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Studying the expression of *P36* (*LACK*) gene in a strain of *Cutaneous Leishmania* (*L. tropica*) and its production as recombinant protein

Thesis presented for master degree in Biotechnology

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الإهداء . .

إلى نبع الحب الصادق الدافئ

إلى رمز العطاء والتضحية والتفاني اللامتناهي

إلى سر وجودي ومصدر فخري واعتزازي

إلى قدوتي الأولى ونبراسي الذي يضيء دربي

هدية الرحمن . .والدي الحبيبين

لكما كل حبي وامتناني

حفظكما الله . . ودمتما لي ذُخرا

الإهداء . .

إلى أشقاء الروح والجسد

إلى من تحلو الحياة وتطيب بوجودهم

إلى أصدقاء طفولتي ونبع ذكرياتي

إلى سندي ومتكاي عند الشدائد

إلى أغلى البشر . . إخواني وأخواتي الأحباء

عين الله ترعاكم

وحفظ لكم أزواجكم وأولادكم

بسم الله الرحمن الرحيم

"وَمَا تَوفِيقِي إِلَّا بِاللهِ عَلَيْهِ تَوَكَّلْتُ وَإِلَيْهِ أُنِيبُ"

شكر وتقدير

الحمد لله حمداً كثيراً طيباً مباركاً فيه مباركاً عليه . .الحمد لله كما ينبغي لجلال وجهه وعظيم سلطانه

والصلاة والسلام على أشرف الخلق والمرسلين سيدنا محمد وعلى آله وصحبه وسلم

أتوجه بخالص الشكر والتقدير إلى السادة أعضاء لجنة الحكم: **الدكتور محمد معروف والدكتور محمود قويدر والدكتورة سلام لاوند** لتفضلهم بتحكيم هذا البحث.

كما أتوجه بعظيم الشكر والتقدير والامتنانلأساتذتي المشرفين،

الدكتور محمود قويدر الذي تفضل بالإشراف على هذا البحث من بدايته إلى نهايته، فكان نعم المرشد والموجه. أسأل الله أن يبقيه ذخراً ونبراساً.

والدكتور شادي سكرية الذي تفضل أيضاً بالإشراف على هذا البحث ومتابعته بأدق تفاصيله، والذي منحني الكثير من وقته وجهده وعلمه فكان لي نعم المعلم والناصح والصديق، والذي لولا دعمه وإرشاده لما تم إنجاز هذا العمل بنجاح. أسأل الله له مزيداً من الإنجاز والنجاح والتألق والتميز.

كما أتقدم بجزيل الشكر والعرفان **للدكتور عبد القادر عبادي** لتفضله بالمشاركة في الإشراف وتعاونه العلمي لإنجاز هذا البحث، إذ لم يبخل بالمساعدة والتوجيه، وقدم لي الكثير فكان نعم القدوة لي بعمله الجادالدؤوب وإصراره على تحقيق أهدافه، زاده الله رفعةً وعلماً.

كل الشكر أقدمه لعمادة كلية الزراعة في جامعة دمشق متمثلةً بالسيد عميد كلية الزراعة **الأستاذ الدكتور محمد أيمن السعدي، الأستاذ الدكتور موسى عبود** وكيل الشؤون العلمية.

كل الشكر والتقدير لجميع الأساتذة المدرسين في ماجستير التقانة الحيوية، كلية الزراعة- جامعة دمشق.

كما أتقدم بخالص الشكر والامتنان إلى كلية العلوم- قسم علم الحياة الحيوانية- مخبر الدراسات العليا للدعم الذي قدموه والسماح لي بإنجاز هذا البحث لديهم.

كل الشكر أيضاً لهيئة الطاقة الذرية السورية- قسم البيولوجيا الجزيئية والتقانة الحيوية للسماح لي بإنجاز بعض مراحل البحث لديهم.

ولا يفوتني التوجه بالشكر لجميع الزملاء والزميلات في مخبر الدراسات العليا- قسم علم الحياة الحيوانية- كلية العلوم.

وفي الختام، أتوجه بأسمى عبارات الشكر الممزوجة بالحب والوفاء لرفيقاتي وصديقات دربي الغاليات اللواتي كنَّ دائماً بجانبي داعمات ومشجعات وداعيات لي بالخير. حفظهنَّ الله وجعلهنَّ من سعداء الدنيا والآخرة.

أَسأَلُ المَوْلَى عَزَّ وَجَلَّ أَنْ يجعلَهُ عَمَلاً مُتَقَبَّلاً وَعِلْماً نافِعاً يُنتَفَعُ بِهِ

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List of Abbreviations

AMP	Ampicillin
AP	Alkaline phosphatase
APS	Ammonium Persulfate
bp	Base Pair
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BSA	Bovine Serum Albumin
CL	Cutaneous Leishmaniasis
E. coli	*Escherichia coli*
FBS	Fetus Bovine Serum
GFP	Green Fluorescent Protein
IB	Inclusion Body
IFN-γ	Interferon-gamma
IL	Interleukin
IPTG	Isopropylthio-D- galactoside
kDa	Kilo Dalton
LACK	*Leishmania* Homologue Of Receptors For Activated CKinase
MCL	Mucocutaneous Leishmaniasis
µg	Microgram
µl	Microliter
ML	Mucosal Leishmaniasis
OD	Optical Density
NBT	Nitro Blue Tetrazolium
NK	Natural Killer Cells
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
rpm	Round Per Minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SLA	Soluble *Leishmania* Antigen
SDS	Sodium Dodecyl Sulfate

SDS-PAGE	SDS- Poly Acrylamide Gel Elcetrophoresis
TAE	Tris Asetic acid EDTA
TBST	Tris-Buffered Saline-Tween 20
TEMED	Tetramethylethylenediamine
Th1	T-helper type-1 cells
TMB	Tetra Methyl Benzidine
V	Volt
VL	Visceral Leishmaniasis
WB	Western Blot
×g	1×g corresponds to normal acceleration due to gravity (9.81 m/s)

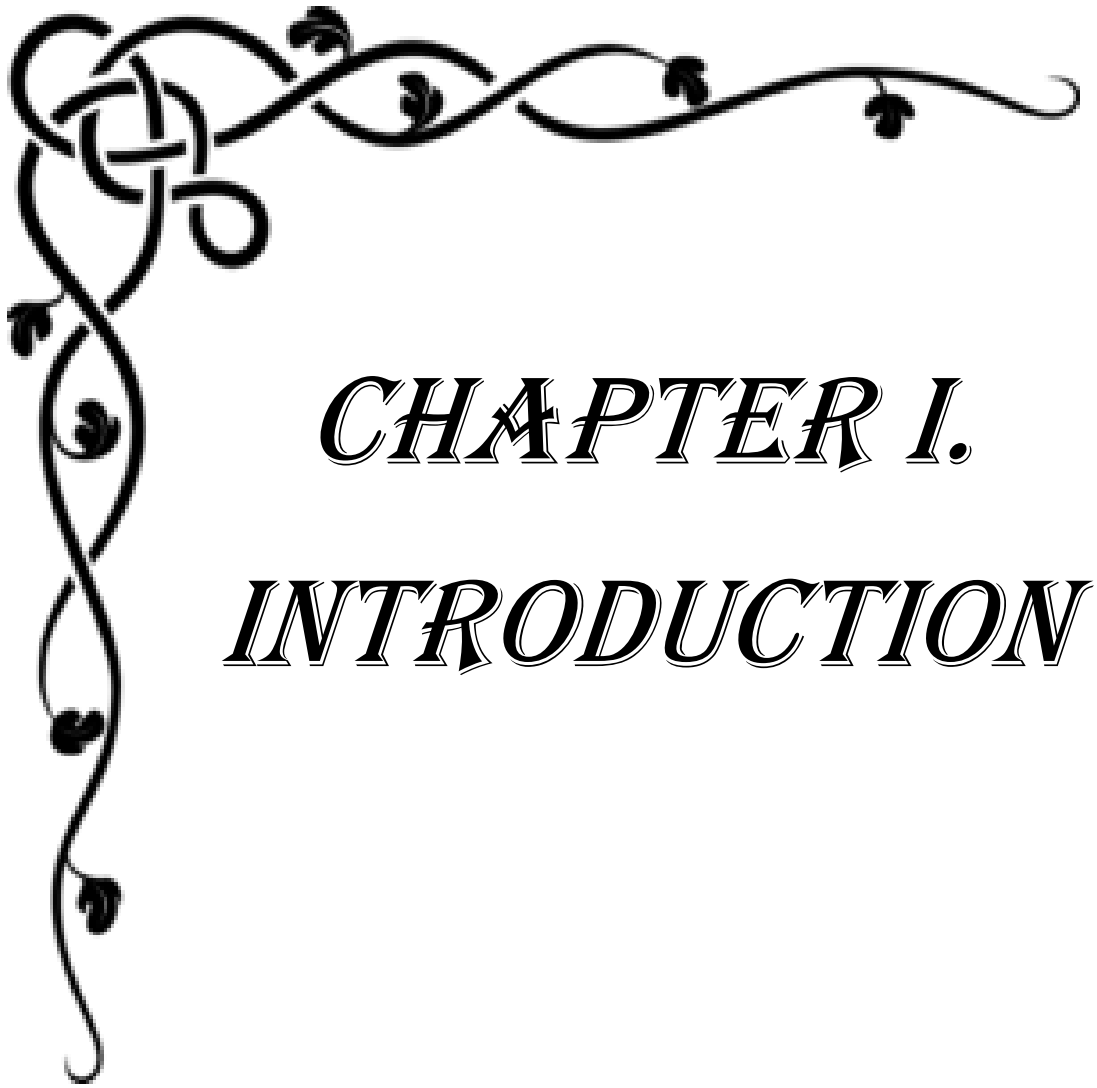
Abstract

Cutaneous Leishmaniasis (CL) is a group of diseases caused by a protozoan parasites of the genus *Leishmania* afflicting millions of people worldwide. Unfortunately, no ideal therapy for CL has been identified which emphasize the need for vaccine development. A suitable leishmaniasis vaccine candidate molecule must be immunogenic and have surface expression in amastigote, the infective stage for mammals. LACK (*Leishmania* homologue of receptors for activated Ckinase) antigen is a 36-kDa protein which provokes a very early immune response against *Leishmania* infection. The location of the P36/LACK antigen on the parasite surface, its immunogenicity and its expression in promastigote and in amastigote of *Leishmaniaspp*. make it a potential candidate for CL serodiagnosis and vaccine. There are several reports on the expression of P36/LACK through different life-cycle stages of genus *Leishmania*, but only a few of them have focused on *L. tropica*.

The present study provides details of the cloning, DNA sequencing and gene expression of *P36/LACK*in this parasite species. Furthermore, *P36/LACK*gene was subcloned in an expression vector, recombinant protein was produced, extracted and purified. First, several local isolates of *Leishmania* parasite were typed using PCR technique. After that,*P36/LACK*gene was amplified and cloned into a vector for sequencing. Afterwards, the expression of this molecule in logarithmic and stationary growth phase promastigotes, as well as in amastigotes, was evaluated by Reverse Transcription-PCR (RT-PCR) technique. The typing result confirmed that all our local isolates belong to *L. tropica*.*P36/LACK*gene sequence was determined and high similarity was observed with the sequences of other *Leishmania* species. Furthermore, the expression of *P36/LACK*gene in both promastigotes and amastigotes forms was confirmed. Additionally, in the present work, an expression system to produce recombinant *L. tropica*-P36/LACK protein was developed. *P36/LACK* gene was subcloned into the expression vector pRSET. The new plasmid construct (pRSET-*P36/LACK*) was efficiently able to express P36/LACK protein in the cytoplasm of *E. coli*. P36/LACK purification was achieved by one step metal affinity chromatography. The purity of the resulted protein was monitored by SDS-PAGE separation and coomassie blue staining, and its identity was confirmed by immunoblotting using specific anti-6×His tag antibody. Recombinant P36/LACK protein was detected by sera from CL patients, suggesting its usefulness for CL serological diagnosis and perhaps as a vaccine for this endemic disease in Syria.

Keywords:

Cutaneous Leishmaniasis, *L. tropica*, LACK protein, Promastigotes, Amastigotes, Cloning, Protein expression.



CHAPTER I.

INTRODUCTION

1. Introduction

1.1. Leishmaniasis

1.1.1. Leishmaniasis disease

Leishmaniasis, a vector-borne disease that is caused by obligate intra-macrophage protozoa, is endemic in the tropical and subtropical regions of Africa, Asia, the Mediterranean, Southern Europe (old world) and South and Central America (new world). This disease is characterized by both diversity and complexity (Herwaldt, 1999). It is caused by more than 20 leishmanial species and is transmitted to humans by ~30 different species of phlebotomine sand flies (Chappuis *et al.*, 2007). Leishmaniasis is probably second only to malaria in importance as a protozoan disease causing human suffering (Phillip G. Lawyer, 2000).

Human disease is usually described as occurring in three primary manifestations: visceral, mucocutaneous and cutaneous . Other manifestations have been described as leishmaniasis recidivans, diffuse cutaneous leishmaniasis and post-*kala-azar* dermal leishmaniasis (Grevelink and Lerner, 1996; Phillip G. Lawyer, 2000).

Visceral Leishmaniasis (VL) is the most severe form of the disease. It is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia. This form is caused by *L. donovani* and L. infantum in the Old World and L. chagasi in the New World.

Mucocutaneous Leishmaniasis (MCL) produces lesions, which can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities. This form is caused mainly by *L. braziliensis* and *L. mexicana.*

Cutaneous leishmaniasis (CL) in the Old World can be caused by any of the four following species, each with different disease progression characteristics: L. infantum can also visceralize and cause the lethal visceral leishmaniasis; L. major is often self-

curing (Morizot *et al.*, 2007); L. aethiopica and L. tropica are recalcitrant to treatment. In the New World, CL is caused by L. mexicana, L. guyanensis, L. amazonensis and L. braziliensis. CL is the most common form of leishmaniasis worldwide, representing 50–75% of all new cases(Grevelink and Lerner, 1996; Hadighi *et al.*, 2006; Modabber *et al.*, 2007).

These species have the same symptoms and life cycles but differ in their geographic distribution. Both species L. major and L. tropica can produce large numbers of skin ulcers on the exposed parts of the body, such as the face, arms and legs, causing serious disability and leaving the patient permanently scarred. The infection site is usually localized to the site in which the sand fly bite occurs (Odiwuor *et al.*, 2011).

1.1.2. Epidemiology of Cutaneous Leishmaniasis in Syria

Generally, Leishmaniasis currently threatens 350 million men, women and children in 88 countries around the world. It is found in Africa, Asia, Europe, North America and South America (Awasthi *et al.*, 2004). In 2000, it was estimated that more than 12 million individuals were infected by the various *Leishmania* species in 88 countries, and an approximate incidence of 0.5 million cases of VL and 1.5 million cases of CL (Awasthi *et al.*, 2004; Olivier *et al.*, 2005).

The most common manifestations are cutaneous lesions; According to the World Health Organization (WHO), 90% of CL cases occur in seven countries; Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syrian Arab Republic. (Herwaldt, 1999; WHO, 2007).

In Syria, CL caused by *L. tropica* is still endemic in its traditional home of Aleppo, but also in Edlib, Lattakia, Tortous, Hama and the city of Damascus. It now represents about 90 % of all CL cases and is one of the most important public health

problems in the Syrian Arab Republic, especially in Aleppo. The population density in urban areas of the Syrian Arab Republic has increased, as well as population movement across the country, which could explain the rise in cases. As a recent survey estimated that 20 % of cases are not reported or treated, a considerable human reservoir exists. CL caused by *L. major* is less common and occurs in rural areas close to Damascus, Deir al Zour and Al Hasakeh (Jorge Alvar1., 2012) (Fig.1). According to WHO, 22,882 cases/year of CL was reported in Syria between 2004-2008 (Rioux *et al.*, 1990; Desjeux, 1992; Tayeh *et al.*, 1997; WHO, 2007; Jorge Alvar1., 2012).

In the Eastern Mediterranean Region an average of 100,000 cases in the last 11 years and more than 120,000 cases in the last 3 years have been reported. In the context of the Syrian crisis the cutaneous leishmaniasis form caused by *L. tropica* is the most important in terms of risk of being introduced in neighbouring countries. It also presents more treatment failures (up to 20% of cases may become chronic). In Syria, both *L. tropica* and *L. major* are endemic and transmission will continue. The main reservoirs of *L. major* are rodents, and humans are the main reservoir for *L. tropica*. (http://www.emro.who.int/neglected-tropical-diseases/information-resources-leishmaniasis/cl-factsheet.html). In September 2012, a cutaneous leishmaniasis outbreak began among Syrian refugees in Lebanon. For 948 patients, *L. tropica* in 85% and *L. major* in 15% of patients were identified (http://wwwnc.cdc.gov/eid/article/20/10/14-0288_article).

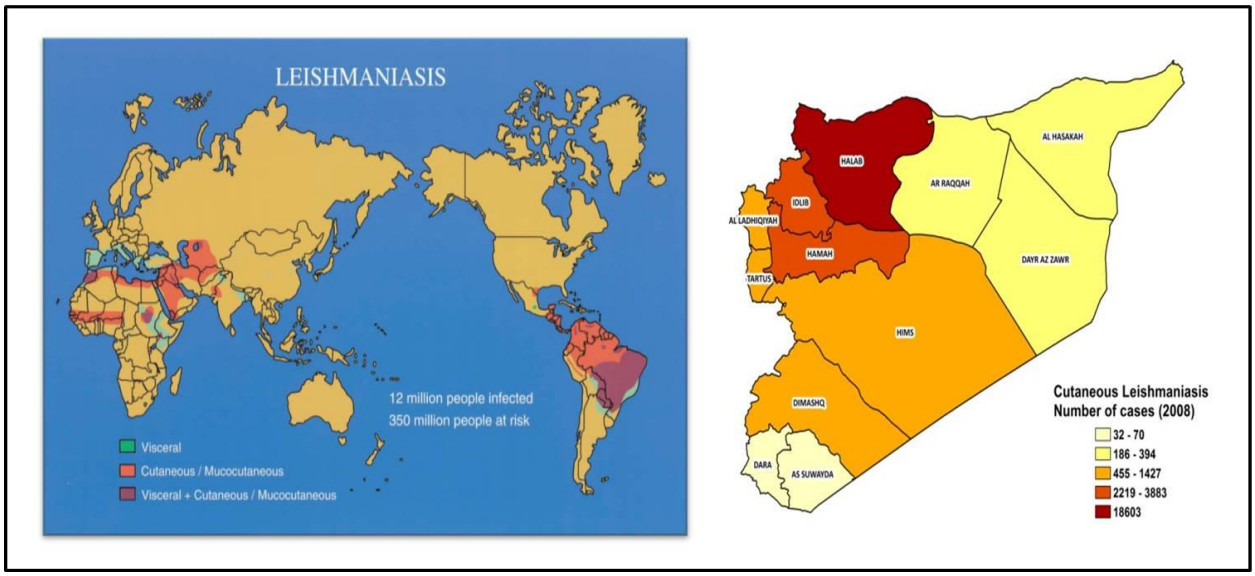


Figure 1. Epidemiology of leishmaniasis.
World map highlighting areas where cutaneous, visceral, and mucocutaneous leishmaniasis is endemic (on the left). In addition to the number of CL cases in Syria (on the right). **(Handman, 2001; Jorge Alvar1., 2012)**

1.1.3. The vector and life cycle of the parasite

Over 500 species of sand flies have been described from various parts of the world, with most being assigned to three genera: *Phlebotomus* and *Sergentomyia* of the Old World, and *Lutzomyia* of the New World. Vectors in the Old World come primarily from the genus *Phlebotomus*; and in the New World, exclusively from *Lutzomyia*. Only a few dozen species are known to be vectors of human disease (Lewis, 1971; Grevelink and Lerner, 1996) (Fig. 2).

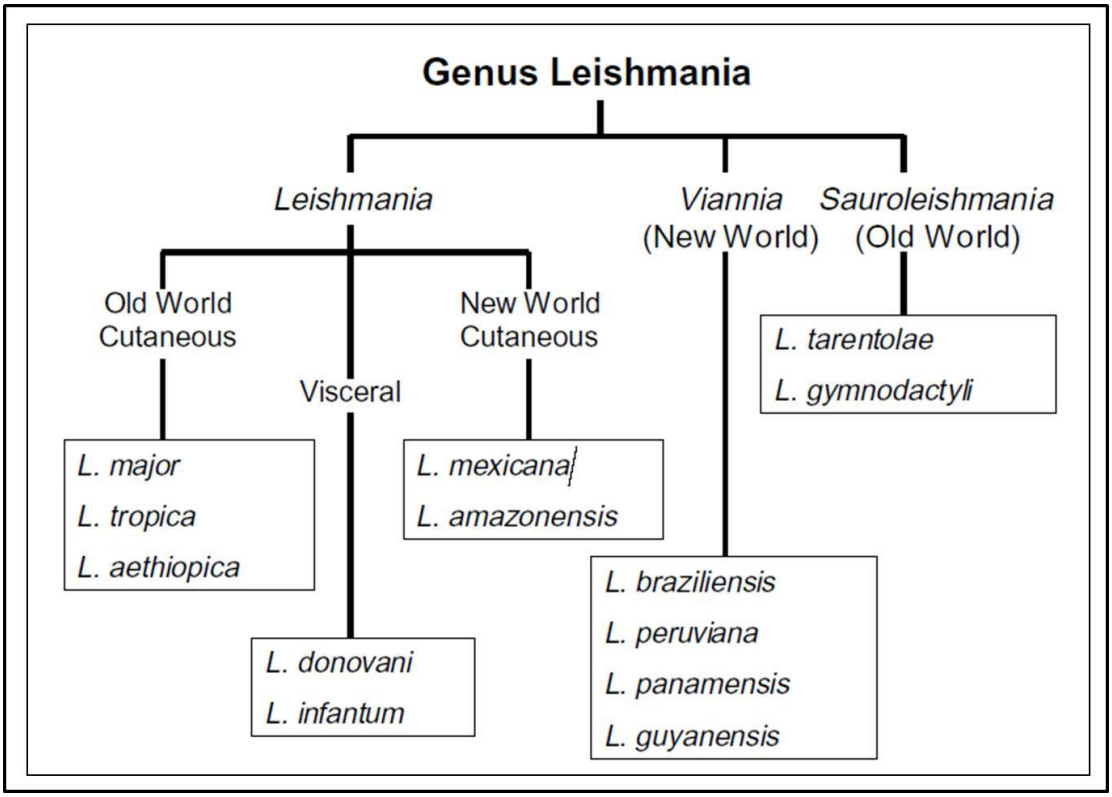


Figure 2. Outline classification of Leishmania illustrating the three subgenera. The list of named species is not comprehensive; over 30 species have been named in the genus including many that are non-pathogenic or of minor medical importance (of limited range or small numbers of recorded cases). The species named above include some of the better known species that are the focus of biomedical research. Parasites in the subgenera *Leishmania* and *Viannia* infect mammals, whereas the *Sauroleishmania* infect reptiles as their vertebrate hosts. (Bates, 2007)

The parasite has a digenetic life-cycle alternating between a mammalian host and insect vectors, phlebotomine sandflies (Diptera: Psychodidae, subfamily Phlebotominae). These are small (usually 1.5–2 mm body length) insects that are principally found in tropical and subtropical regions. Females of two sand fly genera, *Phlebotomus* and *Lutzomyia*, are of medical importance as the only proven vectors of *Leishmania* species pathogenic for humans (Killick-Kendrick, 1999) (Fig.3).

Two distinct developmental stages of Leishmania are recognized. Promastigotes are found within the sandfly and have an elongated shape and long flagellum. Promastigotes can be further classified as procyclic promastigotes, which multiply in the gut of the sandfly, or as the infective metacyclic promastigotes, which are found in

the mouth parts and anterior gut and do not divide. These differentiate into round or oval amastigotes, which lack flagella, once in the host (Olivier *et al.*, 2005) (Fig. 3).

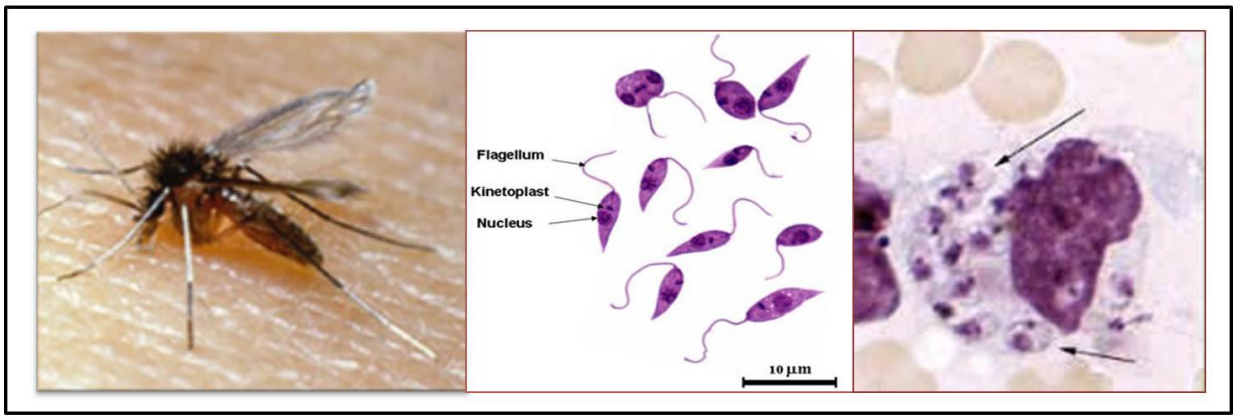


Figure 3. The parasite and its vector.
Picture shows *Leishmania* parasite vector; (phlebotomine sandfly insect) (on the left). *Leishmania spp.* promastigote form (in the middle), and amastigote form, inside an infected macrophage, pointed out with arrows (on the right). (http://www.cdc.gov/parasites/leishmaniasis/)

When an infected Phlebotomid sand fly takes a blood meal it infects the vertebrate host with metacyclic promastigote forms. Within a short time the promastigotes are taken up by macrophages, the first line of defense of the immune system. The promastigote then loses its flagella and transforms into the amastigote form. Once internalized in a phagosome the macrophage lysosome fuses with the phagosome to from a phagolysosome containing the parasite (Bates, 2007; Volf, 2012) (Fig. 4).

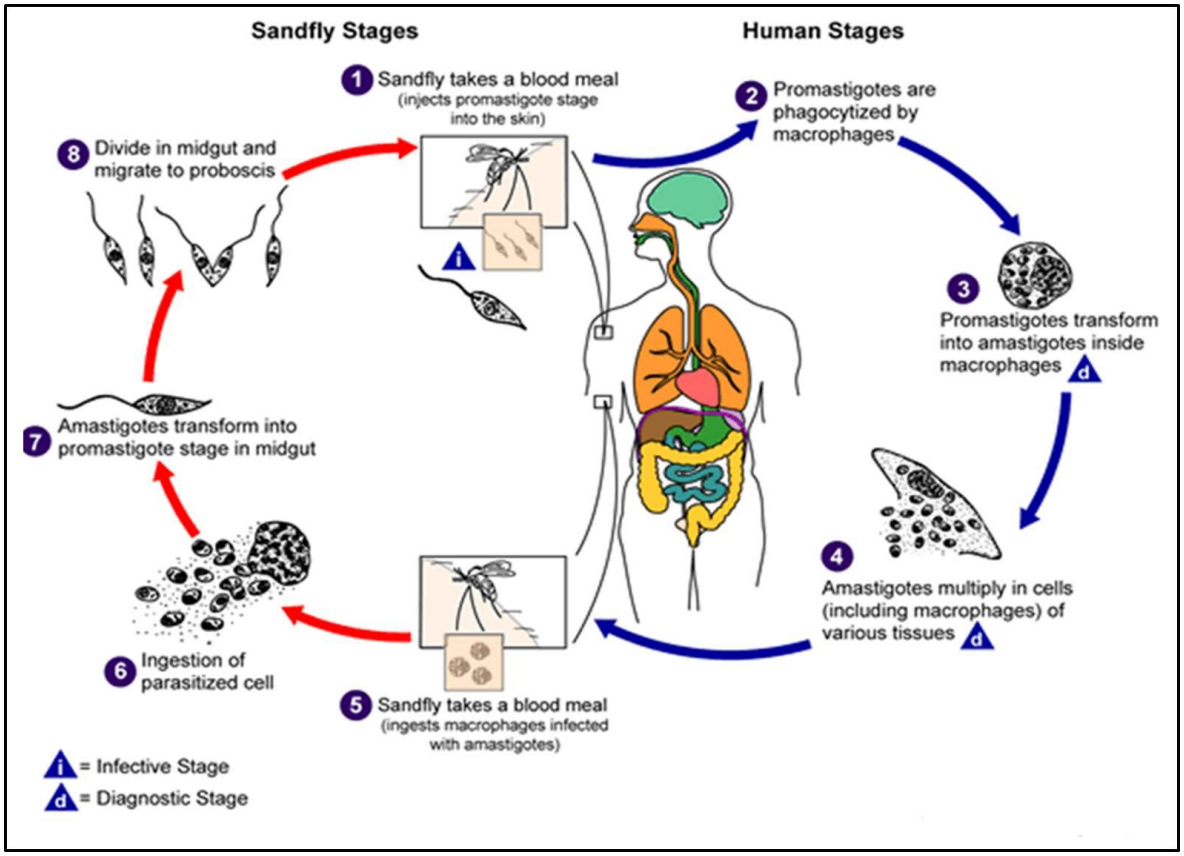


Figure 4. Diagram illustrates Leishmania parasite life cycle.
Diagram illustrates *Leishmania* parasite life cycle in both the vertebrate host (human) and the invertebrate vector (phlebotomine sandfly insect). (http://www.cdc.gov/parasites/leishmaniasis/biology.html)

1.2. Immune response to *Leishmania* infection

Leishmaniasis is characterized by a spectrum of disease phenotypes that corresponds to the strength of the host's cells-mediated immune response. Both susceptible and resistant phenotypes exist within human populations (Grevelink and Lerner, 1996). The resistance is conferred by T-helper type-1 (Th1) cells while the susceptibility is conferred by T-helper type-2 (Th2) cells (Awasthi *et al.*, 2004).

