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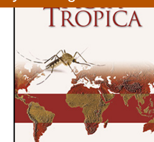

The role of *GlcNAc-PI-de-N-acetylase* gene by gene knockout through homologous recombination and its consequences on survival, growth and infectivity of *Leishmania major* in *in vitro* and *in vivo* conditions

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ABSTRACT

At present, there are no efficacious vaccines or effective drugs against leishmaniasis; therefore new and innovative control methods are urgently required. One way to achieve this important goal is through using reverse genetic engineering to evaluate important enzymes, proteins and macromolecules. One of the most important enzymes for Glycosylphosphatidylinositol (GPI) biosynthetic pathways is *GlcNAc-PI-de-N-acetylase* (*GPI12*). The molecular constructs were cloned in *Escherichia coli* strain Top 10 and confirmed by molecular methods and were transfected by electroporation into *Leishmania major*. We demonstrated that two alleles of the *GPI12* gene in *L. major* were successfully removed and enabling the generation of a null mutant, which supports the idea that *GPI12* is not an essential gene for the growth and survival of *Leishmania* and the homozygous knockouts of *Leishmania* are able to survive. We were able to produce a mutant parasite that caused no damaged to the host. Further investigations are essential to check the safety profile in laboratory animals.

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1. Introduction

Leishmaniasis is a major public health problem occurring primarily in tropical and sub-tropical countries (Alvar et al., 2012). The control and prevention of leishmaniasis, especially in the case of the zoonotic form, has been unsuccessful in most countries (Croft et al., 2006; Khatami et al., 2007). No efficacious and safe vaccine has yet been found for human leishmaniasis and, treatment is the only major choice available to help control cutaneous leishmaniasis (CL). At present, pentavalent antimonial compounds are the standard treatment for all forms of leishmaniasis, but the use of these drugs are limited due to long duration of use, side-effects, low efficacy and increasing resistance.

One way to begin to create new treatments is through the evaluation, by reverse genetic engineering, of important enzymes, proteins and macromolecules. Ever since 1990, reverse genetic tools have been used to study the genes in *Leishmania* (Clayton, 1999). Several reports have shown that some of these genes perform essential roles in different aspects such as survival, growth, differentiation, and infectivity. In *Leishmania major*: metalloprotease GP63 (Hassani et al., 2014), ATG5 protein (Williams et al., 2012), NAD(P)H cytochrome b5 oxidoreductase (Mukherjee et al., 2012), signal peptidase type I (Taheri et al., 2010), thymidine kinase (Thiel et al., 2008), lipophosphoglycan (LPG) (Späth et al., 2003) and leishmanolysin (Joshi et al., 2002); in *Leishmania donovani*: nitroreductase (Wyllie et al., 2013), centrin (Selvapandiyan et al., 2004) and ornithine decarboxylase (Jiang et al., 1999); and in *Leishmania mexicana*: hexose transporter gene (Feng et al., 2013), cysteine proteinases (Williams et al., 2006) and thymidine kinase (Garami and Iiq, 2001).

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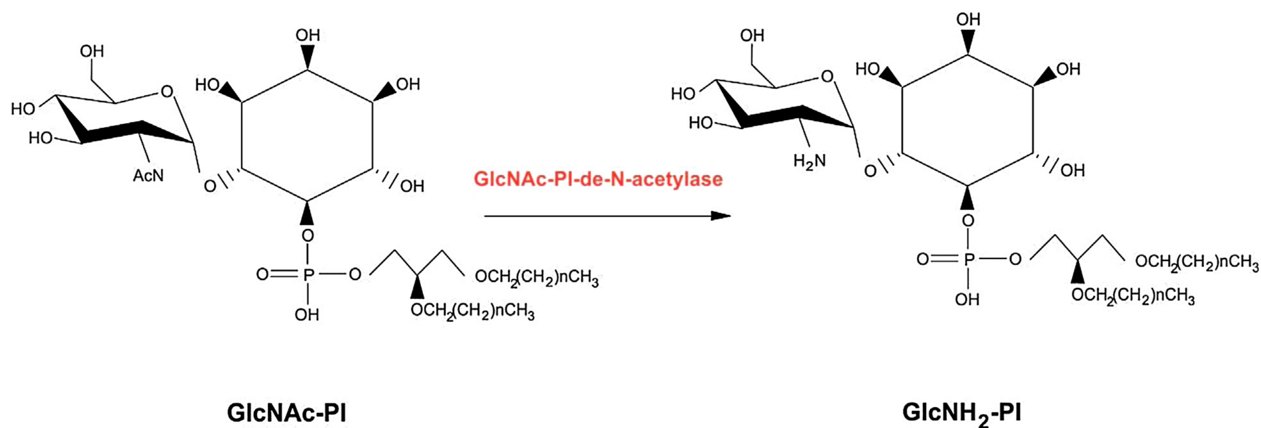


Fig. 1. The second step of GPI biosynthetic pathway.

In this study, the role of the *GlcNAc-PI-de-N-acetylase* (*GPI12*) gene has been evaluated in the growth, survival and infectivity of *L. major* by deletion of the gene through homologous recombination.

There are several cell surface glycoproteins of *Leishmania* that are anchored to the cell surface by Glycosylphosphatidylinositol (GPI) glycolipids. This glycocalyx consists of GPI anchored lipophosphoglycans (LPG), GPI anchored glycoproteins, and free GPIs. The role of these molecules is to protect the parasite from degrading enzymes such as a hydrolase and the alternate complement pathway (Naderer et al., 2004; Späth et al., 2000). The conserved protein glycan that is linked to GPIs is Ethanolamine-*P*-Mana1-2Mana1-6Mana1-4GlcNH2 and is attached to the D-*myo*-inositol ring of phosphatidylinositol (PI) (Ferguson et al., 1999). GPI anchors are synthesized in the endoplasmic reticulum membrane at different stages and then attached to the proteins. So the GPI anchored proteins are made then transferred through the secretory pathway to the plasma membrane. In GPI biosynthesis the first step is transferring *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to PI as a result, the GlcNAc-PI is formed. The catalysing enzyme in this reaction is GPI-*N*-acetylglucosaminyltransferase (GPI-GnT). Then, GlcNAc-PI is de-*N*-acetylated to form $GlcNH_2$-PI by *GlcNAc-PI-de-N-acetylase* (Chandra et al., 2010) (Fig. 1).

In *Trypanosoma brucei* bloodstream forms GPI biosynthesis is necessary; and has been examined as a potential as a drug target (Ferguson, 2000). Trypanosomal *GlcNAc-PI-de-N-acetylase* enzyme is zinc metalloenzyme (Urbaniak et al., 2005) and *GlcNAc-PI-de-N-acetylase* enzymes from *T. brucei* (Sharma et al., 1997) and *L. major* (Smith et al., 1997) were studied *in vitro* and little difference found compared to their human enzyme substrate. Investigation of specific substrate GlcNR-PI analogue of GlcNAc-PI showed that when this analogue was used no de-*N*-acetylated was observed in these parasites but there was no effect on the human enzyme (Smith et al., 2001).

