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Original Article

Designing and Cloning Molecular Constructs to Knock Out *N-Acetylglucosamine Phosphatidylinositol De-N-Acetylase* (GPI12) Gene in *Leishmania major* (MRHO/IR/75/ER)

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Abstract

Background: Leishmaniasis represents a major public health concern in tropical and sub-tropical countries. At present, there is no efficacious vaccine against the disease and new control methods are needed. One way to access this important goal is to knock out genes of specific macromolecules to evaluate the effect of deletion on the growth, multiplication, pathogenesis and immunity of the parasite. The aim of this study was to design and clone molecular constructs to knock out *N-acetylglucosamine phosphatidylinositol de-N-acetylase* (*GPI12*) gene in *Leishmania major*.

Methods: For designing and making molecular constructs, we used pLEXSY-neo2 and pLEXSY-hyg2 vectors. The molecular constructs were cloned in *E. coli* strain Top10. The molecular constructs were transfected by electroporation into *L. major* in two stages.

Results: The molecular constructs were confirmed by Colony PCR and sequencing. The recombinant strains were isolated by selective antibiotics, after which they were confirmed by PCR, Southern and Western blots.

Conclusion: Recombinant parasites were created and examined for subsequent study. With the use of molecular constructs, it was possible to remove and study gene *GPI12* and to achieve a live recombinant *Leishmania* parasite that maintained the original form of the antigenic parasites. This achievement can be used as an experimental model for vaccine development studies. Further investigations are essential to check this model in a suitable host.

Introduction

Leishmaniasis represents a major public health concern in the Eastern Mediterranean Region(1). There are 2 main types of leishmaniasis, cutaneous (CL) and visceral (VL), both have zoonotic and anthroponotic transmission, of which, CL is the commonly observed. Each of these types is found in several foci in every endemic country (2). The prevention and control of leishmaniasis has been unsuccessful in almost all countries, especially in the case of zoonotic foci. Up until now, no efficacious and safe vaccine has been found for all form of leishmaniasis. Treatment; however, is the only major choice to control CL. Currently, pentavalent antimonial compounds are the standard treatment, but the prescribe of these drugs are limited due to their side-effects, long duration of use, low efficacy and increasing resistance (3, 4).Therefore, the need for a new innovative control method is of prime importance.

Nevertheless, a controlled vaccination with living strains has met some success. One way to begin to create new vaccine and drugs is through the evaluation of important enzymes, proteins and macromolecules by reverse genetic engineering. In this type of research, the gene of a specific macromolecule is deleted and then the effect of its deletion is evaluated on the growth, multiplication and pathogenesis of the parasite. lipophosphoglycans (LPG), zinc metalloprotease (gp63), proteophosphoglycan (PPG) and parasite surface antigen-2 (PSA-2/gp46) are like attachments in the host to the macrophage cells and which lead to the development of the parasites inside the host. All of these ligands are attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor (5). The second step of GPI biosynthetic pathway is catalysed by *N-acetylglucosamine phosphatidylinositol de-N-acetylase* (*GlcNAc-PI-de-N-acetylase or GPI12*). This step is an important step that required for all GPI biosynthetic pathways.(6) In these step *GPI12* de-N-acetylated *N-acetylglucosamine phosphatidylinositol* (*GlcNAc-PI*) to produce *glucosamine phosphatidylinositol* (*GlcNH2-PI*). At present, we found no research conducted on the effect of the deletion of this gene on *Leishmania* spp.

Virulent strains overcome the immunization, and the infections incurred often prove to be troublesome in a naturally acquired disease. Thus, currently, most research is concentrated on discovering a live-vaccine strategy which would protect the body in case the natural immunizing system fails to defend the body. Gene targeting and deleting of the essential metabolic genes is the general method applied for engineering conditionally viable *Leishmania* (7).

In gene targeting technology, in vivo homologous recombination enables the replacement of a target genomic region with an exogenous DNA fragment that contains a region homologous to the targeted locus. This technology is indispensable for analysis of the gene's function. To acknowledge its importance, the discovery of the principles of gene targeting in mice was awarded a Noble Prize in 2007. Recently, this technology has been applied to gene therapy, gene studies, and the production of transgenic plants (8).

Homologous gene replacement offers a powerful tool for altering and testing a gene's function (9). Targeted gene replacement via homologous recombination has been an invaluable tool for genetic dissection of the salient metabolic and virulence pathways in *Leishmania* species (10) as well as for many other protozoan parasites (11). The general experimental approach for genetic manipulation of model organisms is essentially the same: DNA sequences of sufficient length direct homologous recombination by flanking the targeted gene referred to here as 5'- and 3'- targeting sequences (TS) are independently isolated and joined to build an alternative gene (i.e. drug resistance gene) that allows the selec-

tion of those cells in which the appropriate integration event has occurred. The most common method for generating *Leishmania* and other parasite gene targeting constructs involves the sequential cloning of 5'-TS and 3'-TS DNAs into a vector encoding in a drug resistance cassette flanked by the restriction sites (Fig. 1) (12).

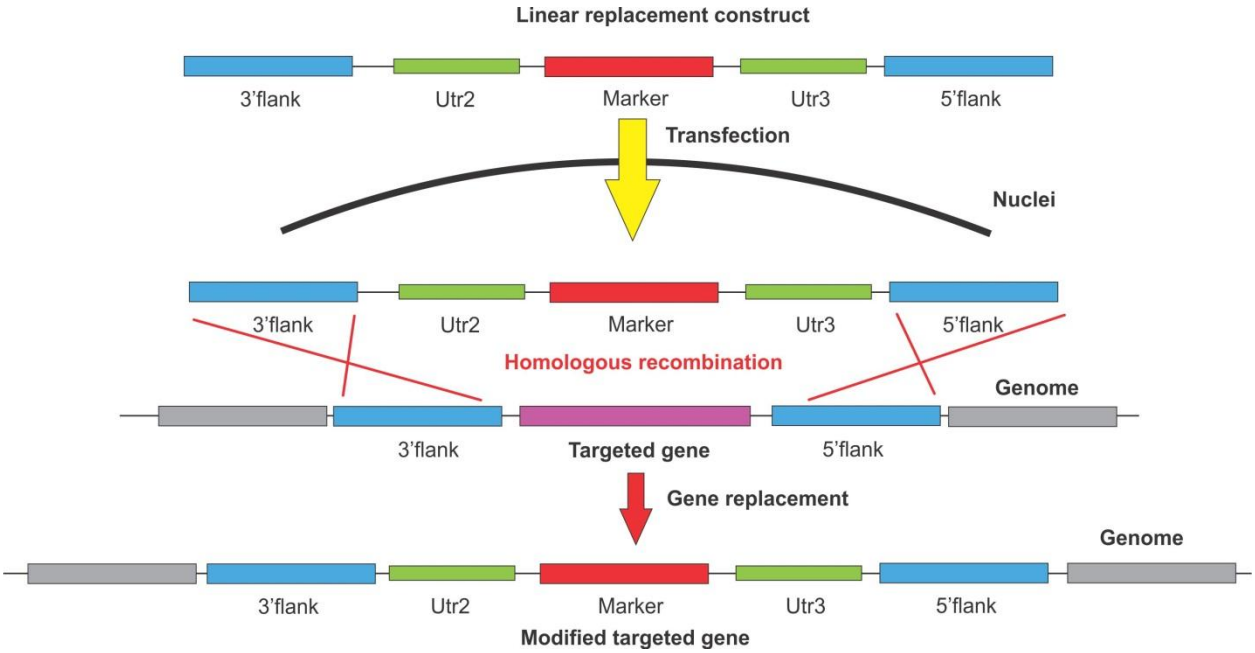


Fig. 1: A schematic diagram of the principles of gene targeting

We deleted *GPI12* gene in *L. major* using the homologous recombination method. By using standard methods, the parasite then became a homozygote for this gene. This gene, however, was not a viable one and thus to achieve a live recombinant *Leishmania* parasite that maintained the original form of the antigenic parasites. Recombinant parasites were created and examined for subsequent study. This achievement can be used in vaccine development studies.