Interleukin-12 (IL-12) produced by macrophages and dendritic cells and interferon-gamma (IFN-γ) produced by natural killer cells (NK), and previously activated T cells, promote the development of Th1 cells, whereas IL-4 induces the development of Th2 cells. The Th1 subpopulation, important for induction of leishmaniasis resistance, produce IFN-γ and tumour necrosis factor-alpha (TNF-α)

which play an important role in cellular immune responses against intracellular pathogens by activating macrophages for intracellular killing of pathogens (Liew *et al.*, 1999). On the other hand, Th2 cells produce IL-4, IL-5, IL-10, and IL-13, and are associated with leishmaniasis susceptibility in *L. major* infection murine models (Chatelain *et al.*, 1992; Wang *et al.*, 1994; Chatelain *et al.*, 1999). Macrophage activation by IFN-γ has been recognized as the major mechanism of *Leishmania* killing (Carvalho *et al.*, 2005). IFN-γ activates macrophages to express inducible nitric oxide synthetase type-2 (iNOS2), the enzyme catalyzing the formation of nitric oxide which kills the intracellular amastigotes. In contrast, Th2 immune response limits the action of Th1 functions via IL-10 and IL-4, which deactivate macrophages helping intracellular parasite growth and disease progression (Awasthi *et al.*, 2004). In human, IL-10, a key macrophage deactivating cytokine, is not restricted to Th2 cells and can be produced by several types of T helper populations (Sornasse *et al.*, 1996) (Fig. 5).

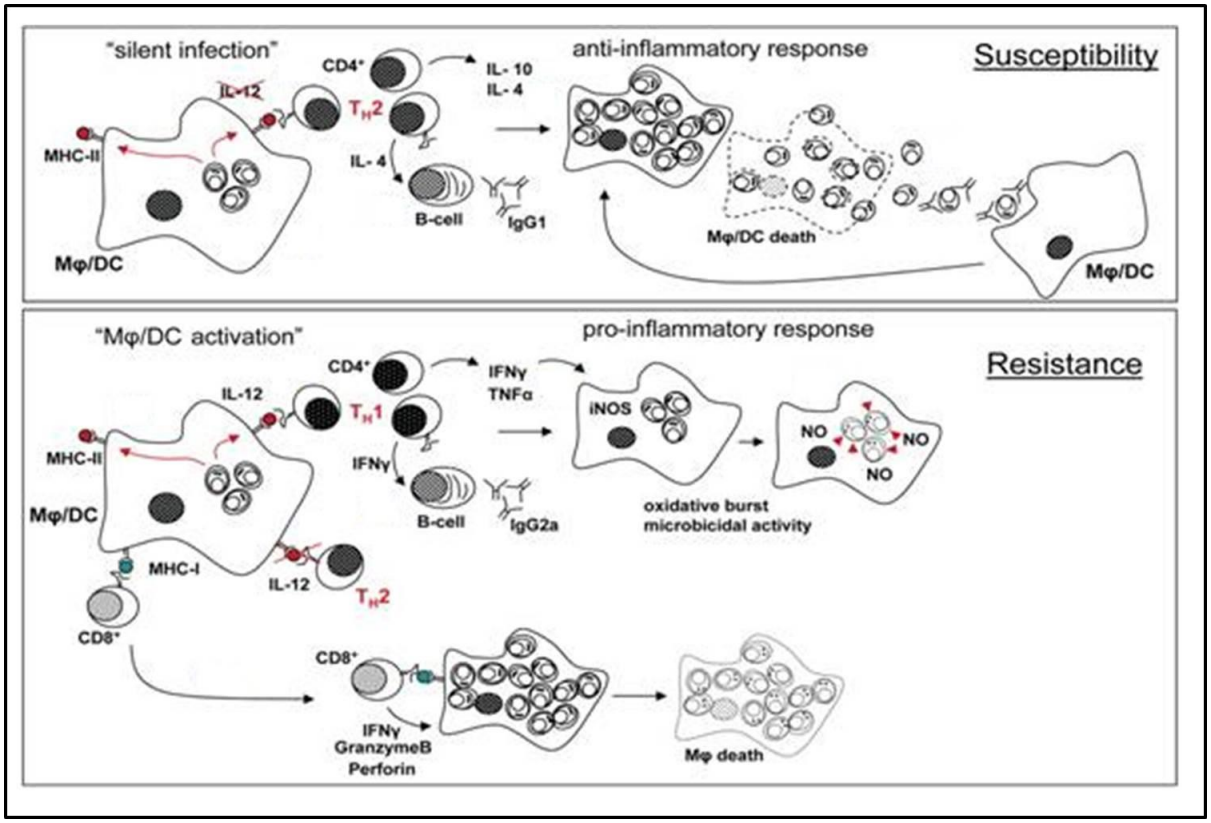


Figure 5. Diagram illustrates both susceptible and resistant phenotypes of immune response against Leishmania infection.
Susceptibility is associated with Th2 cells which produce IL-4 and IL-10 and cause macrophage deactivation helping parasites growth and disease progression. Resistance associated with Th1 cells which produce IFN-γ and TNF-α which activate macrophages for intracellular killing of pathogens.
(http://edoc.hu-berlin.de/dissertationen/schroeder-juliane-2010-09-29/HTML/chapter1.html)

T-cell receptor types have also proved interesting in the immune response to *Leishmania*. In most cell-mediated responses, T-cells bearing α β receptors are much more predominant than those expressing γ δ receptors. In contrast, during the early phase of the response to CL, 30 % of the T-cells may express γ δ receptors (Lima *et al.*, 1994). As a relatively large number of γ δ T-cells are seen in other granulomatous disease, such as leprosy, it is likely that these cells are probably important in the formation of granulomas (Modlin *et al.*, 1989).

Being a parasite, *Leishmania* ensures its own survival by modulating host immune system either by inducing immunosuppression or by promoting pro-parasitic host functions. A detailed knowledge of this host-parasite interaction would help in

designing prophylactic and therapeutic strategies against this infection (Kemp, 1997; Awasthi *et al.*, 2004).

1.3. Treatment methods of Cutaneous Leishmaniasis

The control of leishmaniasis remains a problem principally a zoonotic infection, except in epidemics where it is anthroponotic, interruption of transmission is difficult, though not impossible. No vaccines exist for either VL, CL or MCL and chemotherapy is associated with severe toxic side effects and increasing parasite drug resistance (Croft and Yardley, 2002; Bari and Rahman, 2003).

Current regimes use the pentavalent antimony (Pentostam) and meglumine antimoniate (Glucantime) as primary therapy, which have been the mainstay of therapy for leishmaniasis, but clinical treatment failure is becoming increasingly common in many areas; in addition, these drugs are associated with important adverse effects (Grogl *et al.*, 1992; Herwaldt and Berman, 1992). A number of other drugs may be employed, depending upon the species of *Leishmania* concerned and the resources available to the health professionals involved (Croft and Yardley, 2002).

Recommended secondary treatment employs a variety of drugs. The most widely used of these are amphotericin B and pentamidine. Amphotericin B is highly active but has extensive toxicity complications. However, amphotericin B therapy is associated with fever, nausea, vomiting, malaise, anemia, and renal toxicity. Pentamidine has significant potential side effects including hypoglycemia followed by diabetes mellitus, hypotension, nausea, vomiting, abdominal pain, and headache (Pearson and Sousa, 1996). The newer formulations of amphotericin B are too expensive to use for the majority of endemic countries (Croft and Yardley, 2002; Bari and Rahman, 2003). A new anti-leishmanial, miltefosine, may be used in the future. In short, there remains a pressing need for new anti-leishmanials (Croft and Yardley,

2002). A number of new drugs and therapeutic approaches are being studied. Although preliminary results appear promising with some, the clinical experience is still not sufficient to warrant their general use (Pearson and Sousa, 1996). A wide variety of therapeutic modalities have been employed for cutaneous leishmaniasis. However, none has been demonstrated to be good enough as the first-line therapeutic agent to treat patients in all the epidemiological scenarios (Bari and Rahman, 2003).

1.4. Leishmaniasis Vaccines

Methods used for prevention or control of leishmaniasis have included eradication of the vector or its habitat, destruction of animal reservoirs, treatment of human reservoirs, and vaccination. Technical difficulties such as drug resistance, financial constrains, and operational difficulties have impaired progress toward effective control of leishmaniasis (Grevelink and Lerner, 1996). Vaccination trials in animal models, human beings, or both have been performed with virulent promastigotes, promastigotes attenuated or killed with either gamma irradiation, heat, or mutagen; and specific antigens purified from promastigotes (Grevelink and Lerner, 1996). The development of an effective, noninfectious vaccine is problematic. A previous study revealed that live parasites could be recovered from the site of immunization as well as from draining lymph nodes of animals inoculated with killed promastigotes.

The genetic background of the murine host has a significant influence on the outcome of the infection (Liese *et al.*, 2008). In spite of the immunological cross-reactivity. However, there is some evidence that immunity against one *Leishmania* species fails to confer protection against another one, i.e. immunity appears to remain essentially species specific (Melby, 1991). The species-specific only protection rule is not absolute (Mahmoudzadeh-Niknam, 2004). Cross-species protection in human

beings with different species of *Leishmania* is rare and have been documented only between L. tropica and L. major (Grevelink and Lerner, 1996).

In vitro studies on human and murine cells have confirmed that certain cytokines (e.g. IFN-γ, IL-12) will favour maturation of Th1 responses whereas others (e.g. IL-4, IL-10) support Th2 development. If similar immunoregulatory mechanisms operate in mouse and man, design of vaccines against human leishmaniasis should aim at introducing powerful Th1 like responses. Importantly, once generation of either Th1 or Th2 has started, the immune response seems to be locked in this pattern, even when it is harmful to the host. Therefore new vaccines against leishmaniasis should be designed in a way that they generate controlled Th1 like primary responses (Kemp, 1997).

1.5. Molecular determinants of *Leishmania*

Leishmaniasis disease progresses from infection to symptomatic phase to host death or recovery. The outcome of each phase is depicted to result from the interactions of a distinct group of parasite molecules with a specific host immune compartment. The first group consists of invasive/evasive determinants, which are largely parasite cell surface and secreted molecules (e.g. LPG, GP63, GPI). The second group of parasite molecules consists of "pathoantigenic" determinants unique parasite epitopes present often within otherwise highly conserved cytoplasmic molecules (e.g. HSP60,70,83) (Chang and McGwire, 2002). Investigation on the molecular determinants of virulence have focused on several glycoproteins and glycolipids, including lipophosphoglycan (LPG) (Ryan *et al.*, 1993), the major surface glycoprotein GP63 (Medina-Acosta *et al.*, 1989), glycoinositol phospholipids (GIPLs) (McConville *et al.*, 1990), and members of the HSP70 family of stress/heat-shock proteins (MacFarlane *et al.*, 1990). The third group of parasite molecules is

hypothetically perceived as vaccine determinants (e.g. LACK, KMP-11). Their interactions with the host immune system lead to the elimination or reduction of parasites to effect a clinical cure (Chang and McGwire, 2002).

1.6. P36/LACK protein

The LACK (*Leishmania* homologue of receptors for activated C kinase) antigen is a 36-kDa protein expressed in promastigote and amastigote forms of different *Leishmania* species (Coelho *et al.*, 2003). LACK is an analog of the RACK (receptor for activated C-kinase) proteins present in eukaryotes that are members of the family of WD40 repeat proteins. These proteins mediate protein–protein interactions serving as adaptors for several multicomplex proteins involved in signaling pathways (Gomez-Arreaza *et al.*, 2011). These macromolecules are evolutionarily conserved tryptophan aspartate motif proteins and have diverse but critical functions in eukaryotes, including signal transduction, RNA processing, and cell cycle control (Kelly *et al.*, 2003). The molecular function of LACK in *Leishmania* is not clear although the immunological response to this molecule has been well studied and used for experimental vaccine studies in the mouse model (Gomez-Arreaza *et al.*, 2011). This protein has been shown to be highly conserved in *Leishmania* species examined to date. Because the response to LACK is a very early event in a *Leishmania* infection, it could also include innate elements of immunity (Maasho *et al.*, 2000). The LACK amino acid sequence, including an immunodominant epitope located between amino acids 158 and 173, is highly conserved (Coelho *et al.*, 2003). It has been clearly demonstrated that LACK is essential for the viability of the parasite and to establish the parasite in the host. LACK mutants of *L. major* with diminished levels of this protein fail to develop lesions in susceptible mice and have reduced capacity to reproduce in macrophages *in vitro*. In addition to its cytoplasmic and on the external

surface of the membrane localization, LACK was recently found to also be actively secreted via exosomes. All these findings suggest that LACK could have several functions, its plasminogen binding capacity representing a novel function of this protein. This function could be important in *Leishmania*–host interaction (Gomez-Arreaza *et al.*, 2011).

1.7. Immune response to P36/LACK antigen

LACK is among the most extensively studied *Leishmania* molecules (Kubar and Fragaki, 2006). In Leishmaniasis disease, high levels of IFN-γ and TNF-α are produced (Bacellar *et al.*, 2002), macrophages are activated (Carvalho *et al.*, 1985), and the subsequent non modulated immune response is known to be associated with tissue damage. The recombinant *Leishmania* antigens LACK induce production of IL-10 and is able to downregulate IFN-γ production (Carvalho *et al.*, 2005). The immune modulatory properties of recombinant LACK antigen in CL and ML patients were evaluated. Addition of LACK to SLA stimulated PBMC of CL and ML patients enhanced IL-10 production. Addition of LACK to SLA-stimulated cultures decreased IFN-γ levels by 58 % in CL patients and by 30 % in MCL patients. Neutralization of IL-10 abrogated the downregulatory effect of LACK. The modulatory properties of LACK are due to induction of IL-10 production and may be helpful for attenuating chronic inflammatory diseases (Carvalho *et al.*, 2005). It is known that LACK antigen presents a single epitope (amino acid 156–173) responsible for the induction of IL-10 synthesis (Bourreau *et al.*, 2002). LACK is known to induce a high frequency of CD4 T cells that secrete IL-4 in BALB/c mice, which are known to be associated with exacerbation of *L. major* infection (Malherbe *et al.*, 2000). Moreover, the LACK-specific response has recently been correlated with a downregulation of monocyte activity in human CL (Antonelli *et al.*, 2004).

The LACK antigen of *L. major* induced comparable IL-4 production in V beta 4-V alpha 8 $CD4^+$ T cells. Thus, the IL-4 required for Th2 development and susceptibility to *L. major* is produced by a restricted population of V beta 4-V alpha 8 $CD4^+$ T cells after cognate interaction with a single antigen from this complex parasite. The IL-4 produced rapidly by these $CD4^+$ T cells induces within 48 hours a state of unresponsiveness to IL-12 among parasite-specific $CD4^+$ T cell precursors by downregulating the IL-12 receptor beta 2 chain expression (Louis *et al.*, 1998).

1.8. P36/LACK as vaccine

The newer vaccines under consideration comprise recombinant DNA-derived antigens and peptides. Some of the target antigens are species and life cycle stage specific, while others are shared by promastigotes and amastigotes, some are conserved among *Leishmania* species, while others are not.

A similarly conserved antigen, the *Leishmania* homologue of the receptor for activated C kinase (LACK), which is expressed by both promastigotes and amastigotes has been shown to protect mice from infection, in particular when administered with IL-12 as an adjuvant. Interestingly, LACK is also the major target for Th2 responses in susceptible BALB/c mice, and BALB/c mice made tolerant to LACK are resistant to infection. The significance of this finding for the use of LACK as a vaccine in humans remains to be elucidated (Handman, 2001). The LACK antigen has been used as a tool to investigate various immunity-related mechanisms. It has been tested in several immunization experiments (Mougneau *et al.*, 1995; Melby *et al.*, 2001; Okuno *et al.*, 2002; Coelho *et al.*, 2003), providing heterogeneous results. The immunization of mice with LACK, delivered as either protein or DNA (Mougneau *et al.*, 1995; Kubar and Fragaki, 2005), protects against cutaneous *L. major* challenge by redirecting the early T-cell response away from a pathogenic

IL-4 response and towards a protective Th1 response. By contrast, no protection against the other *Leishmania spp*. tested is achieved in mice, despite the induction of a Th1 response (Melby *et al.*, 2001; Okuno *et al.*, 2002; Coelho *et al.*, 2003). In human patients, as in mice, responses to LACK depend strongly on the infecting *Leishmania* species (Kubar and Fragaki, 2006). The protective effect of the LACK vaccine was mediated by IL-12-dependent IFN-γ production, which was sufficient to overcome the early pathogenic IL-4 response observed in this infection model (Melby *et al.*, 2001). Vaccination with L. donovani p36 (LACK) DNA primed for a strong in vitro and in vivo parasite-specific Th1 response but did not confer protection against parasite challenge. This is in contrast to results from earlier studies with a different murine model of cutaneous leishmaniasis, where the L. major a truncated (24-kDa) version of the 36-kDa LACK antigen, delivered either as recombinant protein (with IL-12 adjuvant) or as DNA vaccine, was highly effective in protecting against cutaneous L. major challenge (Mougneau *et al.*, 1995; Gurunathan *et al.*, 1997).

1.9. Current production methods of recombinant proteins

The use of vaccines has had great impact on the ability to control and prevent infectious diseases. The ability to use small, defined parts of a pathogen and produce that subunit in a non-pathogenic host will increase the safety of vaccines. Subunit vaccine candidates typically consist of surface proteins or polysaccharides. The use of recombinant DNA technology has simplified the production of subunit vaccines. Protein-based subunit vaccines can consist of synthetic peptides, recombinant proteins or gene fragments encoding the protein immunogens (Table 1).

Table 1: Different vaccine types and some of their characteristics

Vaccine type	Characteristics
Whole cell vaccines	
Live, attenuated bacteria or viruses	+ Humoral and cellular immune responses - Risk of reversion to virulent strain - Undefined composition
Killed, inactivated bacteria or viruses	+ No risk of infection (replication deficient, non-infectious) - Less powerful than live vaccines - Undefined composition
Subunit vaccines	
Purified components from pathogen	+ Well-defined composition - Requires large-scale cultivation of pathogen - Poor immunogens, need of adjuvant
Synthetic peptides	+ Well-defined composition + No risk of pathogenicity - Short half-life - Need of adjuvant
Recombinant proteins	+ Well-defined composition + No risk for pathogenicity + Possibility for cost-efficient production and purification - Primarily humoral immune response - Need of adjuvant
Live vectors: Bacterial	+ Humoral and cellular immune responses + Possibility to develop oral vaccines - Risk of reversion to virulent forms for attenuated pathogens
Live vectors: Viral	+ Humoral and cellular immune responses - Risk of reversion to virulent forms for attenuated pathogens
Nucleic Acid vaccines: DNA	+ Cellular and humoral immune responses + Cost-efficient production + *In vivo* amplification systems available - Inefficient transfection - Risk of integration into host genome not completely excluded
Nucleic Acid vaccines: RNA	+ No risk of integration into host genome + Do not have to enter nucleus for translation + *In vivo* amplification systems available - Unstable - Quite expensive production

(http://www.diva-portal.org/smash/get/diva2:8775/FULLTEXT01.pdf)

The basics of this technology is to transfer a gene encoding an antigen, responsible for inducing immune responses sufficient for protection, to a non-

pathogenic host, thereby making the production of the antigen safer and generally more efficient (Fig.6). There are several advantages in using recombinant subunit vaccines. No pathogen is present in the production and purification procedure, thus making the production procedure less hazardous. By using recombinant DNA technology, the production and purification procedure can be carefully designed to obtain high yields of a well-defined product.

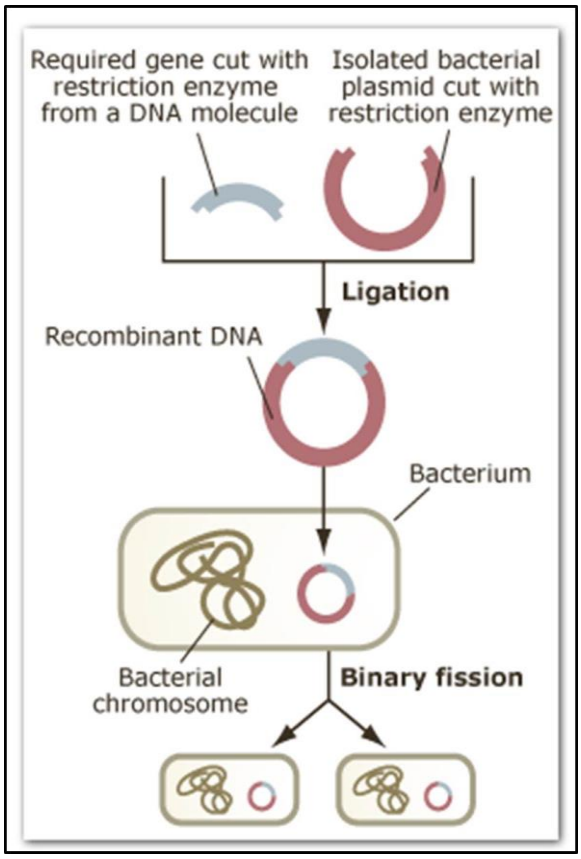


Figure 6. Recombinant DNA technology.
Recombinant DNA technology enables individual fragments of DNA from any genome to be inserted into vector DNA molecules, such as plasmids, and individually amplified in bacteria. (http://tle.westone.wa.gov.au/content/file/c0a7f9da-2dc9-b549-578a-0f1a8921d148/1/bio_science_3b.zip/content/001_dna/page_14.htm)

1.9.1. Gene construct

The gene construct can be designed either for secretion of the expressed protein into the growth medium or the periplasm machinery (Abrahmsen *et al.*, 1986; Uhlen *et al.*, 1988; Hansson *et al.*, 1994) or for intracellulary production of the protein. Intracellular production can be used when expressing proteins with tendency to

aggregate inside cells, or when expressing proteins containing transmembrane regions (Sjolander *et al.*, 1993; Robert *et al.*, 1996). Intracellular expression might lead to the formation of inclusion bodies, i.e. protein aggregates, which requires solubilization and refolding to obtain the product in a soluble and active form (Babiuk, 1999). Some advantages of inclusion body formation are that the product normally is protected from proteolytic degradation, and that high production levels often are obtained.

1.9.2. Production hosts

There are four major host cell systems commonly used to produce recombinant proteins; (i) bacterial expression systems, (ii) yeast expression systems, (iii) insect cell expression systems, and (iv) mammalian expression systems. Each host cell system has individual advantages and disadvantages. Bacterial expression systems are the most convenient to use. Expression levels of recombinant proteins in bacteria are in general high. Bacterial expression systems are suitable to use when no post-translational modifications of the protein are required. Several bacterial systems are available, but Escherichia coli is the dominating and also the best characterized. Many alternatives for optimization of protein expression in bacteria have been reported e.g. choice of strain, transcriptional and translational regulation, and also the possibility to include signal sequences that will target the protein to periplasm or culture media (Makrides, 1996). Since foreign proteins may be rapidly degraded in the bacterial host, the host can be engineered to reduce the proteolysis (Murby *et al.*, 1996; Babiuk, 1999).

1.9.3. Affinity tags

Using affinity fusion partners will make efficient recovery of the expressed recombinant gene product possible. The 6 X His tag is one of the most common tags

used to facilitate the purification and detection of recombinant proteins and a range of products. Chelating Sepharose, when charged with Ni^{2+} ions, selectively binds proteins if complex forming amino acid residues, in particular histidine, are exposed on the protein surface. 6 X His fusion proteins can be easily bound and then eluted with buffers containing imidazole (Arnold, 1991; Lopatin and Varlamov, 1995).

1.10. Amis of the research

The sustained expression of a recombinant antigen is an attractive approach for protection against leishmaniasis. The P36/LACK antigen is of particular interest as a vaccine candidate because of the prominent role it plays in the immunopathogenesis. According to the facts that, Leishmaniasis spreading in Syria, which is mostly caused by *L. tropica*, the absence of studies on strains which belong to this species, and effective vaccines against it are not available, therefore, The aim of this present study was to:

- determine the sequence of *P36/LACK* gene of local isolate of *L. tropica*.

- study the expression of *P36/LACK* gene in both promastigotes and amastigotes forms.

- produce recombinant P36/LACK protein to be used later for the detection of leishmaniasis infection and as experimental vaccine against CL.

CHAPTER II. MATERIALS AND METHODS

2. Materials and Methods

2.1. Parasites and strains typing

2.1.1. Sampling

Samples (five isolates) were collected from patients visiting the Dermatology Hospital in Damascus, with lesions suspected to CL. Positive samples were collected directly from skin scars of the patients on slides. In addition, aspirated samples were taken from lesions for culturing in a semi-solid (RPMI with agar) culture medium (Mugasa *et al.*, 2010).

2.1.1. Microscopic examination

Smears on glass slides, were air dried, fixed with methanol for a few seconds and stained with Giemsa. Then, the stained smears were examined using light microscope 100 × (Olympus, Japan) (Chulay and Bryceson, 1983; Pearson and Sousa, 1996).

2.1.3. In vitro cultivation of *Leishmania* parasite

In vitro cultivation of the parasite *Leishmania* is a useful approach for yielding amounts of the parasite suitable for many different purposes. One of the primary goals of culturists has been to achieve the long-term maintenance of active and dividing populations of different *Leishmania* species. *Leishmania* promastigotes were first grown on biphasic medium (liquid phase added to solid one; RPMI with agar), then transferred liquid monophasic medium.

Used materials:

- Agar, NaCl (Prolabo, France).
- RPMI-1640 medium (Sigma, USA).
- L-Glutamine, Penicillin-Streptomycin (Cytogen, USA).
- Fetus Bovine Serum (FBS) (Sigma, USA).

Procedure:

- Aspirated sample is added to inclined agar (3.5 g agar, and 1.6 g NaCl per 100 ml dH_2O, autoclaved for 15 min, 121 ^oC), with RPMI supplemented with 10 % heat-inactivated FBS, and 100 U/ ml L-Glutamine and Penicillin-Streptomycin.
- Incubation at 26 ^oC until promastigotes forms are distinguished.
- After few days, one drop of media is examined using inverted microscope 40 × (Optika, Italy).
- Once promastigotes were formed, promastigotes are passed to fresh RPMI supplemented with 10 % FBS, and 100 U/ ml L-Glutamine and Penicillin-Streptomycin, and incubated at 26 °C.
- To obtain axenic amastigotes, promastigotes in stationary phase in 25 cm^2 ventilated flask were incubated at 37 °C and pH 5.8 with 5 % CO_2. for about 48 hour (Sereno and Lemesre, 1997; Saar *et al.*, 1998; El Fakhry *et al.*, 2002; Barak *et al.*, 2005).

2.1.4. DNA extraction

DNA was extracted from 5 ml of *Leishmania in vitro* culture (~16×10^6 cell/ml). Promastigote forms of the parasite were harvested and washed with (1 ×) phosphate-buffered saline (PBS) pH 7.4, pelleted by centrifugation at 3000 rpm for 15 minutes at room temperature. Then the genomic DNA was isolated using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions.

Procedure:

- Add 600µl of Nuclei Lysis Solution, and pipet to lyse the cells. Pipet until no visible cell clumps remain.
- To the room temperature sample, add 200µl of Protein Precipitation Solution and vortex vigorously at high speed for 20 seconds. Chill sample on ice for 5 minutes.
- Centrifuge for 4 minutes at 13,000–16,000 × g. The precipitated protein will form a tight white pellet.
- Carefully remove the supernatant containing the DNA (leaving the protein pellet behind) and transfer it to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol.
- Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.
- Centrifuge for 1 minute at 13,000–16,000 × g at room temperature. The DNA will be visible as a small white pellet. Carefully decant the supernatant.