The goal of the present study is to delete the *GPI12* gene and evaluate the *in vivo* and *in vitro* role of this gene and effect of its deletion on the growth, survival and infectivity of *L. major*.

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in accordance with the Ministry of Health and Education of Iran Animal Care Guidelines and approved by the Ethics and Animal Care Committee of Kerman University of Medical Sciences under Ethics Committee no. KMU.REC.1394.1.

2.2. Parasite

L. major Friedlin strain MRHO/IR/75/ER was isolated from infected BALB/c mice and then cultivated in DMEM (Dulbecco's Modified Eagles Medium) high glucose (4.5 g/l) media (Biosera, France) with 10% foetal bovine serum (Gibco, UK), 40 mM HEPES, 2 mM L-glutamine and 10,000 units of penicillin (base) and 10,000 μg of streptomycin (base)/ml (Sigma, Germany) and incubated at 26 °C. Log phase promastigotes (after 3 passages) were used for transfection. Stationary phase promastigotes were used for *in vitro* cell line J774 macrophage infectivity test also for *in vivo* infection.

2.3. The construction of the replacement vectors

In order to design and make the molecular construct we used pLEXSY-neo2 and pLEXSY-hyg2 vectors (Jena Bioscience, Germany). These vectors contain gene markers which cause aminoglycoside phosphotransferase and hygromycin phosphotransferase, respectively, after they enter the genome. The selection of these transfected parasites was performed through constructs which contain these markers that can be isolated by Neomycin and Hygromycin antibiotics.

These vectors are made for the expression or gene knock-in in *Leishmania* parasites which are required in order to modify for gene knock out.

In order to perform trans-splicing and polyadenylation on marker genes, utr2 and utr3 areas were required. Hence, we had rearranged the order of the areas and place the flank gene *GPI12* area (which we intend to delete) after the utr2 and utr3 areas. The sequence of these vectors were evaluated by web cutter2 and neb cutter2 software so that we could find suitable locations for the enzyme place. We chose the enzymes *Bgl*II and *Kpn*I in the left flank area and the enzymes *Fse*I and *Bsrg*I in the right flank area.

The flank area with enzymes were synthesized in the pGEM-b1 vector by Bioneer company (South Korea) then we digested the flank areas by the above mentioned enzymes and situated them in the pLESXY vectors. The molecular constructs were cloned in the *E. coli* strain Top10, which was confirmed by molecular methods (colony PCR and sequencing) and were transfected by electroporation into *L. major*. As for the transfection, a DNA fragment extracted from recombinant vectors containing the antibiotic resistance genes (Neomycin and Hygromycin B) which were flanked by 5\F and 3\F sequences, was used. Both of vectors were digested with *Swa*I to produce linear fragments for transfection (5\F-NEO-3\F and 5\F-HYG-3\F, termed NEO^r and HYG^r cassettes). The fragments were

purified from the gel (agarose gel extraction Kit, Jena Bioscience, Germany) before electroporation.

2.4. Transfection

Log phase wild-type (WT) promastigotes parasites were centrifuged (3000 rpm, 10 min, at 4 °C), washed in PBS buffer 2 times (1.75 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 0.25 mM KCl; pH 7.2) and parasites were resuspended at 4×10^7 parasites/ml in cold HypoOsmolaric Buffer (HOB, Eppendorf) and kept on ice for 10 min (0.4 ml per transfection). Then, 10 μg DNA was added to 20 μl IsoOsmolaric Buffer (IOB, Eppendorf) to 0.4 ml cells, mixed well and transferred to electroporation cuvette kept on ice. It was pulsed with Multiporator (Eppendorf, Germany) for $d = 2$ mm cuvettes at 1000 V and 160 μs and returned to wet ice for exactly 10 min. Electroporated parasites were kept in a liquid medium without antibiotics for one day prior to selection.

To isolate the clones that contained the resistance gene, transfected parasites were gathered by centrifugation (3000 rpm, 10 min, at 4 °C) and plated on to semi-solid DMEM media containing 1% agar in the presence of 50 μg/ml LEXSY Neo antibiotic (Jena Bioscience, Germany). The isolated clones were cultured in a liquid medium with of 50 μg/ml LEXSY Neo antibiotic. For the second round of the transfection, both LEXSY Neo antibiotic at 50 μg/ml and LEXSY Hygro antibiotic at 100 μg/ml (Jena Bioscience, Germany) were used for the selection of homozygote clones. For negative control in tests, WT parasites were electroporated without DNA.

2.5. Genotypes confirmation of mutant parasite

DNA was extracted from the promastigotes by Tissue (plus) SV mini kit (GeneAll, South Korea). To confirm the correct integration of the resistance gene into the *GPI12* site, Southern blot and PCR were performed. The presence of the resistance gene was confirmed by PCR and by using specific forward and reverse primers (forward: Regn1F 5-GAGGGTGACGGTGCCTATTT-3 and reverse: Regn1R 5-CCTTCCCGCTTCAGTGACAA-3). The forward primer (Regn1F) was for a sequence outside the targeted gene while the reverse primer (Regn1R) anneals within the NEO^r gene and the forward: A3804 5-CCGATGGCTGTGTAGAAGTACTCG-3 anneals within the HYG^r gene and reverse: Reg2R 5-GAAACAGGATGGTACGGCTTGGA-3 for a sequence outside the targeted gene.

Probes (HYG^r and NEO^r) were produced by labelling the PCR product with Biotin by using Biotin PCR Labelling Core Kit (Jena Biosience, Germany) according to manufacturer's instructions. To confirm the presence of the resistance gene in the mutant's genome and make a probe, 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. In Southern blot test, DNA from WT and mutant parasite were digested with *Fse*I and *Hind*III restricted enzymes and separated on a 0.8% agarose gel then they were transferred on to a nylon membrane (Roche, Germany) (Sambrook and Russell, 2001).

2.6. Detection of mRNA by RT-PCR

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

2.7. Western blot of GPI12 protein

Promastigotes were centrifuged (3000 rpm for 15 min) and the supernatant discarded and the pellets washed twice with PBS buffer. The pellets were then lysed using SDS-PAGE (2×) sample buffer (4.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 0.05% w/v bromophenol blue, 5% v/v 2-mercaptoethanol) that were added to the pellets on ice and then boiled for 5 min (Bolhassani et al., 2011). Proteins from WT and mutant parasites were separated by SDS-PAGE in a 12% polyacrylamide gel (SDS gel electrophorese; Clever, UK). For western blot, the separated proteins were transferred onto the PVDF transfer membrane (Sigma, Germany) by using a Bio-Rad semi-dry blotting system. The PVDF membrane was soaked with TBST solution (150 mM NaCl, 10 mM Tris–HCl, pH 7.4 and 0.1% Tween 20) with 3% skimmed milk for 2 h and then reacted with antibodies against the linear epitopes *GPI12* protein. These epitopes were from the Genscript Company (USA) for producing antibodies and purification in rabbits. The membrane was washed and the secondary antibody (Goat anti-rabbit IgG alkaline phosphatase conjugate) was added and incubated, after washing, the substrate solution (NBT/BCIP) was added and incubated at room temperature in dark conditions.

