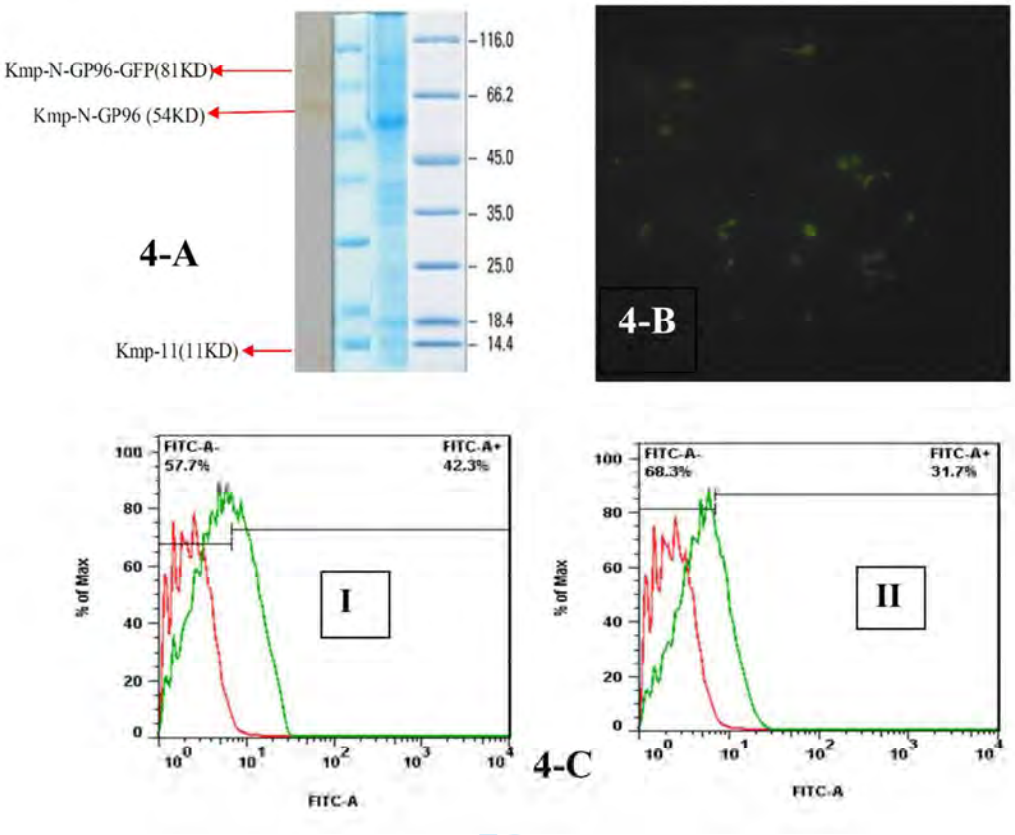

Kmp-N-GP96-GFP(81KD)
Kmp-N-GP96 (54KD)
Kmp-11(11KD)
116.0
66.2
45.0
35.0
25.0
18.4
14.4
4-A
4-B
FITC-A-
57.7%
FITC-A+
42.3%
I
% of Max
FITC-A
FITC-A-
68.3%
FITC-A+
31.7%
II
4-C

Fig.4

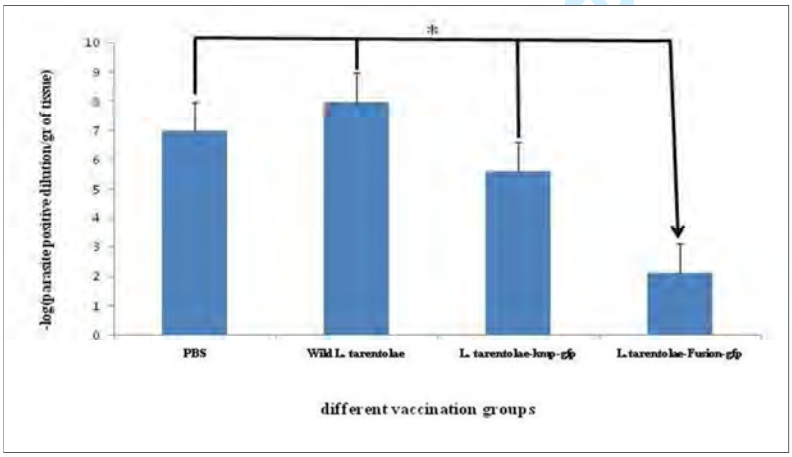

-log(parasite positive dilution/gr of tissue)
PBS
Wild L. tarentolae
L. tarentolae-kmp-gfp
L.tarentolae-Fusion-gfp
different vaccination groups

Fig.5

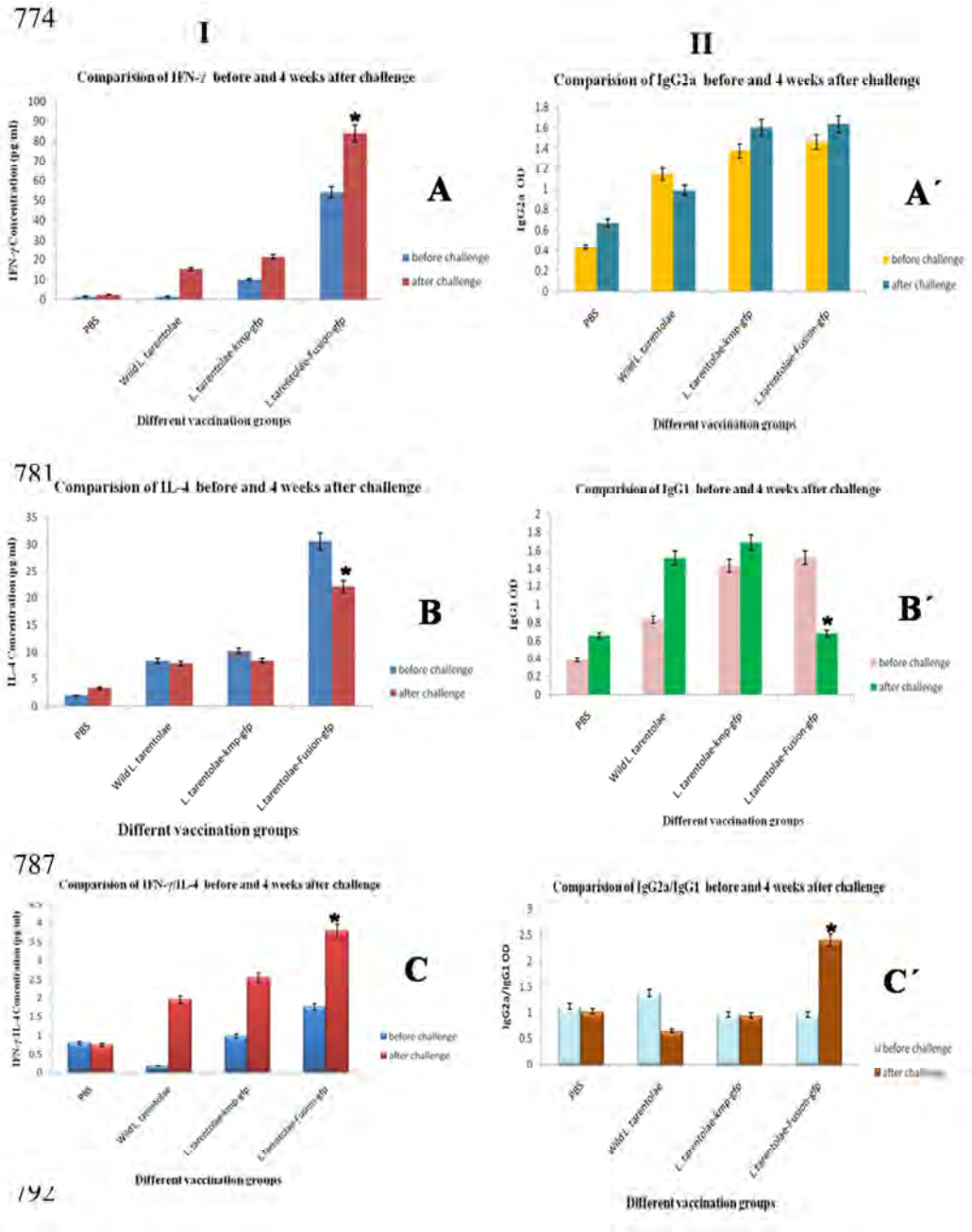
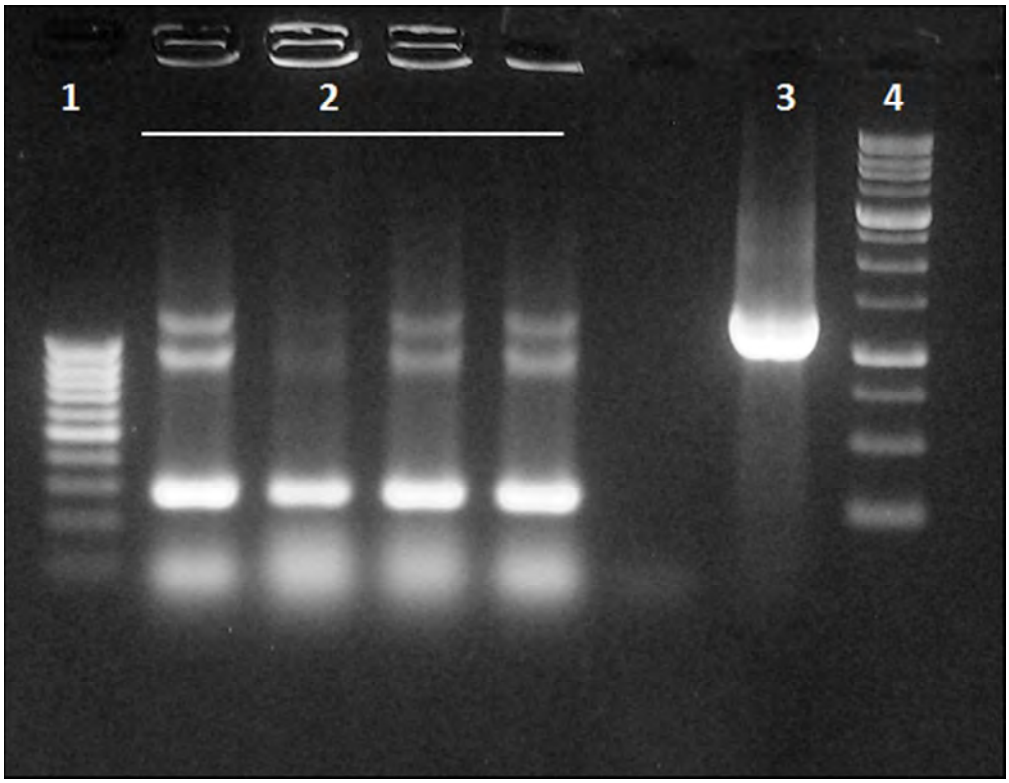