The aim of this study was to design and clone molecular constructs to knock out *N-acetylglucosamine phosphatidylinositol de-N-acetylase* (*GPI12*) gene in *Leishmania major*.

Materials and Methods

Designing construct

For designing and making the molecular construct, we used pLEXSY-2 vectors (pLEXSY-hyg2 and pLEXSY-neo2) (Jena Bioscience, Germany). These vectors contain gene markers that cause encoding aminoglycoside phosphotransferase and Hygromycin phosphotransferase, after entering the genome. These markers are used to help select such transfected parasites by Hygromycin and Neomycin antibiotics.

These vectors are made for expression or gene knock-in in *Leishmania*. In order to achieve our goal (i.e. gene knock out), the structure of the vectors had to be modified. Utr2, utr3 areas are required for the trans-splicing and polyadenylation of the gene marker. Utr2, utr3 and gene markers were existed in pLEXSY-2 vectors (pLEXSY-neo2 and pLEXSY-hyg2) (Fig. 2D). In order to find suitable areas to place the enzyme, the full sequences of these vectors were analysed by the application of Web cutter and Neb cutter software.

We considered the left flank area for the *Bgl*II and *Kpn*I enzymes and the right flank area for *Fse*I and *Bsr*gI (Fig. 2A). Using the sequences of the *Leishmania major* genome available in the gene bank, helped us analyse and select the appropriate left and right flank areas.

The *GPI12* gene was located in chromosome 9, Lmjchr9, LMJF_09_0040 749 bp and encoded a protein with 249 amino acids. We selected sequence of 10375 to 10674 for the left flank area, and the sequences of 11,583 to 12,082 as the right. The *Bgl*II and *Kpn*I enzymes were located on the two ends of the left flank area, while the *Fse*I and *Bsr*gI enzymes were located at the two ends of the right flank area.

The flank areas with their enzymes were synthesized in the pGEM-b1 vector (Fig. 2A, 2B) by Bioneer Company (South Korea). However, the *Swa*I enzyme was placed at the beginning and the end of each flank area for the linearization of the constructs after cloning.

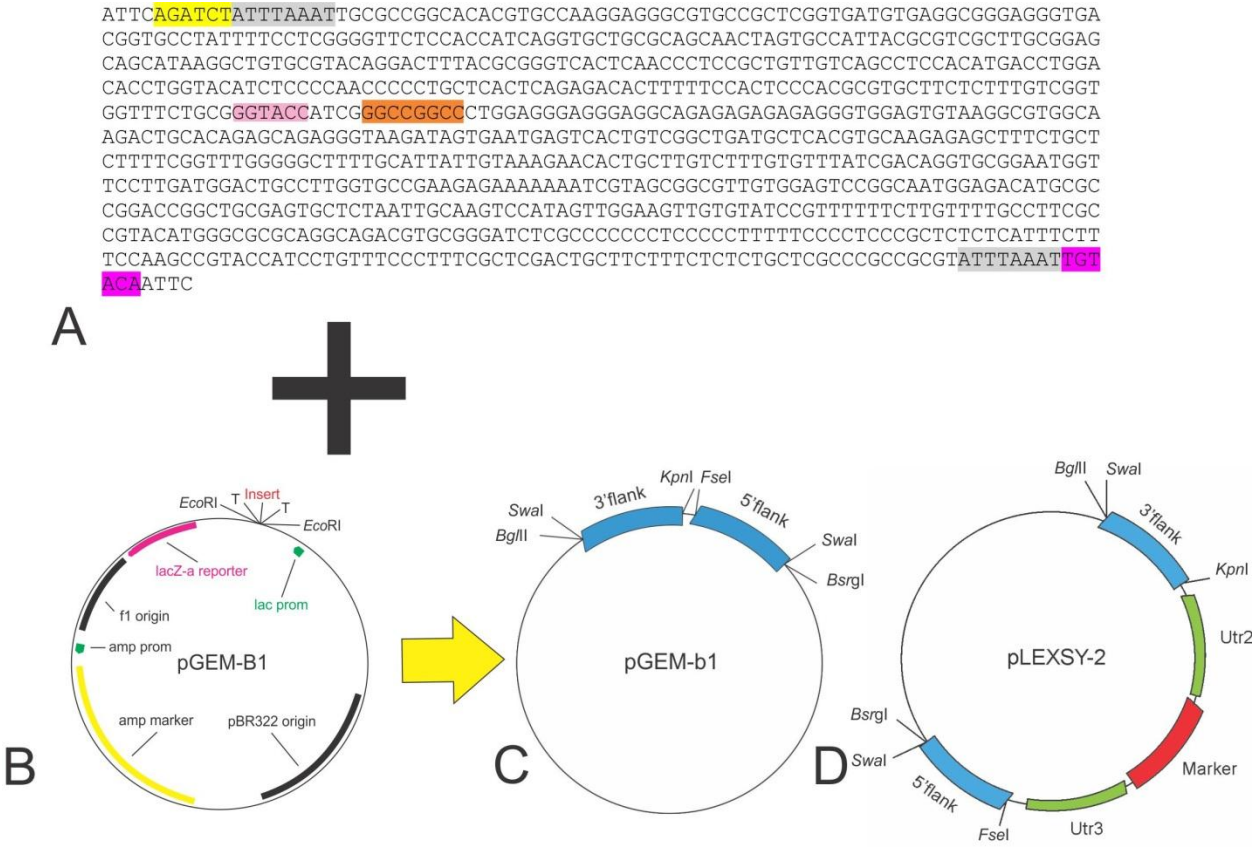


Fig. 2: (A) The flank area with enzymes sequences (yellow:*Bgl*II, gray:*Swa*I, pink:*Kpn*I, orange:*Fse*I, violet:*Bsr*gI). (B) The map of the pGEM-b1 vector. (C) pGEM-b1 vector that contain the flank regions and specific restricted enzymes for cloning. (D) The pLexsy-2 vector contain construct after cloning

Parasite

L. major strain MRHO/IR/75/ER a reference strain from the Center for Research and Training in Skin Diseases and Leprosy (CRTSDL) was used in all experiments. The parasites were isolated from the lesions of infected BALB/c mice and cultivated at 26 ^{0}C in a Dulbecco's Modified Eagles Medium (DMEM) high glucose (4.5g/L) media (Biosera, France), supplemented with 40 mM HEPES ,10% heat-inactivated fetal bovine serum (FBS)(Gibco, UK), 2 mM L-glutamine

and 10.000 units of penicillin (base) and 10.000 µg of streptomycin (base)/ml as penicillin G sodium salt and streptomycin sulfate in 0.85% saline (all from Sigma, Germany). After 2–3 passages, log-phase promastigotes were used for the transfection.