- Add 600µl of room temperature 70 % ethanol, and gently invert the tube several times to wash the DNA. Centrifuge for 1 minute at 13,000–16,000 × g at room temperature.
- Carefully aspirate the ethanol using either a drawn Pasteur pipette or a sequencing pipette tip. The DNA pellet is very loose at this point, and care must be used to avoid aspirating the pellet into the pipette.
- Invert the tube on clean absorbent paper, and air-dry the pellet for 10–15 minutes.
- Add 100µl of DNA Rehydration Solution, and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube.
- DNA concentration was determined for a diluted sample (1:50 v/v) by spectroscopy at 260/280 nm wave length.
- Store the DNA at -20 °C.

2.1.5. PCR reaction

Polymerase Chain Reaction (PCR) was performed by using CSB2/CSB1 primers for species determination, which are specific to the conserved region of *Leishmania* minicircle kinetoplast DNA (kDNA) (Ray, 1989; Yurchenko *et al.*, 1999) (Table 2).

Leishmanial DNA isolated from reference strains *L. major* (MHOM/SY/91/LEM2397) and *L. tropica* (MHOM/SY/90/LEM2066) was used as a positive control (Reference strains were a gift from French National Reference Centre for *Leishmania* (Montpellier, France). The total volume of PCR reaction is 30 µl including (1 ×) Go Taq Buffer, dNTPmix (~0.2mM each dNTP), GoTaq DNA polymerase (1 U) (Promega, UAS), and DNA template 0.25 µg.

Table 2: Oligonucleotide sequence (Alpha DNA)

Protocols	Annealing Temp.	Primers name	Sequence	Length
Parasite typing primers	54 °C	CSB2	5'-CGAGTAGCAGAAACTCCCGTTCA-3'	23
		CSB1	5'-ATTTTTCGCGATTTTCGCAGAACG-3'	24
Housekeeping gene primers	60 °C	Lmr60s-F	5'-ATGCCGCCGAAGTTCGACCC-3'	20
		Lmr60s-R	5'-CGTCGCCACACGGTTCTTGA-3'	20
Gene amplification primers	60 °C	LACK-FL-F	5'-ATGAACTACGAGGGTCACCT-3'	20
		LACK-FL-R	5'-CTCGGCGTCGGAGATGGACC-3'	20
		LACK-F	5'-GCAGCTGTTCAAGATCAACG-3'	20
		LACK-R	5'-GGATCAGGTTGTCCTTGTGG-3'	20
Gene cloning primers	55 °C	P36-F(*Bam*HI)	5'-TATATAGGATCCATGAACTACGAGGGTCACCT-3'	32
		P36-R(*Eco*RI)	5'-TATATAGAATTCTTACTCGGCGTCGGAGATGG-3'	32
Gene sequencing primers	55 °C	P36(seq)-F1	5'-ATGTGGGACCTGCGCAATGG-3'	20
		P36(seq)-F2	5'-GACGGCGCGGCGCTGTTGTG-3'	20
		P36(seq)-R1	5'-GGTGCTCAGGTCCCACAACA-3'	20
		P36(seq)-R2	5'-GTGCTTCAGGAACTTGCGCT-3'	20
pDrive cloning vector primers	55 °C	FP	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'	20
		RP	5'-TCACACAGGAAACAGCTATGAC-3'	20
pRSET primers	55 °C	T_7F	5'-TAATACGACTCACTATAGGG-3'	20
		T_7R	5'-TAGTTATTGCTCAGCGGTGG-3'	20

Used materials:

- 5× Go Taq Buffer.
- 5 units/µl GoTaq DNA polymerase (Promega, UAS).
- 10 mM dNTPmix (~0.2mM each dNTP).
- Primers (10 µM).
- Double-distilled water (ddH_2O) (Table 3).

Procedure:

- PCR reaction was performed in the PeQlab thermocycler (PEQLAP, Germany) with 40 cycles, using an initial heating 94 °C for 2 min. Each cycle was divided into three stages: denaturation (94°C–30 sec), annealing (54 °C–1 min), and elongation (72 °C–1 min). The last cycle followed by final extension step at 72 °C for 10 min (Table 3).

Table 3: PCR mixture and applied program

Reagent	Quantities	Final con.
Go Taq buffer (5×)	10 µl	1×
dNTP mix (10mM each dNTP)	1 µl	0.2mM each dNTP
CSB2 primer (10µM)	2.5 µl	0.5µM
CSB1 primer (10µM)	2.5 µl	0.5µM
GoTaq DNA polymerase (5U/µl)	0.25 µl	1.25u
DNA template	2 µl	<0.5µg/50µl
ddH_2O to	50µl	

Step	Temperature	Time
Initial denaturation	94 °C	2 min
Denaturation	94 °C	30 sec
Annealing	54 °C	1 min
Extension	72 °C	1 min
Final extension	72 °C	10 min
Number of cycles	40	

2.1.6. Agarose gel electrophoresis

Agarose gel electrophoresis is used to determine the size of DNA fragments. PCR products were separated on 0.8 % agarose gel. The length of the PCR products is about 750 bp.

Used materials:

- 50× TAE buffer (2 M Tris-borate; 242 g, glacial acetic acid; 57 ml, 0.5 M EDTA, pH 8.0; 100 ml. Dilute to 1 L with distilled water).
- Running Buffer (0.5× TAE): Dilute 50× TAE to 0.5× and use the amount appropriate for the particular gel apparatus.
- Ethidium Bromide 1mg/ml (Sigma, USA).
- Agarose (Sigma).
- 6× DNA Loading Buffer (Promega, USA).
- DNA length standard (1 kb DNA Ladder; Promega, USA).

Procedure:

- Agarose gel prepared by dissolving 0.8 g of agarose powder in 100 ml TAE buffer, the mixture was heated until boiling so all agarose was dissolved in the buffer.
- The agarose was cooled enough ~60 ^{o}C before it was poured in the gel tray with well comb fixed properly, after adding ~10 µl of ethidium bromide.
- When the gel hardens the comb was removed carefully then 0.5× TAE buffer was added to cover the gel.
- Samples were prepared by adding 1 µl of 6× DNA loading buffer to every 5 µl of PCR product and loaded to the gel wells, DNA marker was also added to the first well of the gel.

- The electrophoresis was run at constant voltage of 80 V for 30 min then it was examined under the UV light.

2.2. Cloning and sequencing of *P36/LACK* gene

2.2.1. Amplification of *P36/LACK* gene

P36/LACK gene was amplified with LACK-FL-F/LACK-FL-R specific primers, which were designed according to *P36/LACK* gene sequence of *L. major* (GenBank accession number AF363975.1) (Table 2).

Procedure:

- The PCR reaction mixture is illustrated in table 4 and the used primers in table 2. PCR reaction was performed in the PeQlab thermocycler (PEQLAB, Germany), using an initial heating 95 ^{o}C for 2 min followed by 35 cycles. Each cycle was divided into three stages: denaturation (95 ^{o}C – 1 min), annealing (55 ^{o}C – 30 sec), and elongation (72 ^{o}C – 1 min). After the reaction, the material was kept at 72 °C for 5 min, and the amplified product was then stored at 4 °C until use (Table 4).

Table 4: PCR mixture and applied program

Reagent	Quantities	Final con.
Go Taq buffer (5×)	10 µl	1×
dNTP mix (10mM each dNTP)	1 µl	0.2mM each dNTP
Forword primer (10µM)	2.5 µl	0.5µM
Reveers primer (10µM)	2.5 µl	0.5µM
GoTaq DNA polymerase (5U/µl)	0.25 µl	1.25u
DNA template	2 µl	<0.5µg/50µl
ddH_2O to	50µl	

Step	Temperature	Time
Initial denaturation	95 °C	2 min
Denaturation	95 °C	1 min
Annealing	55 °C	30 sec
Extension	72 °C	1 min
Final extension	72 °C	5 min
Number of cycles	35	

2.2.2. Purification of PCR products

PCR products were purified using Invisorb Fragment CleanUp kit (Stratec molecular, Germany). The Invisorb columns contain a unique silica membrane, which has selective bonding properties, and are designed to give high end-concentration of purified DNA fragments for subsequent reactions.

Procedure:

- Binding of DNA fragments:

- For PCR mixtures up to 50 µl add 250 µl Binding Buffer to the PCR sample and mix very well by pipetting or vortexing. Transfer the sample completely onto a Spin Filter and centrifuge for 3 min at 13.400 ×g (12.000 rpm).
- For PCR mixture > 50 µl up to 100 µl add 500 µl Binding Buffer to the PCR sample and mix very well by pipetting or vortexing. Transfer the sample completely onto a Spin Filter and centrifuge for 1 min at 13.400 ×g (12.000 rpm). Remove the filtrate and centrifuge again for 2 min.

- Elution of DNA fragments:

- Place the Spin Filter into a new 1.5 ml Receiver Tube. Add at least 10 µl Elution Buffer directly onto the center of the Spin Filter. Incubate for 1 min at room temperature. Centrifugation for 1 min at 9.300 ×g (10.000 rpm).

2.2.3. Cloning of *P36/LACK* gene into pDrive cloning vector

The purified PCR products were cloned into pDrive cloning vector (QIAGEN, Germany). The 5'-terminal base of the PCR primers can affect addition of an A overhang to PCR products by *Taq* DNA polymerase. This plasmid is characterized by several features (Fig. 7):

- The linear form makes it ready-to-use for direct ligation of PCR products.
- Ampicillin and kanamycin genes which are used for positive colonies selection.
- Restriction endonuclease recognition sites around the cloning site, allowing easy restriction analysis of recombinant plasmids.
- T7 and SP6 promoters on either side of the cloning site, allowing in vitro transcription of cloned PCR products as well as sequence analysis using standard sequencing primers.
- Origin of replication which enables plasmid replication within *E. coli*.

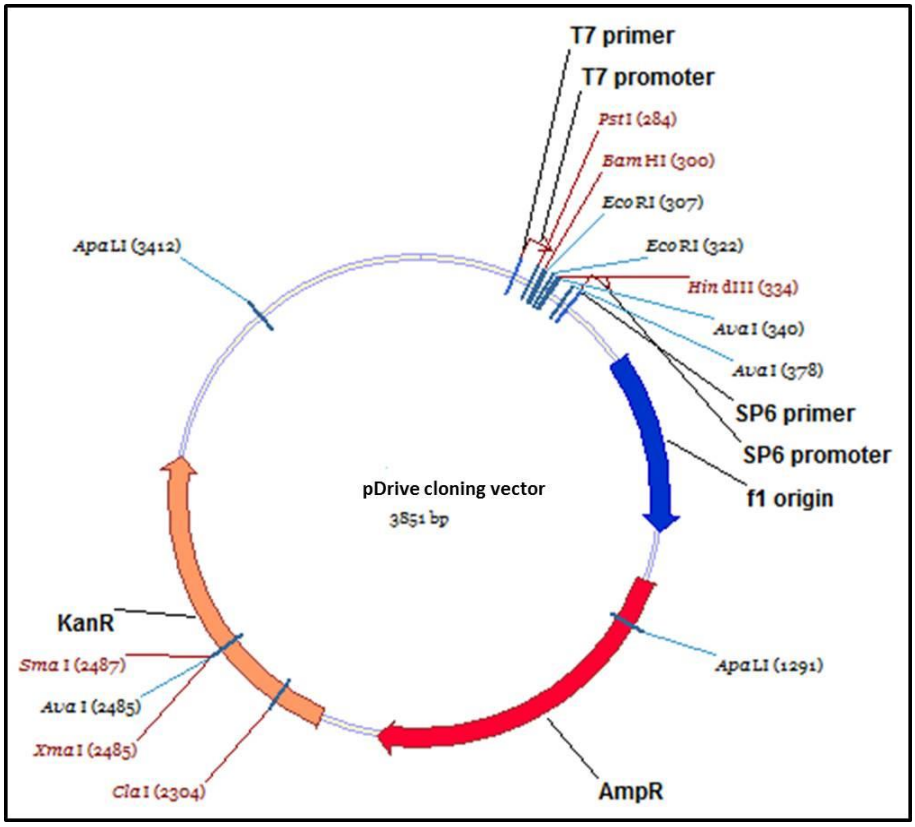


Figure 7. Schematic structure of pDrive cloning vector.
*Designed by Vector NTI program.

Procedure:

- Thaw 2 × Ligation Master Mix, pDrive cloning vector DNA, and distilled water (provided) and place on ice. It is important to mix the solutions thoroughly before use. Keep 2 × Ligation Master Mix on ice and immediately store at –20 °C or –70 °C after use.
- Prepare a ligation-reaction mixture as follow (Table 5):

Table 5: Ligation reaction mixture

Component	Volume
pDrive cloning vector (50 ng/µl)	1 µl
PCR product	2 µl
Distilled water	2 µl
Ligation Master Mix 2 ×	5 µl
Total volume	10 µl

- Briefly mix the ligation-reaction mixture then incubate for 2 h at 16° C.

2.2.4. Sequencing of *P36/LACK* gene

The construct pDrive-*P36/LACK* was sequenced by Genetic Analyzer system ABI-310 using universal specific primers FP/RP (Table 2) for the used plasmid (Applied Biosystems, USA).

2.2.5. Comparison of *P36/LACK* sequences between *Leishmania spp.*

The nucleotide sequences of P36/LACK gene in a local isolate of L. tropica (GenBank accession number: KM042903) was compared with reported lack sequences in L. donovani, L. major, L. amazonensis, L. brazilliensis, L. chagasi, L. infantumand, L. mexicana and from three recently reported L. tropica Iranian isolates (GenBank accession numbers: AF363974.1, AF363975.1, AF363977.1, AF363978.1, U27569.1, U49695.1, AF363976.1, JX305923, JX305924, and KC763809, respectively). Sequence alignment and dendrogram prediction were performed using Geneious v4.8 software, available from www.geneious.com.

2.3. Studying the expression of *P36/LACK* gene

2.3.1. Total RNA Extraction

Total RNA was extracted from 5 ml of *Leishmania* promastigotes; stationary phase (~16×10^6 cell/ml) and logarithmic phase (~12×10^6 cell/ml), as well as from amastigotes (~16×10^6 cell/ml). The extraction was carried out using SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions.

Procedure:

- Harvest the cells and pellet it by centrifugation at 3000 rpm for 15 min.
- Wash the pelleted cells with 2 ml sterile 1 × phosphate buffer saline (PBS).
- Add 175 µl of RNA Lysis Buffer to the washed cells, dispersing the pellet and mixing well by vortexing and/or pipetting.

- Up to 1×10^6 cells will lyse easily in 175 µl of RNA Lysis Buffer. 1×10^6–5×10^6 cells will need to be passed through a 20-gauge needle to shear the genomic DNA. Repeat 4 to 5 times. Expel the lysate into a 1.5 ml tube.
- Add 350 µl of RNA Dilution Buffer (blue) to 175 µl of lysate. Mix by inverting the tube 3–4 times. Place in a water bath or heating block at 70 °C for 3 min. Incubating longer than 3 min may compromise the integrity of the RNA.
- Centrifuge at 12,000–14,000×*g* for 10 min at 20–25 °C.
- Transfer the cleared lysate solution to a fresh microcentrifuge tube by pipetting. Avoid disturbing the pelleted debris.
- Add 200 µl of 95% ethanol to the cleared lysate, and mix by pipetting 3–4 times. Transfer this mixture to the Spin Column Assembly. Centrifuge at 12,000–14,000×*g* for 1 min.
- Take the Spin Basket from the Spin Column Assembly, and discard the liquid in the Collection Tube. Put the Spin Basket back into the Collection Tube.
- Add 600 µl of RNA Wash Solution to the Spin Column Assembly. Centrifuge at 12,000–14,000 ×*g* for 1 min.
- Empty the Collection Tube as before and place it in a rack. For each isolation to be performed, prepare the DNase incubation mix by combining 40 µl Yellow Core Buffer, 5 µl 0.09 M $MnCl_2$ and 5 µl of DNase I enzyme per sample in a sterile tube (in this order). Prepare only the amount of DNase incubation mix required and pipet carefully. Mix by gentle pipetting; do not vortex. Keep the DNase I on ice while it is thawed. Apply 50 µl of this freshly prepared DNase incubation mix directly to the membrane inside the Spin Basket. Make sure that the solution is in contact with and thoroughly covering the membrane. The incubation solution is yellow to make this easier to visualize.
- Incubate for 15 min at 20–25 °C. After this incubation, add 200 µl of DNase Stop Solution to the Spin Basket, and centrifuge at 12,000–14,000×*g* for 1 min.
- Add 600 µl of RNA Wash Solution (with ethanol added) and centrifuge at 12,000–14,000×*g* for 1 min.
- Empty the Collection Tube, and add 250 µl of RNA Wash Solution, centrifuge at high speed for 2 min.
- Transfer the Spin Basket from the Collection Tube to the Elution Tube, and add 100 µl of Nuclease-Free Water to the membrane. Be sure to completely cover the surface of the membrane with the water. Place the Spin Basket Assemblies in the centrifuge with the lids of the Elution Tubes facing out. Centrifuge at 12,000–14,000×*g* for 1 min. Remove the Spin Basket and discard. Cap the Elution Tube containing the purified RNA and store at -70°C.

Total RNA concentration was measured at 260 nm wave length using spectrophotometer (Jenway, England), and 500 ng was loaded into 1 % agarose gel

electrophoresis to inspect its integrity and quality after extraction. Furthermore, 100 ng of total RNA was applied as a negative template in a PCR reaction using *lack* gene specific primers; LACK-F/LACK-R (Table 2). Then, PCR products were examined on 1 % agarose gel to assure the purity of our RNA of any genomic DNA contaminants.

2.3.2. cDNA Synthesis

Complementary DNA (cDNA) is DNA synthesized from a messenger RNA (mRNA) template in a reaction catalyzed by the enzymes reverse transcriptase and DNA polymerase. Reverse transcriptase naturally occurs in retroviruses, operates on a single strand of mRNA, generating its complementary DNA based on the pairing of RNA base pairs (A, U, G and C) to their DNA complements (T, A, C and G respectively) (Fig. 8).

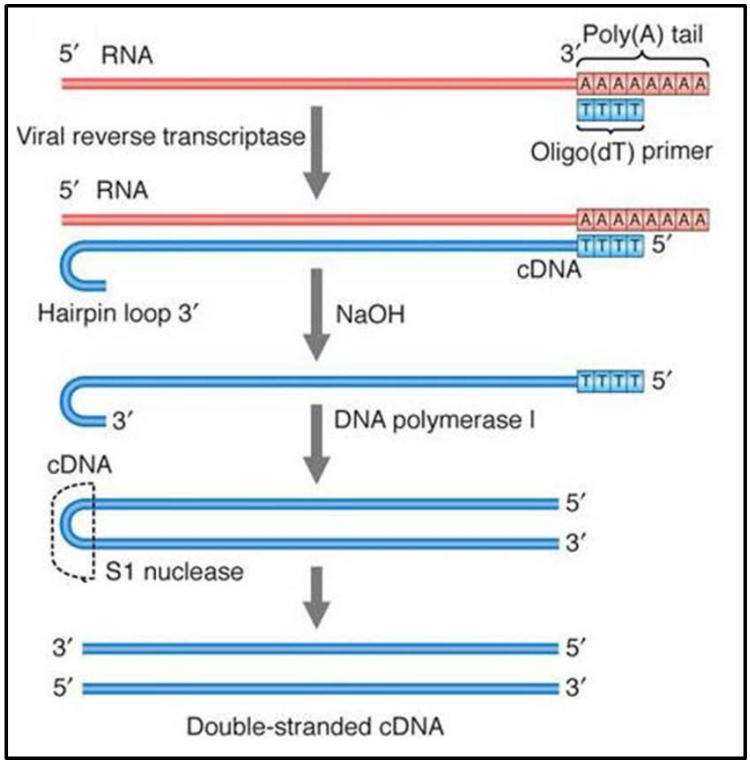


Figure 8. Illustrative scheme of cDNA synthesis procedure.
(http://barleyworld.org/sites/default/files/figure-11-02.jpg)

The obtained total RNA (0.5 µg) was reverse transcribed into single-stranded cDNA using First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions, and the resulted cDNA (60 ng) was used as PCR template.

Procedure:

- Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order (Table 6):

Table 6: cDNA synthesis mixture 1

Template RNA	**total RNA**	**0.1-5 µg**
Primer	**random hexamer primer**	**1 µl**
Water, nuclease-free		**Up to 11 µl**
Total volume		**11 µl**

- Mix gently, centrifuge briefly and incubate at 65 °C for 5 min. Chill on ice, spin down and place the vial back on ice.
- Add the following components in the indicated order (Table 7) to the mixture 1:

Table 7: cDNA synthesis mixture 2

5× Reaction Buffer	**4 µl**
RiboLock™ RNase Inhibitor (20 u/µl)	**1 µl**
10 mM dNTP Mix	**2 µl**
M-MuLV Reverse Transcriptase (20 u/µl)	**2 µl**
Total volume	**20 µl**

- Mix gently and centrifuge.
- For random hexamer primed synthesis, incubate for 5 min at 25 °C followed by 60 min at 37 °C.
- Terminate the reaction by heating at 70 °C for 5 min.

The resulted cDNA (60 ng) was used as PCR template using LAC-F/R specific primers (Table 2), which amplify 230 bp of *P36/LACK* gene, and Lmr60s-F/Lmr60s-

R primers (Table 2), which amplify 200 bp of the encoding gene for 60s ribosomal protein L12 (GenBank accession number: XM_001683687) as a positive control for cDNA synthesis. Three independent experiments were undertaken for the selected genes in all described samples.

Used materials:

- 2×PCR Master Mix kit (Fermentas, Canada).
- Primers (10 µM).
- Double-distilled water (ddH_2O) (Table 8).

Procedure:

- The PCR reaction mixture is illustrated in table 8 and the used primers in table 2. PCR reaction was performed in the PeQlab thermocycler (PEQLAB, Germany), using an initial heating 95 ^{o}C for 2 min followed by 35 cycles. Each cycle was divided into three stages: denaturation (95 ^{o}C –30 sec), annealing (60 ^{o}C – 30 sec), and elongation (72 ^{o}C – 1 min). After the reaction, the material was kept at 72 °C for 5 min, and the amplified product was then stored at 4 °C until use (Table 8).

Table 8: PCR mixture and applied program

Reagent	Quantities
Master Mix	25 µl
LAC-F primer	2.5 µl
LAC-R primer	2.5 µl
cDNA template	2.5 µl
Water to	50 µl

Step	Temperature	Time
Initial denaturation	95 °C	2 min
Denaturation	95 °C	30 sec
Annealing	60 °C	30 sec
Extension	72 °C	1 min
Final extension	72 °C	5 min
Number of cycles	35	

- PCR products were analyzed by 0.8 % agarose gel. Fragment sizes were determined with bands of a DNA length standard (BenchTop 100 bp DNA Ladder. Promega, USA).

2.4. Subcloning, expression and purification of recombinant P36/LACK protein

2.4.1. Bacterial strains

Competent *E. coli* strains TOP10 (Invitrogen, USA) and BL21-Gold (DE3) (Novagen, Germany) were used in *P36/LACK* gene cloning and protein expression, respectively (Table 9).

Table 9: Bacterial cell lines used in this study

Strain	Genotype	Description	Source
E. coli TOP10	F^- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG λ-	Cloning host	Invitrogen
BL21-Gold (DE3)	F^- ompT hsdS(rB– mB–) dcm+ Tetr gal λ(DE3) endA Hte	Expression host	Novagen

2.5. Plasmids

pDrive cloning vector (described previously in section 2.2.3) was used for *P36/LACK* gene cloning. pRSET plasmid (Invitrogen, USA) was used for subcloning and protein expression. This plasmid is characterized by several features (Fig. 9):

- pUC-derived expression vectors that is designed for high-level protein expression and purification from cloned genes in *E. coli*.
- T7 promoter which provides high levels of expression of DNA sequences cloned into the pRSET vectors
- DNA inserts are positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG translation initiation codon, a polyhistidine tag that functions as a metal binding domain in the translated protein, allows simple purification of recombinant proteins by Immobilized Metal Affinity Chromatography.
- Ampicillin resistant gene which is used for positive colonies selection.
- Origin of replication which enables plasmid replication within *E. coli*.
- Restriction enzymes sites *Bam*HI, *Eco*RI which are necessary for *P36/LACK* gene cloning.
- T_7F/T_7R primers binding sites which are used for PCR colony screening.

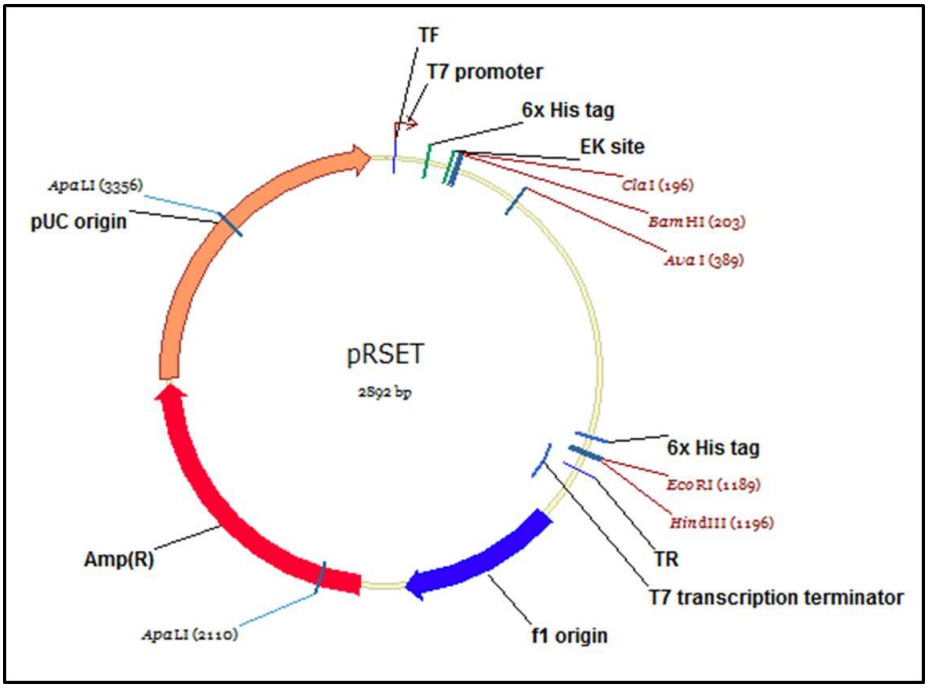


Figure 9. Schematic structure of pRSET plasmid.
*Designed by Vector NTI program.

2.4.3. Culture media

LB Broth Lennox medium (For 1L: 10 g Tryptone, 5 g Yeast Extract and 5 g NaCl, pH 7) is a nutritionally rich medium originally developed for growth and maintenance of recombinant *E. coli* strains (Roth, Germany). It was used as general bacterial cultivation and maintenance medium. For bacterial inoculation and selection, petri dishes containing LB agar medium was prepared by adding 15 g agar per 1L of LB Broth Lennox medium. SOC medium (2 % Tryptone, 0.5 % Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2 mM glucose) is suitable for use in the final step of cell transformation to obtain maximal transformation efficiency of *E. coli* (Invitrogen, UAS). Ampicillin antibiotic was used in the media, at final concentration 100 µg/ml, for the selection of positive colonies which contain the plasmid (Sigma, USA).

2.4.4. Construction of pRSET-P36/LACK Plasmid construct

2.4.4.1. Restriction reaction

Subcloning is a technique used to move a particular gene of interest from a parent vector to a destination vector in order to further study its functionality (Fig. 10).

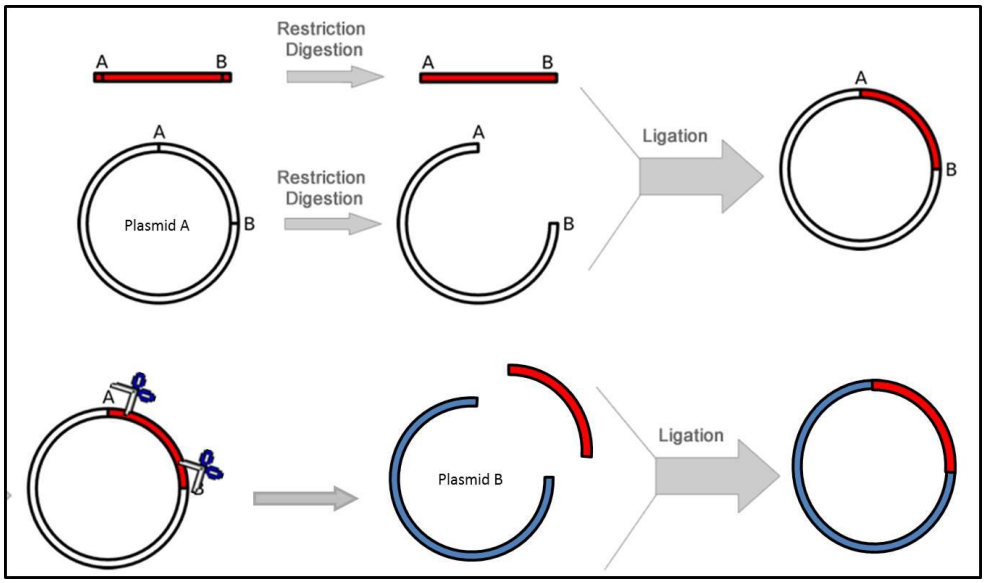


Figure 10. Subcloning procedure.