I
Comparision of IFN-γ before and 4 weeks after challenge
IFN-γ Concentration (pg/ml)
A
before challenge
after challenge
PBS
Wild L. tarentolae
L. tarentolae-kmp-gfp
L.tarentolae-Fusion-gfp
Different vaccination groups
II
Comparision of IgG2a before and 4 weeks after challenge
IgG2a OD
A´
Comparision of IL-4 before and 4 weeks after challenge
IL-4 Concentration (pg/ml)
B
Differnt vaccination groups
Comparision of IgG1 before and 4 weeks after challenge
IgG1 OD
B´
Comparision of IFN-γ/IL-4 before and 4 weeks after challenge
IFN-γ/IL-4 Concentration (pg/ml)
C
Comparision of IgG2a/IgG1 before and 4 weeks after challenge
IgG2a/IgG1 OD
C´

Figure.6



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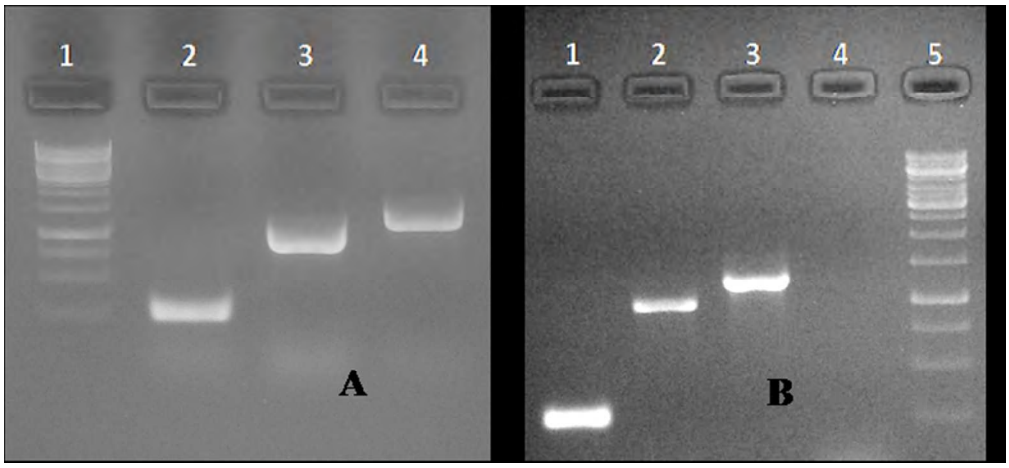

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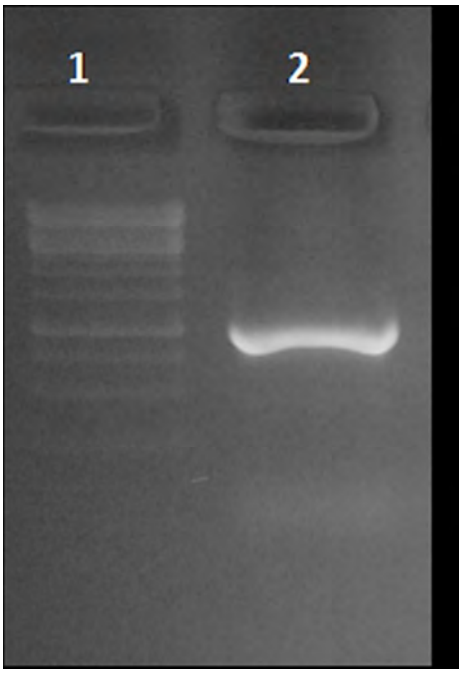

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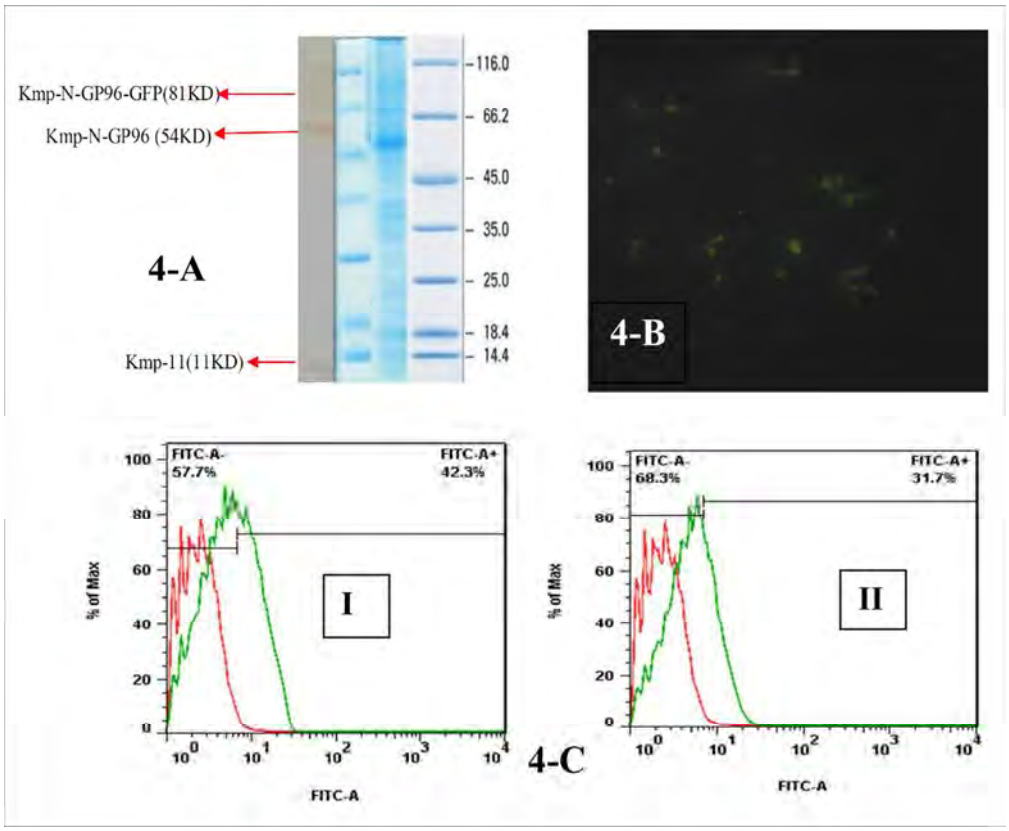

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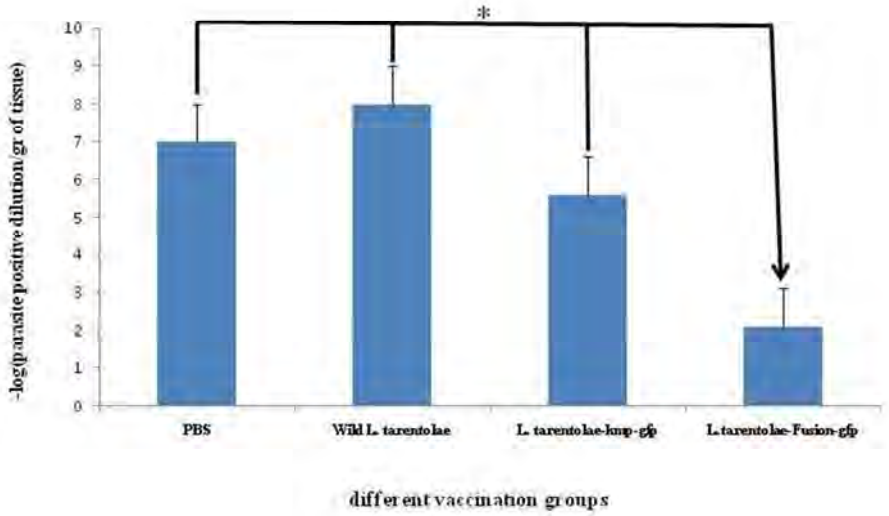