Prokaryote host culture

We used *E.coli* top 10 (Invitrogen, UK) for competent cell preparation and transformation in all experiments. The preparation was carried out according to the standard method on LB media (13).

Preparing inserts and vectors

The plasmid pGEM-B1 contains sequences of flank areas which preform PCR by primer M13 (M13for; 5'-TGTAAAACGACGGCCAG-3', M13rev; 5'-CAGGAAACAGCTATGAC-3') (Fig. 3A). PCR fragments were cut with *Bgl*II and *Kpn*I enzymes (Fermentas, USA) and the PCR product was divided to 3 pieces of 600, 300 and 100 bp. A 300 bp fragment (Fig. 3B) was extracted from the gel, which was 3'flank. Also, pLEXSY-neo2 was cut by two enzymes: *Bgl*II and *Kpn*I (Fig. 3D). The vectors were cut with the enzymes extracted from the gel and ligated with 3'flank by T4 DNA ligase (Fermentas, USA). Ligation was transformed to the competent cell for cloning the vectors that contained 3'flank (pLEXSY-neo2/R1). To confirm the colonies containing the desired plasmid, Colony PCR was performed.

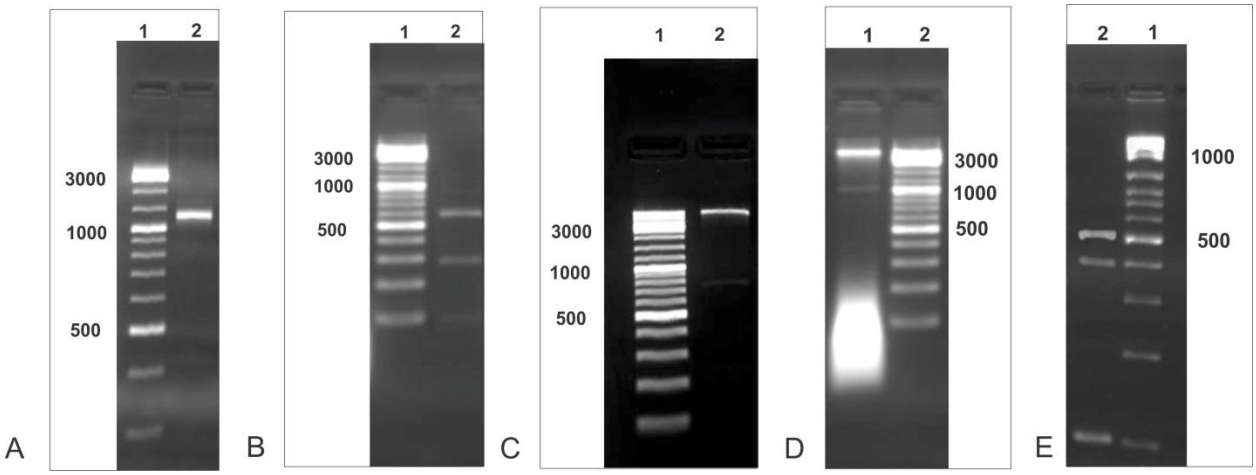


Fig. 3: In all figures, lane 1 shows molecular size marker. (A) The sequences of flank areas on which PCR was performed by primerM13; Lane 2 shows the amplified ~1kb band. (B) The PCR fragments which were cut with *Bgl*II and *Kpn*I enzymes. The PCR product which was divided into 3 pieces of 600, 300 and 100 bp; Lane 2 shows the 300 bp fragment. (C) The pLEXSY vector, which was cut by *Fse*I and *Bsr*gI enzymes; Lane 2 shows the ~ 0.7 kb fragment is separated from the vector. (D) The pLEXSY vector, which was cut by *Bgl*II and *Kpn*I enzymes. Lane 2 shows the ~1 kb fragment is separated from the vector. (E) The PCR fragments which were cut with *Fse*I and *Bsr*gI enzymes. The PCR product which was divided into 3 pieces of 500, 400 and 100 bp; Lane 2 shows the 500 bp fragment

PLEXSY-neo2/R1 was extracted via the alkaline lysis method. The PCR fragments were cut by *Fse*I and *Bsr*gI enzymes (New England Biolabs, UK). The plasmid pGEM-B1 contains sequences of flank areas, which preform PCR by primer M13. The PCR product was then divided into 3 pieces of 500, 400 and 100 bp (Fig. 3E). The 500 bp fragment was extracted from the gel and became 5'flank. PLEXSY-neo2/R1 was cut by two enzymes: *Fse*I and *Bsr*gI (Fig. 3C). The vectors, which were cut by the enzymes extracted from the gel, were ligated with 5'flank by T4 DNA ligase (Fermentas, USA).

The transformation and cloning were performed in the competent cell and the colonies were confirmed by Colony PCR. The vectors which contained 3'flank and 5'flank (pLEXSY-neo2/R1/R2) (Fig. 2D) were constructed and sequenced. This process was repeated for the pLEXSY-hyg2/R3/R4 construction as well.

Plasmid extraction via the alkaline lysis method

A single colony of transformed bacteria was inoculated into 2 ml of rich LB medium containing ampicillin antibiotic. The culture was incubated for 16 h at 37 °C with vigorous shaking; 1.5 ml of the culture was then poured into a microfuge tube and centrifuged for 30 seconds at maximum speed at 4 °C. The medium was removed by aspiration leaving the bacterial pellet as dry as possible. The bacterial pellet was resuspended in 100 µl of ice-cold alkaline lysis solution I (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0) by vigorous vortexing. 200 µl of freshly prepared alkaline lysis solution II (0.2 N NaOH , 1% (w/v) SDS) was added to each bacterial suspension. The tube was closed tightly and its contents were mixed by inverting the tube five times rapidly. 150 µl of ice-cold alkaline lysis solution III (60.0 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, and 28.5 ml H_2O) was then added. The tube was inverted several times to mix the alkaline lysis solution III and the viscous bacterial lysate. The tube was stored on ice for 3-5 min. The bacterial lysate was centrifuged at maximum speed for 5 min at 4 °C in a microfuge. Then, the supernatant solution was transferred to a fresh tube. The nucleic acids were precipitated from the supernatant by adding 2 volumes of ethanol at room temperature. The solution was mixed by vortexing and then allowed to stand at room temperature for 2 min. The precipitated nucleic acids were collected by centrifugation at maximum speed for 5 min at 4°C in a microfuge. The supernatant was removed by gentle aspiration. The tube was placed in an inverted position on a paper towel to allow all of the fluid to drain away. The tube was then opened at room temperature until the ethanol had completely evaporated and no fluid was visible in the tube. The nucleic acids were then dissolved in 50 µl of DNAse free water.