2.8. In vitro *studies*

The murine macrophage (MQ) cell line J774 was grown in DMEM high glucose (4.5 g/l) media (Biosera, France), with 10% foetal bovine serum (Gibco, UK), 40 mM HEPES, 2 mM L-glutamine and 1% PenSterp in tissue culture flask and incubated at 37 °C in 5% CO_2 for 3 days. After preparing the macrophages, we counted them and then added 500,000 MQ cells to each well of a microplate and incubated them at 37 °C in 5% CO_2 for 1 h to attach them to the plate.

For infecting the MQ, the stationary phase promastigotes from WT and mutants were added at the ratio of 5:1 (parasites to MQ) and again incubated at 37 °C in 5% CO_2 for 1 h. To exclude the free parasites, the supernatants were peptized and then 400 μl of complemented DMEM medium was added to each well and at 6, 24, 48 and 72 h of the experiment, the contents of the wells were removed by pipetting and transferred to a microtube and then centrifuged (1800 rpm, 5 min, 4 °C) and the supernatant discarded. Then, the sediment was smeared on a slide and fixed with methanol and stained by Giemsa and observed under a microscope.

One hundred MQ were evaluated in each coverslip then the percentage of the infected MQ and the mean number of parasites per MQ were calculated. The experiments were performed three times at different time points.

2.9. In vivo *studies*

Forty female BALB/c mice weighing 20–25 g and aged 6–8 weeks were obtained from the animal house of Kerman University of Medical Sciences. The animals were fed with standard food and water and keep under standard conditions: temperature 20–23 °C, 55 ± 10% relative humidity with a 12 h light and dark cycle, in a pathogen free environment. They were randomly allocated into 2 groups ($n = 20$).

Each group was inoculated subcutaneously at the base of the tail with 1×10^6 stationary promastigotes of mutant or WT parasites. The lesion size was measured weekly, using a metric calliper. Mice were euthanized at different time intervals and tissues including lymph node and skin from the base of the tail were gathered from each group (three mice randomly per group selected at each specific period) and evaluated for live parasites by culturing in DMEM medium with 10% FBS (and LEXSY Neo, HYGR antibiotics for mutant

parasites). Also, PCR was performed to confirm the mutant parasites.

2.10. Quantification of parasite load

To quantify the parasite load, lymph node and skin from the base of the tail were collected from two mice from each group at different time points post infection. Genomic DNA was extracted from 20 mg of tissue with Tissue (plus) SV mini (GeneAll, South Korea). Reaction was performed by using 100 ng of the DNA, 5 pmol of RV1 (forward: 50-CTTTTCTGGTCCCGCGGGTAGG-30) and RV2 (reverse: 50-CCACCTGGCCTATTTTACACCA-30) primers (Lachaud et al., 2002) and 5 μl Qiagen QuantiFast SYBR Green Mastermix (Qiagen, Germany) in a total volume of 12 μl with the following amplification program: 95 °C for 5 min, 25 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s in an Rotor Gene Q real time system (Qiagen, Germany). The DNA of normal mouse skin at the base of the tail and WT were used as negative and positive controls, respectively. For drawing the standard curve the WT DNA in dilution series 0.1–10^5 parasites was used.

2.11. Statistical analysis

The statistical analysis between the two groups done by Student's *t*-test using SPSS software. The differences were considered significant at $p < 0.05$. The data was presented as means ± SD.

3. Results

3.1. The genotypic analysis

For verifying the knock out *GPI12* gene by resistance genes we evaluated the DNA of mutants by PCR and Southern blot tests. Antibiotic resistance gene integration was confirmed by using primers Regn1R and Regn1F. Fig. 2 shows 1.9-kb amplicon representing NEO^r (Fig. 2A, lane 2, 3); yet, WT has no specific amplified band (Fig. 2A, lanes 4, 5). Fig. 2B shows 1.4-kb amplicon representing HYG^r integration (Fig. 2B, lanes 2, 4) and WT has no specific amplified band (Fig. 2B, lanes 3, 5).

For Southern blot, DNA from WT and mutants was digested with *Fse*I and *Hind*III and hybridized with the Biotin-labelled NEO^r and HYG^r probe (Fig. 3A, C, and D). The results showed that neomycin probe was hybridized to a 4.4-kb fragment and the hygromycin probe was hybridized to a 4.6-kb fragment in the mutant parasites. In contrast no hybridization of probes with WT genomic DNA was seen (Fig. 3B).

3.2. Morphological properties of the parasites

The size of promastigotes in the WT and mutant was measured and compared daily using a microscope eyepiece micrometre after staining with Giemsa. The light microscopy observation shows that mutant parasites are not different in their shape and size in comparison to the WT parasites.

3.3. The gene expression of GPI12

The expression of *GPI12* was examined by RT-PCR. As it is shown in Fig. 4, the amplification of a 328 bp band in the cDNA of the WT promastigotes confirmed the presence of *GPI12* and its expression (Fig. 4, lanes 2 and 3); however, in the cDNA of the mutant parasite, no specific amplified band was observed and the absence of *GPI12* and its expression was confirmed (Fig. 4, lanes 4 and 5).

3.4. Protein production

To check the protein production in the mutants and WT parasites, Western blot analysis was performed. Anti- *GPI12* linear epitopes were able to recognize the 28.8 kDa protein band relevant to *GPI12* in the WT parasites (Fig. 5, lanes 2 and 3) and the mutant parasites (Fig. 5, lanes 4 and 5) no specific band confirmed the absence of the *GPI12* protein product. This confirms the data obtained with RT-PCR.

3.5. *In vitro* study

J774 cell line MQ was used to examine the influence of *GPI12* disruption on the infectivity and survival of *L. major* in *in vitro* condition. In Giemsa stained infected cells, each parasite was distinctly observed by the light microscope. Six hours after infection, the mutant parasites were attached and entered the macrophages (41.67 ± 1.12%; $n = 3$), although at a lower level in contrast to the WT parasites (97.67 ± 1.52%; $n = 3$). Within the 2 days post-infection, the mutants parasites were able to infect the MQ, however, the total number of the infected MQ was lower in comparison with the WT parasites ($p < 0.05$) (Fig. 6A). The percentage of the infected MQ with mutant parasites decreased by 72 h after the infection.

The total number of the parasites in one hundred macrophages was also recorded at different time points. The parasite count was lower in the mutants (613.33 ± 24; $n = 3$) in comparison with the WT parasites (1325.66 ± 25.54; $n = 3$) 6 h after the infection (Fig. 6B). The difference between the number of the mutant parasites and the WTs was more evident at 72 h after the infection ($p < 0.05$). The total number of the parasites inside the MQs decreased differently in both cases. It seems that the lack of the *GPI12* has direct influence on the parasite loads in the MQ. This result suggests that *GPI12* has an important role in the attachment, survival and replication of parasites in MQ.

3.6. *In vivo* study

We evaluated the ability of the mutant parasites in causing infection and lesions in BALB/c mice (Fig. 7A). The mice infected with wild-type *L. major* begun to develop lesions after 4 weeks and the skin at the base of the tail lesion increased (Fig. 7C and D). The mutant parasites could not create cutaneous lesions in the skin at the base of the tail of the infected mice even at six months after infection (Fig. 7B). At different time points (2, 4 and 8 weeks), in the two groups (wild and mutant) the infected lymph nodes were cultured in a complete media without or with (for mutant) any antibiotic, and motile and live parasites were detected.