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195x125mm (96 x 96 DPI)

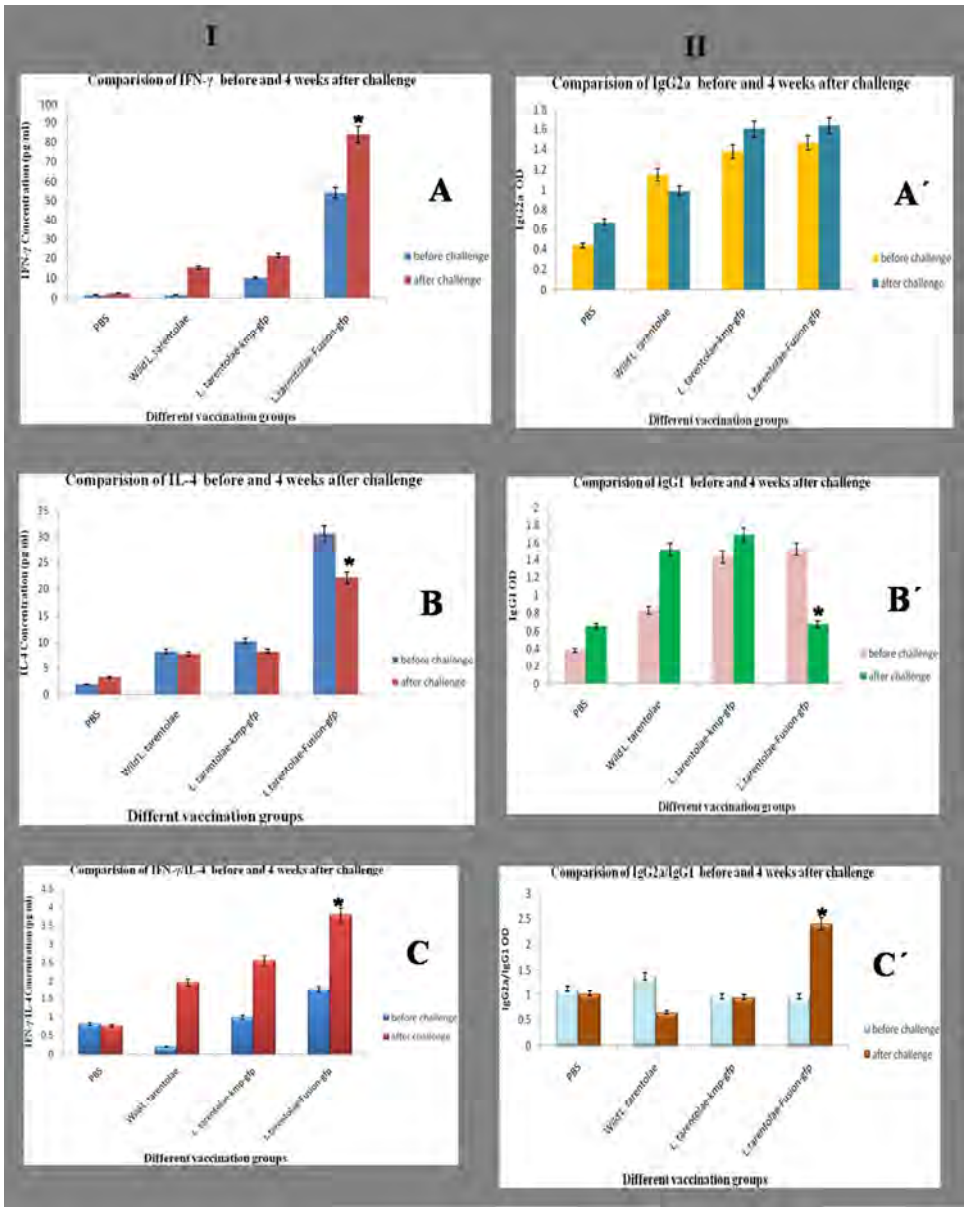

Figure.6. Analysis of the specific cellular and humoral response factors in vaccinated and control groups before and after challenge. (I) Cytokine production by splenocytes in vaccinated and control groups .A: IFN-γ production by splenocytes in vaccinated and control groups after stimulation with F/T Leishmania tarentolae-KMP11-NTGP96-GFP ($p < 0.05$);B: IL-4- γ production by splenocytes in vaccinated and control groups after stimulation with F/T Leishmania tarentolae-KMP11-NTGP96-GFP ($p < 0.05$);C: IFN- γ /IL-4 ratio in vaccinated and control groups after stimulation with F/T Leishmania tarentolae-KMP11-NTGP96-GFP ($p < 0.05$). (II)Analysis of the specific IgG isotypes in vaccinated and control groups. A´: Specific IgG2a antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups ($p < 0.01$); B´: Specific IgG1 antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (*$P < 0.01$ compared to PBS); C´: IgG2a/IgG1 ratio in vaccinated and control groups before and 4 weeks after challenge (*$P < 0.01$ compared to PBS).

Fig.1. Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes in a single PCR reaction (multiplex PCR) on 1.2 % (w/v) agarose gel.Lane1:100 bp DNA Ladder; Lane 2: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively);Lane 3: single expanded band of KMP11-NTGP96 fusion (approximately 1293 bp). Lane4:1kb DNA ladder.

Fig.2. Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes in PCR reaction with using: A. recombinant *Leishmania tarentolae* DNA as the template and genes specific primers that Lane1: 1kb DNA ladder; Lanes 2, 3, 4: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively) ;and **B.** recombinant parasite cDNA as the template and genes specific primers that Lanes 1, 2, 3: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively); lane 4: wild *leishmania tarentolae* as the negative control; Lane5: 1kb DNA ladder.

Fig.3. Electrophoresis of the amplified fragments (1 Kb) of specific targeting of the expression cassette into the ssu locus in PCR reaction with using recombinant Leishmania tarentolae DNA as the template and vector specific primers on 1.2 % (w/v) agarose gel. Lane1: 1kb DNA ladder; Lanes 2: expanded bands of 1Kb.

Fig.4. Expression of the fusion gene by *Leishmania tarentolae*. (A) Western blot analysis for evaluating expression of the KMP11-NTGP96-GFP fusion protein. (B) Expression of EGFP by recombinant *Leishmania tarentolae* promastigotes.(C)Percentage of the EGFP positive population in *Leishmania tarentolae* promastigotes transfected with either pLEXSY-KMP11-NTGP96-GFP (I) or pLEXSY-KMP11-GFP (II) that shown in green lines in comparison to wild type parasite that shown in red lines as determined by flowcytometry.

Fig.5. spleen parasite burden in all groups following immunization and infectious challenge with *Leishmania infantum*. The parasite number in spleen was evaluated at 4 weeks after challenge.

Figure.6. Analysis of the specific cellular and humoral response factors in vaccinated and control groups before and after challenge. (I) Cytokine production by splenocytes in vaccinated and control groups .A: IFN- γ production by splenocytes in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP ($p< 0.05$);**B:** IL-4- γ production by splenocytes in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP ($p< 0.05$);**C:** IFN- γ /IL-4 ratio in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP ($p< 0.05$). **(II)Analysis of the specific IgG isotypes in vaccinated and control groups. A´:** Specific IgG2a antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups ($p< 0.01$); **B´:** Specific IgG1 antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (*P < 0.01 compared to PBS); **C´:** IgG2a/IgG1 ratio in vaccinated and control groups before and 4 weeks after challenge (*P < 0.01 compared to PBS).

Protective Efficacy of Live *Leishmania tarentolae* Expressing KMP11-NTGP96-GFP fusion as a vaccine candidate against visceral Leishmaniasis

Vahid Nasiri [2], Abdolhossein Dalimi [1], Fatemeh Ghaffarifar [1], Azam Bolhassani [3]

[1] Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

[2] Department of Parasitology, Razi Vaccine and Serum Research Institute, Alborz, Iran

[3] Department of Hepatitis & AIDS, Pasteur Institute of Iran, Tehran, Iran

Correspondance: Abdolhossein Dalimi, Department of Parasitology and Entomology, Faculty of Medical Sciences, Tarbiat Modares University, P.O.Box: 14115-111, Tehran, Iran (e-mail: dalimi_a@modares.ac.ir).

Short Running: recombinant *Leishmania tarentolae* -KMP11-NTGP96-GFP vaccine

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Keywords: KMP-11, NT-GP96, *Leishmania tarentolae, Leishmania infantum*, visceral leishmaniasis,vaccine.