Transformation procedure

Five ml of LB media was inoculated in *E. coli* and left to grow for 16 h at 37 °C in a shaker incubator. 0.1 ml of overnight culture was inoculated into a sterile tube containing 2 ml of LB broth. The culture was shaken at 37 °C until the cell density reached a mid-log growth phase (about 5 x 10^7 cells/ml), this took 2 to 4 h. The culture was then chilled on ice for 10 min. The cell suspension was spun at 2500 rpm in a centrifuge for 5 min at 4 °C and the supernatant discarded. Cells were resuspended in 200 µl of the ice-cold sterile 0.1 M $CaCl_2$. The cell suspension was placed in an ice bath for 10 min, then centrifuged at 2500 rpm for 5 min at 4 °C, and the supernatant discarded. Gently the cells were resuspended in 50 µl of sterile ice-cold $CaCl_2$ using pre-cooled pipettes and the competent cells remained on the ice. Ten to 40 ng of plasmid were added to the competent cells and the mixture incubated on ice for a further 30 min. The reaction was transferred to a 42 °C water bath for exactly 1.5 min, and incubated on ice for 2 min. 100 µl of LB broth was added without antibiotic to each tube and incubated at 37 °C for 1 h in a shaker incubator to allow cells to recover and express the antibiotic resistance marker. The appropriate quantity of cells was then spread on plates, which contained a LB agar media with ampicillin antibiotic. All plates were incubated overnight at 37 °C (Fig. 5E).

Parasite transfection

Parasite transfection was done according to the manufacturer's instruction (Jena Biosience, Germany) for electroporation of *Leishmania* with Eppendorf Multiporator. Mid-log phase wild-type (WT) promastigotes were harvested

by centrifugation (3000 rpm, 10 min, 4 ^{0}C), washed 2 times in PBS (8 mM Na_2HPO_4, 1.75 mM KH_2PO_4, 0.25 mM KCl, 137 mM NaCl; pH 7.2) and then resuspended at 4 × 10 ^{7} parasite/ml in cold HypoOsmolaric Buffer (HOB, Eppendorf) and kept on ice for 10 min (0.4 ml per transfection). Ten µg DNA (linear replacement construct) was added in 20 µl IsoOsmolaric Buffer (IOB, Eppendorf) to 0.4 ml cells, mixed well and transferred to a 2 mm electroporation cuvette (long electrodes) which was kept on ice. Cuvettes were pulsed using a Multiporator (Eppendorf, Germany) at 1000 V and 160 µsec and then returned to ice for exactly 10 min. The electroporated cells were kept in the liquid media in absence of antibiotics for 24 h prior to selection. To identify the resistant clones, transfected parasites were centrifuged (3000 rpm, 10 min, 4 ^{0}C) and plated onto semi-solid DMEM media which contained 1% noble agar in the presence of 50 µg/ml LEXSY Neo antibiotic (Jena Bioscience, Germany). Single isolated clones were cultured in liquid medium in the presence of 50 µg/ml LEXSY Neo antibiotics. After the second round of transfection, both LEXSY Neo antibiotic (at 50 µg/ml) and LEXSY Hygro antibiotic (at 100 µg/ml) (Jena Bioscience, Germany) were used to homozygote the colonial selection. In each experiment, wild-type parasites were electroporated without DNA and were used as a negative control.

Genotypes confirmation of mutant parasites

Genomic DNA was isolated from log-phase promastigotes by using Tissue (plus) SV mini kit (GeneAll, South Korea). PCR and Southern blot analysis were performed to confirm correct integration of the antibiotic-resistance gene into the *GPI12* site. The presence of an antibiotic-resistance gene was approved by PCR and by using specific primers forward and reverse. Forward: Regn1F 5-GAGGGTGACGGTGCCTATTT-3 and reverse: Regn1R 5-CCTTCCCGCTTCAGTGACAA-3). Forward primer Regn1F is designed for a sequence outside the targeted gene while the reverse primer (Regn1R) anneals within the neomycin resistance gene (NEO^r) gene and forward:A3804 5- CCGATGGCTGTGTA-GAAGTACTCG-3 anneals within the hygromycin resistance gene (HYG^r) gene and reverse: Reg2R 5-GAAACAGGATGGTACGGCTTGGA-3 for a sequence outside the targeted gene(14). NEO^r and HYG^r probe was generated with Biotin PCR Labelling Core Kit (Jena Biosience, Germany) by labelling the PCR product with biotin, which was done according to the manufacturer's instructions. To check the presence of neomycin-resistance genes in the mutants and to make a probe, specific internal primers of neomycin-resistance gene (forward: neoF 5-GACTGGTTGCTATTGGGCGA-3; reverse neoR 5- GAATCCAGAAAAGCGGCCAT-3) and hygromycin-resistance gene (forward hygf 5- CTCGGAGGGCGAAGAATCTC-3; reverse hygr 5- TCGTCCATCACAGTTTGCCA-3) were used (14). For Southern blot analysis, genomic DNA from mutant and wild type parasites were digested with *HindIII* and *Fse*I and later separated on a 0.8% agarose gel. DNA fragments were transferred onto a positively charged nylon membrane (Roche, Germany). Hybridization and detection of signals were carried out according to the standard method (13).

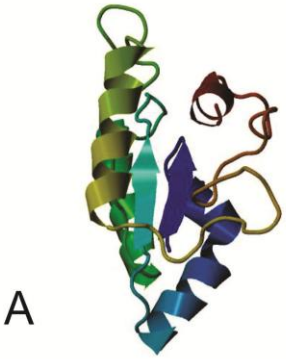
Detection of GPI12 gene mRNA

Total RNA from log-phase parasites were isolated using total RNA Purification Kit (Jena Biosience, Germany). The cDNA was synthesized from 10 ng of total RNA using the RocketScript RT PreMix with oligo-dT primers. The cDNA was PCR amplified with specific primer of *GPI12* gene (forward= CDf 5-GACTTGCAAGACGGCATGTG -3; reverse =CDR 5-AGACTGATGCGGGTGGAATG-3)(14).

Western blot analysis of GPI12 protein

GPI12 L. major protein sequence was derived from a gene bank. Linear epitopes analysis was performed by Optimum Antigen TM design

tool software and by SVMtrip software from the University of Nebraska (Fig. 4B). The epitopes, which had minimal cross-reactivity with the rabbit, were selected (Fig. 4C). These epitopes were obtained commercially from Genscript (USA) to produce antibodies and purification in rabbits (Fig. 4D).