The insert was subcloned into pRSET plasmid by digesting both, pDrive-P36 and pRSET with *Bam*HI and *Eco*RI restriction enzymes (Fermentas, Canada). In general, 10 units of restriction enzyme were used to digest 1 µg of DNA (fragment or plasmid) at 37 ºC for 2 h.

Used materials:

- Restriction enzymes *Bam*HI and *Eco*RI and 10× Tango buffer (Fermentas, Canada).
- ddH2o.

Procedure:

- The digestion reactions were set as follow in table 10.
- The mixture was incubated at 37 °C for 2 h.
- The enzymes activity was inhibited by heating the mixture microtube in a water bath for 15 min at 65 °C.

Table 10: Digestion reaction of both cloning and expression vectors

pDrive-P36 plasmid		pRSET plasmid	
Plasmid DNA (5 µg)	14 µl	Plasmid DNA (5 µg)	20 µl
Tango buffer 10× Final concentration 2×	10 µl	Tango buffer 10× Final concentration 2×	10 µl
*Bam*HI (10U/µl)	5 µl	*Bam*HI (10U/µl)	5 µl
*Eco*RI (10U/µl)	5 µl	*Eco*RI (10U/µl)	5 µl
ddH_2O	16 µl	ddH_2O	10 µl
Reaction final volume 50 µl			

2.4.4.2. Purification of digestion reaction products

After digestion reaction, digested products were subjected to 0.8 % agarose gel electrophoresis. The interest gene and pRSET plasmid were extracted from agarose gel and purified using QIAquick Gel Extraction kit (QIAGEN, Germany) according to the manufacturer's instructions.

Procedure:

- Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
- Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg ~ 100 µl). For >2 % agarose gels, add 6 volumes Buffer QG.
- Incubate at 50 °C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel.
- After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
- Add 1 gel volume of isopropanol to the sample and mix.
- Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold.
- To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin/apply vacuum again.
- If the DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 0.5 ml Buffer QG to the QIAquick column and centrifuge

for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

- To wash, add 0.75 ml Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

  Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2–5 min after addition of Buffer PE.

- Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min at 17,900 x g (13,000 rpm) to remove residual wash buffer.
- Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
- To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.
- If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

2.4.4.3. Ligation reaction

The purified insert and pRSET were ligated using T4 DNA ligase (Amersham, USA). The DNA ligase catalyzes the formation of covalent phosphodiester linkages, which permanently join the nucleotides together. After ligation, the insert DNA is physically attached to the backbone and the complete plasmid can be transformed into bacterial cells for propagation.

Used materials:

- Ready-to-Go T4 DNA ligase (Amersham, USA).
- ddH_2O.

Procedure:

- The enzyme and its buffer were added to the microtube which contains the plasmid and the DNA fragment (ration 1:1) according to the manufacture instruction of the kit.
- The ligated mixture (final volume 10 µl) in the microtube was incubated for 2 h at 16 ºC.

- The enzyme activity was inhibited by heating the microtube in a water bath for 10 min at 65 °C.

2.5. Bacterial transformation

Genetic transformation occurs when a host organism takes in foreign DNA and expresses the foreign gene (Fig. 11).

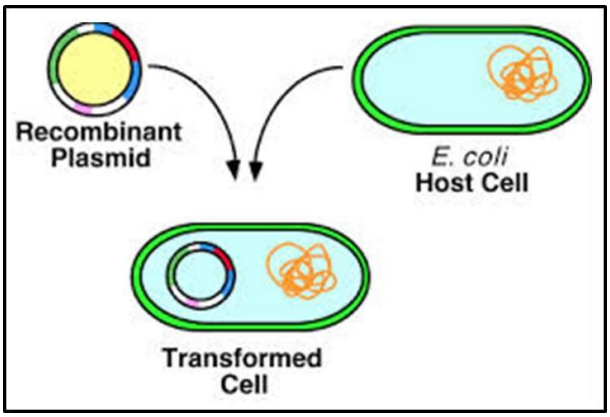


Figure 11. Bacterial transformation.

(http://calvinwong315.blogspot.com/2011_11_01_archive.html)

2.5.1. Preparation of electro-competent cells

E. coli cells are made electro-competent by thorough washing to ensure the removal of all medium salts, so the electric charge is not conducted through the medium and electroporation is carried out at 0°C to minimize heat damage to the cells. Freshly prepared electro-competent *E. coli* TOP10 cells were transformed with the new plasmid construct pRSET-P36 by electroporation.

Used materials:

- Glycerol 10%.
- ddH_2O.

Procedure:

- First day: starter culture of cells was prepared by selecting a single colony of *E. coli* TOP10 from fresh LB plate and inoculating a 10 ml of LB medium at 37 °C in shaker overnight.

- Second day: 250 ml LB medium in 1 L Erlenmeyer flask was inoculated with the 10 ml starter culture and grew in 37 °C shaker until the OD_{600} reaches ~ 0.5 - 0.6.
- The culture was incubated on ice for 1 h with swirling occasionally to ensure even cooling.
- The culture was split into six parts by pouring about 40 ml into ice cold 50 ml conical tubes. The cells were harvested by centrifugation at 6000 ×g for 5 min at 4 °C.
- The supernatant was discarded and each pellet was resuspended in 40 ml of ice cold ddH_2O.
- The cells were harvested by centrifugation at 6000 ×g for 5 min at 4 °C.
- The supernatant was discarded and each pellet was resuspended in 20 ml of ice cold ddH_2O.
- Resuspensions were combined into 3 centrifuge conical tubes. The cells were harvested by centrifugation at 6000 ×g for 5 min at 4 °C.
- The supernatant was discarded and each pellet was resuspended in 10 ml of ice cold 10 % glycerol. Each suspension was transferred to one conical tube.
- The cells were harvested by centrifugation at 6000 ×g for 5 min at 4 °C.
- The supernatant was carefully aspirated with a sterile Pasteur pipette. Each pellet was resuspended in 500 µl of ice cold 10 % glycerol by gently swirling. The final OD_{600} of the resuspended cells should be ~ 200-250.
- The cells were aliquoted into sterile chilled 1.5 ml microtubes and stored in the -80 °C freezer or in the liquid nitrogen.

2.5.2. Transformation by electroporation

Electroporation is a mechanical transfection method that uses an electrical pulse to create temporary pores in cell membranes through which substances like nucleic acids can pass into cells. It is a highly efficient strategy for the introduction of foreign DNA into host cells. The bacterial cells were transformed by Eppendorf Electroporator 2510.

Used materials:

- Competent cells *E. coli* TOP10 (Invitrogen USA).
- LB/Amp medium.
- SOB medium: 2 % Tryptone, 0.5 % Yeast extract, 10 mM NaCl, 2m M KCl.
- Mg^{+2} ions: 1 M $MgCl_2$, 1 M $MgSO_4$.

- SOC medium: SOB medium supplemented with 20 mM Glucose and 20 mM Mg^{+2}.
- 1 mm electroporation cuvette (BioRrad, England).

Procedure:

- An aliquot of electro-competent *E. coli* TOP10 (40 µl) was thawed up on ice and a 2 µl of the ligation mixture was introduced for transformation.
- The bacteria was filled up in a chilled 1 mm electroporation cuvette and incubated on ice for 1 min.
- The electroporation was accomplished with 1.8 kV/cm.
- The bacteria suspension was rinsed with 1 ml of SOC medium from the cuvette and incubated in 1.5 ml microtube for 1 h at 650 rpm shaking at 37 °C.
- The transformed bacteria were platted out on a LB-agar-plate in the presence of ampicillin.
- The agar plate was incubated overnight at 37 °C.
- After the growth of transformed *E. coli*, a number of single, isolated colonies were picked for plasmid miniprep after confirming the entry of the plasmid construct by colony PCR screening using T_7F/T_7R primers which were designed especially for pRSET plasmid and positive ones were confirmed using (T_7F/P36(seq)-R2) primers.

2.5.3. Plasmid Miniprep

This procedure is based on the alkaline lysis and used to isolate plasmid DNA from bacterial cell suspensions. Plasmids are relatively small supercoiled DNA molecules while bacterial chromosomal DNA is much larger and less supercoiled. This difference allows for selective precipitation of the chromosomal DNA and cellular proteins from plasmids and RNA molecules.

Plasmid constructs were isolated from some positive clones by GeneJET Plasmid Miniprep Kit (Thermo Scientific, Canada), after being grown in LB/ampicillin medium, according to the manufacturer's instructions.

Procedure:

- Growth of Bacterial Cultures
- Pick a single colony from a freshly streaked selective plate to inoculate 1-5 ml of LB medium supplemented with the appropriate selection antibiotic.

Incubate for 12-16 h at 37 C while shaking at 200-250 rpm. Use a tube or flask with a volume of at least 4 times the culture volume.

- Harvest the bacterial culture by centrifugation at 8000 rpm in a microcentrifuge for 2 min at room temperature. Decant the supernatant and remove all remaining medium. Do not overload the column:

  For high-copy-number plasmids do not process more than 5 ml of bacterial culture. For low-copy-number plasmids it may be necessary to process larger volumes of bacterial culture (up to 10 ml) to recover a sufficient quantity of DNA.

- Resuspend the pelleted cells in 250 µl of the Resuspension Solution. Transfer the cell suspension to a microcentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
- Add 250 µl of the Lysis Solution and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear.
- Add 350 µl of the Neutralization Solution and mix immediately and thoroughly by inverting the tube 4-6 times.
- Centrifuge for 5 min to pellet cell debris and chromosomal DNA.
- Transfer the supernatant to the supplied GeneJET spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate.
- Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
- Add 500 µl of the Wash Solution to the GeneJET spin column. Centrifuge for 30-60 sec and discard the flow-through. Place the column back into the same collection tube.
- Repeat the wash procedure using 500 µl of the Wash Solution.
- Discard the flow-through and centrifuge for an additional 1 min to remove residual Wash Solution.
- Transfer the GeneJET spin column into a fresh 1.5 ml microcentrifuge tube. Add 50 µl of the Elution Buffer to the center of GeneJET spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 2 min at room temperature and centrifuge for 2 min.
- Discard the column and store the purified plasmid DNA at -20 C.

2.5.4. Preparation of chemically competent cells

Chemically competent cells are calcium chloride treated to facilitate attachment of the plasmid DNA to the competent cell membrane. The competent cell is alternatively heated in a water bath, this opens the pores of the cell membrane allowing entry of the

plasmid. Chemically competent cells are the best solution for general cloning and subcloning application.

Used materials:

- TSS buffer (0.9×) 50 ml: 5 g PEG 8000, 1.5 ml 1 M $MgCl_2$, 2.5 ml DMSO, LB medium, pH 6.5.

Procedure:

- First day: Bacterial cells of *E. coli* BL21-Gold (DE3) were cultured on 10 ml LB medium overnight at 37 °C.
- Second day: the overnight culture was diluted into 25-50 ml of fresh LB medium in a 200 ml flask, and then incubated at 37 °C until the OD_{600} reached 0.2-0.5.
- The culture was split into two 50 ml conical tubes and incubated on ice for 10-30 min.
- The tubes were centrifuged for 5 min at 6000 ×g and 4 °C, the supernatant was discarded.
- The pellet was resuspended in chilled TSS buffer. The volume used of TSS was 10 % of the culture volume.
- 100 µl aliquots were added to chilled 1.5 ml microtubes and stored at – 80 °C.

2.5.5. Chemical transformation

Chemical competence is conferred to *E. coli* by re-suspension in $CaCl_2$ solution at 0 °C. Under these conditions, the Ca^{2+} ion is thought to create pores in the membrane, assist binding of the DNA to the cell membrane and mask the negative charge on the DNA, easing its passage through the hydrophobic cell membrane. The DNA is forced into the cells by applying a short 42 °C heat shock, which results in a thermal current that sweeps the DNA into the cells. Confirmed plasmid construct was used to transform by heat chock *E. coli* BL21-Gold (DE3) cells.

Used materials:

- Competent cells *E. coli* BL21-Gold (DE3) (Novagen, Germany).
- LB/Amp medium.
- SOC medium.

- Water bath at 42 °C.

Procedure:

- Pure plasmid (~10 ng) was added to100 µl of chemical competent cells and incubated on ice for 20 min.
- The mixture was transferred to water bath at 42 °C for 30 sec, and then it was returned back to the ice for 2 min.
- 1 ml of SOC medium was added and incubated for 1hr at 37 °C with shaking.
- The transformed bacteria were platted on a LB-agar-plate with ampicillin.
- The agar plate was incubated overnight at 37 °C.

2.6. Expression and extraction of recombinant protein

Protein expression of recombinant protein P36/LACK was performed by inserting the plasmid construct pRSET–P36 into the protein expression host *E. coli* BL21-Gold (DE3). Expression of the gene of interest from pRSET is controlled by the strong phage T7 promoter. The T7 expression system relies upon T7 RNA polymerase. While T7 RNA Polymerase is not endogenous to bacteria, some strains of *E. coli*, like BL21 (DE3), have been engineered to carry the gene encoding for this RNA Polymerase in a piece of DNA called the DE3 bacteriophage lambda lysogen. The *lac* repressor represses expression of T7 RNA polymerase. Addition of isopropyl β-D thiogalactoside (IPTG), lactose analog, stops the inhibition of *lac* promoter and allows expression of T7 RNA polymerase. Once sufficient T7 RNA polymerase is produced, it binds to the T7 promoter and transcribes the gene of interest (Fig.12).

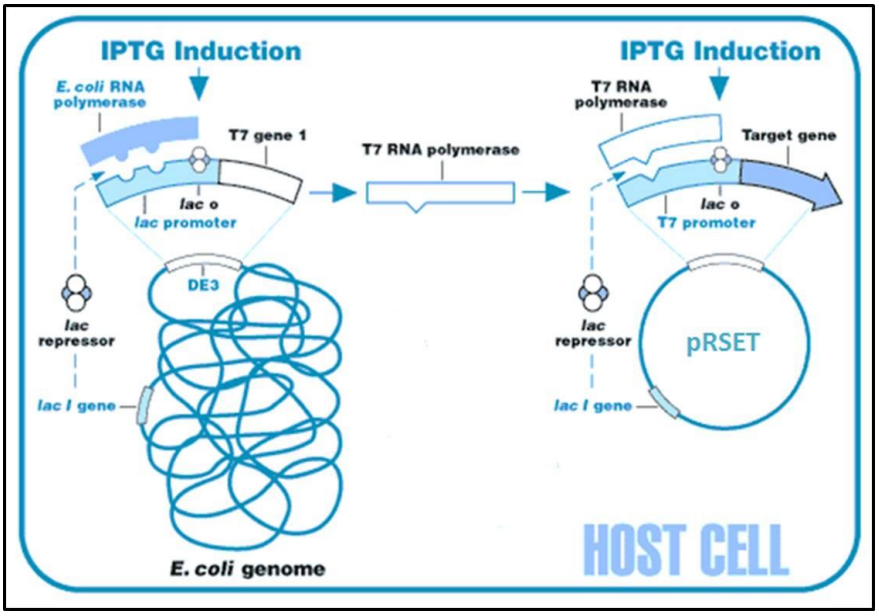


Figure 12. Induction of protein expression of T7 promoter system by IPTG.

(http://www.quora.com/How-does-IPTG-induced-gene-expression-work-at-a-molecular-level)

Used materials:

- LB/Amp medium.
- 1M IPTG (Promega, USA).
- Binding buffer (0.2 M Sodium phosphate buffer, 200 mM Imidazole, and 8 M Urea)

Procedure:

- First day: 10 ml of LB/Amp medium was inoculated with single colony from recently plated transformation and incubated at 250 rpm shaking overnight at 37 °C.
- Second day: 250 ml of LB/Amp medium was inoculated with 2.5 ml starter culture and grew at 37 °C shaker until the OD_{600} reaches ~ 0.5-0.7, then 0.5 mM IPTG was added to the culture and incubated for 16 h at 37 °C shaker.
- Third day: The 250 ml culture were transferred to 50 ml conical tubes, then centrifuged at 12000 ×g for 10 min at 4 °C.
- The supernatant was discarded and the cells pellet was weighed.
- A 5 ml of binding buffer was added to every 1 g of sediment then it was lysed by sonicator (Lab sonic) according to the following program (2 min, pulse 15 sec duty/45 sec repose, amplitude 40 %) on ice.
- The lysate was cleared by 20 min centrifugation at 8,000 rpm and 4 °C.
- Then the supernatant which contains the cytoplasmic proteins was kept at 4 °C.

2.7. Protein purification by affinity chromatography

Using fast protein liquid chromatography (FPLC) AKTA prime plus system (GE Life Science, UK), recombinant P36/LACK was purified from the cytoplasmic extract using a 5 ml column of Nickel charged Nitrilotriacetic acid (NTA) superflow Sepharose (QIAGEN, Germany).

Used materials:

- Phosphate buffer 8× (pH 7.4): 80 mM Na_2HPO_4, 4 M NaCl, 80 mM NaH_2PO_4.
- 2 M Imidazole (pH 7.4).
- Binding buffer: 1× Phosphate buffer, 20 mM Imidazole, 8 M Urea.
- Elution buffer: 1× Phosphate buffer, 500 mM Imidazole, 8 M Urea.
- HiTrap Chelating HP column 5 ml (GE Life Sciences, UK).
- HiPrep™ 26/10 desalting column (GE Life Sciences, UK).
- Protein concentrator tubes Vivaspin6 (cutoff 10 kDa) (VivaScience, Fisher Scientific, UK).

Procedure:

- The device AKTA prime plus (GE Life Sciences, UK) was used for the purification process. The device's tubes were washed by ethanol 20% to get rid of any contaminating proteins then washed by filtered distilled water to get rid of ethanol. After that, the nickel-charged column was attached to the device.
- Protein samples were prepared to be homogenous with binding and elution buffers by adding Phosphate buffer 8× in final concentration 1×, and Imidazole 2 M in a final concentration 20 mM.
- Purification column was washed with 20 ml of binding buffer.
- Purification column was washed with 20 ml of elution buffer.
- Purification column was equilibrated again with 20 ml of binding buffer.
- Protein samples were applied to the column with a flow rate adjusted to 5 ml/min, during this, flow through was collected; which is the unbound protein of the bacterial extracts.
- Purification column was washed with 20 ml of binding buffer.
- The recombinant bound proteins were eluted by passing 20 ml of elution buffer through the column.
- Purified proteins were desalted using buffer exchange column and concentrated using concentrator tubes (cutoff 10 kDa).

- Protein concentration was determined by Bradford protein assay and a stock of 1–5 mg/ml is prepared in 50 % glycerol and stored at -20°C.
- To confirm the success of the purification, pure proteins and flow through proteins were separated through 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.8. Protein assay

The concentration of the purified protein was determined by Bradford method.

Used materials:

- BSA 1 µg/µl.
- 5× dye reagent Bradford (BioRad, USA).
- Whatman #1 filter (BioRad, USA).

Procedure:

- Dye reagent Bradford (1×) was prepared by diluting 1 part concentrated dye reagent with 4 parts ddH_2O. Then it was filtered through Whatman #1 filter to remove particulates.
- Five dilutions of a protein standard were prepared, which are representative of the protein solution to be tested. The linear range of the assay for BSA is 0.2-1 µg/µl.
- 10 µl of each standard and sample solution were added into a clean, dry microplate. Protein solutions are normally assayed in duplicate.
- 200 µl of diluted dye reagent were added to each well and mixed well, then the micoplate was incubated at RT for at least 5 min.
- Absorbance will increase over time thus samples should be incubated at RT for no more than 1 h.
- Absorbance was measured at 595 nm wave length using (Thermo Labsystem/well wash 4 Mk2).

2.9. Protein gel electrophoresis

After protein purification, samples should be resolved by denaturing SDS-PAGE. If stained with a dye such as Coomassie brilliant blue, the intensity of the bands will usually be proportional to the amount of protein. This allows the purity of the sample to be estimated and whether the purified protein is of the expected size.

The purity of the P36/LACK was evaluated in a Coomassie-stained SDS-PAGE (Laemmli, 1970). Protein samples were separated by SDS-PAGE using a Bio-Rad

Mini-Protean Tetra Cell system (Bio-Rad, USA) following the manufacturer's instructions. Gels were prepared using stacking gel 5 % and running gel 12 %. After electrophoresis, the gel was stained in Coomassie brilliant blue buffer (45 % Methanol, 10 % Acetic acid, 0.25 % Coomassie R250) for 2 h and then washed several times in destaining buffer.

Used materials:

- Acrylamide/Bis–Acrylamide 30 % (Roth, Germany).
- 1× tetramethylethylenediamine (TEMED) (Roth, Germany).
- Tris buffer 0.5 M, pH 6.8 (BDH laboratories, England).
- Tris buffer 1.5 M, pH 8.8 (BDH laboratories, England).
- SDS 10 % (BIO BASIC INC., Canada).
- Ammonium persulfate 10 % (APS) (Pharmcia Biotech, Sweden).
- Electrophoresis buffer 1× (for 1L: 3.03 g Tris base and 14.41 g Glycine, 1 g SDS).
- Staining solution 1× (for 400 ml: ~500 mg Coomassie brilliant blue R250 (Sigma, USA), 200 ml glacial acetic acid, 120ml methanol and 80 ml ddH_2O).
- Destaining solution 1× (for 1 L: 300 ml methanol, 100 ml glacial acetic acid and 600 ml ddH2O).
- 5× protein loading buffer (16 mM Tris, 10 % SDS, 25% Glycerol, 0.01 % Bromophenol Blue, 5 % β-Mercaptoethnol, pH 6.8).
- Protein molecular weight ladder (Chromatein Prestained Protein Ladder; Vivantis, Malaysia).

Procedure:

- A 10 ml of 12% running gel was prepared according to the table.11:

Table 11: Running gel composition

Components	Volume
30% Acrylamide/Bis–Acrylamide	5 ml
1.5 M Tris buffer (pH 8.8)	2.5 ml
ddH2O	2.4 ml
10 % SDS	100 µl
10 % APS	50 µl
TEMED 1×	6 µl

- The running gel was poured between the two glass plates until polymerization.
- A layer of diluted SDS (0.1 %) was added to the top of the gel.
- After the gel was completely polymerized, the diluted SDS layer was removed and distilled water was added for washing.
- A 5 ml of 5 % stacking gel was prepared according to the table 12:

Table 12: Stacking gel composition

Components	Volume
30 % Acrylamide/Bis–Acrylamide	850 µl
0.5M Tris buffer (pH 6.8)	1.25 ml
ddH_2O	2.850 ml
10% SDS	50 µl
10% APS	25 µl
TEMED 1×	3 µl

- The stacking gel was poured above the polymerized running gel.
- Appropriate comb was inserted between the glass plates, then gel was allowed to completely polymerize for 15 min.
- After polymerization the comb was pulled out straight upwards and the wells were washed out well by distilled water.
- The gel casting stand was moved to sink and filled with the electrophoresis buffer.
- Samples preparation: 5 µl of 5× loading buffer; which contains β-Mercaptoethnol that contributes in protein denaturation, was added to every 20µl of protein sample in microtubes
- Protein samples were heated in boiled water bath for 5 min, in order to break the disulfide bonds in the proteins.
- A 20 µl of protein sample was loaded in each well and protein marker was also added to the first well of the gel.
- After loading the protein samples in the gel, a voltage of 85 V was applied, when the samples pass through the running gel the voltage was raised to 110 V till the end of the electrophoresis.
- The gel was stained in staining solution for 1 h with stirring.
- The gel was transferred from staining to destaining solution with stirring.

2.10. Immunoblotting of P36/LACK protein

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to specifically detect

its antigen) was introduced by Towbin, *et al.* (Towbin *et al.*, 1979) and is now a routine technique for protein analysis. The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture. Western blotting can give qualitative data about that protein.

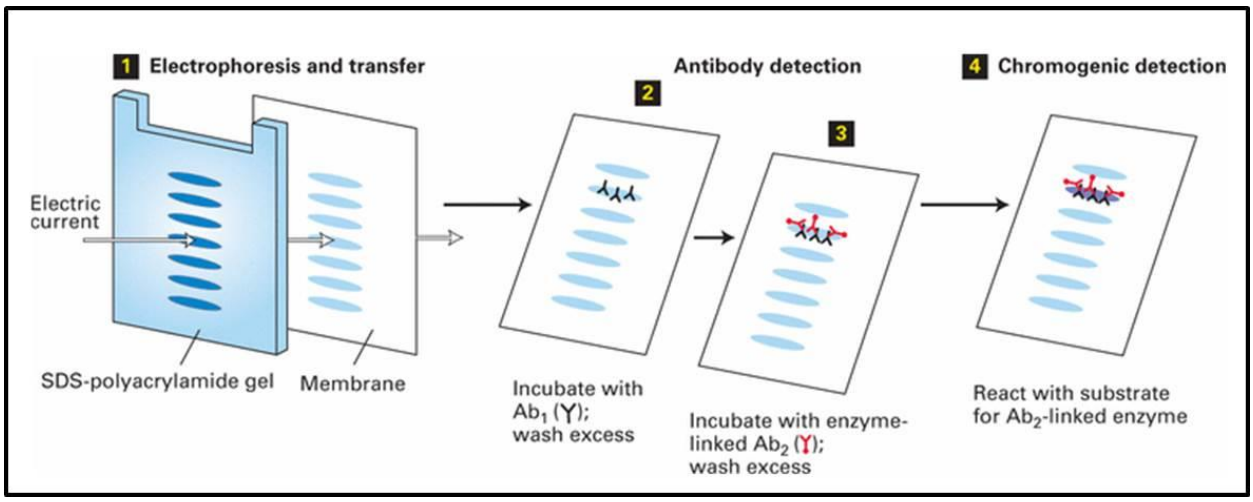


Figure 13. Western blot procedure.
(http://www.western-blot.us/procedure-of-western-blot)

Used materials:

- Transfer buffer (25 mM Tris, 190 mM Glycine, 20 % methanol, pH 8.3).
- Tris-buffered saline with Tween 20 (TBST) buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.1 % Tween 20).
- Blocking buffer (3 % BSA in TBST).
- Substrate buffer (100 mM Tris base, 100 mM NaCl and 5 mM $MgCl_2$, pH 9.5).
- 0.2 μm nitro-cellulose membranes (Whatman, Germany).
- Primary antibodies: 1/5000 dilution of rabbit anti-His antibody (Bethyl Laboratories, USA).
- Secondary antibodies: 1/2000 dilution of goat anti-rabbit antibody conjugated to alkaline phosphatase (AP) (Bethyl Laboratories, USA).
- 1/1000 dilution of serum from CL patients.
- Secondary antibodies: 1/5000 dilution of goat anti-human antibody conjugated to AP (Thermo scientific, Canada).
- Nitro blue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Sigma, USA).
- Distilled water.

Procedure:

Transferring the protein from the gel to the membrane:

- Place the gel in 1 × transfer buffer for 10-15 min.
- Assemble the transfer sandwich and make sure that no air bubbles are trapped in the sandwich. The blot should be on the cathode and the gel on the anode.
- Place the cassette in the transfer tank and place an ice block in the tank.
- Transfer at 100 V and 250 mA for 1.5 h.

Antibody incubation:

- Briefly rinse the blot three washes for 5 min with TBST.
- Block in 3 % BSA in TBST for 1 h at room temperature, or at 4 ^{o}C overnight.
- Rinse the blot three times for 5 min with TBST.
- Incubate in the primary antibody solution for 1-3 h at room temperature. Or alternatively, incubate blots with 1/1000 dilution of serum from CL patients.
- Rinse the blot three times for 5 min with TBST.
- Incubate in the AP conjugated secondary antibody solution for 1 h at room temperature. (Choose the correct secondary antibody according to the primary one).
- Rinse the blot three times for 5 min with TBST.

Detection:

- Apply the NBT/BCIP chromogen substrate in AP buffer to the blot with shaking.
- When bands come into view, stop the reaction by rinsing the blot for 5 min with distilled water.

CHAPTER III.

RESULTS

3. Results

3.1. Typing of local isolates

Typing was accomplished using PCR technique and species-specific primers CSB2/CSB1 (Table 2). PCR products were separated on 0.8 % agarose gel (Fig. 14). PCR result was considered positive when a single band of correct size ~750 bp was observed, which corresponds to *L. tropica* kDNA minicircle (line 2), while the band with 560 bp corresponds to *L. major* (line 1). This result confirmed the identity of the analyzed parasites in comparison with the reference strains.