In addition, we compared the number of the parasites in the skin at the base of the tail and the lymph nodes at 4 to 12 weeks after infection. The DNA obtained from these two sites was extracted to check parasite burden quantification by real-time PCR as shown in Fig. 8. The differences were significant at different time points ($p < 0.05$).

4. Discussion

The *GPI12* gene located in chromosome 9, Lmjchr9, LMJF_09_0040 749 bp. This gene encodes a protein with 249 amino acids. *GlcNAc-PI-de-N-acetylase* is an essential step required for all GPI biosynthetic pathways. The goal of present study was to examine the role of *GPI12* in the differentiation, growth and infectivity of *L. major* through gene disruption. We showed that two alleles of *GPI12* gene in *L. major* were successfully removed and were enabled to generate the null mutant, which supports the idea that *GPI12* appears to not be an essential gene for the

growth and survival of *Leishmania* and the homozygous knockouts of *Leishmania* are able to survive.

In this study, we observed that the mutant promastigotes had the same morphological properties in comparison with the WT promastigotes.

An *in vitro* study using murine MQ cell line J774, showed that by the use of recombinant parasites the entry rate and infecting ability was decreased.

As for the mutant parasites, they were unable to produce skin lesions even six months post-infection in the BALB/c mice. This data confirms our observation in the *in vitro* cultured MQ.

In general, the mutant parasites that grow within macrophages were divided into three categories: (1) mutant parasites that cause latency ulcers in sensitive mice, which are a defect in the early stages of infection. Such parasites are unable to perform the process of differentiation of procyclic promastigotes into infective metacyclic promastigotes and their invasion and survival in macrophages. The amastigote forms of these mutant parasites pass a natural course of proliferation and survival in the MQ cells. (2) Those parasites which can be differentiated into amastigotes within macrophages and in mice but which do not survive, and as a result the infection disappears after a relatively short time. (3) Those parasites that are capable of living without causing any harm (Myler

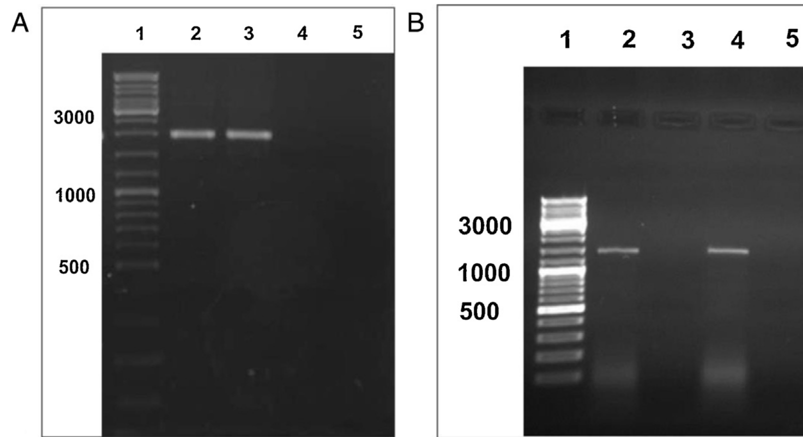


Fig. 2. Confirmation of antibiotic resistance gene integration by PCR in mutant parasites: (A) isolated clones with PCR using primers Regn1R and Regn1F. Lanes 2 and 3 show the specific band of 1.9 kb in mutant parasites with NEO^r, In the WT, as shown in lanes 4 and 5, no specific band was seen, Lane 1 is the ladder of DNA. (B) This shows 1.4-kb amplicon which represents HYG^r integration in lanes 2 and 4 while WT DNA has no specific band in lanes 3 and 5 and Lane 1 is the ladder of DNA.

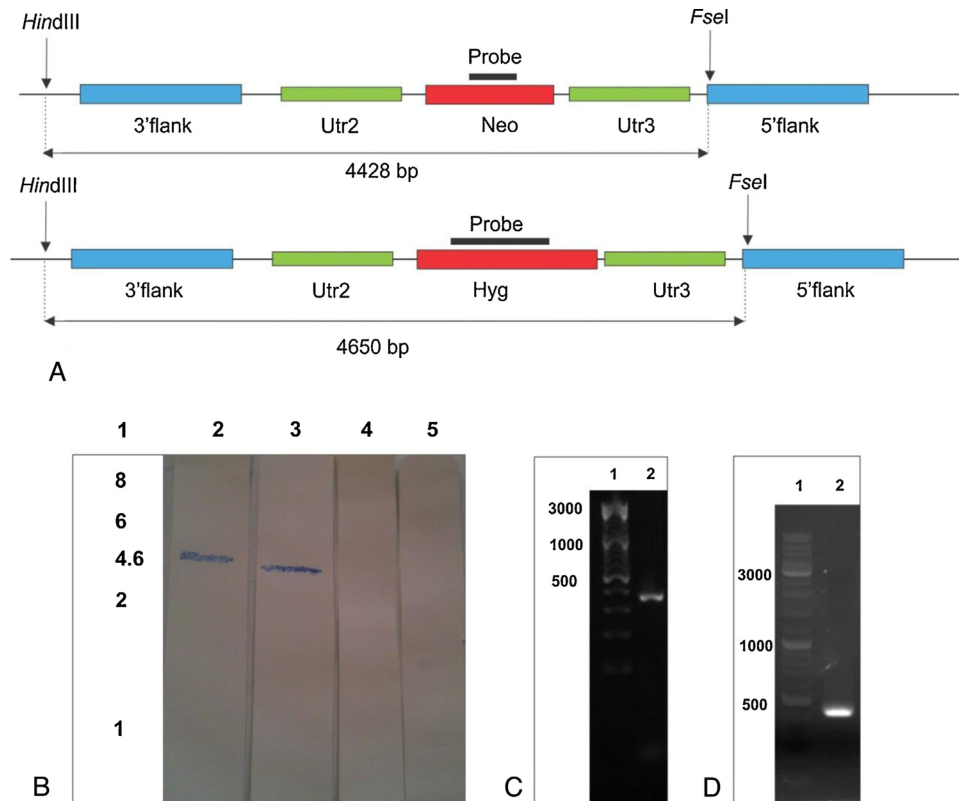


Fig. 3. Southern blot analysis of genomic DNA. (A) Restriction enzyme sites. (B) Genomic DNA derived from *L. major* wild-type and mutant parasite were digested with *fse*I and *Hind*III and hybridized with NEO^r and HYG^r gene as probe. Lane 1 is the DNA molecular weight marker. Lane 2—mutants hybridized with HYG^r. Lane 3—mutants hybridized with NEO^r. Lane 4—*L. major* wild-type parasite as the control for HYG^r. Lane 5—*L. major* wild-type parasite as the control for NEO^r. (C) NEO^r and (D) HYG^r probe.