SUMMARY

Leishmaniases are neglected tropical diseases that cause human infections varying from self-healing cutaneous lesions to mucosal diffuse cutaneous and visceral forms. It has been reported that KMP-11 is fully protective antigen and induces very high cellular immune response and NT-GP96 acts as a strong biologic immunologic adjuvant. The use of the non-pathogenic *Leishmania tarentolae* as a live vaccine vector to deliver specific *Leishmania* antigens is a recent approach. KMP-11 and NT-GP96 genes cloned into the pJET1.2/blunt cloning vector and then into pEGFP-N1 expression vector. The KMP-11, NT-GP96 and GFP fused in pEGFP-N1 and subcloned into *Leishmania*n pLEXSY-neo vector. Finally this construct transferred to *Leishmania tarentolae* by electroporation. Tranfection confirmed by SDS-PAGE, WESTERN blot, flowcytometry and RT-PCR. Protective Efficacy of this construct evaluated as a vaccine candidate against visceral Leishmaniasis. Parasite burden, humoral and cellular immune responses were assessed before and at 4 weeks after challenge. Results indicated that immunization with *Leishmania tarentolae*-KMP11-NTGP96-GFP provides significant protection against visceral leishmaniasis and was able to induce an increased expression of IFN-γ and IgG2a. Following challenge, a reduced parasite load in the spleen of the KMP11-NTGP96-GFP immunized group was detected. These results suggest that *Leishmania tarentolae*-KMP11-NTGP96-GFP could be considered as a potential tool in vaccination against visceral leishmaniasis.

INTRODUCTION

Leishmaniases include a wide variety of diseases that range in severity of self-healing cutaneous leishmaniasis to fatal disseminated visceral leishmaniasis that affect about 12 million people worldwide(1). As per WHO report, nearly 200,000 to 400,000 new cases of VL occur annually with 20,000 to 30,000 deaths per year (2). Already, there is no any effective vaccine for human leishmaniases and challenge with the disease is restricted only to chemotherapy of some resistance parasites with limited number of toxic drugs(3,4). Several antigens such as KMP-11, LeIF, GP63 ,p36/LACK, CP A-B, LD1, PSA-2, TSA/LmSTI1, PFR2, GP46, HASPB1, LCR1, and A2 have been tested as vaccine candidates(2), but they have not given any completely satisfied results.

Among various *Leishmania* molecules that have been identified as potential candidate antigens for second-generation vaccines, KMP-11 has attracted much attention because of its highly antigenicity for murine, canine and human T cells(5–8). KMP-11 is found in all kinetoplastid protozoa (9) and is highly conserved (> 95% homology) in all *Leishmania* species, suggesting an essential role for this protein in the biology of the parasite(10). Studies on KMP-11 protein have shown that KMP-11 has clearly three immunological roles: B-cell immunostimulatory, inducer lymphocyte proliferation and response cytotoxic and immunoprotective in animal models (11, 12). The ability of KMP-11 protein to induce proliferation of T lymphocytes was demonstrated (13, 14). Ramirez showed that immunization of BALB/c mice with an attenuated strain of *Toxoplasma gondii* expressing the Leishmania KMP-11 protein , induces a specific immune response and immunoprotective in such animals (15). Some findings suggest that the KMP-11 protein may be involved with

mobility in both the parasite and in binding to the host cell. So, it could be considered as a candidate for vaccine production antigen (16).

GP96 is a member of the HSP90 family and plays important roles in innate and adaptive immune responses, besides protein folding and assembly(17, 18). Among these functions, its ability to bridge the innate and adaptive immune systems has attracted extensive interest. Immunotherapies using HSPs to generate specific antitumor responses have been evaluated in clinical studies. GP96 and its N-terminal fragment, but not the C-terminal fragment showed adjuvant effects in enhancing the peptide-specific CTL response against HBV infection and HCC, and this response was peptide concentration dependent(19).

Live attenuated vaccines are the gold standard for protection against intracellular pathogens(20). Since 1986, it has been shown that *Leishmania* protozoan could be used to express foreign genes(21, 22). Among Trypanosomatidae family, *Leishmania tarentolae* is a nonpathogenic parasite of the lizard *gecko Tarentolae annularis* and has been developed as a new potential eukaryotic expression system, as proven in the expression of erythropoietin and tissue plasminogen activator .This parasite can differentiate into amastigote, but is not able to persist long enough within mammalian macrophages and thus can be used as a vaccine vector to deliver specific *Leishmania* antigens(23–25).

The main aim of the present study was to evaluate the protective efficacy of live recombinant *Leishmania tarentolae* expressing KMP11-NTGP96-GFP fusion as candidates for live engineered recombinant vaccine against visceral leishmaniasis in BALB/c mice.

MATERIALS AND METHODS

DNA and Plasmids constructs

Genomic DNA of Iranian strain of *Leishmania infantum* (MCAN/IR/07/Moheb-gh.) promastigotes was extracted by commercial DNA Extraction kit (Bioneer, Korea) .A pBluescript-GP96 plasmid containing the Xenopus GP96 DNA (accession number AY187545, 2552 bp) was kindly provided by Dr. Jacques Robert (University of Rochester Medical Center, USA).*Escherichia coli* strains TOP10 (Novagene Co.), pJET1.2/blunt cloning vector (Clone JET™ PCR Cloning Kit ,Fermentas), pEGFP-N1 (Invitrogen Co) and pLEXSY-neo(Jena Bioscience, GmbH) expression vectors were used in this study.

Amplification of KMP-11

Oligonucleotide primers were designed based on the KMP- 11 gene sequences (accession number KF150697, 279 base pair) as follows:

Forward primer: 5'- AGA TCT ACC ATG GCC ACC ACG TAC GAG GAG-3'that ACC ATG: Kozak sequence and AGA TCT: *Bgl II* cut site.

Reverse primer: 5'- GAA TTC CTT GGA TGG GTA CTG CGC AGC-3' that GAA TTC: *EcoRI* cut site and primers without stop codon.

The PCR amplification with Pfu DNA polymerase (Vivantis) and DNA of *leishmania infantum* was done according to: 95ºc for 3 min as initial denaturation, 35 cycles at 95ºc for 30s, 60ºc for 30s, 72ºc for 30s and then 72ºc for 10 min as final extension.

Amplification of NT-GP96

The forward and reverse primers for amplifying the NT-GP96 of Xenopus GP96 DNA (accession number AY187545, 1014 base pair) were designed as following:

Forward primer: 5'- CGG GAA TTC GAA GAT GAC GTT GAA -3' that GAA TTC: *EcoRI* cut site.

Reverse primer: 5'- AT GGT ACC TTT GTA GAA GGC TTT GTA-3' that GGT ACC: *KpnI* cut site.

The following program was used for PCR amplification of NT-GP96 with using pBluescript-GP96 plasmid and Pfu DNA polymerase (Vivantis): 95ºc for 5 min as initial denaturation , 30 cycles at 95ºc for 1min, 62 ºC for 2min and 72 ºC for 1.5 min and then 72ºc for 20 min as final extension. Correct insertion confirmed by PCR, restriction enzymes digestion and sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing.

Design of the pLEXSY- KMP 11 -NTGP96-GFP construct

Cloning of KMP 11 -NTGP96 in pEGFP-N1 expression vector

First the KMP-11and NT-GP96 PCR products were ligated into pJET1.2/blunt cloning vector and transformed into *E.coli* TOP10 strain .Then, the plasmids pJET- KMP-11 and pEGFP-N1 were digested by *Bgl II* and EcoRI restriction enzymes. The digested bands of KMP-11 (279 bp) and pEGFP-N1 were purified by gel purification kit (Vivantis Co.) and KMP-11 fragment ligated into digested pEGFP-N1 expression vector and transformed into *E.coli* TOP10 strain .The recombinant plasmids pEGFP- KMP 11 and pJET-NT-GP96 were digested by *EcoRI* and *KpnI* restriction enzymes and the digested bands of NT-GP96 fragment (1014 bp) ligated into digested pEGFP-N1- KMP 11 and transformed into *E.coli* TOP10 .PCR amplifications were performed on the recombinant colonies using forward of KMP-11and reverse of NT-GP96 genes primers. Colonies containing the recombinant plasmid were selected and recombinant plasmids were extracted by Vivantis plasmid extraction kit and digested by *Bgl II* / *EcoRI* (for KMP-11), *EcoRI* / *KpnI* (for NT-GP96) and *NheI* / *KpnI* (for KMP 11 -NTGP96 fusion) restriction enzymes (Fermentas Co.) for digestion confirmation.

Subcloning of KMP 11 -NTGP96 Fusion in pJET1.2/blunt cloning vector

The forward primer of KMP-11 and reverse primer of NTGP96 for amplifying the KMP 11 - NTGP96 Fusion (1293 base pair) were designed as following:

Forward primer: 5'- GGA TCC ACC ATG GCC ACC ACG TAC GAG GAG-3'that ACC ATG: Kozak sequence and GGA TCC: *BamHI* cut site.

Reverse primer: 5'- AT GGT ACC TTT GTA GAA GGC TTT GTA-3' that GGT ACC: *KpnI* cut site.