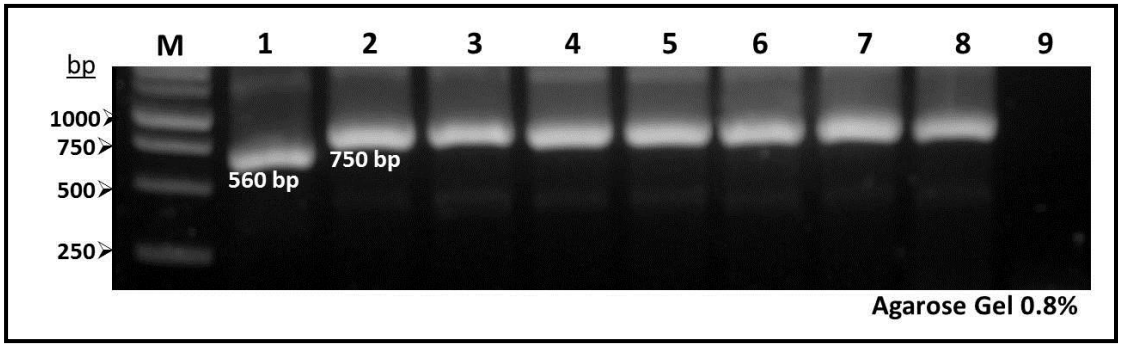


Figure 14. PCR result of parasite typing.
PCR result from tested *Leishmania* isolates and from two reference strains using species-specific kDNA minicircle CSB2/CSB1 primers. Lane M: marker (1kb DNA ladder); Lanes 1and 2 reference strains *L. major* and *L. tropica*, respectively. Lanes 3-8: local isolates of *L. tropica*. Lane 9: negative control.

3.2. Cloning of *P36/LACK* gene into pDrive cloning vector

Full-length *P36/LACK* gene was amplified from *L. tropica* genomic DNA using two specific primers LACK-FL-F/LACK-FL-R (Table 2). The amplified gene is about 1000 bp in length (Fig. 15).

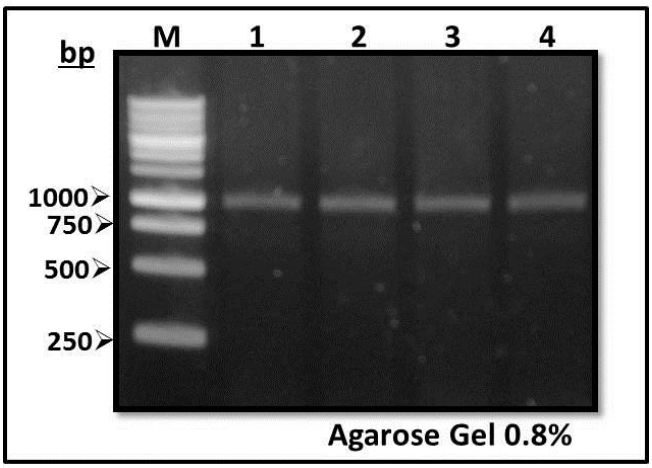


Figure 15. P36/LACK gene amplification.
Amplification of *P36/LACK* gene. PCR reaction was performed on genomic DNA extracted from *L. tropica*, using *P36/LACK* specific primers (lanes 1-4). Lane M: marker (1kb DNA ladder).

The amplified gene was purified and inserted into pDrive cloning vector through ligation reaction (Fig. 16).

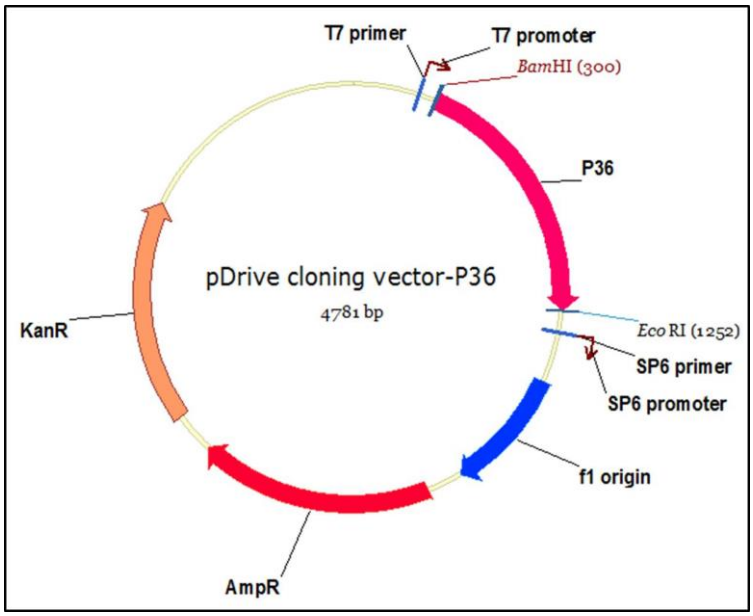


Figure 16. Schematic structure of pDrive cloning vector-P36/LACK construct.
*Designed by Vector NTI program.

pDrive-*P36/LACK* construct was used to transform competent *E. coli* TOP10 cells by electro shock and grown colonies on selective plates, containing ampicillin, were screened by PCR using two plasmid specific primers (FP/RP) (Table 2). Successful cloning in pDrive plasmid was confirmed by colony PCR using a combination between a vector-specific primer and an insert-specific primer. Such reaction

confirms the insert orientation, since cloning using pDrive cloning vector is not a technique that will retain orientation. The positive pDrive-*P36/LACK* construct was used for the subsequent subcloning into pRSET expression vector.

3.3. Sequencing of *P36/LACK* gene

Plasmid constructs from positive colonies were prepared and sent for sequencing. pDrive-*P36/LACK* construct was sequenced using Genetic Analyzer system ABI-310 (Applied Biosystems, USA) at the department of Molecular Biology and Biotechnology at the AECS. Sequencing result was compared with the *P36/LACK* sequence from different *Leishmania* species. Sequence alignment analysis revealed the presence of similar *P36/LACK* sequence form all tested *L. tropica* local isolates, and the resulted sequence (Fig. 17) was sent for registration in NCBI database (GenBank accession number: KM042903).

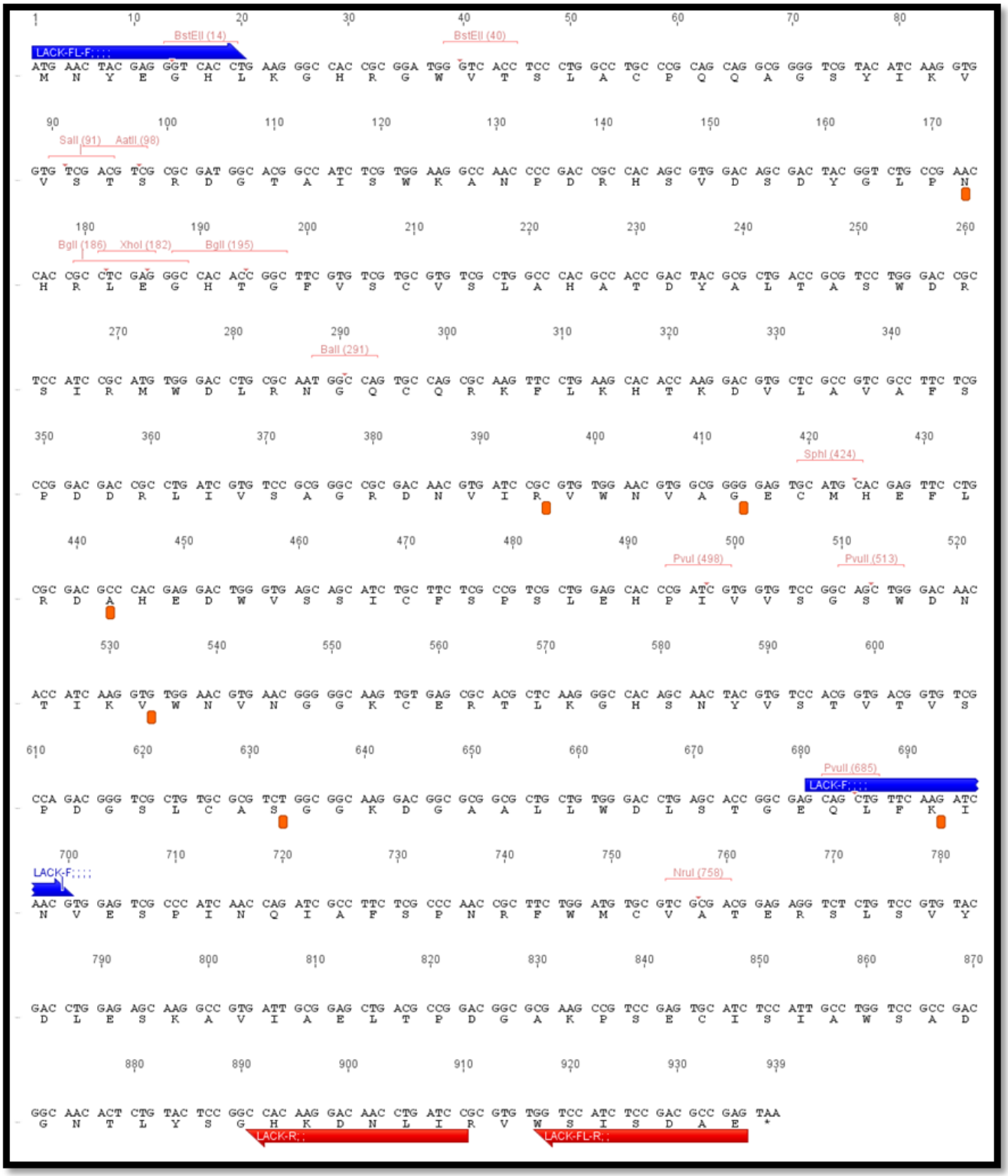


Figure 17. Nucleotide and deduced amino acid sequences of L. tropica P36/LACK gene.
The nucleotide sequence is shown in the 5'-3' direction, numbered from the first base of start codon as the position 1. Deduced amino acid sequences are located below the nucleotide sequence. Positions of *P36/LACK* gene specific primers and of several cutting restriction enzymes from the most commonly used ones are shown. Dotes below the sequence indicate the nucleotides that differ between *L. tropica* and other species.

3.4. Comparison of *P36/LACK* sequences between *Leishmania spp*.

The nucleotide sequence of *L. tropica P36/LACK* from the local isolates (KM042903) was aligned with other reported *P36/LACK* sequences from different *Leishmania spp*., as well as with three previously published *L. tropica P36/LACK* gene sequences (JX305923, JX305924, and KC763809). Sequence alignment of *P36/LACK* genes from these records revealed that the local isolates (KM042903) manifest three *L. tropica* hallmark nucleotide substitutions; an A instead of G (A/G) at position 173 (resulting in an asparagine instead of a serine), a G/C at 414 (glycine) and a T/C at 633 (serine). However, in the fourth position at 396 C/A (Arginine), the *P36/LACK* gene from the local isolates differs from all other *L. tropica* isolates and resembles the other *Leishmania* species *P36/LACK* genes by possessing an A instead of C. Interestingly, the *L. tropica* isolate (KC763809), which found to be very close to our local isolates in the dendrogram of *lack* genes, was found to share with the local isolates a unique substitution; a G/A (valine) at position 534 comparing with other strains including those from *L. tropica*. Another unique substitution for our local isolate is a C/G (alanine/glycine) at position 443, and this change seems to be tolerated by *L. tropica* at this site since an S (G or C) was found in this location of the isolate (KC763809) from the same species. One confusing notice was regarding the G (lysine) at position 693, which characterizes all *Leishmania* species including *L. tropica* local isolates and the isolate (JX305924) but not the isolate (JX305923), which has an A (lysine) substitution. However, it seems that both possibilities are also acceptable for this site in *L. tropica* since an R (G or A) was found in this location in the isolate (KC763809) from the same species (Fig. 17).

The result of the alignment of the *P36/LACK* gene sequences from all records was presented in a dendrogram (Fig. 18). Remarkably, the sequence of lack gene has

almost perfect matching among *L. tropica* isolates. The cluster of similarity in this species could be easily distinguished from two other clusters; the first which is closer in similarity and contains *L. donovani*, *L. chagasi*, and *L. infantum*, and a second distant cluster gathering *L. amazonensis, L. major, L. brazilliensis, and L. mexicana.* However, *P36/LACK* gene seems to be a highly conserved gene in *Leishmania* genius since a relatively great degree of similarity (reaching 98.1 %) was observed between the farthest two sequences in the dendrogram; the local isolates of *L. tropica* and *L. mexicana* (Fig. 18).

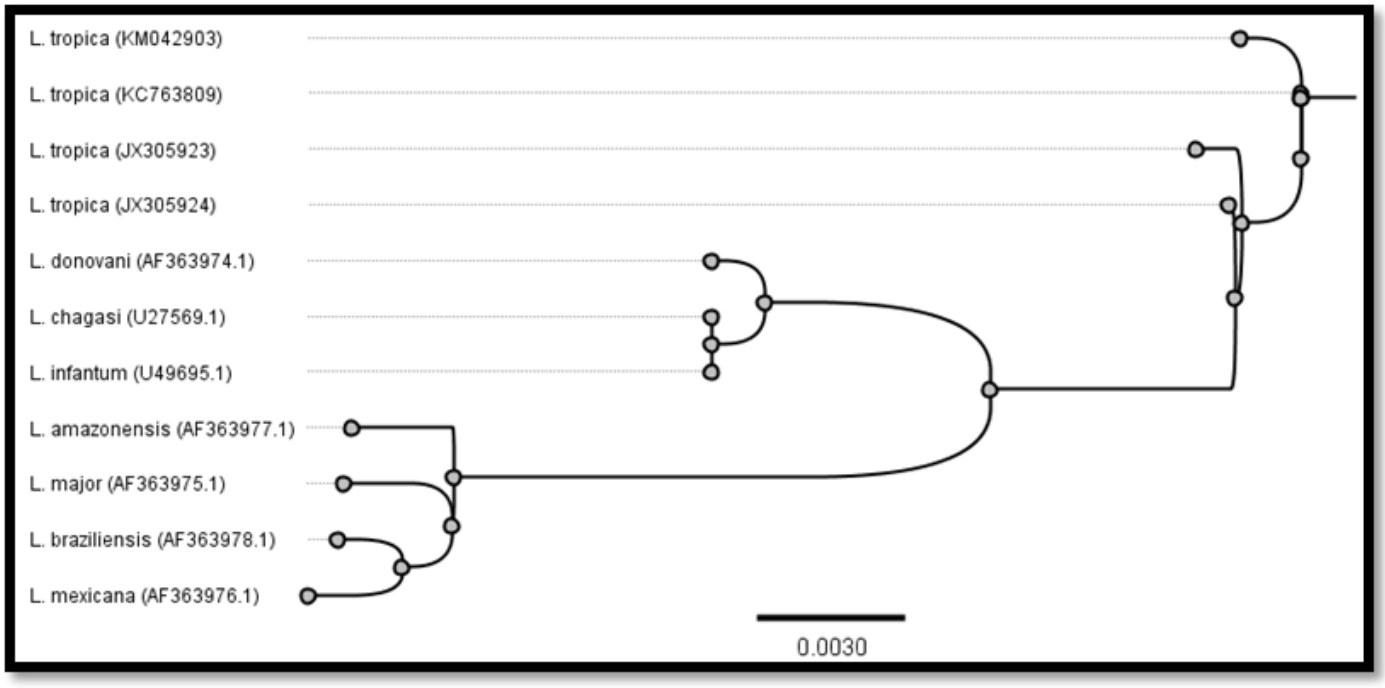


Figure 18. A dendrogram of P36/LACK gene sequences.
A dendrogram of *P36/LACK* gene sequences from different *Leishmania* species that were aligned with the local *L. tropica P36/LACK* gene (KM042903).

3.5. Total RNA Extraction, cDNA Synthesis and RT-PCR

Total RNA was extracted successfully from cultured promastigotes (from stationary and logarithmic phases) and axenic amastigotes. The concentration of RNA was determined by measuring the absorbance of a diluted RNA sample at 260 nm wave length. 100 ng of RNA was used for electrophoresis (0.8 % agarose gel). Furthermore, total RNA electrophoresis illustrated smear of mRNA and three bands

of rRNA, showing an acceptable integrity and purity of the prepared RNA (Fig. 19A, B, and C). Moreover, the purity of the RNA from genomic DNA contaminants was confirmed by PCR reaction, using *P36/LACK* gene specific primers LACK-FL-F/ LACK-FL-R (Table 2) where a negative amplification is expected (Fig. 19D).

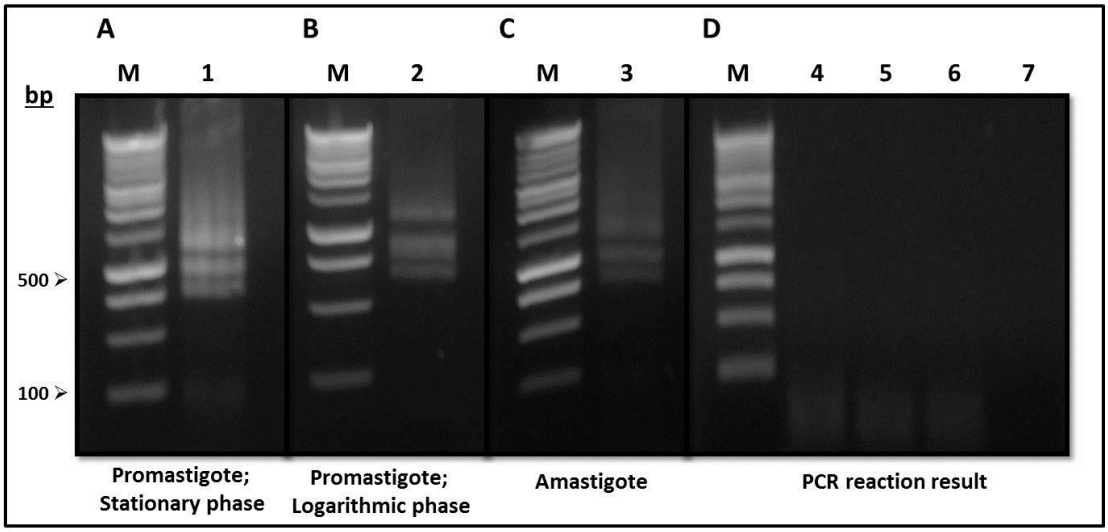


Figure 19. Evaluation of the quality and purity of the extracted total RNA.
Evaluation of the quality and purity of the extracted total RNA. A, B and C: depict smear of mRNA and three bands of rRNA reflecting the excellent extraction of total RNA in promastigotes; stationary and logarithmic phases, and axenic amastigotes (Lanes 1, 2 and 3), respectively. D: Result of PCR reactions confirming the purity of extracted total RNA from genomic DNA contaminants in promastigotes; stationary and logarithmic phases, and axenic amastigotes (Lanes 4, 5 and 6), respectively. Lane 7: negative control for PCR reaction.

Then, 500 ng of RNA was used for cDNA synthesis. Total RNA was reverse transcribed, and the synthesized cDNA was used as template to amplify a small domain from *P36/LACK* full-length gene using LACK-F/LACK-R primers (Table 2). This resulted in a single band of 230 bp in all tested conditions, confirming the presence of *P36/LACK* gene transcripts in both amastigotes and promastigotes; stationary and logarithmic phases. Additionally, the integrity of the cDNA have been checked by amplifying 60s ribosomal protein L12 housekeeping gene which resulted in a single band of 200 bp refers to positive control transcripts (HKG) (Fig. 20).

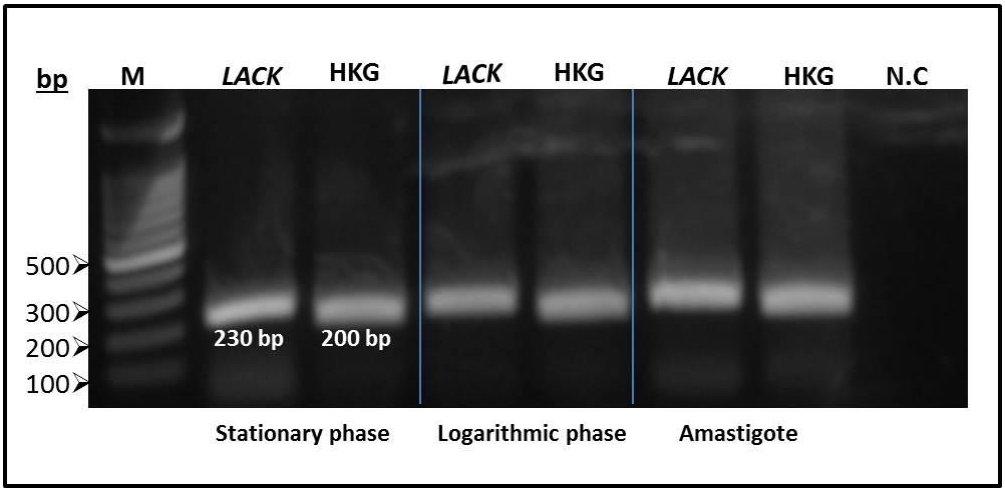


Figure 20. PCR result of synthesized cDNA.
PCR reaction was applied on cDNA of *P36/LACK* gene of promastigotes; stationary and logarithmic phases, and amastigotes, respectively, and cDNA of 60s ribosomal protein L12 gene (a positive control) of promastigotes; stationary and logarithmic phases, and amastigotes, respectively. The PCR product size is approximately 230 bp of the target gene (*LACK*), and 200 bp of the housekeeping gene (HKG). Lane M: marker (BenchTop 100 bp DNA ladder); and lane 7: negative control.

3.6. Subcloning of *P36/LACK* gene into pRSET plasmid

P36/LACK gene was transferred from cloning vector (pDrive) to expression vector (pRSET) by subcloning technique (Fig. 21). The plasmid pRSET is a T7 promoter-dependent system which produces cytoplasmic N-terminal His6-tagged proteins in *E. coli* cells (Fig. 20B). Tagging of recombinant proteins is indispensable for the subsequent steps of purification and detection.

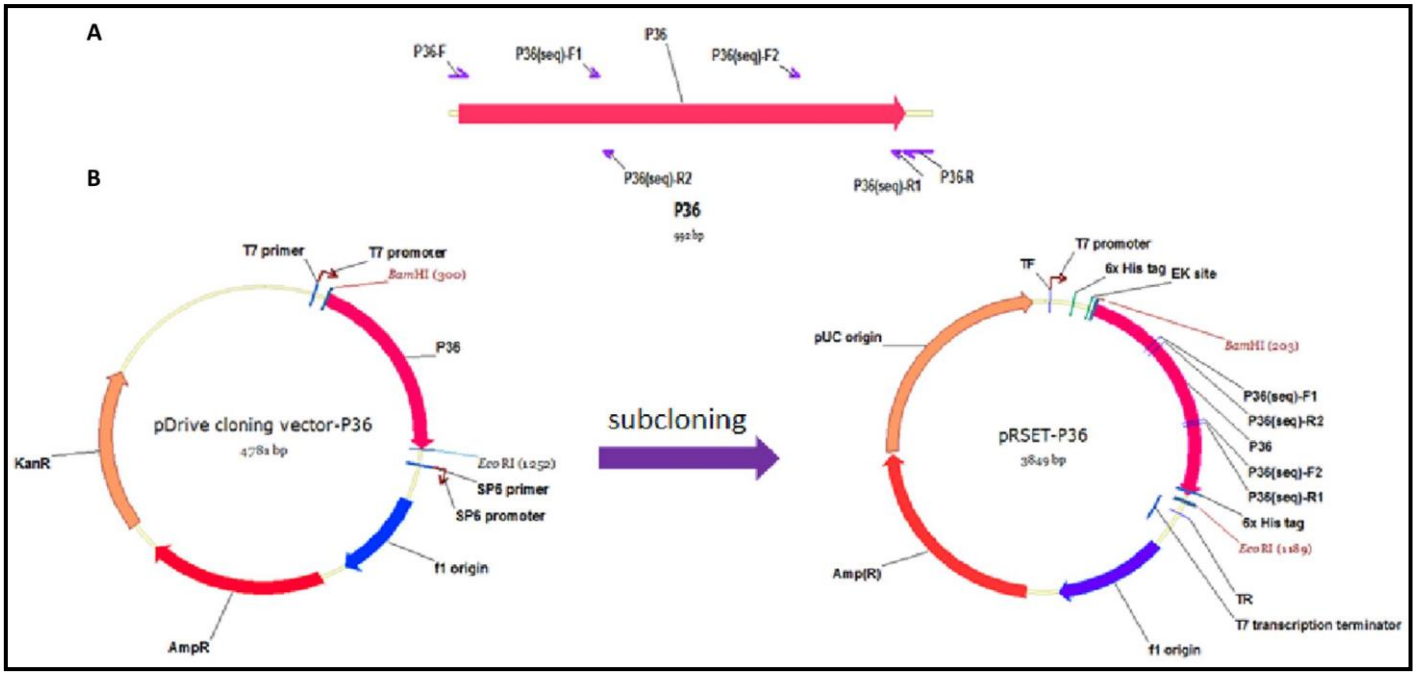


Figure 21. Scheme of the P36/LACK gene and pDrive cloning vector plasmid used in the cloning, and pRSET plasmid used in sub cloning.
(A) *P36/LACK* gene and the positions of different primers used in the cloning and sequencing. (B) Maps of the resulted plasmid constructs (pDrive-*P36/LACK*) for cloning and sequencing and (pRSET–*P36/LACK*) for expression where the position of the inserted P36 gene is indicated. The most important elements of the plasmids are indicated. These include T7 promoter, 6XHis tag downstream the two restriction sites (*Bam*HI and *Eco*RI) used for *P36/LACK* gene ligation. Position of the primers used for PCR positive colonies screening and for sequencing is shown.

*Designed by Vector NTI program.

pDrive-*P36/LACK* construct was digested with *Bam*HI and *Eco*RI restriction enzymes (Fig. 22A). Digestion of this DNA construct resulted in a sticky ended fragment (992 bp) and linearized pDrive (3789 bp). Similar digestion reaction, with the same two restriction enzymes *Bam*HI/*Eco*RI, was performed on pRSET plasmid resulting in a linearized full-length pRSET plasmid (2857 bp) (Fig. 22B). the ready sticky ended fragment (*P36/LACK*) was directly ligated with the linearized pRSET plasmid. Ligated products (pRSET-*P36/LACK*) were used to transform competent *E. coli* TOP10 cells by electric shock and positive colonies on the selective plates were screened by PCR using pRSET-specific forward primer (T_7F) and P36-spicefic sequencing reverse primer (P36(seq)-R2). This approach enabled distinguishing between two types of colonies; empty pRSET-containing colonies and pRSET-

P36/LACK -containing colonies which gave a fragment of 506 bp due to the presence of the insert gene within (Fig. 22C).

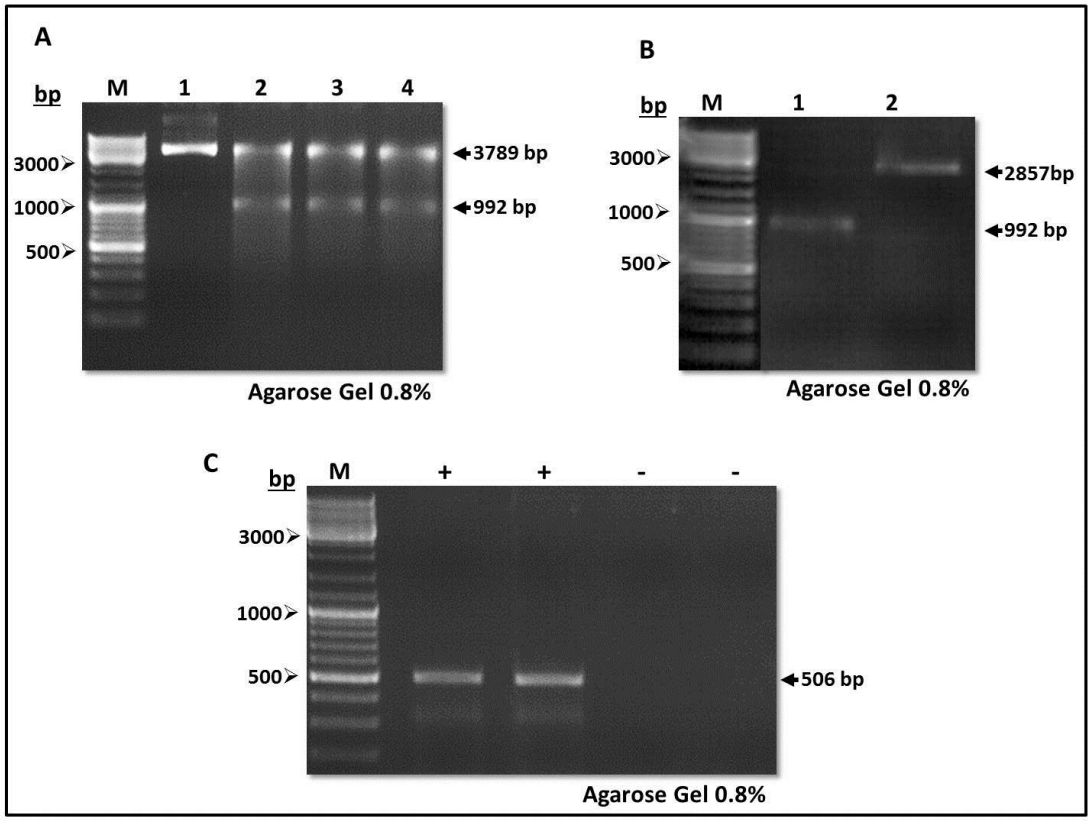


Figure 22. Subcloning of P36/LACK gene.
(A) Digestion of pDrive cloning vector-*P36/LACK* after cloning and sequencing for subcloning; lane 1: the purified construct (pDrive cloning vector-*P36/LACK*) without digestion as a control; lane 2: the construct digested using *Bam*HI; lane 3: the construct digested using *Eco*RI; lane 4: the construct digested using both *Bam*HI and *Eco*RI restriction enzymes. (B) Shows the purity of digested *P36/LACK* gene (lane 1) and digested pRSET plasmid (lane 2) using both *Bam*HI and *Eco*RI restriction enzymes for ligation reaction. (C) Results of colony PCR screening performed on 4 randomly selected clones after *E. coli* TOP10 transformation with the products of the ligation reaction (pRSET-*P36/LACK*). DNA molecular weight marker (M).