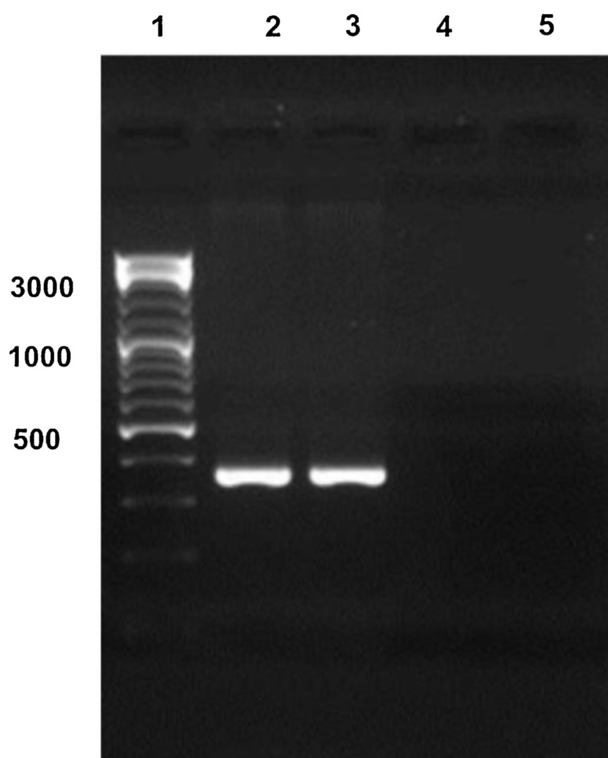


Fig. 4. Expression analysis of *GPI12*in mutant and WT parasites. The RT-PCR tests using the cDNA of WT (lanes 2 and 3) and mutant parasites (lanes 4 and 5) with *GPI12* specific primers. The amplified 328 bp band confirmed mRNA *GPI12* expression only in the wild strain. Lane 1 shows the molecular ladder.

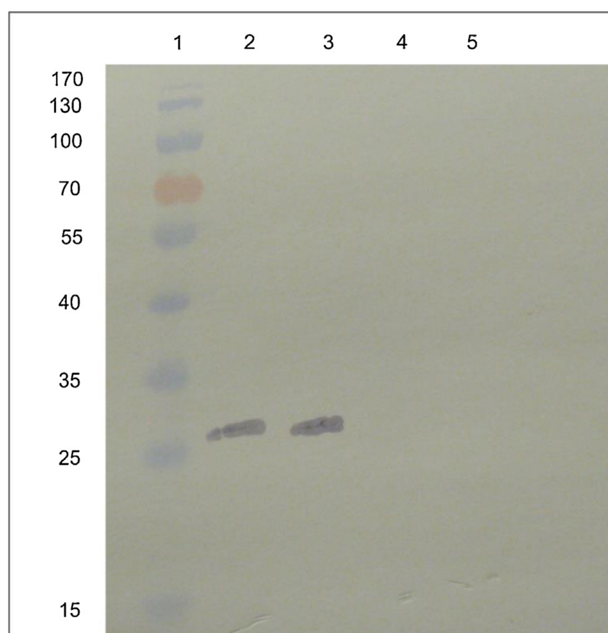


Fig. 5. Western blot test. The *GPI12* detected by using anti- *GPI12* linear epitopes able to recognize the 28.8 kDa protein band in WT parasite (lanes 2 and 3) however, in mutant parasites there were no specific protein bands (lanes 4 and 5). Lane 1 presents the protein marker.

and Fasel, 2008). According to the present research recombinant parasites are placed in the third group.

According to different reports, the essential genes in the parasite life cycle that have been identified in the multi-level segmentation are as follows: (a) genes that are essential for live promastigotes in culture. It includes the deletion of both alleles of the genes, which is not that easily performed. They are associated with gene amplification, Trisomy or Tetrasomy, or they are observed in circle shapes and their proliferation happens as extra-chromosomal, which later remains in the cell. These group of genes are cited as genes such as DHFR-TS (Cruz et al., 1991; Cruz et al., 1993), alpha tubulin (Curotto de Lafaille and Wirth, 1992), Crk1 (Mottram et al., 1996) and trypanothione reductase (Dumas et al., 1997). In some cases, deleting a gene only in the presence of a copy of that in episomal form is possible for example Topoisomerase gene (Balaña-Fouce et al., 2008). (b) Parasite genes which are essential in the differentiation of the pro-