A

B

MHSITVVCISVVLALLLLLWMKISNLAGTSVRSDVLFVFAHPDDEAMFFTPLLHSLRTQRVTVHFLCLSNGNYAGMGKEREKE
LYASGAFFGVQRRNIRVVDHADLQDGMCNIWSPLLIRREIESYMQKAGNISTIVTFDKYGVSGHPNHIAVHNGVRELKENM
PPGILHLQLRTRSLLWKYVGLLAVLPYALWSSTCVSRASFVAVIPPASVWESMAAMRKHAGQLVWFRYLFVIFSSYTYVNEIEEL

NO	Start	Antigenic Determinant	Length	Antigenicity/ Surface/ Hydrophilicity	Coil	Amphipathic	Synthesis	Mus_musculus Rattus_norvegicus Oryctolagus_cuniculus blast		
1	95	CRRNIRVVDHADLQD	14	2.34/0.71/0.48	Y	Y	N	50%	42%	42%
2	154	CHNGVRELKENMPPG	14	1.70/0.86/0.36	Y	Y	N	64%	64%	64%
3	31	VRSDVLFVFAHPDDC	14	1.31/0.57/-0.10	Y	N	N	42%	42%	64%
4	236	CIFSSYTYVNEIEEL	14	0.60/0.57/-0.33	Y	N	N	56%	56%	57%
5	117	CLLIRREIESYMQKA	14	0.52/0.64/0.32	N	Y	N	71%	71%	56%

C

D

CRRNIRVVDHADLQD

CHNGVRELKENMPPG

Fig. 4: (A). 3D structure of *GPI12* protein extracted from ExPASy protein portal.(B) Linear epitope analysis was performed by software.(C) Epitopes which had been blasted and had minimal cross-reactivity with the rabbit were selected.(D) These selected epitopes were ordered to produce antibodies

Promastigote forms were harvested by centrifugation at 3000 rpm for 15 min and washed in PBS. The pellets were immediately lysed in 2x SDS–PAGE sample buffer (2% w/v SDS , 4.5 mM Tris–HCl, pH 6.8, 10% v/v glycerol, 5% v/v 2-ercaptoethanol, 0.05% w/v bromophenol blue) on ice and then boiled for 5 min. Samples from both the wild type and mutant *L. major* were separated by SDS–PAGE in on a 12% (w/v) polyacrylamide gel (SDS gel apparatus; Clever, UK). For Western blot analysis, the resolved proteins were transferred onto PVDF transfer membrane (Sigma, Germany) using a Bio-Rad semi dry blotting system. The membrane was pre-equilibrated overnight with TBST solution (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) containing 3% skimmed milk and then primary antibodies against the linear epitopes *GPI12* protein was added and incubated. The PVDF membrane was washed with TBS solution(10 mM Tris–HCl (pH 7.4),

150 mM NaCl) and the secondary antibody (Goat anti-rabbit IgG alkaline phosphatase conjugate) (abcam, UK) was reacted and incubated, after washing, and a substrate solution (NBT/BCIP) was added followed by incubation at room temperature in the dark.

Results

Molecular confirmation of constructs

To confirm the colonies containing the desired plasmid, Colony PCR was performed. Colony PCR is a convenient high-throughput method for determining the presence or absence of inserted DNA in plasmid constructs (15). The transformants can be lysed in water with a short heating step. This initial heating step causes the release of the plasmid DNA from the cell, so it can serve as a template for the amplification reaction. A sterile toothpick was twirled in an individual colony, then it was inserted into 20µl of water in a new tube, it was twirled against the side wall, then the toothpick was streaked onto a grid location on the LB plate containing ampicillin and a backup of the colonies was gathered (Fig. 5f). This was repeated for as many colonies as required, 3 - 5 times is usually sufficient for each construct. The primers, which were designed to specifically target the inserted DNA, can be used to determine whether the construct contains the DNA fragment of interest. Colonies containing pLEXSY-neo2/R1 vectors (Fig. 5E) were confirmed by Colony PCR and by using specific primers forward and reverse. Forward: Regn1F 5'-GAGGGTGACGGTGCCTATTT-3' and Reverse: Regn1R 5'-CCTTCCCGCTTCAGTGACAA-3'. Fig. 5A shows 1.9-kb amplicon representing colonies containing pLEXSY-neo2/R1 vector (lane 5) and other colonies without these vectors have no specific amplified band. To confirm that pLEXSY-neo2/R1/R2 vectors were present in the colonies, Colony PCR was performed by specific primers forward and reverse. Forward: A1432 5'-GCATGGCGATGCCTGCTTGC-3'; Reverse: Reg2R 5'-GAAACAGGATGGTACGGCTTGGA-3'. Fig. 5B, shows 1.5-kb amplicon representing colonies containing the pLEXSY-neo2/R1/R2 vector (lane 5) and another colonies without these vectors which have no specific amplified band. To confirm that the pLEXSY-hyg2/R3 vector was present in the colonies, Colony PCR was performed by specific primers forward and reverse. Forward: P1442 5'-CCGACTGCAACAAGGTGTAG-3'; Reverse: A264 5'-CATCTATAGAGAAGTACACGTAAAAG-3'. Fig. 5C shows 0.5-kb amplicon representing colonies containing pLEXSY-hyg2/R3 vector (lane 7) and the other colonies, which lack these vectors and have no specific amplified band. To confirm that the pLEXSY-hyg2/R3/R4 vector was present in the colonies, Colony PCR was performed by specific primers forward and reverse. Forward: A3804 5'- CCGATGGCTGTGTAGAAGTACTCG-3'and Reverse: Reg2R 5'-GAAACAGGATGGTACGGCTTGGA-3'. Fig. 5D, shows 1.4-kb amplicon representing colonies with these constructs (lane 5).

Linear replacement construct

After the recombinant plasmids were confirmed by sequencing, a DNA fragment from recombinant plasmids containing the antibiotic-resistance gene (Neomycin and Hygromycin B) flanked by 5' and 3'F sequences was used for transfection. Both vectors were digested with *Swa*I to produce linear replacement fragments (5'F-NEO-3'F and 5'F-HYG-3'F, termed NEO^r and HYG^r cassettes). After the digestion pLEXSY-neo2/R1/R2 vector with *Swa*I, four fragments were produced: 3860, 2864, 1094 and 96 bp, among which 3860 bp was the specific fragment (Fig. 6A). pLEXSY-hyg2/R3/R4 vector, after being digested with *Swa*I, produced the following four fragments:4082,2868,1094, and 96 bp, among which 4082 bp was the specific fragment (Fig. 6B). The Linear replacement construct were purified from the gel before electroporation (agarose gel extraction kit, Jena Bioscience, Germany).