3.7. Expression and Purification of P36/LACK protein in *E. coli*

Production of P36/LACK as recombinant protein was obtained after transformation of *E. coli* BL21-Gold (DE3) cells with the confirmed pRSET-*P36/LACK* plasmid construct. Cells were grown in LB medium supplemented with antibiotic and protein expression was then induced by IPTG. The produced recombinant protein was precipitated in inclusion bodies in bacterial cells, and could easily be recovered from bacterial cells by one step of disruption using a sonicator,

followed by centrifugation to eliminate cell debris. Disruption step should be accomplished in a buffer high concentration of suitable detergent (8 M Urea) to recover the recombinant protein from inclusion bodies.

Purification of P36/LACK from cytoplasmic extract was done on immobilized-metal affinity chromatography, after treatment with urea to dissolve the formed inclusion bodies, using Nickel-charged NTA column installed on AKTA prime system. The UV-detector, supplemented with this system, enabled real-time monitoring of the different steps of P36/LACK purification (Fig. 23A and 23B). In both of these purification curves, and after sample injection, two distinctive peaks could be observed. The first high peak indicate the flow-through of bacterial lysate, whereas the next low peak indicate the pure P36/LACK protein. The obvious difference between tow purification curves is the quantity obtained of P36/LACK protein; low quantities the protein was obtained when using PBS (I) in comparison when using PBS with urea (II) (Fig. 23C).

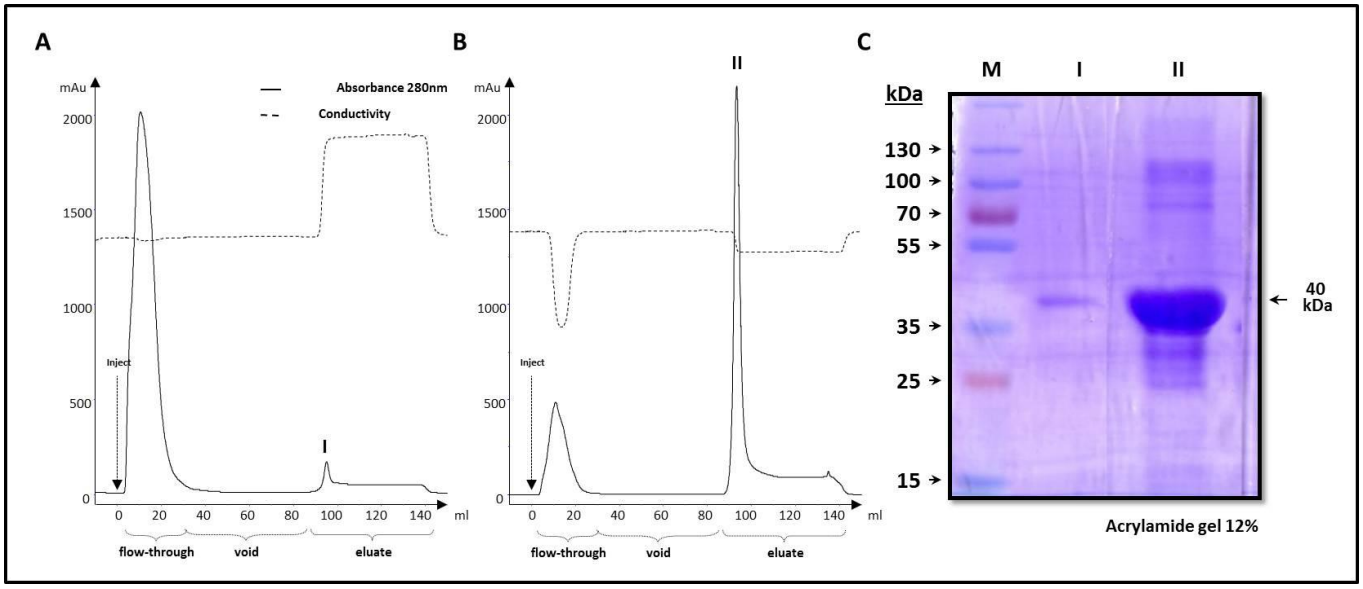


Figure 23. The expression and purification of P36/LACK protein.
(A) and (B) diagram of purification procedure using Ni^{+}-NTA column installed on FPLC AKTA prime system. Continuous line represents the absorbance of the eluate and the dashed line represents the conductivity of the eluate, different purification steps are shown below and peaks of the flow-through sample and of purified P36/LACK are indicated. In diagram A, extraction and purification were accomplished using PBS, while in diagram B extraction and purification were accomplished under denaturing conditions using Urea. (C) SDS-PAGE (acrylamide 12 %) of the purified protein samples, with PBS (lane I) or with PBS+Urea (lane II).

The protein expression and purification procedures of recombinant P36/LACK were followed by SDS-PAGE separation and coomassie brilliant blue staining (Fig. 24A), and immunoblotting for purity assessment (Fig. 24B and 24C). Interestingly, a remarkable expression of P36/LACK protein (40 kDa) could be observed after IPTG induction and incubation for 16 h at 37 °C (Fig. 24A, lane 3). However, a trace of P36/LACK protein could still be seen in flow-through (lane 4) resulting in a high purification yield of the recombinant protein (lane 5). Although, expressed protein was totally purified from bacteria cytoplasmic extract by column purification which yielded 90 % pure P36/LACK. The yield of purified recombinant protein estimably reached 2 mg/ml of bacteria culture.

In immunoblotting, pure P36/LACK protein on 12 % acrylamide gel was transferred to 0.2 µm nitrocellulose membrane, then the membrane was processed in

two different ways. P36/LACK protein detection was accomplished using rabbit anti-His6-AP conjugated antibody (1/2000) or using CL patient's serum (1/1000).

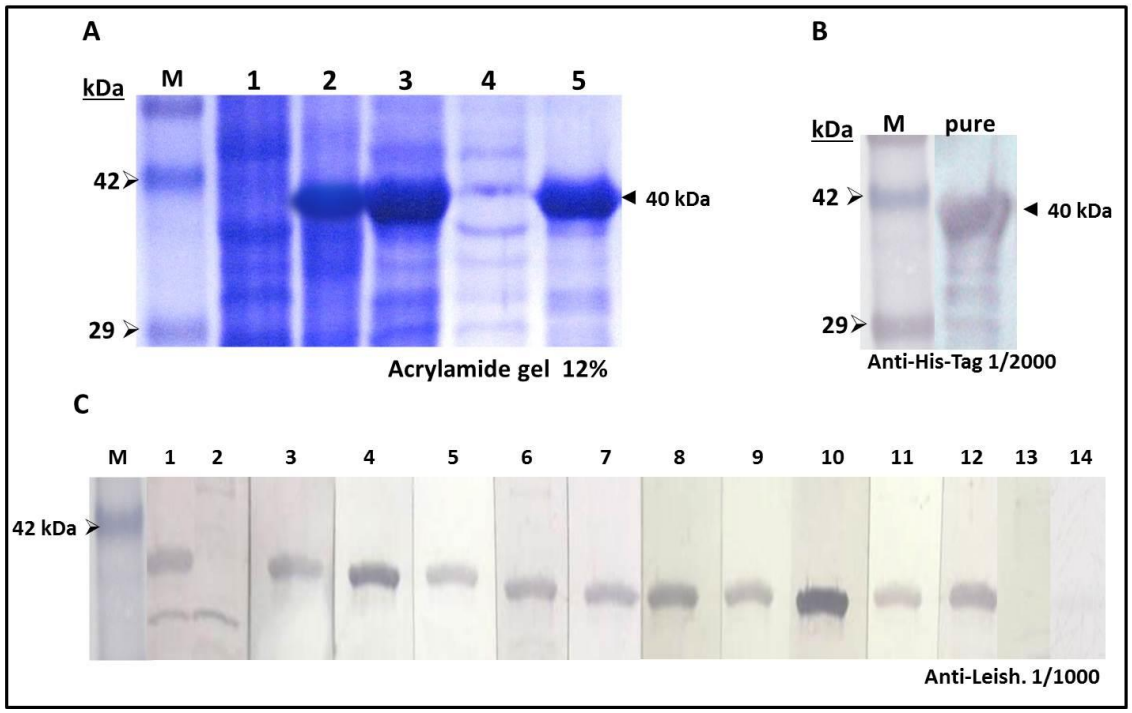


Figure 24. SDS-PAGE analysis and Immunoblotting of P36/LACK protein.

Protein migration in SDS-PAGE (acrylamide 12 %) of protein samples obtained after different steps of purification. Total cytoplasmic extract after 16 h of IPTG induction (lane 3; while lanes 1 and 2 show before and after induction respectively), and purified P36/LACK protein (lane 5). Detection of migrated proteins was done ether by coomassie brilliant blue staining (A) or by WB detection using rabbit anti-His antibody (1/2000) (B) or using positive serum from CL patients (1/1000) (C) (lanes 1, and 3-12 different positive serums; lanes 2, and 13 GFP protein as a control with positive serum and negative serum respectively; and lane 14 purified P36/LACK with negative serum). The location of P36/LACK in the gel is indicated and defined as 40 kDa compared to the protein molecular weight ladder in the first lane (M).

Anti-His antibodies can specifically detect and join with recombinant P36/LACK protein fused with six polyhistidine tag. This specific connection give qualitative information about the production and purification of target protein. Obtaining one detected band with the expected weight (40 kDa) confirms the protein purity and quality, and gives an idea about its quantity (Fig. 24B). Furthermore, CL patient's serum was used in P36/LACK protein detection to verify and confirm the quality of P36/LACK protein, which should be detected by anti-leishmania antibodies in this serum (Fig. 24C). 11 CL patient's sera were used in protein detection as positive sera. And as expected, it was resulted in one band with 40 kDa (lanes 1 and 3-12). On the

other hand, no bands were appeared after processing with serum from non-infected human (lane 14). Moreover, to verify the specific detection of P36/LACK protein by anti-leishmania antibodies, the recombinant green fluorescent protein (GFP) was used in immunoblotting and processed with positive and negative sera. In result, no bands with the expected weight (~24 kDa) of GFP protein were appeared after processing with positive serum (lane 2) and negative serum (lane 13). These results confirm the successful production and purification of P36/LACK protein with an acceptable amounts and correct protein configuration. Thus, recombinant P36/LACK protein could be a useful tool for further procedures such as CL diagnosis and CL vaccines development.

CHAPTER IV.

DISCUSSION

AND

CONCLUSION

4. Discussion

Leishmaniasis remains a public health problem worldwide. In spite of its huge health impact on the populations in vast areas, leishmaniasis is one of the most neglected diseases. No safe and effective vaccine currently exists against any form of human leishmaniasis. The spectrum and efficacy of available antileishmanial drugs are also limited (Kobets *et al.*, 2012). Current control is based on chemotherapeutic treatments which are expensive, toxic and associated with high relapse and resistance rates. Vaccination remains the best hope for control of all forms of the disease, and the development of a safe, effective and affordable antileishmanial vaccine is a critical global public-health priority (Mutiso *et al.*, 2013). The development of effective and affordable vaccine against leishmaniasis is highly desirable. Although considerable progress has been made over the last decade in understanding immune mechanisms underlying potential candidate antigens, including killed, live attenuated parasites, crude parasites, pure or recombinant *Leishmania* proteins or DNA encoding leishmanial proteins, as well as immunomodulators from sand fly saliva, very few candidate vaccines have progressed beyond the experimental stage. As such there is no vaccine against any form of human leishmaniasis. In recent years, however, much interest has been stimulated towards vaccination against leishmaniasis focused mainly on cutaneous leishmaniasis with fewer attempts against visceral leishmaniasis (Nagill and Kaur, 2011).

Although a great deal of knowledge has been gained from studies on the immunobiology of leishmaniasis, there is still no universally acceptable, safe, and effective vaccine against the disease (Okwor *et al.*, 2012). As a second generation vaccines against leishmaniasis several antigens have been identified and characterized that might be potential vaccine candidates (Coler and Reed, 2005). According to

previous studies, P36/LACK protein is one of these candidates (Khamesipour *et al.*, 2006; Nagill and Kaur, 2011; Kobets *et al.*, 2012).

In this study, we showed by the molecular typing with PCR technique that our *Leishmania* local isolates are belonging to *L. tropica*. Such method is widely used for distinguishing the different species of *Leishmania*, simply by amplifying highly conserved sequence blocks of kDNA minicircles (Ray, 1989; Yurchenko *et al.*, 1999). Recently, PCR is used for visceral and cutaneous leishmaniasis diagnosis (Gomes *et al.*, 2007). In compared studies of PCR assays, PCR based on kDNA was the most sensitive diagnostic assay for CL and recommended for routine diagnosis (Bensoussan *et al.*, 2006). Efforts to design new diagnostic methods for leishmaniasis have centered on molecular biology techniques. The one technique that appears to show the most promise for distinguishing past from current infection is the PCR for kDNA (Grevelink and Lerner, 1996). Species-specific primers for almost all *Leishmania* parasites have been developed. The method is extremely sensitive and when combined with the monoclonal antibody testing appears able to differentiate between past infection (antibody positive/PCR negative) and current infection (antibody positive/PCR positive) (de Bruijn and Barker, 1992; Smyth *et al.*, 1992).

The genomic DNA of one isolate belonging to *L. tropica* was extracted and *P36/LACK* gene was amplified, cloned and sequenced. *L tropica P36/LACK* nucleotide sequences from the local isolate was identical and manifest a great similarity with *L. tropica P36/LACK* gene from published records. This provides further confirmation of the identity of our local isolates based on the analysis of *P36/LACK* gene. Furthermore, *P36/LACK* gene from local isolate showed significant homology to *P36/LACK* sequence from several other species including *L. donovani, L. major, L. amazonensis, L. brazilliensis, L. chagasi, L. infantum and L. mexicana*,

implying that P36/LACK protein may have an important functions for the parasite life cycle. This high similarity of *P36/LACK* nucleotide sequences may refer to identical amino acid sequence among the different *Leishmania spp.* Melby *et al* (2001) had shown that *L. donovani* P36/LACK deduced amino acid sequence was identical to the published *L. chagasi* and *L. infantum* sequences (GenBank accession numbers U27569 and U49695, respectively) and differed at only 1 or 2 amino acids from the *L. major, L. mexicana, L. amazonensis,* and *L. braziliensis* sequences they obtained (Melby *et al.*, 2001). In this work, P36/LACK from our local strains was found to differ in two amino acids from other *Leishmania spp.* because of nucleotides substitution at position 173 and 443.

We demonstrated that *P36/LACK* gene was expressed in logarithmic and stationary phases present procyclic and metacyclic promastigotes, as well as in amastigotes in *L. tropica*. P36/LACK protein is believed to be expressed by all *Leishmania spp.* in both promastigote and amastigote stages (Courret *et al.*, 1999; Kubar and Fragaki, 2006). Recently, Hajjaran *et al* (2012) study approved that LACK protein is one of the over expressed proteins in *L. tropica* promastigotes through performing a comparative proteomics analysis on two different strains of *L. tropica* (Hajjaran *et al.*, 2012). In this study, the expression of *P36/LACK* gene was established at mRNA level in different stages of *L. tropica* parasite life cycle. But it will obviously be essential to validate these findings at protein level when the antibody is available, as post-transcriptional mechanisms have been shown to regulate protein levels in *Leishmania* (Kramer, 2012). The continuous expression and production of P36/LACK protein reflects its important role in various stages of the parasite life cycle, and his major role was confirmed by several studies; P36/LACK is required for vertebrate parasitization, plasminogen activation, and can function as an

aggravating factor in animal host causing early phase infection. In addition, P36/LACK can induce both proliferative and cytokine responses in peripheral blood mononuclear cells (Maasho *et al.*, 2000; Okuno *et al.*, 2002; Kelly *et al.*, 2003; Gomez-Arreaza *et al.*, 2011). To date, there have been numerous attempts at developing a successful vaccine against leishmaniasis (Dunning, 2009). The protective role of P36/LACK as a vaccine in humans is not straightforward (Maasho *et al.*, 2000). On the other hand, P36/LACK administered with IL-12 is able to direct the T cells toward a Th1 response in mice (Mougneau *et al.*, 1995). However, driving of the immunodominant P36/LACK response toward a Th1 phenotype could be the key for preventing the ill effects of Th2 responses (Maasho *et al.*, 2000).

Immunization with recombinant antigens or plasmid DNA encoding *Leishmania* antigens represents a promising approach to vaccinate against leishmaniasis. This DNA vaccines can induce both humoral and cell-mediated immune responses and results in long lasting immunity (Gurunathan *et al.*, 1997). Both models, P36/LACK DNA and recombinant P36/LACK protein could induce protection in mice that were immunized and challenged with *L. major* promastigotes (Gurunathan *et al.*, 1997). However, DNA vaccines have shown great promise in animal models, but have not yet proven their utility in humans. There have been no clinical trials beyond phase-II to test DNA vaccines in humans. Thus, a major challenge for DNA vaccine candidates, remains the demonstration of safety and efficacy in humans in both clinical trial and field settings.

These results which demonstrated a successful application of P36/LACK DNA/Protein vaccine in the infectious disease models open up an avenue for the development of vaccines against Old World cutaneous leishmaniasis caused by *L. tropica*. In the current work, and according to the previous reports, a high-level

expression system of His6-tagged *L. tropica* P36/LACK was established in *E. coli*. After *P36/LACK* gene amplification, cloning and sequencing, the genomic DNA was used as a template in PCR reaction, since *P36/LACK* gene does not contain introns (*L. major P36*; GeneBank accession number AF363975.1). Cloning was performed using pDrive cloning vector which provides superior performance through the direct ligation of the blunt-ended PCR products, allowing sequence analysis using standard sequencing primers. Following sequence confirmation, DNA insert was subcloned into pRSET plasmid using specific restriction enzymes. The pRSET vector is a pUC-derived expression vector designed for high-level protein expression and purification from cloned genes in *E. coli*. The presence of the T7 promoter provides high level expression of the down-stream cloned gene (Studier and Moffatt, 1986; Studier *et al.*, 1990). In addition, DNA inserts are positioned downstream and in frame with a sequence that encodes an N-terminal polyhistidine tag that functions as a metal binding domain in the translated protein (Schoepfer, 1993). High level of recombinant P36/LACK protein expression was obtained in *E. coli* BL21-Gold (DE3). However, the product precipitated in cytoplasmic inclusion bodies after induction at 37 °C . Protein aggregation is a major bottleneck during the bacterial production of recombinant proteins. In general, the induction of gene expression at sub-optimal growth temperatures improves the solubility of aggregation-prone polypeptides and minimizes inclusion bodies formation (Vera *et al.*, 2007). Choosing appropriate conditions for cell growth and induction can influence the formation of these insoluble structures (Strandberg and Enfors, 1991), especially, temperature which affects directly proteins stability and confirmation (Privalov, 1979). Increased temperature has been found to stimulate aggregation in several cases (Chalmers *et al.*, 1990). Low temperature affects the quality of the recombinant protein, especially

within the insoluble cell fraction. The fraction of aggregated protein (i.e. IB) was largely decreased at low temperatures (below 37 ^{o}C), and the conformational quality was improved (Vera *et al.*, 2007). Traditionally, very high concentrations of denaturing buffers (reaching 9 M) are usually used to solubilize proteins in the inclusion bodies. Urea is one of classic denaturants used for inclusion bodies dissolving and refolding aggregated proteins (Rudolph and Lilie, 1996; Dunbar *et al.*, 1997; Falconer *et al.*, 1998). On the other hand, polyhistidine-tagged fusion proteins that form IBs can be extracted with urea and purified directly by nickel-chelate affinity chromatography. Removal of contaminating proteins and refolding by exchange to non-denaturing buffer conditions can then be performed directly on the purification column just before the elution step (Colangeli *et al.*, 1998; Palmer and Wingfield, 2004). To overcome IBs formation in future, a new system which combines improved expression, solubility screening and purification efficiency can be used. The system is based on newly constructed vector, which generates a protein with an N-terminal His tagged green fluorescent protein (GFP) fusion to allow rapid quantitation of soluble protein (Liu and Naismith, 2009). Previous studies reported the use of GFP as an excellent expression tag for fusion proteins, which can improve their expression while preserving their function and native-like structure. Fusion protein method allows the purification and the detection of a protein of interest even when no specific antibody is available (Liu and Naismith, 2009; Al-Homsi *et al.*, 2012).

Previous published works described production of recombinant P36/LACK protein from other different *Leishmania spp.* for application as an experimental vaccine with or without adjuvant against VL or CL caused by different *Leishmania spp.* such as *L. amazonensis* (Coelho *et al.*, 2003), *L. mexicana* (Gomez-Arreaza *et al.*, 2011), *L. major* and *L. infantum* (Khamesipour *et al.*, 2006). Another study

determined the ability of LACK DNA to induce protection after challenging with *L. major* promastigotes. The induced protection was similar to that achieved by P36/LACK protein and rIL-12, but superior to P36/LACK protein without rIL-12 (Gurunathan *et al.*, 1997).

Moreover, the recombinant P36/LACK protein presents a new applicable approach for CL serological diagnosis since this protein gave a positive result in immunoblotting using many different serums from CL patients. As well, recombinant P36/LACK may be able to detect CL disease which is caused by different *Leishmania* species since P36/LACK is a conserved antigen between different *Leishmania* species. But this point still needs more investigations. Additionally, recombinant P36/LACK presents a tool for developing one of CL candidates vaccine derived from *L. tropica*, and more steps can be achieved for production and testing of P36/LACK as a recombinant protein and/or DNA vaccine.

In summary, *L. tropica P36/LACK* gene sequence was determined and compared with many other *Leishmania ssp. P36/LACK* gene expression was checked in different stages of the parasite life cycle. Finally, a new approach was developed for *L. tropica* P36/LACK production as a recombinant protein. Recombinant P36/LACK may be useful as a diagnostic tool since good positive results were obtained with western blot using CL patient's serum. In addition, recombinant P36/LACK may be useful in the development of an efficient vaccine against Old World cutaneous leishmaniasis.

Conclusion

Our study confers a base for producing the recombinant LACK protein since it is of particular interest as a vaccine candidate against CL. It focuses on:

- Determining *P36/LACK* gene sequence in *L. tropica* (local isolates), and registration the resulted sequence in database.
- Comparing its sequence with those reported in data base for other *Leishmania spp.*
- Verifying of *P36/LACK* gene expression at mRNA level in two different phases of the parasite life cycle; logarithmic and stationary phase of promastigotes, as well amastigotes.
- Development of a new approach for *L. tropica* LACK production as a recombinant protein, which may be useful as a diagnostic tool. As well, recombinant LACK may be useful in the development of an efficient vaccine against Old World cutaneous leishmaniasis.

Perspectives

It would be of great importance in the future to pursuit this interesting project through conducting investigations in the following domains:

- Studying the expression of *P36/LACK* gene in *L. tropica* in real time using quantitative Real-time PCR (qRT-PCR).
- Validate *P36/LACK* gene expression at protein level when the antibody is available, as post-transcriptional mechanisms have been shown to regulate protein levels in *Leishmania*.
- Using a new system for recombinant P36/LACK protein production based on newly constructed vector (pRSET-GFP) to allow rapid quantitation of soluble protein and avoid protein precipitation in inclusion bodies.
- Testing the produced protein as a diagnostic tool for cutaneous leishmaniasis.
- Testing the produced protein as a vaccine against cutaneous leishmaniasis.

CHAPTER V.

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Original Article

Sequencing and Gene Expression Analysis of *Leishmania tropica LACK* Gene

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Abstract

Background: *Leishmania* Homologue of receptors for Activated C Kinase (LACK) antigen is a 36-kDa protein, which provokes a very early immune response against *Leishmania* infection. There are several reports on the expression of *LACK* through different life-cycle stages of genus *Leishmania*, but only a few of them have focused on *L.tropica*.

Methods: The present study provides details of the cloning, DNA sequencing and gene expression of *LACK* in this parasite species. First, several local isolates of *Leishmania* parasites were typed in our laboratory using PCR technique to verify of *Leishmania* parasite species. After that, *LACK* gene was amplified and cloned into a vector for sequencing. Finally, the expression of this molecule in logarithmic and stationary growth phase promastigotes, as well as in amastigotes, was evaluated by Reverse Transcription-PCR (RT-PCR) technique.

Results: The typing result confirmed that all our local isolates belong to *L.tropica*. *LACK* gene sequence was determined and high similarity was observed with the sequences of other *Leishmania* species. Furthermore, the expression of *LACK* gene in both promastigotes and amastigotes forms was confirmed.

Conclusion: Overall, the data set the stage for future studies of the properties and immune role of *LACK* gene products.

Introduction

Leishmaniasis, a vector-borne disease caused by obligate intra-macrophage protozoa, which is endemic in large areas of the tropics, subtropics and the Mediterranean basin, and has been identified as a category 1 disease by the World Health Organization (WHO) (1). *Leishmania* spp. cause a wide variety of diseases that range in severity from self-healing cutaneous leishm-aniasis (CL) to fatal visceral leishmaniasis (2). It is caused by more than 20 leishmanial species and is transmitted to humans by ~30 different species of phlebotomine sandflies (3, 4). CL is caused by many species of *Leishmania*; *L.major*, *L.aethiopica* and *L.tropica*, in the Old World (5). Nearly 90% of CL cases occur in Afghanistan, Algeria, Brazil, the Islamic Republic of Iran, Peru, Saudi Arabia and Sudan in addition to Syria (1). Two distinct developmental stages of *Leishmania* are recognized. Promastigotes are found within the sandfly. Promastigotes can be further classified as procyclic promastigotes, which multiply in the gut of the sandfly, or as the infective metacyclic promastigotes (Infective stage), found in the mouthparts and anterior gut and do not divide. These differentiate into round or oval amastigotes (Diagnostic stage), which *LACK* flagella, once in the host (6).

Leishmania homologue of receptors for activated C kinase (LACK) antigen is a 36-kDa protein expressed in promastigotes and amastigotes forms of different *Leishmania* species (7), which is a member of the family of WD40 repeat proteins. These macromolecules are evolutionarily conserved tryptophan-aspartate motif proteins and have diverse but critical functions in eukaryotes, including signal transduction, RNA processing, and cell cycle control (8). *LACK* is essential for the parasite viability and the parasite establishment in the host. In addition to its cytoplasmic localization, *LACK* was recently found to also be actively secreted, and this secretion occurring via exosomes (9). The function of *LACK* in *Leishmania* is not clear, although the immunological response to this molecule has been well studied and used for experimental vaccine studies in the mouse model (9). Since the response to *LACK* is a very early event in a *Leishmania* infection, it could also include innate elements of immunity (9). *LACK* antigen has been used as a tool to investigate various immunity-related mechanisms. It has been tested in several immunization experiments, providing heterogeneous results. The immunization of mice with *L.donovani LACK* protein protects against cutaneous *L.major* challenged (7, 10, 11). In human patients, as in mice, response to *LACK* depends strongly on the infecting *Leishmania* species (12).

In consideration of CL distribution in Syria, which is mostly caused by *L.tropica*, and the prominent protective role of *LACK* protein as a vaccine candidate against leishmaniasis, we aimed to determine the sequence of *LACK* gene of local isolate of *L.tropica* and to study the expression of *LACK* gene in both promastigotes and amastigotes forms.

Materials and Methods

Parasites Sampling

Samples were collected from patients visiting the Dermatology Hospital in Damascus, with lesions suspected to CL. Positive samples were collected directly from skin scars of the patients on slides. In addition, aspirated samples were taken from lesions for culturing in a semi-solid culture medium (13).

Microscopic examination

Smears on glass slides, were air dried, fixed with methanol for a few seconds and stained with Giemsa. Then, the stained smears were examined using light microscope 100×(Olympus, Japan)(3, 14).