The PCR amplification with Pfu DNA polymerase (Vivantis) and pEGFP-N1 - KMP 11 - NTGP96 Fusion plasmid and the following amplification program was done: 95ºc for 5 min as initial denaturation, 30 cycles at 95ºc for 1min, 60 ºC for 2min and 72 ºC for 1.5 min and then 72ºc for 20 min as final extension.

The KMP 11 -NTGP96 Fusion PCR product band was ligated into Pjet1.2 cloning vector and transformed into *E.coli* TOP10 strain. Correct insertion confirmed by PCR, restriction enzymes digestion and sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing.

Sub cloning of KMP 11 -NTGP96 Fusion in pLEXSY-neo *Leishmania* expression vector

The recombinant plasmid pJET- KMP 11-NTGP96 Fusion was digested by *BamHI / KpnI* and pLEXSY-neo was digested by *BglII/KpnI* restriction enzymes and the KMP 11 -NTGP96 Fusion band ligated into digested pLEXSY-neo and ligation transformed into *E.coli* TOP10 strain competent cells and dispersed onto LB agar plates containing 100 µg/ml of ampicillin at 30˚c for overnight. After overnight incubation at 30ºc, colonies that appeared on the agar plate were detected and for confirmation, PCR amplifications were performed on these colonies using forward of KMP-11and reverse of NT-GP96 genes primers and also by using both KMP-

11 and NT-GP96 genes forward and reverse primers at a single PCR reaction as a multiplex PCR. Correct insertion confirmed by PCR, restriction enzymes digestion and sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing.

Sub cloning of Green Florescent Protein (GFP) gene in pLEXSY- KMP 11 -NTGP96

The plasmids pEGFP-N1and pLEXSY- KMP 11 -NTGP96 were digested by *NotI* and *KpnI* restriction enzymes and the digested band of GFP fragment (741 bp) ligated into digested pLEXSY- KMP 11 -NTGP96 and transformed into *E.coli* TOP10.pLEXSY-KMP11-NTGP96-GFP correct insertion confirmed by PCR, restriction enzymes digestion and sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing.

Design the pLEXSY- KMP 11 -GFP construct

The plasmids pEGFP- KMP-11 and pLEXSY were digested by *BglII* and *NotI* restriction enzymes and the digested band of KMP-11-GFP fragment (1020 bp) ligated into digested pLEXSY and transformed into *E.coli* TOP10.pLEXSY-KMP11 -GFP correct insertion confirmed by PCR, restriction enzymes digestion and sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing.

Cultivation and transfection of *Leishmania tarentolae*

The *Leishmania tarentolae* Tar II (ATCC 30143) strain was cultivated in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), at 26 ◦C. For transfection, 3.5×10^{7} log-phase parasites were washed and re-suspended in 350 µl of electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose; pH 7.5) and mixed with 50 µl H_2O containing 10 µg of linearized pLEXSY-KMP11-NTGP96-GFP with *SwaI* restriction enzymes(Fermentas, USA), stored on ice for 10 min, and

electroporated (Bio-Rad Gene Pulser Ecell, Germany) at 450 V and 500 mF as described previously(26). Then, the electroporated promastigotes were added to in 5ml of RPMI-20% FCS medium without any selective drug and incubated for 24 h at 26ºC.After this period recombinant parasites selected by adding 50 µg/ml of Geniticine (G418)(Sigma, USA) to overnight growth culture of transected parasites. The growth of cells highly resistant to Neomycin was observed after 7–10 days.

Screening of recombinant *Leishmania tarentolae* colonies and confirmation of the KMP11-NTGP96-GFP Fusion Gene Expression

PCR confirmation

To confirm the integration of the KMP11-NTGP96-GFP Fusion into the *ssu* locus of *Leishmania genome*, PCR was performed by using genomic DNA of transgenic strains as a template and *ssu* forward primer F3001 (5'- GATCTGGTTGATTCTGCCAGTAG-3') and reverse primer A1715 hybridizing within the 5'UTR of the target gene (5'- TATTCGTTGTCAGATGGCGCAC-3') with annealing temperature 60ºC according to the LEXSY Kit protocol (Jena bioscience, Germany). One of the primers hybridizes within the expression cassette and other hybridizes to the *ssu* sequence not present in the plasmid. Integration of the expression cassette into the *ssu* locus yielded a 1 kb fragment that was not obtained in the control reactions with the genomic DNA of *Leishmania tarentolae* wild type.

RNA Extraction and Reverse-Transcription PCR

Total RNA extracted from promastigote of *Leishmania tarentolae* with the RNX Plus kit (Cinnagene ®) according to the manufacturer's instructions. The RNA concentration and quality was assessed by both UV absorbance and electrophoresis on the 2% agarose gel. The total RNA reverse was transcribed to cDNA (with using RevertAid™ H Minus Reverse Transcriptase,

Fermentas®) and this cDNA was used as template DNA for RT-PCR amplification. To detect the KMP11, NT-GP96 and Fusion, PCR reactions were carried out using specific primer pairs to amplify each gene separately. The RT-PCR product was analyzed by electrophoresis on a 1.2% agarose gel.

Flow Cytometry and Fluorescence Microscopy analysis

Analysis promastigote forms of *L.tarentolae*- KMP11-NTGP96-GFP were examined for GFP expression by Epifluorescence microscopy. Promastigotes were centrifuged in 3000 rpm for 10 min and after washing once with PBS, cells were re-suspended in PBS and mounted on microscope slides. Expression of EGFP protein was evaluated by Epifluorescent microscopy. For flow cytometric measurement, parasites at two different growth phases (logarithmic and stationary phases) were centrifuged at 3000 rpm for 10 min, washed with PBS and then resuspended at 10^{6} cell/ml in PBS and stored on ice. Cells were analyzed on a FACS caliber flow cytometer (BD: Becton Dickinson, Franklin Lakes, NJ) equipped with a 15 mV, 488 nm, air-cooled argon ion laser. 50,000 events were recorded and EGFP expression in transgenic *Leishmania tarentolae* was measured in comparison with wild type (WT) parasites.

SDS–PAGE and Western Blot Analysis

Promastigote forms of the transformed *L. tarentolae*- KMP11-NTGP96-GFP and the wild type parasites were harvested by centrifugation at 3000 rpm for 15 min and washed in PBS and the pellets were lysed in 5X SDS-PAGE sample and then boiled for 5 min. Samples were then loaded on a 12.5% SDS-PAGE. The gels were transferred onto a nitrocellulose membrane and Western blotting was performed according to the standard procedure(27).

Mice, Immunization Schedules and challenge infection

Female inbred BALB/c mice, 7 week-old were acquired from the Animal Breeding Facility Centre of Razi Vaccine and Serum Research Institute, Karaj, Iran. They were housed in clean cages and fed ad libitum. The immunization experiments were carried out in four groups of mice (n= 15 at each group) and all tests were done in triplicate. The first group received PBS only and Group 2 immunized with 2×10^7 Wild *Leishmania tarentolae* as the control; group 3 vaccinated with 2×10^7 recombinant *L. tarentolae*-KMP11-GFP; group 4 vaccinated with 2×10^7 recombinant *L.tarentolae*-KMP11-NTGP96-GFP. All groups were immunized subcutaneously via footpad. Four weeks after the last immunization, all animals were challenged with 10^7 stationary phase of *Leishmania infantum* strain JPCM5 (MCAN/ES/98/LLM-877) virulent promastigotes by intra peritoneal injection.

Determination of Parasite Burden

Five mice from each group were sacrificed at 4 weeks after challenge and parasite burden in the spleens was quantitatively determined by serial dilutions method. Briefly, A piece of spleen was excised, weighed and then homogenized with a tissue grinder in 2 ml of RPMI-1640 medium (Sigma, USA) supplemented with 20% heat-inactivated fetal calf serum. Under sterile conditions, serial dilutions ranging from 1 to 10^{-20} were prepared in wells of 96 well microtitration plates. After 3, 7 and 14 days after incubation at 26ºC, plates were examined with an inverted microscope at a magnification of 40×. The presence or absence of mobile promastigotes was recorded in each well. The final titer was the last dilution for which the well contained at least one motile parasite. The number of parasites per gram was calculated in the following way: parasite burden = $-\log_{10}$ (parasite dilution/tissue weight)(28, 29).

Determination of Antibody response

Before challenge and 4 week after challenge, all groups of mice were bled retro-orbitally and the levels of anti-soluble Leishmania antigen(SLA) IgG1 and IgG2a Abs were evaluated using ELISA method according to the manufacturer's instruction (Mouse IgG2a&1 detection kit, ebioscience, USA).

Cytokine Assays

To determine the levels of IFN- γ and IL-4, in each group of experiment five mice were sacrificed before and also 4 weeks after challenge and spleen of them were removed and homogenized in PBS. After erythrocytes lysis using ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2-EDTA), splenocytes were washed with PBS and resuspended in RPMI-10% FCS. Cells were then seeded at a density of 3.5×10^6 cells/ml in the presence of *Leishmania tarentolae*-KMP11-NTGP96-GFP Freeze/Thawed (25 mg/ml). Concanavalin A (Con A; 5 mg/ml) and medium alone were used as the positive and the negative control respectively. Plates were incubated for 72h at 37ºC in 5% CO2 humidified atmosphere for IFN- γ and IL-4 measurement. The IFN- γ and IL-4 production in supernatants of splenocytes cultures was measured by ELISA kits (U-CyTech, Netherlands), according to the manufacturer's instructions. All experiments were run in triplicates.