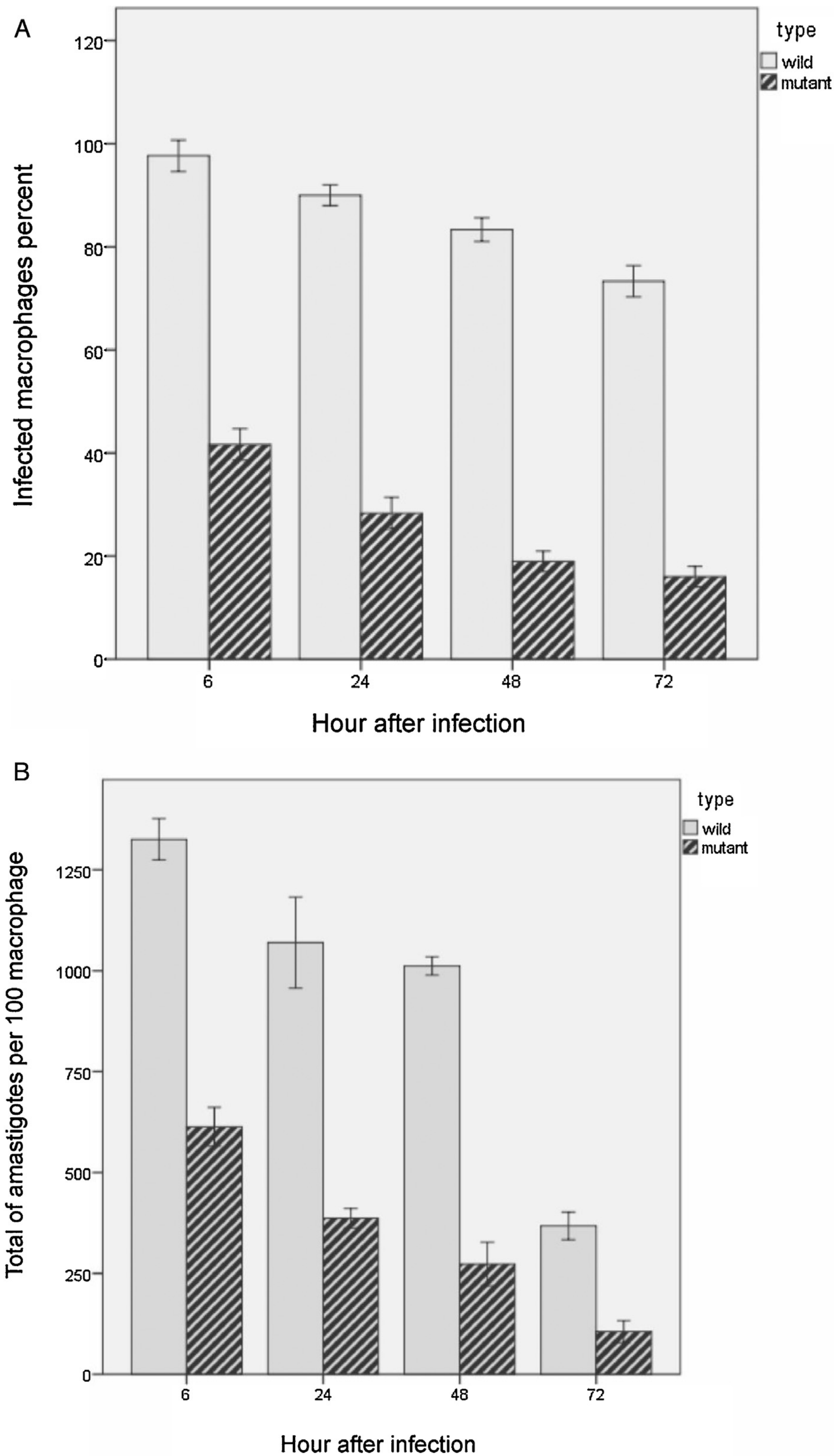


Fig. 6. (A) Percentage of MQ infected with WT and mutant parasites in 6, 24, 48 and 72 h. The total number of the MQ and infected MQ were calculated. Data was reported as the mean ± SD. (B) The total number of the parasites within one hundred MQ cells at different time points post-infection. All tests were performed in triplicate.

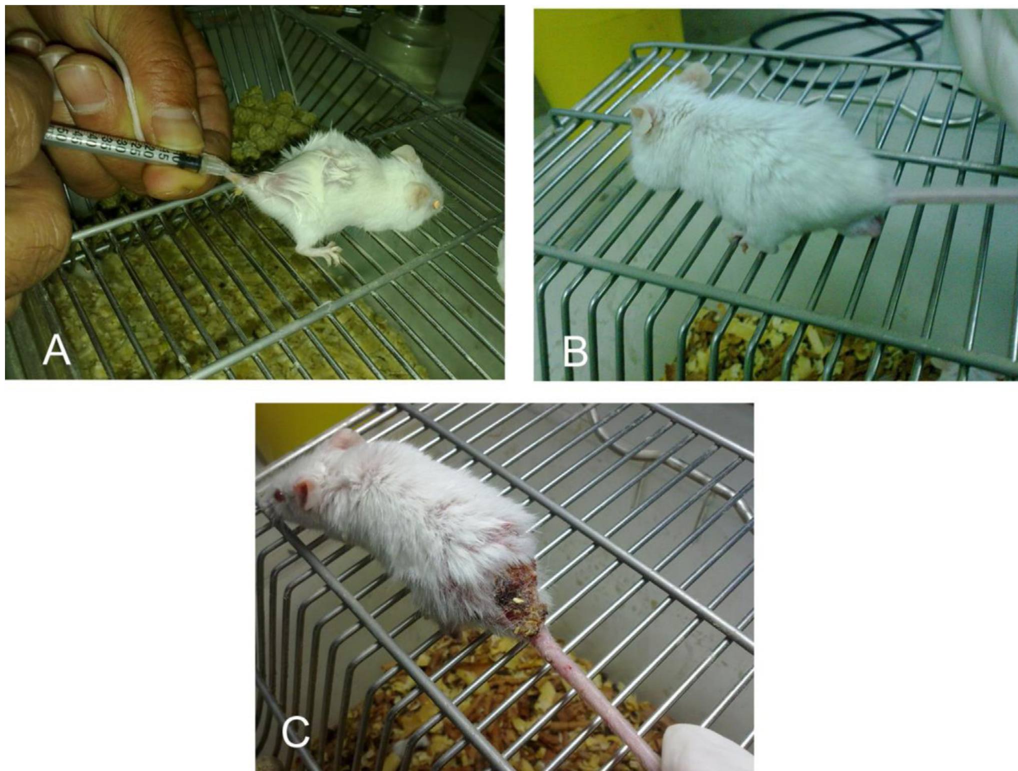
mastigote to amastigote or even the proliferation of amastigotes, LIT1 (Huynh et al., 2006) gene is placed in this group. (c) Genes that play an important role in the pathogenesis of macrophages; GDPMP gene (Garami and Iiq, 2001) for example. *Leishmania* with molecules such as LPG, GP63 and cysteine protease is capable of establishing infection in parasitophorous vacuole in macrophages (Chang and McGwire, 2002). It seems that the *GPI12* gene could be placed in the third group.

A common feature among most species of *Leishmania* is their ability for survival and reproduction among acidic phagolysosomes

which contain hydrolase enzymes. This is one of the necessary conditions for the parasite's infectivity and for the severity of diseases caused by *Leishmania* proliferation within macrophages. In fact, mutations in genes which are involved in the primary infection of macrophages and the survival of the cells reduced the severity and delayed the wound formation (Huynh et al., 2006).

In this study we were able to isolate live and motile mutant parasites from infected lymph nodes which were cultured in complete media. It is likely that these genes are not necessary for the replication and viability of promastigotes in *in vitro* culture. There are other reports of genes with similar results such as the cysteine proteinase B gene, which acts as a virulence factor and is not necessary for *in vitro* growth and differentiation (Mottram et al., 1996). Another example is *L. major* Δlpg2 null mutant which could not generate *in vivo* disease (Kébaïer et al., 2006).

Virulence is influenced by two different groups of parasite molecules: one group is the vast majority of products secreted by the parasite surface and the other is intracellular molecules encompassing the so-called pathoantigens and the generally conserved proteins in the cytoplasm. This causes an immune response against intracellular parasite antigens.



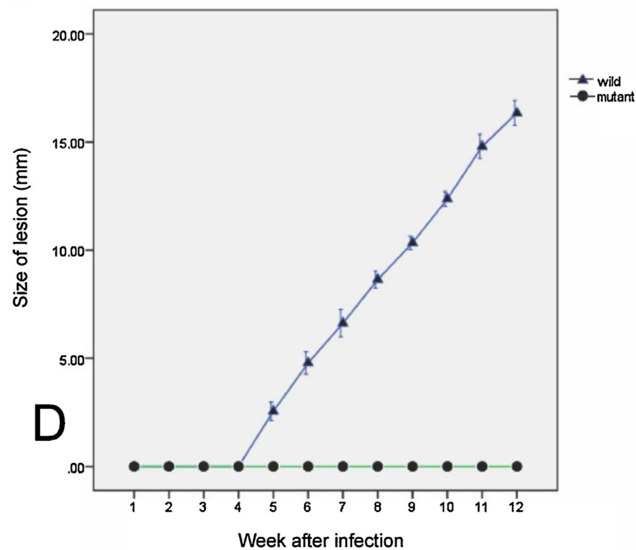


Fig. 7. *In vivo* study. (A) Mice infected with parasites in the skin at the base of the tail. (B) The mutant parasites could not create cutaneous lesions until after six months of infection. (C) Mice infected with wild-type *L. major* begun to develop lesions after 4 weeks and increased. (D) In the two groups (wild and mutant), the lesion size in the skin at the base of the tail was measured.