In vitro cultivation of Leishmania

Cultures were obtained by needle aspiration from the border of the lesions. The aspirated fluid was discharged into the culture tubes contain a semisolid medium; agar and RPMI-1640 (Sigma, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS),100U/ml L-Glutamine and 100U/ml penicillin-streptomycin (Cytogen, Germany). The cultures were incubated at 26°C. After a few days, one drop of media was examined using inverted microscope 40× (Optika, Italy). Once promastigotes were formed, the cultures were transferred to RPMI-1640 supplemented with FBS, L-Glutamine and penicillin-streptomycin as described above. To obtain axenic amastigotes, promastigotes in stationary phase in 25cm^2 ventilated flask were incubated at 37°C and pH 5.8 with 5% CO_2. for about 48hour (15-18).

DNA extraction

DNA was extracted from 5ml of *Leishmania* in vitro culture (~16×10^{6}cell/ml). Promastigotes were harvested and washed with (1×) phosphate-buffered saline (PBS) pH7.4, pelleted by centrifugation at 3000rpm for 15minutes at room temperature. Then, genomic DNA was isolated using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions.

PCR amplification

Polymerase Chain Reaction (PCR) was performed using CSB2 5'-CGAGTAGCAGAAAC TCCCGTTCA-3' and CSB1 5'-ATTTTTCGCG ATTTT CGCAGAACG-3' primers for species determination, which are specific to the conserved region of *Leishmania* minicircle kinetoplast DNA (kDNA)(19, 20). Leishmanial DNA isolated from reference strains *L.major* (MHOM/SY/91/LEM2397) and *L.tropica* (MHOM/SY/90/LEM2066) was used as a positive control (Reference strains are a gift from French National Reference Centre for-*Leishmania*(Montpellier, France). The total volume of PCR reaction is 30µl including (1×) Go Taq Buffer, dNTPmix (~0.2mM each dNTP), GoTaq DNA polymerase (1u) (Promega, UAS), and DNA template 0.25µg. DNA amplification was performed in the PeQlab thermocycler (PEQLAP, Germany) with 45 cycles, using an initial heating 94°C for 2min. Each cycle was divided into three stages: denaturation (94°C–30sec), annealing (54°C–1min), and elongation (72°C–1min). After the reaction, the material was kept at 72°C for 10min. PCR products were separated on 1% agarose gel. Fragment sizes were determined with bands of a DNA length standard (1kb DNA Ladder, Promega, USA). The length of the amplified PCR products is about 750bp.

Cloning and sequencing of LACK gene

LACK gene was amplified with the use of GoTaq DNA polymerase and LACK-FL-F 5'-ATGAACTACGAGGGTCACCT-3' and LACK-FL-R 5'-CTCGGCGTCGGAGATGGACC-3' specific primers. Primers were designed according to *LACK* gene sequence of *L.major* (GenBank accession number AF363975.1). PCR products were purified using Invisorb Fragment CleanUp kit (Stratec molecular, Germany). Then, the purified fragments were cloned into the open plasmid pDrive cloning vector (QIAGEN, Germany), and sequenced by Genetic Analyzer system ABI-310 using universal specific primers for the plasmid; FP (CGCCAGGGTTTTCCCAGTCACGAC) and RP (TCACACAGGAAACAGCTATGAC) (Applied Biosystems, USA).

Comparison of LACK sequences between *Leishmania* spp.

The nucleotide sequences of *LACK* gene in a local isolate of *L.tropica* (GenBank accession number: KM042903) was compared with reported *LACK* sequences in *L.donovani, L.major, L.amazonensis, L.braziliensis, L.chagasi, L. infantum and, L. mexicana* and from three recently reported *L.tropica* Iranian isolates (GenBank accession numbers: AF363974.1, AF363975.1,

AF363977.1, AF363978.1, U27569.1, U49695.1, AF363976.1, JX305923, JX305924, and KC763809, respectively). Sequence alignment and dendrogram prediction were performed using Geneious v4.8 software, available from www.geneious.com.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from 5ml of *Leishmania* promastigotes; stationary phase (~16×10^6cell/ml) and logarithmic phase (~12×10^6cell/ml), as well as from axenic amastigotes (~16×10^6cell/ml). The extraction was carried out using SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. Total RNA concentration was measured at 260nm wavelength using spectrophotometer (Jenway, England), and 500ng was loaded into 1% agarose gel electrophoresis to inspect its integrity and quality after extraction. Furthermore, 100ng of total RNA was applied as a negative template in a PCR reaction using *LACK* gene specific primers; LACK-F 5'-GCAGCTGTTCAAGATCAACG-3' and LACK-R 5'- GGATCAGGTTGTCCTTGTGG-3'. Then, PCR products were examined on 1% agarose gel to assure the purity of our RNA of any genomic DNA contaminants. Hereinafter, total RNA (0.5µg) was reverse transcribed into single-stranded cDNA using First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions, and the resulted cDNA (60ng) was used as PCR template.

In PCR reaction, two couples of specific primers were used. LACK-F/R primers, which amplify 230bp of *LACK* gene, and Lmr60s-F 5'-ATGCCGCCGAAGTTCGACCC-3' and Lmr60s-R 5'-CGTCGCCACACGGTTCTTGA-3' primers, which amplify 200bp of the encoding gene for 60s ribosomal protein L12 (GenBank accession number: XM_001683687) as a positive control for cDNA synthesis. Three independent experiments were undertaken for the selected genes in all described samples.

Results

Typing of local isolates

Typing was accomplished using PCR technique and species-specific primers CSB2/CSB1. PCR result was considered positive when a single band of correct size ~750bp was observed, which corresponds to *L.tropica* kDNA minicircle, while the band with 560bp corresponds to *L.major*. This result confirmed the identity of the analyzed parasites in comparison with the reference strains (Fig. 1).

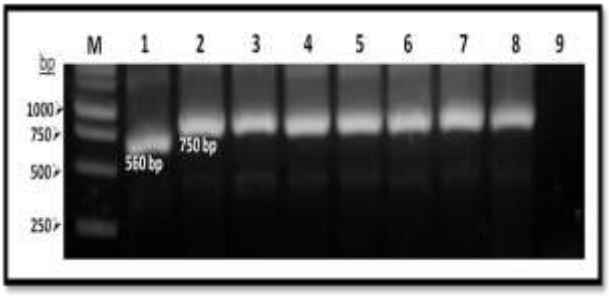

Fig. 1: PCR result from tested leishmania isolates and from two reference strains using species-specific kDNA minicircle CSB2/CSB1 primers. Lane M: marker (1kb DNA ladder); Lanes 1and 2 reference strains *L. major* and *L. tropica*, respectively. Lanes 3-8: local isolates of *L.tropica*. Lane 9: negative control

Cloning and sequencing of LACK gene

Full-length *LACK* gene was amplified from *L.tropica* genomic DNA using two specific primers LACK-FL-F/R. GoTaq DNA polymerase, used in PCR reaction, adds overhang A to each end of the target sequence, which allows the gene insertion into linerized pDrive cloning vector. After bacterial transformation, blue/white colony screening was performed to select positive colonies, confirmed by colony PCR using two plasmid specific primers (FP/RP) resulting in a band of bp (data not shown). Plasmid constructs from positive colonies were prepared and sent for sequencing. Sequence alignment analysis revealed the presence of similar *LACK* sequence form all tested *L.tropica* local isolates, and the resulted sequence

(Fig.2) was sent for registration in NCBI data- base (GenBank accession number: KM042903).

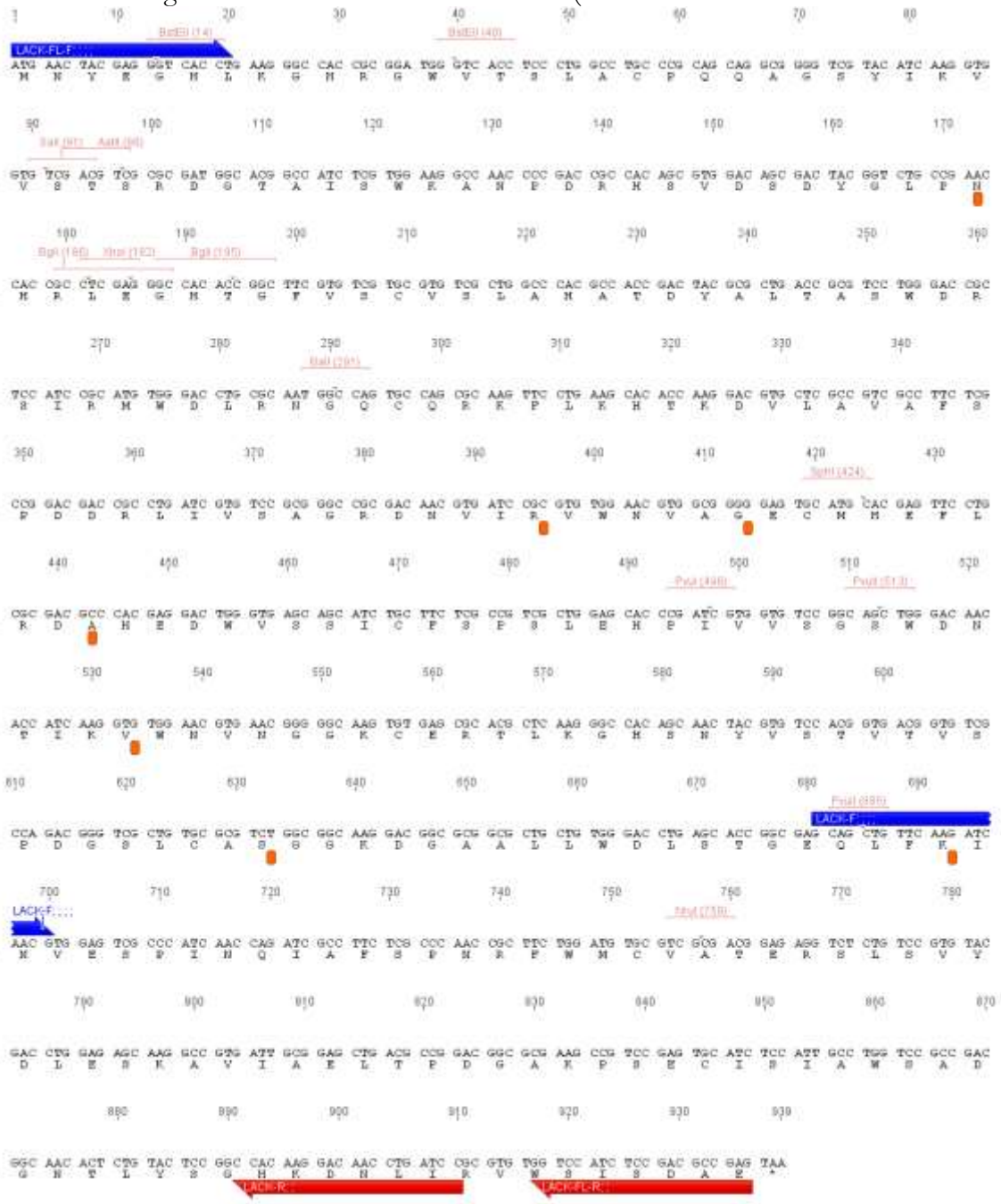

Fig. 2: Nucleotide and deduced amino acid sequences of *L.tropica LACK* gene. The nucleotide sequence is shown in the 5'-3' direction, numbered from the first base of start codon as the position 1. Deduced amino acid sequences are located below the nucleotide sequence. Positions of *LACK* gene specific primers and of several cutting restriction enzymes from the most commonly used ones are shown. Dotes below the sequence indicate the nucleotides that differ between *L.tropica* and other species

Comparison of LACK sequences between Leishmania spp

The nucleotide sequence of *L.tropica LACK* from the local isolates (KM042903) was aligned with other reported *LACK* sequences from different *Leishmania* spp. as well as with three previously published *L.tropica LACK* gene sequences (JX305923, JX305924, and KC763809). Sequence alignment of *LACK* genes from these records revealed that the local isolates (KM042903) manifest three *L.tropica* hallmark nucleotide substitutions; an A instead of G (A/G) at position 173 (resulting in an asparagine instead of a serine), a G/C at 414 (glycine) and a T/C at 633 (serine). However, in the fourth position at 396 C/A (Arginine), the *LACK* gene from the local isolates differs from all other *L.tropica* isolates and resembles the other *Leishmania* species *LACK* genes by possessing an A instead of C. Interestingly, the *L.tropica* isolate (KC763809), which found to be very close to our local isolates in the dendrogram of *LACK* genes, was found to share with the local isolates a unique substitution; a G/A (valine) at position 534 comparing with other strains including those from *L.tropica*. Another unique substitution for our local isolate is a C/G (alanine/glycine) at position 443, and this change seems to be tolerated by *L.tropica* at this site since an S (G or C) was found in this location of the isolate (KC763809) from the same species. One confusing notice was regarding the G (lysine) at position 693, which characterizes all *Leishmania* species including *L.tropica* local isolates and the isolate (JX305924) but not the isolate (JX305923), which has an A (lysine) substitution.

However, it seems that both possibilities are also acceptable for this site in *L.tropica* since an R (G or A) was found in this location in the isolate (KC763809) from the same species (Fig.2).

The result of the alignment of the *LACK* gene sequences from all records was presented in a dendrogram (Fig.3).

Remarkably, the sequence of *LACK* gene has almost perfect matching among *L.tropica* isolates. The cluster of similarity in this species could be easily distinguished from two other clusters; the first which is closer in similarity and contains *L.donovani, L.chagasi,* and *L.infantum,* and a second distant cluster gathering *L.amazonensis, L.major, L.braziliensis, and L.mexicana*. However, *LACK* gene seems to be a highly conserved gene in *Leishmania* genius since a relatively great degree of similarity (reaching 98.1%) was observed between the farthest two sequences in the dendrogram; the local isolates of *L.tropica* and *L.mexicana*(Fig.3).

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted successfully from cultured promastigotes (from stationary and logarithmic phases) and axenic amastigotes. Furthermore, total RNA electrophoresis illustrated smear of mRNA and three bands of rRNA, showing an acceptable integrity and purity of the prepared RNA (Fig.4A, B, and C). Moreover, the purity of the RNA from genomic DNA contaminants was confirmed by PCR reaction, using *LACK* gene specific primers where a negative amplification is expected (Fig.4D).

Then, total RNA was reverse transcribed, and the synthesized cDNA was used as template to amplify a small domain from *LACK* full-length gene using LACK-F/LACK-R primers.

This resulted in a single band of 230bp in all tested conditions, confirming the presence of *LACK* gene transcripts in both amastigotes and promastigotes; stationary and logarithmic phases, as well as a single band about 200bp refers to positive control transcripts (Fig.5).

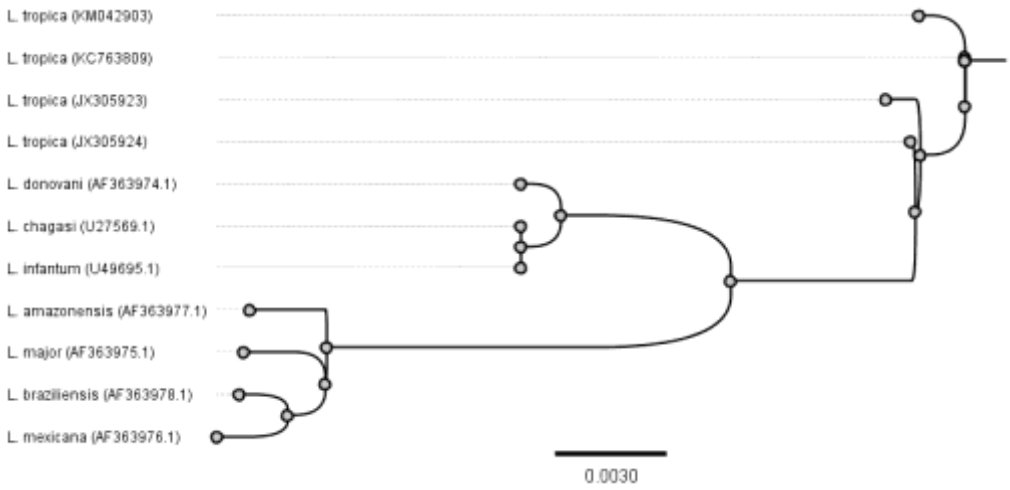

Fig. 3: A dendrogram of *LACK* gene sequences from different *Leishmania* species that were aligned with the local *L.tropica LACK* gene (KM042903)

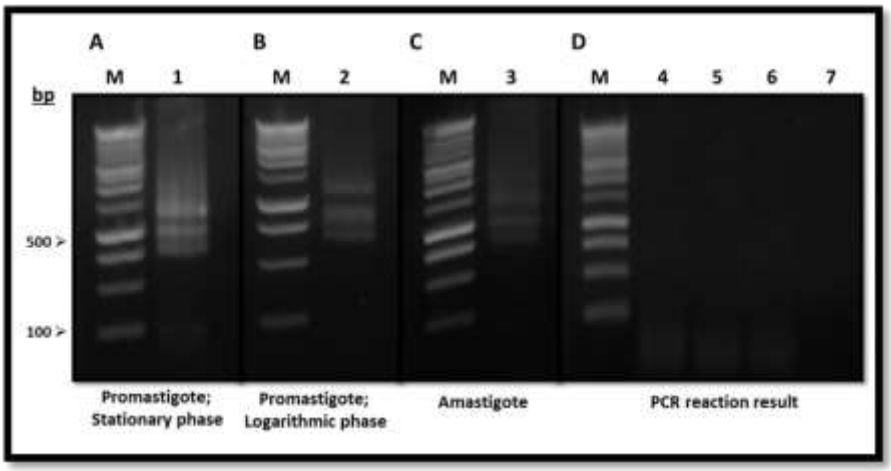

Fig. 4: Evaluation of the quality and purity of the extracted total RNA.**A, B** and **C**: depict smear of mRNA and three bands of rRNA reflecting the excellent extraction of total RNA in promastigotes; stationary and logarithmic phases, and axenic amastigotes (Lanes 1, 2 and 3), respectively. **D**: Result of PCR reactions confirming the purity of extracted total RNA from genomic DNA contaminants in promastigotes; stationary and logarithmic phases, and axenic amastigotes (Lanes 4, 5 and 6), respectively. Lane 7: negative control for PCR reaction

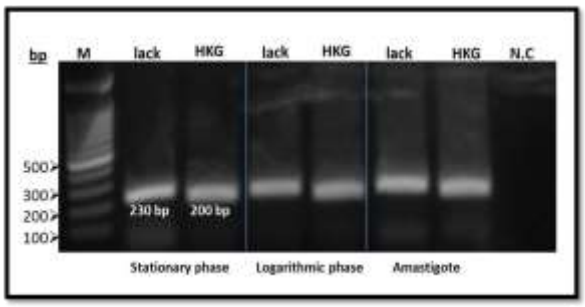

Fig. 5: PCR result of synthesized cDNA. PCR reaction was applied on cDNA of *LACK* gene of promastigotes; stationary and logarithmic phases, and amastigotes, respectively, and cDNA of 60s ribosomal protein L12 gene (a positive control) of promastigotes; stationary and logarithmic phases, and amastigotes, respectively. The PCR product size is approximately 230bp of the target gene (*LACK*), and 200bp of the housekeeping gene (HKG). Lane M: marker (BenchTop 100bp DNA ladder); and lane 7: Negative control

Discussion

In this study, we showed by the molecular typing with PCR technique that all *Leishmania* local isolates were belonging to *L.tropica*. Such method is widely used for distinguishing the different species of *Leishmania*, simply by amplifying highly conserved sequence blocks of kDNA minicircles (19, 20). Recently, PCR is used for visceral and cutaneous leishmaniasis diagnosis (21). In compared studies of PCR assays, PCR based on kDNA was the most sensitive diagnostic assay for CL and recommended for routine diagnosis (22). The genomic DNA of one isolate belonging to *L.tropica* was extracted and *LACK* gene was amplified, cloned and sequenced. *Ltropica LACK* nucleotide sequences from the local isolates were identical and manifest a great similarity with *L.tropica LACK* gene from published records. This provides further confirmation of the identity of our local isolates based on the analysis of *LACK* gene. Furthermore, *LACK* gene from local isolates showed significant homology to *LACK* sequence from several other species including *L.donovani, L.major, L.amazonensis, L.braziliensis, L.chagasi, L.infantum and L.mexicana*, implying that *LACK* protein may have an important functions for the parasite life cycle. This high similarity of *LACK* nucleotide sequences may refer to identical amino acid sequence among the different *Leishmania* spp. Melby et al. (2001) had shown that *L.donovani LACK* deduced amino acid sequence was identical to the published *L.chagasi* and *L.infantum* sequences (GenBank accession numbers U27569 and U49695, respectively) and differed at only 1 or 2 amino acids from the *L.major, L.mexicana, L.amazonensis,* and *L.braziliensis* sequences they obtained (10). In this work, *LACK* protein from our local strains was found to differ in two amino acids from other *Leishmania* spp. because of nucleotides substitution at position 173 and 443.

We demonstrated that *LACK* gene was expressed in logarithmic and stationary phases present procyclic and metacyclic promastigotes respectively, as well as in amastigotes in *L.tropica*. *LACK* protein is believed to be expressed by all *Leishmania* spp. in both promastigote and amastigote stages (12, 23). Recently, Hajjaran et al. (2012) study approved that *LACK* protein is one of the over expressed proteins in Iranian isolate of *L.tropica* promastigotes (24). The continuous expression and production of *LACK* protein reflects its important role in various stages of the parasite life cycle, and his major role was confirmed by several studies; *LACK* is required for vertebrate parasitization, plasminogen activation, and can function as an aggravating factor in animal host causing early phase infection. In addition, *LACK* can induce both proliferative and cytokine responses in peripheral blood mononuclear cells (8, 9, 11, 25). To date, there have been numerous attempts at developing a successful vaccine against leishmaniasis (2). The protective role of *LACK* as a vaccine in humans is not straightforward (25). On the other hand, *LACK* administered with IL-12 is able to direct the T cells toward a Th1 response in mice (26). However, driving of the immunodominant *LACK* response toward a Th1 phenotype could be the key for preventing the ill effects of Th2 responses (25). Immunization with recombinant antigens or plasmid DNA encoding *Leishmania* antigens represents a promising approach to vaccinate against leishmaniasis. These DNA vaccines can induce both humoral and cell-mediated immune responses and results in long lasting immunity (27).Both models, *LACK* DNA and recombinant *LACK* protein can induce protection in mice that were immunized and challenged with *L.major* promastigotes (27). These results, which demonstrated a successful application of *LACK* DNA/Protein vaccine in the infectious disease models, open up an avenue for the development of vaccines against Old World cutaneous leishmaniasis caused by *L.tropica*.

This report is limited to study the sequence and the expression of *LACK* gene at mRNA level in different stages of *L.tropica* parasite life cycle. However, it will obviously be essential to validate these findings at protein level when the antibody is available, as post-transcriptional mechanisms have been shown to regulate protein levels in *Leishmania* (28).

Conclusion

Our study confers a base for producing the recombinant *LACK* protein since it is of particular interest as a vaccine candidate against CL. It focuses on determining *LACK* gene sequence in *L.tropica* (local isolates), comparing its sequence with those reported in database for other *Leishmania* spp. Finally, verifying of *LACK* gene expression at mRNA level in two different phases of the parasite life cycle; logarithmic and stationary phase of promastigotes, as well amastigotes.

Acknowledgments

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Cloning, Expression and Purification of Recombinant *Leishmania Tropica* P36 (Lack) Protein for Serodiagnosis and Vaccine Applications

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Abstract: Cutaneous Leishmaniasis (CL) is a group of diseases caused by a protozoan parasite of the genus *Leishmania* afflicting millions of people worldwide. Unfortunately, no ideal therapy for CL has been identified which emphasize the need for vaccine development. A suitable leishmaniasis vaccine candidate molecule must be immunogenic and have surface expression in amastigote, the infective stage for mammals. The location of the P36 (LACK) antigen on the parasite surface, its immunogenicity and its expression in promastigote and in amastigote of *Leishmania spp.* make it a potential candidate for CL serodiagnosis and vaccine. In the present work, we developed an expression system to produce recombinant *L. tropica*-P36. Using *L. tropica* genomic DNA as template, *P36* gene was amplified by PCR then cloned into the expression vector pRSET. The new plasmid construct (pRSET-P36) was efficiently able to express P36 protein in the cytoplasm of *E. coli*. P36 purification was achieved by one step metal affinity chromatography. The purity of the resulted protein was monitored by SDS-PAGE separation and coomassie blue staining, and its identity was confirmed by immunoblotting using specific anti-6×His tag antibody. Recombinant P36 was detected by sera from CL patients, suggesting its usefulness for CL serological diagnosis and perhaps as a vaccine for this endemic disease in Syria.

Keywords *L. tropica*; P36; pRSET; cloning; protein expression.

Introduction

Leishmaniasis is a neglected disease which affects 12 million people in nearly 90 countries presenting a worldwide public-health problem [1-3]. CL is caused by many species of *Leishmania*; *L. major*, *L. aethiopica* and *L. tropica*, in the Old World [4]. Nearly 90% of CL cases occurs in Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Sudan in addition to Syria [1].

Leishmaniasis is treatable; however, anti-leishmanial therapy is a confusing subject largely because of the complexities of the disease. Unfortunately, no ideal therapy for CL has been identified [5]. Drug resistance and toxicities associated with chemotherapy emphasize the need for a safe and effective vaccine [6]. Vaccine development for parasitic diseases is more difficult than for most bacteria and viruses due to the complexity of the pathogen and its interactions with the vertebrate host. Recently, much interest has been stimulated towards vaccination against leishmaniasis focused mainly on CL with fewer attempts against VL [7].

The recombinant DNA-derived *Leishmania* proteins can be used in diagnosis, therapy, and development of vaccines [8]. Several antigens have been identified and characterized that might be potential vaccine candidates. P36 is a 36-kDa protein, or alternatively named LACK antigen, expressed in promastigotes and amastigotes life stage of different *Leishmania* species [9-11] and P36 is one of the vaccine candidates for leishmaniasis [6, 12]. It has been clearly demonstrated that P36 is essential for the viability of the parasite and for the parasite establishment in the host. The function of P36 in *Leishmania* is not clear although the immunological response to this molecule has been well studied and used for experimental vaccine studies in the mouse model [6,13,14]. In addition, P36 has been used as a tool to investigate various immunity-related mechanisms, and tested in several immunization experiments, providing heterogeneous results [9,11, 15].

Because of CL spreading in Syria, which is mostly caused by *L. tropica*, and the prominent protective role of P36 protein as a vaccine candidate against leishmaniasis, we aimed to produce recombinant P36 protein to be used later as experimental vaccine against CL, as well to be used for the detection of leishmaniasis infection.

Materials and Methods

Bacterial Strains, Growth Conditions and Plasmid

E. coli strains TOP10 (Invitrogen) and BL21-Gold (DE3) (Novagen) were used in cloning and protein expression after transformation by electroporation with the plasmid pRSET (Invitrogen). For general maintenance and protein expression, *E. coli* were grown in Luria Broth (LB; 1 % Tryptone, 0.5 % yeast extract, 171 mM NaCl) (Bio Basic INC) with ampicillin antibiotic (Sigma; 100 g/ml) in orbit-rotating 37°C incubator.

Plasmid Construction, Cloning and Subcloning

L. tropica genomic DNA was extracted. P36 gene was amplified by PCR with two specific primers P36-F (5'-TATATA**GGATCC**ATGAACTACGAGGGTCACCT-3') and P36-R (5'-TATATA**GAATTC**TTAC TCGGCGTCG GAGATG G-3'). Primers were designed according to P36 gene sequence of *L. major* (GeneBank accession number AF363975.1). These primers were designed to amplified the full length gene with the start and stop codons, and to add *Bam*HI restriction site at the 5' end and *Eco*RI at the other end of the PCR amplicon. PCR amplification program consisted of 2 min of denaturation step at 95 °C followed by 35 cycles of short denaturation step at 94 °C for 1 min, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min. Amplified P36 fragment was purified using Invisorb Fragment CleanUp kit (Stratec molecular) then inserted into open plasmid pDrive cloning vector (Qiagen). The insert sequence was confirmed by sequencing. After that, the insert was subcloned into pRSET plasmid by digesting both, pDrive-P36 and pRSET with *Bam*HI and *Eco*RI restriction enzymes (Fermentas). Then, the insert and pRSET were ligated using T4 DNA ligase (Amersham). Freshly prepared electro-competent *E. coli* TOP10 cells were transformed with the new plasmid construct pRSET-P36 by electroporation. Colony PCR screening for positive P36 clones was performed using pRSET specific primers (T_7F/T_7R). Plasmid constructs were isolated from some positive clones by Plasmid Miniprep Kit (Qiagen) after being grown in LB/ampicillin medium. Successful cloning of these plasmid constructs was confirmed by digestion with restriction enzymes and by sequencing.