Ethics Statement

This research was carried out accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Tarbiat Modares university and all animals experiments including maintenance, handling and blood collection were approved by Institutional Animal Care and Research Advisory Committee of Tarbiat Modares university based on the Specific

National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education of Iran.

Statistical Analysis

Statistics were performed using SPSS version 18 and one way ANOVA (Multiple-comparison Tukey post Hoc test) and Student's t-test was employed to assess the significance of the differences between the mean values of control and experimental groups. Differences were considered statistically significant when $p < 0.05$. Data shown represent the mean values ± standard error of the mean (SEM) of three independent experiments

RESULTS

Constructing molecular structures

Following PCR amplification, a 279 bp DNA fragment for KMP-11 and 1014 bp for NT-GP96 were identified by agarose gel electrophoresis. The PCR products were successfully ligated into a pJET1.2/blunt cloning vector and subsequently, KMP-11 fragment was ligated into pEGFP-N1 expression vectors and then recombinant pEGFP-N1- KMP 11 -NTGP96, pJET- KMP 11 -NTGP96, pLEXSY- KMP11-NTGP96 and pLEXSY- KMP11-NTGP96-GFP plasmids were constructed successfully. Following PCR amplifications using pLEXSY- KMP11-NTGP96-GFP plasmids as the template and forward and reverse primers specific for KMP-11 and NT-GP96 genes all three DNA fragment (279 bp for KMP-11, 1014 bp for NT-GP96 and 1293 bp for KMP11-NTGP96 fusion) was identified by agarose gel electrophoresis of the PCR products (Fig. 1).Accurate presence of all constructs confirmed by enzyme digestion, PCR amplification and sequencing.

Confirmation of construction of Recombinant *Leishmania tarentolae* expressing the KMP11-NTGP96-GFP Fusion

Recombinant *Leishmania tarentolae* stably expressing the KMP11-NTGP96-GFP Fusion gene were generated by introducing the linearized pLEXSY- KMP11-NTGP96-GFP vector into the 18S rRNA *ssu* locus of *Leishmania tarentolae*. Specific targeting of the expression cassette into the *ssu* locus was confirmed both by genomic PCR with gene specific primers (Fig.2.A) and vector specific primers (Fig.3) .Amplification of all expected genes in a RT-PCR product, confirmed the expression of KMP11-NTGP96-GFP by *Leishmania tarentolae* at the mRNA level (Fig. 2.B).

Expression of GFP (marker for expression of KMP11-NTGP96-GFP) in recombinant *Leishmania tarentolae* parasites was confirmed by fluorescence microscopy (Fig. 4.B) and also by fluorescence-activated cell sorting (FACS) analysis (Fig. 4.C). The KMP11-NTGP96-GFP expression was also assessed by western blot analysis. As shown in Figure 4.A, immunoreactive bands were detected in recombinant *Leishmania tarentolae* parasites using polyclonal antibody.

Immunization with recombinant *Leishmania tarentolae* and determination of Parasite burden

Four groups of mice were considered for immunization with two subsequent repeats as described in Materials and Methods. The results are shown, obtained from these two independent experiments. The degree of protection against infection was determined by measurement of the parasite burden in the spleen at 4weeks after challenge with *Leishmania infantum*. As shown in Figure 5, immunization using a *Leishmania tarentolae*-KMP11-NTGP96-GFP reduced the infection in the spleen at 4 weeks after challenge in contrast to the control groups (PBS and wild *Leishmania tarentolae*).

Evaluation of IFN- γ /IL-4 ratio after immunization with Live Recombinant parasites

The levels of IFN-γ and IL-4 production were analyzed before and 4 weeks after challenge in the supernatant of the spleen cells culture of all five groups following stimulation with Freeze/Thawed *Leishmania tarentolae*-KMP11-NTGP96-GFP. As shown in Figure 6-I-A, stimulation of isolated splenocytes from vaccinated group with *Leishmania tarentolae*-KMP11-NTGP96-GFP prior and 4 weeks after challenge elicited a significantly higher IFN-γ production than other groups ($p < 0.05$).

The production of IL-4 upon antigen stimulation before and 4 weeks after challenge was also higher in this vaccinated group (Fig. 6-I-B).We further calculated the IFN- γ to IL-4 ratio for each vaccinated group as an indicator of potential immunization (Fig. 6-I-C). The Leishmania specific IFN- γ /IL-4 ratio were higher in *Leishmania tarentolae*-KMP11-NTGP96-GFP vaccinated group compared to the others both at before and 4 weeks after challenge.

IgG antibody isotypes response to Immunization with Live Recombinant *Leishmania tarentolae*

To compare IgG isotypes in different groups, all sera were assayed by ELISA before and 4 weeks after challenge. As shown in Figures 6-II-A´ and 6-II-B´, IgG1 and IgG2a isotypes before challenge were higher in groups that vaccinated with *Leishmania tarentolae*-KMP11-NTGP96-GFP and *Leishmania tarentolae*-KMP11-GFP in comparison to the control groups ($p<0.05$). Also, increased amount of IgG2a was seen in these groups at 4 weeks after challenge. Interestingly, in the group vaccinated with Live *Leishmania tarentolae*-KMP11-NTGP96-GFP, a decreased amount of specific IgG1 was detected in comparison to other vaccinated and control groups. The ratio of IgG2a/IgG1 was significant in *Leishmania tarentolae*-KMP11-NTGP96-GFP as compared with all other groups (Fig. 6-II-C´) ($p<0.05$).

DISCUSSION

Leishmania vaccine development has proven to be a difficult and challenging task, which is mostly hampered by inadequate knowledge of parasite pathogenesis and the complexity of immune responses needed for protection(30). If the patient with visceral leishmaniasis left without any treatment it can be lead to death in 90% of cases. The drugs which are currently used as treatment for leishmaniasis cannot be handled easily due to a number of problems including high toxicity and various side effects(31). Therefore, efforts to introduce new candidates for vaccine production are currently being considered. Despite advances in *Leishmania* genomics and proteomics (32), modern biotechnology for antigen expression, purification and delivery, and the large availability of murine models in the field of experimental immunology, *Leishmania* vaccinology still suffers from several bottlenecks that limit the progress towards effective and universal vaccines(33).

We construct a live recombinant *Leishmania tarentolae* expressing KMP11-NTGP96-GFP fusion as candidates for live engineered recombinant vaccine against visceral leishmaniasis in BALB/c mice. Das et a. (2014) indicated that KMP11 is very excellent target for immunization against Leishmaniasis (34) and our results showed that above prediction about immunological function of KMP11 is correct. On the other side, GP96 as an adjuvant, plays important roles in innate and adaptive immune responses. The immune effect of GP96 is probably dependent on its ability to bind peptide epitopes. It was reported that both the N- and C-terminal fragments of GP96 are able to bind peptides, with the N-terminal fragment behaving at a similar capacity to the full-length GP96(35, 36). Linkage of antigens to HSPs (Hsp70, calreticulin, Hsp60, GP96) represents a potential approach for increasing the potency of DNA

vaccines. For example, vaccines containing full lengthHPV16E7 fused to Mycobacterium tuberculosis HSP70 increased the frequency of E7-specific CD8+ T cells by at least 30 fold relative to vaccines containing the wild-type E7 gene(19). Recently a research indicated that the adjuvant activity of NT (gp96) was more efficient in the induction of immune responses when fused to the C-terminal end of the HCV DNA polytope and the NT (gp96) improved the efficacy of the DNA vaccine, and this immunomodulatory effect was dependent on the position of the fusion(37).All of these research confirms our results that indicated to positive immunological effects of NT-gp96 fusion with KMP11 to make a new effective fusion construct for better stimulation of protective immunity.

In our research, following immunization, a notable pattern was observed on the profile of cytokines and immunoglobulin expression. A significant production of IFN-γ cytokine was detected following immunization with *Leishmania tarentolae*-KMP11-NTGP96-GFP.We also detected considerable levels of IL-4 in immunized mice with this group. These results indicated that our vaccine strategy mediated protection was associated with a mixed Th1/Th2 that is typical of other successful experimental vaccines against visceral leishmaniasis(38). But, the important point is the matter that the level of IFN-γ cytokines were increased after challenge, but this pattern was decreasing about IL-4. Interestingly, the expression ratio of IFN-γ/IL-4 of mice immunized with *Leishmania tarentolae*-KMP11-NTGP96-GFP was significantly higher before and after challenge in comparison to control groups. Also, a significant difference in the expression ratio of IgG2a/IgG1 was observed in mice immunized with this vaccination group.