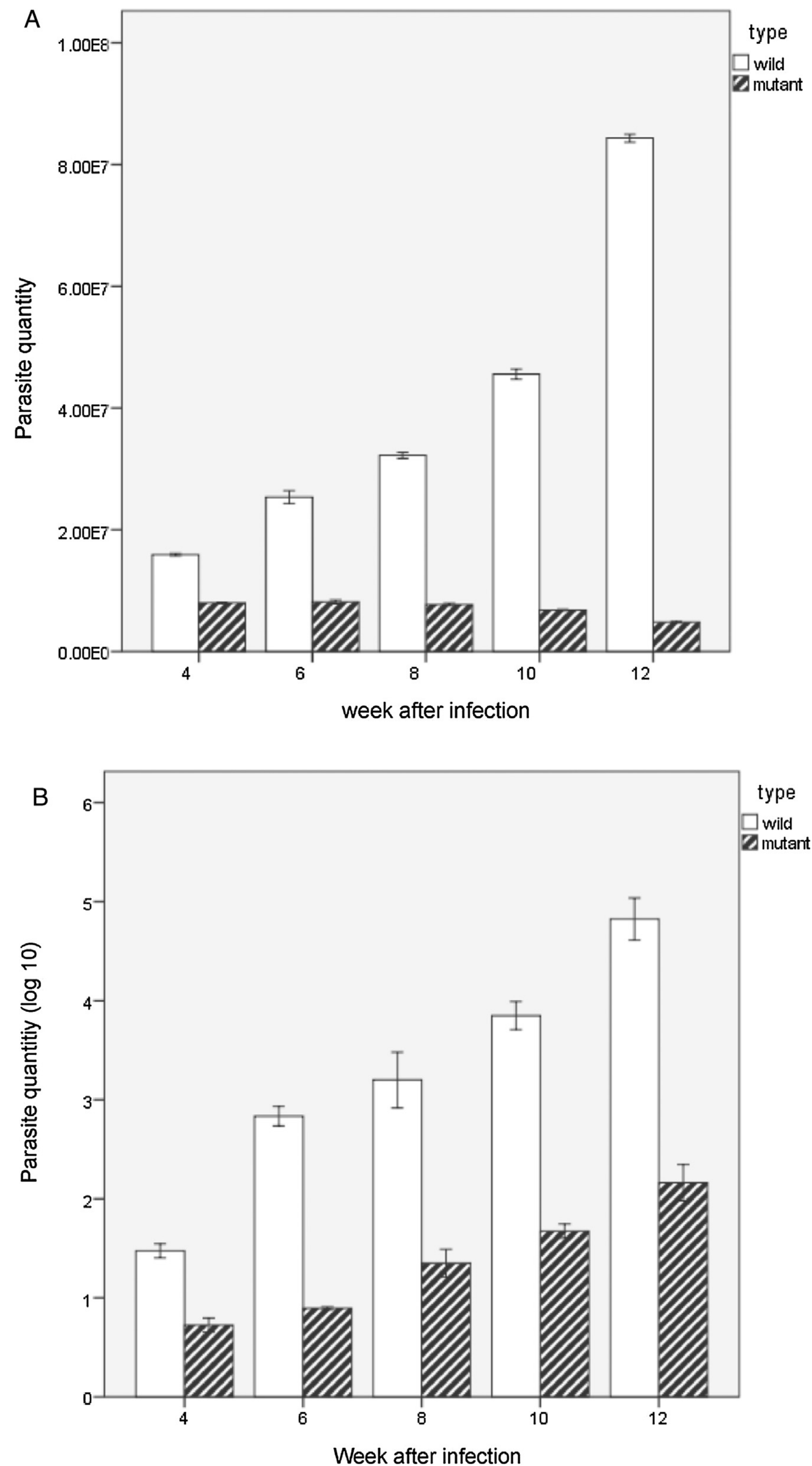


Fig. 8. Real-time PCR quantification of the parasites: The DNA obtained from the two groups of the mice was isolated from the skin at the base of the tail (A) and the lymph nodes (B) to perform parasite load by real-time PCR at different time periods.

The immune responses against a surface or secreted molecules are low. In fact, many of these molecules contribute to parasite infection in the host cells (Chang and McGwire, 2002; Chang et al., 2003).

Today, a new generation of genetically attenuated live vaccines are manufactured and evaluated in *Leishmania*. The salient fact about this generation of vaccines is that they maintain the original form of the parasite antigens, while making specific genetic modifications in the *Leishmania* genome by using gene targeted deletion

technology through homologous recombination. It is possible to produce parasites which lack the genes necessary for long-term survivals or virulences.

In this study, we were able to produce mutant parasite that was unable to damage the host; while at the same time containing pathoantigen proteins which are effective in establishing immunity. Further investigations are essential to check the safety profile in laboratory animals.

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References

Alvar, J.V.I., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., WHO Leishmaniasis Control Team, 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7, e35671.

Balaña-Fouce, R., García-Estrada, C., Pérez-Pertejo, Y., Reguera, R.M., 2008. Gene disruption of the DNA topoisomerase IB small subunit induces a non-viable phenotype in the hemoflagellate *Leishmania major*. BMC Microbiol. 8, 113.

Bolhassani, A., Taheri, T., Taslimi, Y., Zamanilui, S., Zahedifard, F., Seyed, N., Torkashvand, F., Vaziri, B., Rafati, S., 2011. Fluorescent *Leishmania* species: development of stable GFP expression and its application for *in vitro* and *in vivo* studies. Exp. Parasitol. 127, 637–645.

Chandra, S., Ruhela, D., Deb, A., Vishwakarma, R.A., 2010. Glycobiology of the *Leishmania* parasite and emerging targets for antileishmanial drug discovery. Expert Opin. Ther. Targets 14, 739–757.

Chang, K.P., McGwire, B.S., 2002. Molecular determinants and regulation of *Leishmania* virulence. Kinetoplastid Biol. Dis. 1, 1.

Chang, K.P., Reed, S.G., McGwire, B.S., Soong, L., 2003. *Leishmania* model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity. Acta Trop. 85, 375–390.

Clayton, C.E., 1999. Genetic manipulation of kinetoplastida. Parasitol. Today 15, 372–378.

Croft, S.L., Sundar, S., Fairlamb, A.H., 2006. Drug resistance in leishmaniasis. Clin. Microbiol. Rev. 19, 111–126.

Cruz, A., Coburn, C.M., Beverley, S.M., 1991. Double targeted gene replacement for creating null mutants. Proc. Natl. Acad. Sci. U. S. A. 88, 7170–7174.

Cruz, A.K., Titus, R., Beverley, S.M., 1993. Plasticity in chromosome number and testing of essential genes in *Leishmania* by targeting. Proc. Natl. Acad. Sci. U. S. A. 90, 1599–1603.

Curotto de Lafaille, M.A., Wirth, D.F., 1992. Creation of Null/+ mutants of the alpha-tubulin gene in *Leishmania* enriettii by gene cluster deletion. J. Biol. Chem. 267, 23839–23846.

Dumas, C., Ouellette, M., Tovar, J., Cunningham, M.L., Fairlamb, A.H., Tamar, S., Olivier, M., Papadopoulou, B., 1997. Disruption of the trypanothione reductase gene of *Leishmania* decreases its ability to survive oxidative stress in macrophages. EMBO J. 16, 2590–2598.