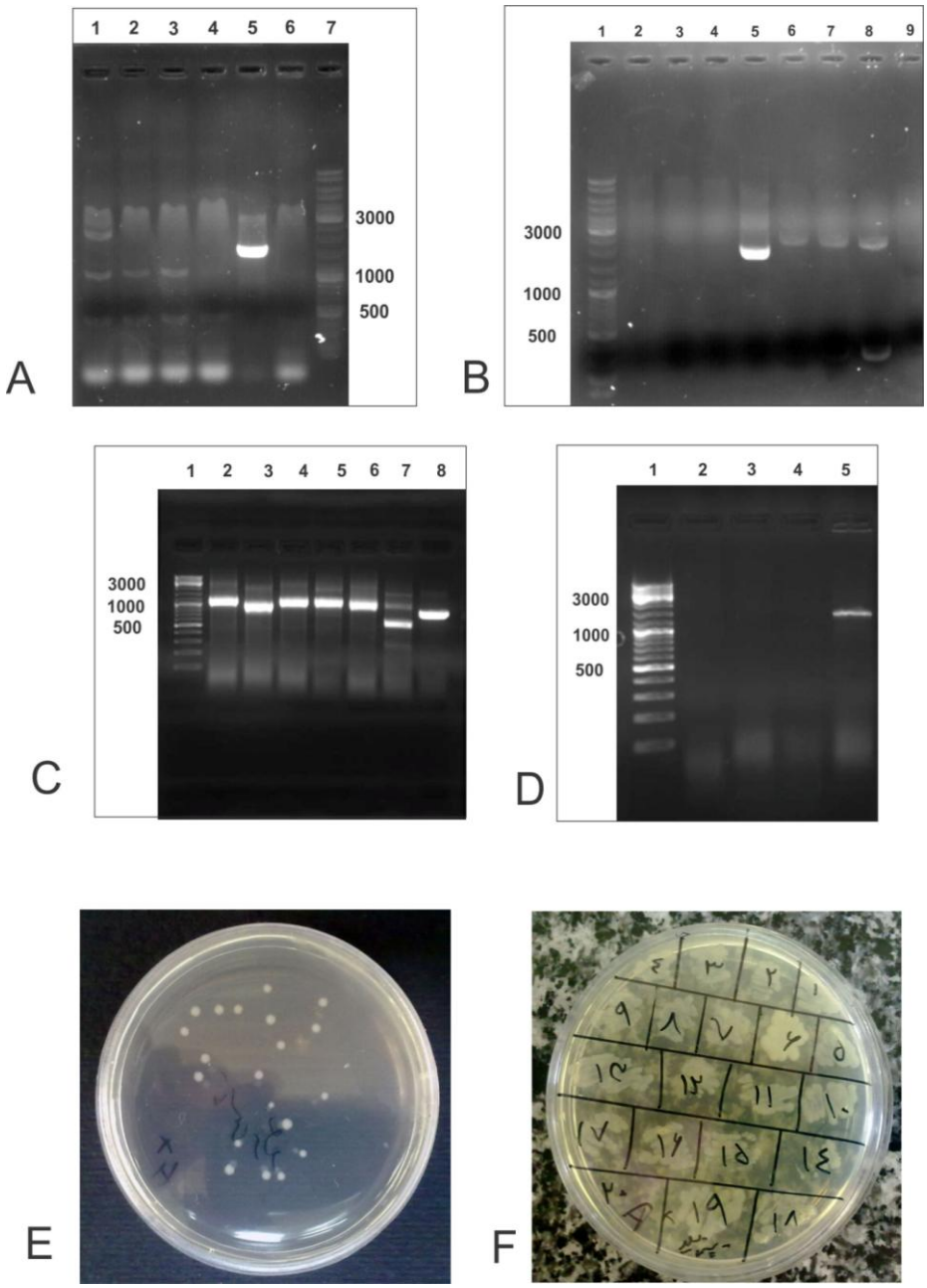


Fig. 5: To confirm colonies containing the desired plasmid, Colony PCR was performed (A) Three colonies were tested using similar primers Regn1R and Regn1F. Lane 5 represents the expected band of 1.9 kb that contain pLEXSY-neo2/R1. Lane 7 shows molecular size marker. (B) Confirm colonies that contain pLEXSY-neo2/R1/R2 vectors Colony PCR performed by specific primers A1432 and Reg2R. Lane 5 represents the expected band of 1.5 kb that contains pLEXSY-neo2/R1. Lane 1 shows molecular size marker. (C) Confirms that pLEXSY-hyg2/R3 used P1442 and A264 primers. Lane 7 shows 0.5-kb amplicon representing colonies, which contain pLEXSY-hyg2/R3 vector. Lane 1 shows molecular size marker. (D) Colonies with pLEXSY-hyg2/R3/R4 vectors are confirmed by these primers: A3804 and Reg2R. Lane 5 shows 1.4-kb amplicon representing colonies with these constructs. Lane 1 shows a molecular size marker. (E) LB agar plate with ampicillin antibiotic along with colonies of *E.coli* top10 contains some constructs after the transformation. (F) The LB agar plate, which is used as a backup plate

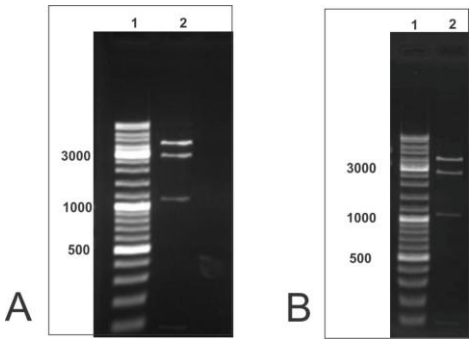


Fig. 6: Vectors were digested with *Swa*I to produce a linear replacement. (A) Lane 2, pLEXSY-neo2/R1/R2 vector produced four fragments after being digested with SwaI: 3860, 2864, 1094 and 96 bp, of which 3860 bp was the specific fragment. Lane1 shows molecular size marker. (B) Lane 2, pLEXSY-hyg2/R3/R4 vector produced the following four fragments after being digested with *Swa*I: 4082, 2868, 1094, and 96 bp, of which 4082 bp is the specific fragment. Lane 1 shows molecular size marker

Genotypic analysis of mutant parasites for antibiotic gene integration

In the first round of transfection, we expected that one allele of *GPI12* was disrupted by NEO construct replacement using the 5'F-NEO-3'F cassette. To generate double mutants, 5'F-HYG-3'F fragment was used to target the second allele of *GPI12*.To find the homozygote clones, a selection procedure was performed on semi-solid medium supplemented with both lexsy NEO and lexsy HYGR. In order to verify the complete *GPI12* ORF replacement by NEO^r gene and HYG^r through homologous recombination, we analysed genomic DNA of mutants using both PCR and Southern blot analysis. Antibiotic specific integration was confirmed using primers Regn1R and Regn1F (14).

For Southern blot analysis, genomic DNA from wild-type and mutants was digested with *Fse*I/*Hin*dIII and hybridized with the Biotin-labelled HYG^r and NEO^r probe (14).

Morphological features of the mutant parasites

The size of wild type and mutant promastigotes after staining with Giemsa was measured by microscope eyepiece micrometre. Light microscopy analysis demonstrated that mutant parasites are not different in size and shape in comparison to the wild-type parasites.

Gene expression of GPI12 in wild-type and mutant parasites

Expression of *GPI12* was studied by RT-PCR. cDNA from wild-type and mutant strains were used in RT-PCR reaction with *GPI12*-specific primers. The amplification of a specific band in cDNA of wild-type *L. major* promastigotes confirmed the presence of *GPI12* mRNA and its expression (Fig. 7. Lanes 2 and 3) but in cDNA of mutant parasites no specific amplified band was observed and the absence of *GPI12* and its expression was confirmed (14).

Protein product in the in wild-type and mutant parasites

To confirm the presence of protein products in the mutants, as well as the wild-type *L. major* as the control, Western blot analysis was performed by using cell lysates from promastigotes. Anti-*GPI12* linear epitopes were able to detect the 28.8kDa protein band corresponding to *GPI12* in the wild type (Fig. 7A. lanes 2 and 3) however, the mutant parasite had no such specific band (Fig. 7A. lanes 4 and 5); confirming the absence of *GPI12* protein product. The obtained data is consistent with RT-PCR of the mutant parasites. To obtain a suitable concentration of the primary antibody, it was tested by Dot Western Blot (Fig. 7B).