The important result is that this group also displayed a significant reduction in the parasite load in spleen four weeks after challenge, that suggesting this modulation in the immune response is capable to control infection. The significant parasite load reduction observed in the *Leishmania*

tarentolae-KMP11-NTGP96-GFP immunized group after challenge could be a result of the increased expression ratio of IFN-γ/IL-4 and IgG2a/IgG1 that indicated to activation of Th1cell mediated immune response that could have an impact on parasite establishment and disease development pattern. Vaccines based on combination of different antigen candidates have been shown to improve protection. In fact, a recombinant Q protein formed by genetic fusion of five parasite intracellular antigens has been successfully tested in dogs(39–41).

The data shown herein indicates that *Leishmania tarentolae*-KMP11-NTGP96-GFP group was able to confer a significant degree of protection against *L. infantum*.

For effective intervention measures to control VL in endemic areas, it is imperative to design a vaccine, which is the most economical way of controlling infectious diseases(2). For many years, many laboratories around the world have worked on the development of an effective vaccine against leishmaniasis(42). Different Vaccination studies in experimental murine models have shown that live attenuated forms of parasites gives better protection when compared to other recombinant antigens(43). *Leishmania tarentolae* is a parasite of the *gecko Tarentolae annularis* and because of its several unique features, including higher specific growth rate compared to mammalian cells, cultivation in low cost media, safety for humans, possibility to introduce several copies of a foreign gene into the parasite genome and production of recombinant proteins with an animal-like N-glycosylation pattern is a feasible eukaryotic expression system for high level production of active recombinant biopharmaceuticals (24, 44). A couple of recombinant pharmaceutical and non-pharmaceutical glycoproteins such as human erythropoietin, tissue plasminogen activator and laminin- 332 have already been produced in this expression system and in all cases, the expressed proteins were biologically active(25, 25, 45). These advantages, in addition to feasibility for constitutive or regulative protein production, make *Leishmania*

tarentolae an attractive host for high level production of heterologous proteins(24, 25, 46, 47) and as an ideal candidate for vaccine against leishmaniases.

In conclusion, this study shows that immunization with live recombinant *Leishmania tarentolae*-KMP11-NTGP96-GFP provides significant protection against visceral leishmaniasis in the BALB/c mice. According to the results and information presented in this study, it seems that using fusion strategy between KMP-11 and GP96 can lead us to making an effective construct for increasing the efficiency of a live recombinant vaccine against visceral leishmaniasis. Designing of effective live recombinant vaccines is attractive in terms of low cost, optimal safety, stability and potency when compared with other vaccination strategy. In addition, further investigation about its efficacy should be done in the susceptible hamster and dog model of visceral leishmaniasis and this strategy could have a broader impact on vaccine development efforts for visceral leishmaniasis worldwide.

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Fig.1. Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes in a single PCR reaction (multiplex PCR) on 1.2 % (w/v) agarose gel.Lane1:100 bp DNA Ladder; Lane 2: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively);Lane 3: single expanded band of KMP11-NTGP96 fusion (approximately 1293 bp). Lane4:1kb DNA ladder.

Fig.2. Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes in PCR reaction with using: A. recombinant *Leishmania tarentolae* DNA as the template and genes specific primers that Lane1: 1kb DNA ladder; Lanes 2, 3, 4: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp

respectively) ;and **B.** recombinant parasite cDNA as the template and genes specific primers that Lanes 1, 2, 3: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively); lane 4: wild *leishmania tarentolae* as the negative control; Lane5: 1kb DNA ladder.

Fig.3. Electrophoresis of the amplified fragments (1 Kb) of specific targeting of the expression cassette into the ssu locus in PCR reaction with using recombinant Leishmania tarentolae DNA as the template and vector specific primers on 1.2 % (w/v) agarose gel. Lane1: 1kb DNA ladder; Lanes 2: expanded bands of 1Kb.

Fig.4. Expression of the fusion gene by *Leishmania tarentolae*. (A) Western blot analysis for evaluating expression of the KMP11-NTGP96-GFP fusion protein. (B) Expression of EGFP by recombinant *Leishmania tarentolae* promastigotes.(C)Percentage of the EGFP positive population in *Leishmania tarentolae* promastigotes transfected with either pLEXSY-KMP11-NTGP96-GFP (I) or pLEXSY-KMP11-GFP (II) that shown in green lines in comparison to wild type parasite that shown in red lines as determined by flowcytometry.

Fig.5. spleen parasite burden in all groups following immunization and infectious challenge with *Leishmania infantum*. The parasite number in spleen was evaluated at 4 weeks after challenge (*$p < 0.05$ compared to PBS).

Figure.6. Analysis of the specific cellular and humoral response factors in vaccinated and control groups before and after challenge. (I) Cytokine production by splenocytes in vaccinated and control groups .A: IFN- γ production by splenocytes in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP (*$p < 0.05$ compared to PBS);**B:** IL-4- γ production by splenocytes in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP (*$p < 0.05$ compared to

PBS);**C:** IFN- γ /IL-4 ratio in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP (*p< 0.05 compared to PBS). **(II)Analysis of the specific IgG isotypes in vaccinated and control groups. A´:** Specific IgG2a antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (P < 0.01 compared to PBS); **B´:** Specific IgG1 antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (*P < 0.01 compared to PBS); **C´:** IgG2a/IgG1 ratio in vaccinated and control groups before and 4 weeks after challenge (*P < 0.01 compared to PBS).