Feng, X., Rodriguez-Contreras, D., Polley, T., Lye, L.F., Scott, D., Burchmore, R.J., Beverley, S.M., Landfear, S.M., 2013. 'Transient' genetic suppression facilitates generation of hexose transporter null mutants in *Leishmania* mexicana. Mol. Microbiol. 87, 412–429.

Ferguson, M.A., 2000. Glycosylphosphatidylinositol biosynthesis validated as a drug target for African sleeping sickness. Proc. Natl. Acad. Sci. U. S. A. 97, 10673–10675.

Ferguson, M.A., Brimacombe, J.S., Brown, J.R., Crossman, A., Dix, A., Field, R.A., Güther, M.L., Milne, K.G., Sharma, D.K., Smith, T.K., 1999. The GPI biosynthetic pathway as a therapeutic target for African sleeping sickness. Biochim. Biophys. Acta 1455, 327–340.

Garami, A., Ilg, T., 2001. Disruption of mannose activation in *Leishmania mexicana*: GDP-mannose pyrophosphorylase is required for virulence, but not for viability. EMBO J. 20, 3657–3666.

Hassani, K., Shio, M.T., Martel, C., Faubert, D., Olivier, M., 2014. Absence of metalloprotease GP63 alters the protein content of *Leishmania* exosomes. PLoS One 9, e95007.

Huynh, C., Sacks, D.L., Andrews, N.W., 2006. A *Leishmania amazonensis* ZIP family iron transporter is essential for parasite replication within macrophage phagolysosomes. J. Exp. Med. 203, 2363–2375.

Jiang, Y., Roberts, S.C., Jardim, A., Carter, N.S., Shih, S., Ariyanayagam, M., Fairlamb, A.H., Ullman, B., 1999. Ornithine decarboxylase gene deletion mutants of *Leishmania donovani*. J. Biol. Chem. 274, 3781–3788.

Joshi, P.B., Kelly, B.L., Kamhawi, S., Sacks, D.L., McMaster, W.R., 2002. Targeted gene deletion in *Leishmania major* identifies leishmanolysin (GP63) as a virulence factor. Mol. Biochem. Parasitol. 120, 33–40.

Kébaïer, C., Uzonna, J.E., Beverley, S.M., Scott, P., 2006. Immunization with persistent attenuated Delta lpg2 *Leishmania major* parasites requires adjuvant to provide protective immunity in C57BL/6 mice. Infect. Immun. 74, 777–780.

Khatami, A., Firooz, A., Gorouhi, F., Dowlati, Y., 2007. Treatment of acute old world cutaneous leishmaniasis: a systematic review of the randomized controlled trials. J. Am. Acad. Dermatol. 57, 335.

Lachaud, L., Marchergui-Hammami, S., Chabbert, E., Dereure, J., Dedet, J.P., Bastien, P., 2002. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. J. Clin. Microbiol. 40, 210–215.

Mottram, J.C., McCready, B.P., Brown, K.G., Grant, K.M., 1996. Gene disruptions indicate an essential function for the LmmCRK1 cdc2-related kinase of *Leishmania mexicana*. Mol. Microbiol. 22, 573–583.

Mukherjee, S., Sen, S.S., Das, S., Bose, M., Roy, J., Adak, S., 2012. NAD(P)H cytochrome b5 oxidoreductase deficiency in *Leishmania major* results in impaired linoleate synthesis followed by increased oxidative stress and cell death. J. Biol. Chem. 287, 34992–35003.

Myler, P.J., Fasel, N., 2008. *Leishmania*: After the Genome. Caister Academic Press.

Naderer, T., Vince, J.E., McConville, M.J., 2004. Surface determinants of *Leishmania* parasites and their role in infectivity in the mammalian host. Curr. Mol. Med. 4, 649–665.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Selvapandiyan, A., Debrabant, A., Duncan, R., Muller, J., Salotra, P., Sreenivas, G., Salisbury, J.L., Nakhasi, H.L., 2004. Centrin gene disruption impairs stage-specific basal body duplication and cell cycle progression in *Leishmania*. J. Biol. Chem. 279, 25703–25710.

Sharma, D.K., Smith, T.K., Crossman, A., Brimacombe, J.S., Ferguson, M.A., 1997. Substrate specificity of the *N*-acetylglucosaminyl-phosphatidylinositol de-*N*-acetylase of glycosylphosphatidylinositol membrane anchor biosynthesis in African trypanosomes and human cells. Biochem. J. 328, 171–177.

Smith, T.K., Sharma, D.K., Crossman, A., Dix, A., Brimacombe, J.S., Ferguson, M.A., 1997. Parasite and mammalian GPI biosynthetic pathways can be distinguished using synthetic substrate analogues. EMBO J. 16, 6667–6675.

Smith, T.K., Crossman, A., Borissow, C.N., Paterson, M.J., Dix, A., Brimacombe, J.S., Ferguson, M.A., 2001. Specificity of GlcNAc-PI de-*N*-acetylase of GPI biosynthesis and synthesis of parasite-specific suicide substrate inhibitors. EMBO J. 20, 3322–3332.

Späth, G.F., Epstein, L., Leader, B., Singer, S.M., Avila, H.A., Turco, S.J., Beverley, S.M., 2000. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. Proc. Natl. Acad. Sci. U. S. A. 97, 9258–9263.

Späth, G.F., Garraway, L., Turco, S.J., Beverley, S.M., 2003. The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. Proc. Natl. Acad. Sci. U. S. A. 100, 9536–9541.

Taheri, T., Salmanian, A.H., Gholami, E., Doustdari, F., Zahedifard, F., Rafati, S., 2010. *Leishmania major*: disruption of signal peptidase type I and its consequences on survival, growth and infectivity. Exp. Parasitol. 126, 135–145.

Thiel, M., Harder, S., Wiese, M., Kroemer, M., Bruchhaus, I., 2008. Involvement of a *Leishmania* thymidine kinase in flagellum formation, promastigote shape and growth as well as virulence. Mol. Biochem. Parasitol. 158, 152–162.

Urbaniak, M.D., Crossman, A., Chang, T., Smith, T.K., van Aalten, D.M., Ferguson, M.A., 2005. The *N*-acetyl-D-glucosaminylphosphatidylinositol De-*N*-acetylase of glycosylphosphatidylinositol biosynthesis is a zinc metalloenzyme. J. Biol. Chem. 280, 22831–22838.

Williams, R.A., Tetley, L., Mottram, J.C., Coombs, G.H., 2006. Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. Mol. Microbiol. 61, 655–674.

Williams, R.A.M., Smith, T.K., Cull, B., Mottram, J.C., Coombs, G.H., 2012. ATG5 Is Essential for ATG8-Dependent Autophagy and Mitochondrial Homeostasis in *Leishmania major*. PLoS Pathog. 8, e1002695.

Wyllie, S., Patterson, S., Fairlamb, A.H., 2013. Assessing the essentiality of *Leishmania donovani* nitroreductase and its role in nitro drug activation. Antimicrob. Agents Chemother. 57, 901–906.