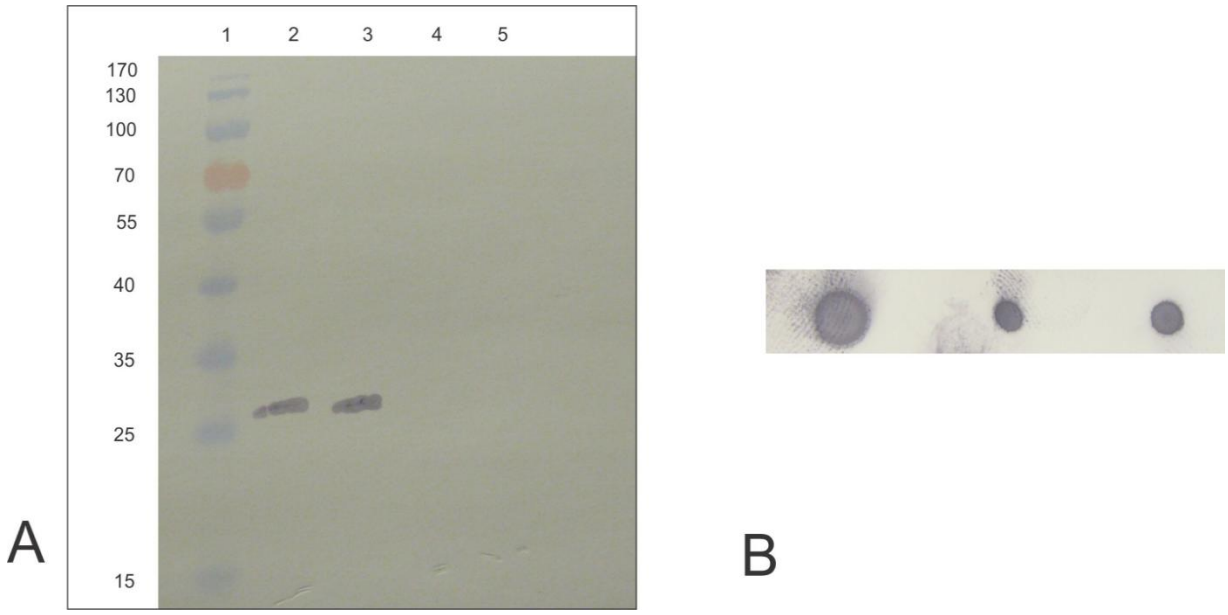


Fig. 7: The expression in parasites as determined by Western blot analysis. (A) Lysates from both the mutant parasites (lanes 4 and 5) and the wild-type (lanes 2 and 3) were separated on 12% SDS–PAGE and transferred onto the membrane. The *GPI12,* which was detected by using anti-*GPI12* linear epitopes, was recognizable as a 28.8 kDa protein band. Lane 1 shows protein size markers (14). (B)To obtain a suitable concentration of the primary antibody, it was tested by Dot Western Blot

Discussion

The purpose of this study was to delete *GPI12* gene in *L. major* by the homologous recombination method. We demonstrated that both alleles of *GPI12* gene in *L. major* were removed. We also showed that they were able to generate null mutants, which supports the idea that the *GPI12* gene is not essential for the survival and growth of *Leishmania*. The homozygous knockouts of *Leishmania* were able to survive. In this study, we adopted those methods that save our time and money.

In the present study, the flank region was synthesized using the pGEM-b1 vector. In a few studies (10, 16, 17), 5 ‘ and 3’ flanking regions of the *Lmjsp* were amplified by PCR from *L. major* genomic DNA using specific primers containing restriction enzyme sites. This is correct, but when the PCR product can be incised with enzymes, incision areas are not visible on agarose gel, and a long time has to be spent on building constructs, and cloning would take very long. However, in our method, the incision areas were visible on the gel and the ligation cloning was shorter.

In this study, we used of the pLexsy-hyg2 and pLexsy-neo2 vectors. Previously these vectors have been used for gene knock-in in *L. major* (18). In order to achieve our goal (i.e. gene knockout), the structures of the vectors had to be modified. Utr2, utr3 areas are required for the trans splicing and polyadenylation of the gene marker. We have shown that the use of these vectors is possible to delete a gene in *L. major*.

In our study, Southern blot analysis was performed to confirm the correct integration of the antibiotic-resistance gene into the gene site. NEO^r and HYG^r probes were generated by labelling the PCR product with Biotin PCR Labelling Core Kit. In such studies, usually radiolabelling probes are used. Non-radioactive detection methods represent some

advantages versus the radioactive methods as they avoid the need for specific waste disposal methods and the licensing and safety concerns connected with the use of radioactive material. Non-radioactive probes are also more stable than the radioactive probes that are made with P^{32} and Southern blotting detection sensitivity is comparable between the two probe types. Exposure times to detect the hybridization signal in non-radioactive detection methods are shorter. Overall, non-radioactive probes are cheaper (19).

Western blotting is a technique that used for identification of specific proteins. In this technique, proteins are separated through vertical gel electrophoresis based on their molecular weight and by type. Separated proteins are then transferred to a membrane, each protein producing a specific band in the membrane. The membrane is blocked and then incubated with specific antibodies. Usually primary polyclonal antibodies are used which are prepared after injection of the complete protein into a suitable host (Fig. 4A).

We however, have used a new method in our own experiment. In Western blot analysis, linear epitopes of protein are exposed to the primary antibody after SDS page is performed. Considering this, we attempted to use only the linear epitopes of *GPI12* protein of *L. major* for antibody production (instead of the complete proteins) which were analysed using software and used after performing necessary check-ups with appropriate host epitopes of these epitopes.

Using the linear epitope antibody instead of a complete protein, removes synthesis protein production and the purification step (20) and reduces the possibility of cross-reactivity with other proteins.

The drugs currently available are not satisfactory, because drug therapy for a long time is expensive and toxicity and drug resistance has been observed. In recent years, the production of an effective vaccine to prevent human *Leishmania* has been considered but not yet made (21). According to an old principle of vaccination, as the vaccine is closer to the nature of a disease, it provides a better immunity. In terms of public health, attenuated vaccines are the gold standard for protection against intracellular organisms, for example, the production of vaccines to protect against rubella, smallpox, measles, etc. Many methods are used for the production of attenuated strains such as in long-term *in vitro* cultures (22), selected for temperature sensitivity (23), chemical mutagenesis (24) and γ-attenuation (25). Although these attenuated strains created protection in a murine model, but because of the possibility of them returning to the wild form are not suitable for use in humans (25). In addition, unknown attenuation leads to loss of effectiveness for protective immunity, because these strains fail to infect or did not express major antigen epitopes for long time (26). The completion of the project to completely sequence the genome of *Leishmania* species, provided the possibility of genetic manipulation for the manufacture of new vaccines. A defined and specific genetic alteration of the *Leishmania* genome can be brought about using a gene targeting strategy through homologous recombination that allows the generation of parasites, which lack the specific genes essential for virulence and long-term survival. The first recombinant parasite generated by gene replacement was *the L. major* dihydrofolate reductase-thymidylate synthase (dhfr-ts) knock out (27). Vaccines made by this method keep the original form of the antigenic parasites.

Virulence is influenced by two groups of parasite molecules: one group consists of a vast majority of the products secreted by the parasite membrane and the other group is intracellular molecules, which encompasses the so-called pathoantigen and generally conserves proteins in the cytoplasm. The patients' immune responses are produced against the parasitical intracellular antigens. In contrast, the immune response produced against surface or secreted molecules is low. Actually,

many of these molecules contribute to the parasite infection in the host cells (28-31).