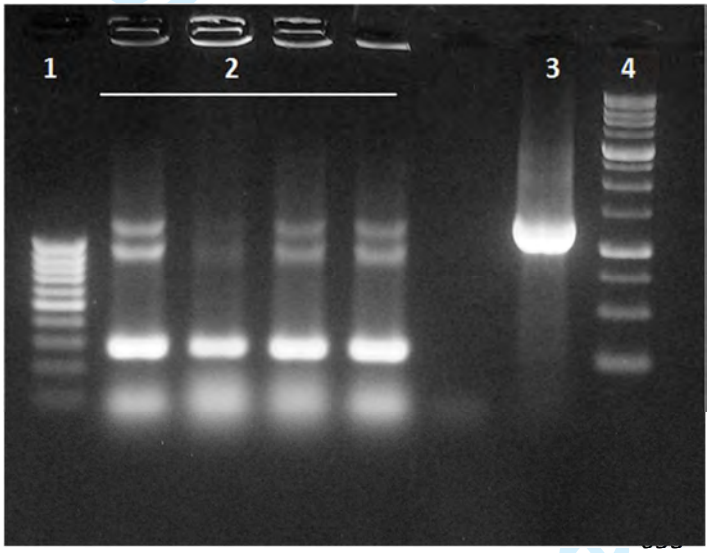

Fig.1

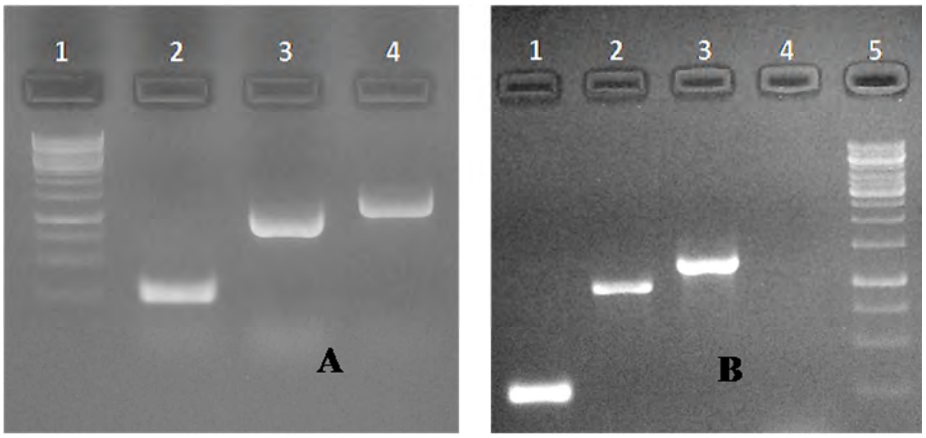

Fig.2

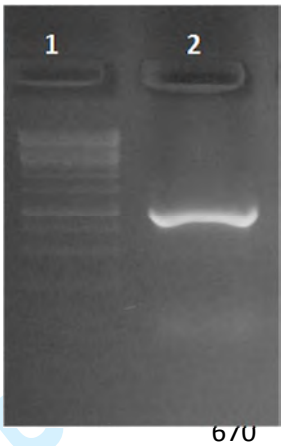

Fig.3

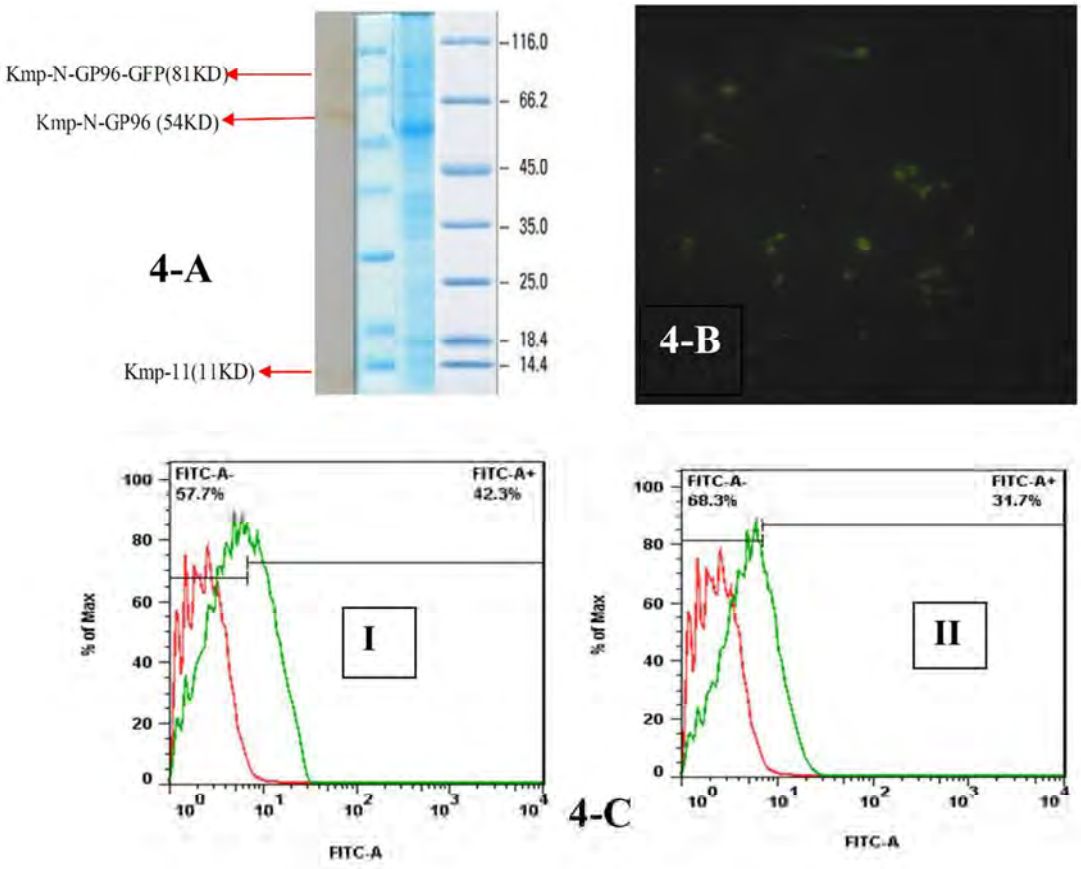

Fig.4

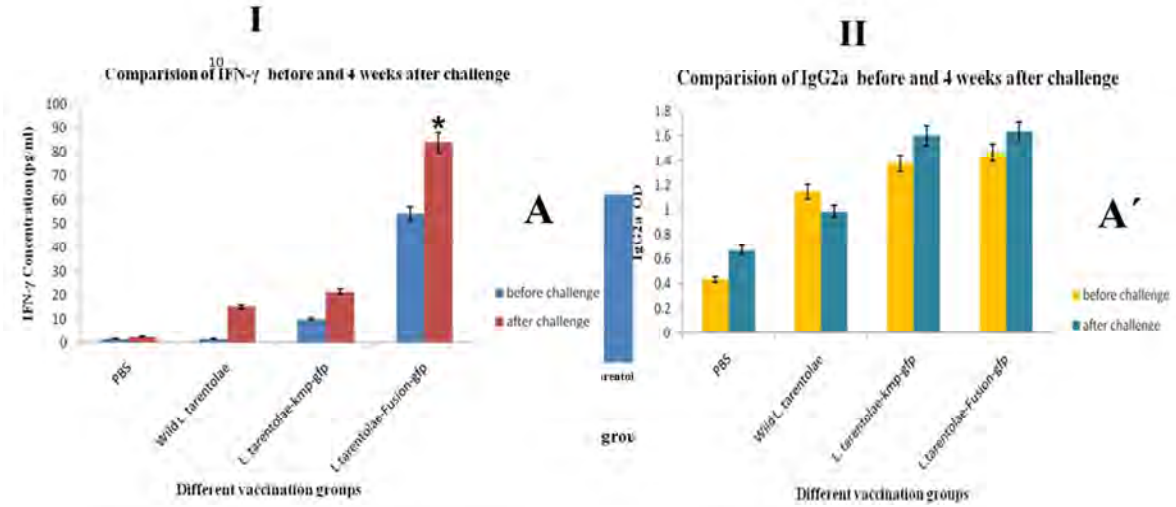
I
II
Comparision of IFN-γ before and 4 weeks after challenge
IFN-γ Concentration (pg/ml)
A
before challenge
after challenge
PBS
Wild L. tarentolae
L. tarentolae-kmp-gfp
L. tarentolae-Fusion-gfp
Different vaccination groups
Comparision of IgG2a before and 4 weeks after challenge
IgG2a OD
A′
before challenge
after challenge
PBS
Wild L. tarentolae
L. tarentolae-kmp-gfp
L. tarentolae-Fusion-gfp
Different vaccination groups

Fig.5

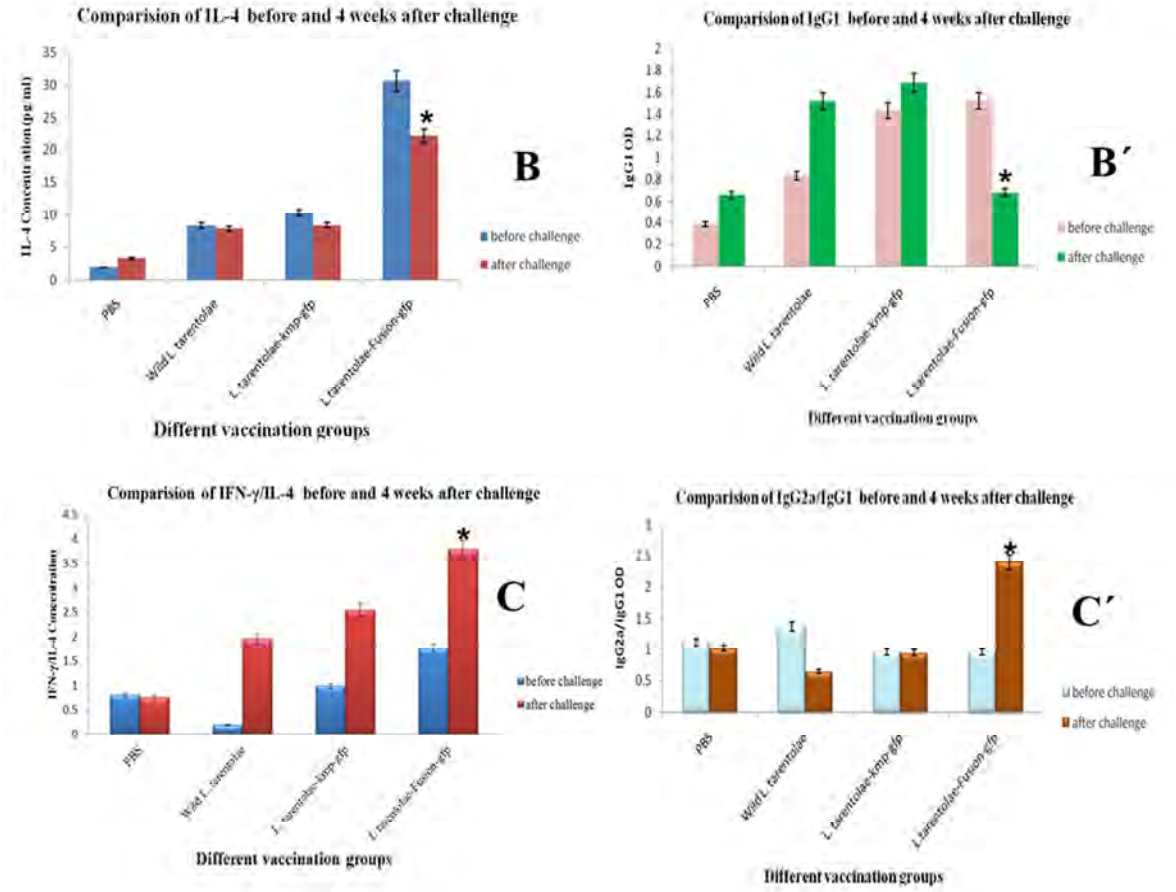
Comparision of IL-4 before and 4 weeks after challenge
IL-4 Concentration (pg/ml)
B
before challenge
after challenge
PBS
Wild L. tarentolae
L. tarentolae-kmp-gfp
L. tarentolae-Fusion-gfp
Differnt vaccination groups
Comparision of IgG1 before and 4 weeks after challenge
IgG1 OD
B′
before challenge
after challenge
PBS
Wild L. tarentolae
L. tarentolae-kmp-gfp
L. tarentolae-Fusion-gfp
Different vaccination groups
Comparision of IFN-γ/IL-4 before and 4 weeks after challenge
IFN-γ/IL-4 Concentration
C
before challenge
after challenge
PBS
Different vaccination groups
Comparision of IgG2a/IgG1 before and 4 weeks after challenge
IgG2a/IgG1 OD
C′
before challenge
after challenge
PBS
Wild L. tarentolae
L. tarentolae-kmp-gfp
L. tarentolae-Fusion-gfp
Different vaccination groups

Figure.6