Conclusion

We were able to create a null mutant parasite that maintained the original form of the antigenic parasites, which may make an effective immunity. Further investigations are essential to check this model in a suitable host.

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References

1. Alvar J VI, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M. WHO Leishmaniasis Control Team. Leishmaniasis Worldwide and Global Estimates of Its Incidence. PLoS ONE. 2012;7(5):e35671.
2. Postigo JAR. Leishmaniasis in the World Health Organization Eastern Mediterranean Region. Int J Antimicrob Agents. 2010; 36 Suppl 1:S62-5.
3. Croft SL SS, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. Clin Microbiol Rev. 2006;19(1):111-26.
4. Khatami A FA, Firooz A, Gorouhi F, Dowlati Y. Treatment of acute Old World cutaneous leishmaniasis: a systematic review of the randomized controlled trials. J Am Acad Dermatol. 2007;57(2):335.
5. Späth GF EL, Epstein L Leader B, Singer SM, Avila HA, Turco SJ, Beverley SM. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. Proc Natl Acad Sci U S A. 2000;97(16):9258-63.
6. Chawla B, Madhubala R. Drug targets in *Leishmania*. J Parasit Dis. 2010;34(1):1-13.
7. Silvestre R C-d-SA , Cordeiro-da-Silva A, Ouaissi A. Live attenuated *Leishmania* vaccines: a potential strategic alternative. Arch Immunol Ther Exp (Warsz). 2008;56(2):123-6.
8. Hidekazu K. Enhancement of Homologous Recombination Efficiency by Homologous Oligonucleotides. Cell Interaction. 2012. p. 233.
9. Capecchi MR. Altering the genome by homologous recombination. Science. 1989;244(4910):1288-92.
10. Cruz A CC, C M Coburn , Beverley SM. Double targeted gene replacement for creating null mutants. Proc Natl Acad Sci USA. 1991;88(16):7170-4.
11. Crabb BS CB, Cooke BM ,Reeder JC, Waller RF, Caruana SR, Davern KM, Wickham ME, Brown GV, Coppel RL, Cowman AF. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell. 1997;89(2):287-96.
12. Fulwiler AL SD, Soysa DR , Ullman B, Yates PA. A rapid, efficient and economical method for generating leishmanial gene targeting constructs. Mol Biochem Parasitol. 2011;175(2):209-12.
13. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual: Cold Spring Harbor Laboratory Press; 2001.
14. Almani PG, Sharifi I, Kazemi B, Babaei Z, Bandehpour M, Salari S, et al. The role of GlcNAc-PI-de-N-acetylase gene by gene knockout through homologous recombination and its consequences on survival, growth and infectivity of *Leishmania major* in in vitro and in vivo conditions. Acta Trop. 2016;154:63-72.
15. Soltani M SA, Sadrebazzaz A, Nassiri M, Tahmoorespoor M. Cloning, Nucleotide Sequencing and Bioinformatics Study of NcSRS2 Gene, an Immunogen from Iranian Isolate of *Neospora caninum*. Iran J Parasitol. 2013;8(1):114-27.
16. Taheri T SA, Salmanian AH, Gholami E, Doustdari F, Zahedifard F, Rafati S. *Leishmania major*: disruption of signal peptidase type I and

its consequences on survival, growth and infectivity. Exp Parasitol. 2010;126:135-45.
17. Huynh C SD, Sacks DL , Andrews NW. A *Leishmania amazonensis* ZIP family iron transporter is essential for parasite replication within macrophage phagolysosomes. J Exp Med. 2006;203(10):2363-75.
18. Bolhassani A TT, Taheri T, Taslimi Y, Zamanilui S, Zahedifard F, Seyed N, Torkashvand F, Vaziri B, Rafati S. Fluorescent *Leishmania* species: development of stable GFP expression and its application for in vitro and in vivo studies. Exp Parasitol. 2011;127(3):637-45.
19. Karcher S. Non-radioactive nucleic acid detection systems. In: Gelvin S, Schilperoort R, editors. Plant Molecular Biology Manual: Springer Netherlands; 1994. p. 309.
20. Li Q HY, Hu Y , Xu Y, Chen J, Fang L, Liu Z, Jiao X. A gene knock-in method used to purify plasmid pSPI12 from *Salmonella enterica* serovar Pullorum and characterization of IpaJ. J Microbiol Methods. 2014;98:128-33.
21. Kedzierski L ZY, Zhu Y, Handman E. *Leishmania* vaccines: progress and problems. Parasitology. 2006; 133 Suppl:S87-112.
22. Mitchell GF HE, Handman E , Spithill TW. Vaccination against cutaneous leishmaniasis in mice using nonpathogenic cloned promastigotes of *Leishmania major* and importance of route of injection. Aust J Exp Biol Med Sci. 1984;62:145-53.
23. Gorczynski RM. Immunization of susceptible BALB/c mice against *Leishmania* braziliensis. II. Use of temperature-sensitive avirulent clones of parasite for vaccination purposes. Cell Immunol. 1985;94(1):11-20.
24. Kimsey PB TC, Theodos CM , Mitchen TK, Turco SJ, Titus RG. An avirulent lipophosphoglycan-deficient *Leishmania major* clone induces CD4+ T cells which protect susceptible BALB/c mice against infection with virulent L. major. Infect Immun. 1993;61(12):5205-13.
25. Rivier D SR, Shah R , Bovay P, Mauel J. Vaccine development against cutaneous leishmaniasis. Subcutaneous administration of radioattenuated parasites protects CBA mice against virulent *Leishmania major* challenge. Parasite Immunol. 1993;15(2):75-84.
26. Streit JA RT, Recker TJ ,Filho FG, Beverley SM, Wilson ME. Protective immunity against the protozoan *Leishmania chagasi* is induced by subclinical cutaneous infection with virulent but not avirulent organisms. J Immunol. 2001;166(3):1921-9.
27. Titus RG G-FF, F J Gueiros-Filho ,de Freitas LA, Beverley SM. Development of a safe live *Leishmania* vaccine line by gene replacement. Proc Natl Acad Sci U S A. 1995;92(22):10267-71.
28. Kwang-Poo Chang, Bradford S McGwire. Molecular determinants and regulation of *Leishmania* virulence. Kinetoplastid Biol Dis. 2002;1(1):1.
29. Chang KP RS, Reed SG , McGwire BS, Soong L. *Leishmania* model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity. Acta Trop. 2003;85(3):375-90.
30. Seyed N ZF, Safaiyan S, Gholami E, Doustdari F, Azadmanesh K, et al. In silico analysis of six known Leishmania major antigens and in vitro evaluation of specific epitopes eliciting HLA-A2 restricted CD8 T cell response. PLoS Negl Trop Dis. 2011; 5(9):e1295.
31. Seyed N, Taheri T, Vauchy C, Dosset M, Godet Y, Eslamifar A, et al. Immunogenicity evaluation of a rationally designed polytope construct encoding HLA-A*0201 restricted epitopes derived from Leishmania major related proteins in HLA-A2/DR1 transgenic mice: steps toward polytope vaccine. PLoS One. 2014;9(10):e108848.