Expression and Purification of P36 Protein

Confirmed plasmid construct was used to transform by heat chock *E. coli* BL21-Gold (DE3) cells. Protein expression of P36 was performed in 250 ml shake flasks by growing the bacteria in LB medium till an optical density of 0.5 to 0.7 was reached and then expression was induced with 0.5 mM isopropyl β-D-thiogaldctoside (IPTG, Promega) for 16 h at 37 °C. After pelleting the cells, the pellet was resuspended in binding buffer (20 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, and 8 M urea), then lysed by sonication (Lab Sonic) on ice and the lysate was cleared by 20 min centrifugation at 8,000 rpm and 4 °C. Using fast protein liquid chromatography (FPLC) AKTA prime plus system (GE life science), recombinant P36 was purified from the cytoplasmic extract using a 5 ml column of Nickel charged Nitrilotriacetic acid (NTA) superflow Sepharose (Qiagen). After washing, the bound proteins were refolded on the column then eluted from the column with the elution buffer (20 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole and 8 M urea). The eluted fraction was dialyzed to eliminate the urea and refold the protein then concentrated on Vivaspin concentrators with a molecular mass cutoff of 10 kDa (Vivascience). The concentration of the purified protein was determined by Bradford method. The purity of P36 was evaluated in a coomassie-stained SDS-

PAGE. Protein samples were separated by SDS-PAGE using a Bio-Rad Mini-Protean Tetra Cell system following the manufacturer's instructions. Gels were prepared using stacking gel 5 % and running gel 12 %. After electrophoresis, the gel was stained in coomassie brilliant blue buffer (45 % methanol, 10 % acetic acid, 0.25 % coomassie R250) for 2 h and then washed several times in destaining buffer.

Immuno-Blotting of P36 Protein

For immuno-blotting, proteins in acrylamide gel were blotted onto 0.2 µm nitro-cellulose membranes (Whatman) using 1 × transfer buffer (800 ml dH2O containing 14.4 g glycine and 3 g tris, pH 8.3 and 200 ml methanol). After incubation in the blocking buffer (3 % BSA in tris buffered saline-tween-20, pH 7.5), blots were incubated once with 1/5000 dilution of rabbit anti-His antibody (Bethyl Laboratories), then incubated with 1/2000 dilution of goat anti-rabbit antibody conjugated to alkaline phosphatase (AP) (Bethyl Laboratories). Final revelation was done using NBT/BCIP (Nitro blue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate; Sigma) chromogen substrate in AP buffer (100 mM tris base, 100 mM NaCl and 5 mM $MgCl_2$, pH 9.5). Alternatively, blots were incubated with 1/1000 dilution of sera from CL patients, then incubated with 1/5000 dilution of goat anti-human antibody conjugated to AP (Thermo scientific) and then reveled as described previously.

Results

Cloning of P36 Gene Into Pdrive Cloning Vector

Full-length P36 gene was amplified from *L. tropica* genomic DNA using two specific primers. The amplified gene is about 1000 bp in length (Fig. 1A). Go*Taq* DNA polymerase, used in PCR reaction, adds an overhang A to the ends of the amplified sequence, which allows P36 gene insertion into linearized pDrive plasmid and the positive construct was used for the subsequent subcloning into pRSET expression vector (Fig. 1B). The plasmid pRSET is a T7 promoter-dependent system which produces cytoplasmic N-terminal His6-tagged proteins in *E. coli* cells. Tagging of recombinant proteins is indispensable for the subsequent steps of purification and detection.

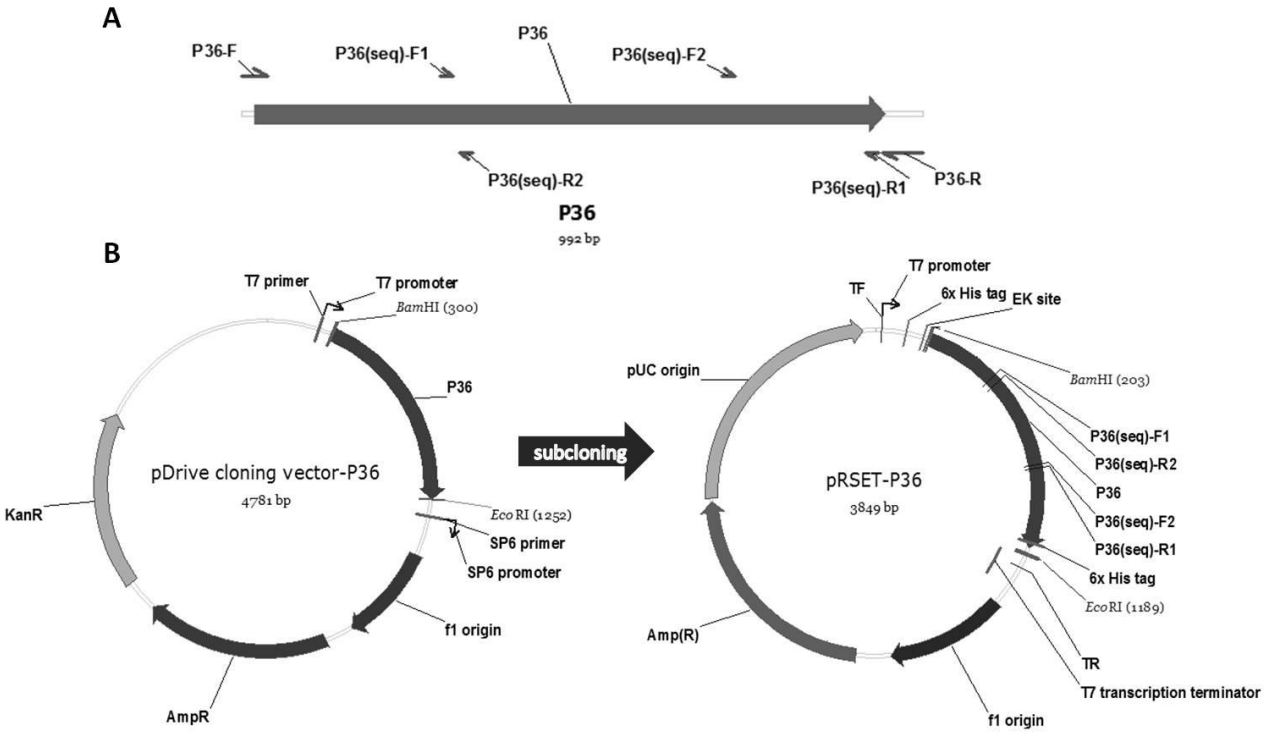

Fig. 1. Scheme of P36 gene, pDrive plasmid used in cloning and pRSET plasmid used in protein expression.

(A) P36 gene and the positions of different primers used in the cloning and sequencing. (B) Maps of the resulted plasmid constructs (pDrive-P36) for cloning and sequencing and (pRSET–P36) for expression where the position of the inserted P36 gene is indicated. The most important elements of the plasmids are indicated. These include T7 promoter, His6 tag downstream the two restriction sites (*Bam*HI and *Eco*RI) used for P36 gene ligation. Positions of the primers used for PCR positive colonies screening and for sequencing are shown.

PCR amplified P36 gene was successfully cloned in pDrive plasmid as confirmed by colony PCR using a combination between a vector-specific primer and an insert-specific primer. Such reaction confirms the insert orientation, since cloning using the A-overhang left by *Taq* DNA polymerase and T-tailed vector is not a technique that will retain orientation. After cloning, P36 was sequenced (Data not shown).

Subcloning of P36 Gene Into Prset Plasmid

P36 was digested with *Bam*HI and *Eco*RI restriction enzymes, resulting in a sticky ended fragment ready for ligation in linearized pRSET plasmid with the same two restriction enzymes (Fig. 2C). Ligated products were used to transform *E. coli* TOP10 cells by electric shock and positive colonies on the selective plates were screened by PCR using pRSET-specific forward primer (T_7F) and P36-spicefic sequencing reverse primer ($P36_{seq}$-R2). This approach enabled distinguishing between two types of colonies; empty pRSET-containing colonies and pRSET-P36-containing colonies which gave a fragment of 506 bp due to the presence of the insert gene within (Fig. 2D).

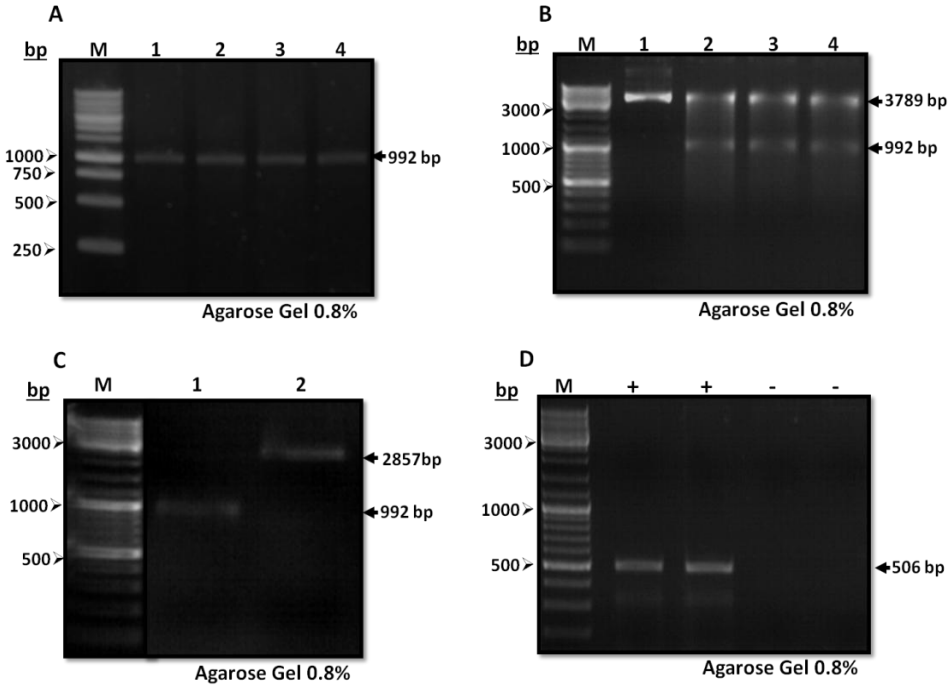

Fig. 2. Amplification, cloning and subcloning of P36 gene.

(A) PCR reaction was performed on genomic DNA extracted from *L. tropica,* using P36 specific primers (lanes 1-4). **(B)** Digestion of pDrive cloning vector-P36 after cloning and sequencing for subcloning; lane 1: the purified construct (pDrive-P36) without digestion as a control; lane 2: the same construct digested using *Bam*HI; lane 3: the construct digested using *Eco*RI; lane 4: the construct digested using both *Bam*HI and *Eco*RI restriction enzymes. (C) Shows the purity of digested P36 gene (lane 1) and pRSET plasmid (lane 2) using both *Bam*HI and *Eco*RI restriction enzymes for ligation reaction. (D) Results of colony PCR screening performed on 4 randomly selected clones after *E. coli* TOP10 transformation with the products of the ligation reaction.

Expression and Purification of P36 Protein In E. Coli

Production of P36 as recombinant protein was obtained after transformation of *E. coli* BL21-Gold (DE3) cells with the confirmed pRSET-P36 plasmid construct. Cells were grown in LB medium supplemented with antibiotic and protein expression was then induced by IPTG. Purification of P36 from cytoplasmic extract was done on immobilized-metal affinity chromatography, after treatment with urea to dissolve the formed inclusion bodies, using Nickel-charged NTA column installed on AKTA prime system. The UV-detector, supplemented with this system, enabled real-time monitoring of the different steps of P36 purification (Fig. 3A and 3B). The protein expression and purification procedures of recombinant P36 were followed by SDS-PAGE and immunoblotting for purity assessment (Fig. 3C and 4). A remarkable expression of P36 could be observed after IPTG induction and incubation for 16 h at 37 °C. Although, expressed protein was totally purified from bacteria cytoplasmic extract by column purification which yielded 90 % pure P36. The yield of purified recombinant protein estimably reached ~500 mg/L of bacteria culture.

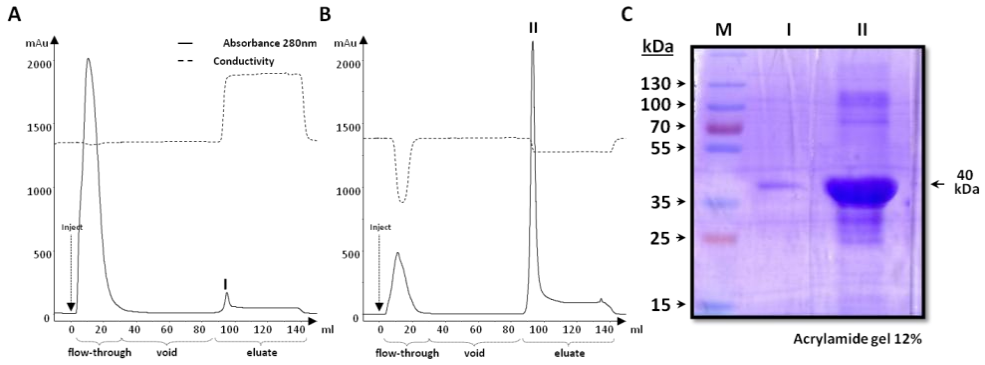

Fig. 3. The expression and purification of P36 protein.

(A) and **(B)** diagrams of purification procedure using Ni+-NTA column installed on FPLC AKTA prime system. Continuous line represents the absorbance of the eluate and the dashed line represents the conductivity of the eluate. Different purification steps are shown below and peaks of the flow-through sample and of purified P36 are indicated. In diagram A, extraction and purification were accomplished using PBS, while in diagram B extraction and purification were accomplished under denaturing conditions using Urea. **(C)** SDS-PAGE (acrylamide 12 %) of the purified protein samples, with PBS (lane I) or with PBS+Urea (lane II).

In immunoblotting, purified P36 protein was detected by anti-His6-AP conjugated antibody (Fig. 4B) or by diluted sera from CL patients (Fig. 4C) in which the recombinant green florescent protein (GFP) was used as a negative control with positive and negative sera.

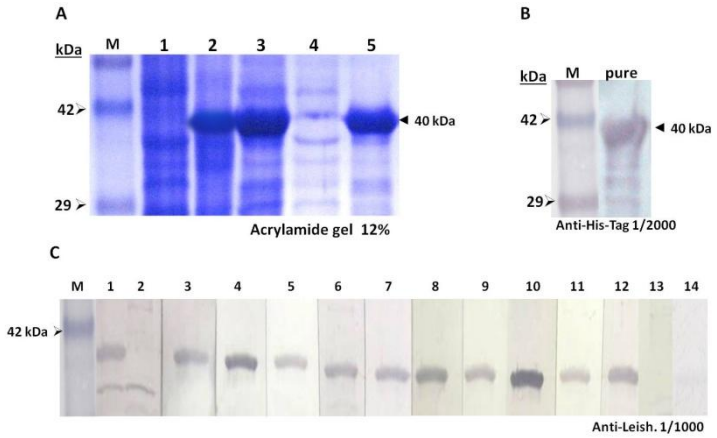

Fig. 4. SDS-PAGE analysis and immunoblotting of P36 protein.

Protein migration in SDS-PAGE (acrylamide 12 %) of protein samples obtained after different steps of purification. Total cytoplasmic extract after 16 h of IPTG induction (lane 3; while lanes 1 and 2 show before and after induction respectively), and purified P36 protein (lane 5) in comparison with flow-through (lane 4). Detection of migrated proteins was done either by coomassie blue staining **(A)** or by immunoblotting using rabbit anti-His antibody (1/2000) **(B)** or using positive serum from CL patients (1/1000) **(C)** (lanes 1, and 3-12 different positive serums; lanes 2, and 13 GFP protein as a control with positive and negative sera respectively; and lane 14 purified P36 with negative serum). The location of P36 in the gel is indicated and defined as 40 kDa compared to the protein molecular weight ladder in the first lane (M).

Discussion

In recent years, great efforts were performed searching for an effective vaccine against Leishmaniasis in particular Cutaneous Leishmaniasis. Although a great deal of knowledge has been gained from studies on the immunobiology of leishmaniasis, there is still no universally acceptable, safe, and effective vaccine against the disease [16]. As a second generation vaccines against Leishmaniasis several antigens have been identified and characterized that might be potential vaccine candidates [6]. According to previous studies, P36 (LACK) protein is one of these candidates [7, 12, 17].

In the current work, a high-level expression system of His6-tagged *L. tropica* P36 was established in *E. coli*. Firstly, *P36* gene was amplified, cloned and sequenced. The genomic DNA was used as a template in PCR reaction, since *P36* gene does not contain introns (*L. major P36*; GeneBank accession number AF363975.1). Cloning was performed using pDrive cloning vector which provides superior performance through the direct ligation of the blunt-ended PCR products, allowing sequence analysis using standard sequencing primers. Following sequence confirmation, DNA insert was subcloned into pRSET plasmid using specific restriction enzymes. The pRSET vector is a pUC-derived expression vector designed for high-level protein expression and purification from cloned genes in *E. coli*. The presence of the T7 promoter provides high level expression of the down-stream cloned gene [18, 19]. In addition, DNA inserts are positioned downstream and in frame with a sequence that encodes an N-terminal polyhistidine tag that functions as a metal binding domain in the translated protein [20]. High level of recombinant P36 protein expression was obtained in *E. coli* BL21-Gold (DE3). However, the product precipitated in cytoplasmic inclusion bodies. Protein aggregation is a major bottleneck during the bacterial production of recombinant proteins. In general, the induction of gene expression at sub-optimal growth temperatures improves the solubility of aggregation-prone polypeptides and minimizes inclusion bodies formation [21]. Choosing appropriate conditions for cell growth and induction can influence the formation of these insoluble structures [22], especially, temperature which affects directly proteins stability and confirmation [23]. Increased temperature has been found to stimulate aggregation in several cases [24]. Low temperature affects the quality of the recombinant protein, especially within the insoluble cell fraction. The fraction of aggregated protein (i.e. IB) was largely decreased at low temperatures (below 37 ^{o}C), and the conformational quality was improved [21]. Traditionally, very high concentrations of denaturing buffers are usually used to solubilize proteins in the inclusion bodies. Urea is one of classic denaturants used for inclusion bodies dissolving and refolding aggregated proteins [25-27]. On the other hand, polyhistidine-tagged fusion proteins that form IBs can be extracted with urea and purified directly by nickel-chelate affinity chromatography. Removal of contaminating proteins and refolding by exchange to non-denaturing buffer conditions can then be performed directly on the purification column just before the elution step [28, 29]. To overcome IBs formation in future, a new system which combines improved expression, solubility screening and purification efficiency can be used. The system is based on newly constructed vector, which generates a protein with an N-terminal His tagged green fluorescent protein (GFP) fusion to allow rapid quantitation of soluble protein [30]. Previous studies reported the use of GFP as an excellent expression tag for fusion proteins, which can improve their expression while preserving their function and native-like structure. Fusion protein method allows the purification and the detection of a protein of interest even when no specific antibody is available [30, 31].

Previous published works described production of recombinant P36 protein from other different *Leishmania spp.*, for application as an experimental vaccine with or without adjuvant against VL or CL caused by different *Leishmania spp.* such as *L. amazonensis* [9], *L. mexicana* [13], *L. major* and *L. infantum* [12]. Another study determined the ability of P36 DNA to induce protection after challenging with *L. major*

promastigotes. The induced protection was similar to that achieved by P36 protein and rIL-12, but superior to P36 protein without rIL-12 [10].

The recombinant P36 protein presents a new applicable approach for CL serological diagnosis since this protein gave a positive result in immunoblotting using many different serums from CL patients. As well, recombinant P36 may be able to detect CL disease which is caused by different *Leishmania* species since P36 is a conserved antigen between different *Leishmania* species. But this point still needs more investigations. Additionally, recombinant P36 presents a tool for developing one of CL candidates vaccine derived from *L. tropica*, and more steps can be achieved for production and testing of P36 as a recombinant protein and/or DNA vaccine.

In summary, a new approach was developed for *L. tropica* P36 production as a recombinant protein. Recombinant P36 may be useful as a diagnostic tool since good positive results were obtained with western blot using CL patient's serum. In addition, recombinant P36 may be useful in the development of an efficient vaccine against Old World cutaneous leishmaniasis.

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الجمهورية العربية السورية

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كلية الزراعة

دراسة التعبير عن الجين (*LACK*) *P36* في سلالة من الليشمانيا الجلدية (الليشمانيا المدارية) وإنتاج البروتين المؤشب LACK

أطروحة أعدت لنيل درجة الماجستير في التقانة الحيوية

إعداد الطالبة

نور محمد أيمن حموده

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هيئة الطاقة الذرية السورية

الملخص

يُعد داء الليشمانية الجلدي أحد الأمراض التي تسببها طفيليات أوالي تنتمي إلى جنس الليشمانيا والذي يهدد ملايين البشر حول العالم. لا يتوفر علاج فعال تجاه الليشمانية الجلدية حتى الآن مما يجعل تطوير لقاح ناجع وفعال تجاهها حاجةً مُلحة. ويُعتقد بأن الجزيئات المناسبة والمرشحة لأن تكون لقاحات ناجحة تجاه الليشمانية يجب أن تكون معروضة على سطح الطفيلي في الطور داخلي السوط Amastigote وهو الطور المُعدي للثدييات. يُعد المستضد P36/LACK ذو الوزن الجزيئي 36 كيلو دالتون مُرشحاً قوياً لاستخدامه في تشخيص الإصابة بداء الليشمانية الجلدي وحتى استخدامه كلقاح وذلك بسبب موقعه على سطح الطفيلي بشكليه داخلي السوط وخارجي السوط Promastigote وقدرته على استثارة استجابة مناعية باكرة جداً تجاه الخمج بطفيلي الليشمانيا. اهتمت العديد من الأبحاث بدراسة التعبير عن المورثة *P36/LACK* لدى طفيلي الليشمانيا خلال أطوار مختلفة من دورة حياة الطفيلي إلا أن القليل منها ركز على دراسة هذا المستضد لدى الليشمانيا المدارية *L. tropica*.

اهتمت دراستنا بتنسيل وسلسلة المورثة *P36/LACK* المُرمزة لهذا المستضد، ودراسة التعبير عن هذه المورثة في طفيلي الليشمانيا المدارية. ومن ثم نقل المورثة إلى حامل تعبير مورثي وإنتاج البروتين بشكل مؤشب recombinant protein واستخلاصه وتنقيته.

بدايةً، نُمط عددٌ من العزلات أو السلالات المحلية من طفيلي الليشمانيا جزيئياً بالاعتماد على التفاعل السلسلي للبوليميراز PCR. ومن ثم، تم عزل DNA الجينومي وتضخيم المورثة *P36/LACK* وإدخالها ضمن حامل من أجل سلسلتها وتحديد التتالي النكليوتيدي لها. بعد ذلك تم دراسة التعبير عن هذه المورثة على مستوى mRNA عن طريق تقانة RT-PCR، وذلك في كل من طوري النمو والاستتباب للشكل خارجي السوط بالإضافة للشكل داخلي السوط. وتبيَّن من خلال التنميط الجزيئي أن العزلات المحلية تعود للنوع *L. tropica*. تم تحديد التتالي النكلوتيدي للمورثة وكان هناك تشابه كبير عند مقارنة هذا التتالي مع أنواع أخرى من الليشمانيا. كما أوضحت دراستنا أن المورثة *P36/LACK* تُعبر عن نفسها في كلا الطورين خارجي وداخلي السوط. طوِّر من خلال هذا العمل نظام تعبير خاص لإنتاج البروتين بشكل مؤشب وذلك عن طريق نقل المورثة إلى حامل تعبير مورثي pRSET، إذ كان التركيب الجديد الناتج (pRSET-*P36/LACK*) قادراً على التعبير عن البروتين P36/LACK في سيتوبلاسما بكتيريا الاشريكية القولونية *E. coli* ومن ثم تم تنقية البروتين المؤشب الناتج باستخدام تقانة الكروماتوغرافيا التآلفية، والتأكد من نقاوته بالرحلان البروتيني SDS-PAGE بعد تلوين الهلامة باستخدام أزرق الكومازي. وللتأكد من هوية البروتين الناتج قمنا بإجراء اختبار التبصيم المناعي immunoblotting باستخدام أضداد نوعية anti-6×His tag antibody موجهة ضد ذيل عديد الهستدين المدمج بالبروتين المؤشب. وأخيراً، اختبرت قدرة مجموعة من أمصال المرضى على التعرف على البروتين P36/LACK النقي بهدف استعماله في التشخيص المصلي للمرض ومن ثم اختباره كلقاح ضد الداء الجلدي المستوطن في سوريا.

الكلمات المفتاحية:

داء الليشمانية الجلدي، الليشمانيا المدارية، البروتين LACK، الطفيلي خارجي السوط، الطفيلي داخلي السوط، التنسيل المورثي، التعبير البروتيني.

الخلاصة

يعد داء الليشمانية واحداً من أهم الأمراض الطفيلية انتشاراً حول العالم؛ حيث ينتشر الداء، بحسب منظمة الصحة العالمية، في حوالي 88 دولة، ويَتَوَطن في أقاليم عدة من العالم القديم والجديد، تمتد من وسط أسيا عبر الشرق الأوسط، وجنوب أوروبا، وإفريقيا إلى أمريكا اللاتينية. يصل معدل الإصابة بداء الليشمانية إلى 1.5-2 مليون إصابة سنوية مع احتمال وقوع /350/ مليون شخص تحت خطر الإصابة. يسبب هذا الداء حوالي 20 نوعاً من طفيلي وحيد خلية من جنس الليشمانيا إجباري التطفل داخل الخلايا (البالعات الكبيرة). ينقل طفيلي الليشمانيا إلى المضيف الفقاري أنثى ذبابة الرمل (الفاصدة).

يعرف لداء الليشمانية ثلاثة أشكال سريرية تختلف عن بعضها من حيث نوع الطفيلي المسبب ونوع النسيج المستهدف والأعراض المرضية وهذه الأشكال السريرية هي:

الليشمانية الحشوية (Visceral Leishmaniasis)

و الليشمانية المخاطية الجلدية (Mucocutaneous Leishmaniasis)

و الليشمانية الجلدية (Cutaneous Leishmaniasis).

تعد منطقة الشرق الأوسط من المناطق التي تتَوَطّن فيها الليشمانية الجلدية، ويسببها في أغلب الحالات النوعان *L. major*، و*L. tropica*. تشترك الأنواع المختلفة لطفيلي الليشمانيا بدورة الحياة، حيث يتعاقب فيها شكلين إعاشيين؛ **الشكل أمامي السوط Promastigote** الذي يأخذ شكلاً متطاولاً مغزلياً، يعيش داخل المضيف الناقل (أنثى حشرة ذبابة الرمل) وفي الوسط الصنعي أي أوساط الزرع المحضونة عند درجة حرارة 26 درجة مئوية؛ **الشكل داخلي السوط Amastigote** الذي يأخذ شكلاً دائرياً أو بيضوياً ذا نواة كبيرة مركزية، يعيش ضمن البالعات الكبيرة للمضيف الفقاري (الإنسان) أو في أوساط الزرع عند درجة الحرارة 37 درجة مئوية.

بالرغم من الأبحاث العالمية الكثيرة التي تحاول التوصل إلى حلول لداء الليشمانية، إلا أن الجهود العلمية لم تتكلل بالنجاح بعد، فما يزال ايجاد لقاح فعال وآمن ضد الداء بعيد المنال. تهتم الدراسات الحديثة بمجموعة من المورثات وبروتيناتها التي تؤدي أدواراً مناعية مهمة عند دخول الطفيلي جسم المضيف الفقاري. لعل أهم هذه البروتينات، تلك المحافظة بين الأنواع المختلفة للطفيلي وتلك التي يعبر عنها في كلا طوري نمو الطفيلي؛ أمامي وداخلي السوط. ومن هذه البروتينات، البروتين P36/LACK ذو الوزن الجزيئي 36 كيلو دالتون، الذي أظهرت

الدراسات دوره الوقائي من الإصابة التجريبية بالطفيلي عند استخدامه في إستراتيجيات تلقيحية متنوعة.

أهمية وهدف البحث:

نظراً لصعوبة مكافحة داء الليشمانية في البلدان التي يستوطن فيها، ونظراً لظهور العديد من سلالات الطفيلي المقاومة للأدوية المستعملة لمعالجة الداء، حيث بات بعضها غير أمن تماماً وغير فعال بشكل كامل، تكتسب الوقاية من الإصابة بداء الليشمانية الجلدي أهمية كبيرة، إلا أن لقاحاً فعالاً يؤمن الوقاية الكاملة من الإصابة مازال غير متوفر حتى الآن بالرغم من الأبحاث العلمية العديدة المهتمة في التوصل لهكذا لقاح.

بما أن معظم الدراسات في مجال إيجاد لقاح لم تهتم بالنوع *L. tropica* الذي أصبح النوع الأكثر انتشاراً في سوريا وربما في منطقة شرق المتوسط، وبالنظر للنتائج الواعدة لاستعمال المورثة *P36/LACK* في استراتيجيات تلقيحية متنوعة والتي أدت إلى وقاية حيوانات التجربة من الإصابة التجريبية، وفي ظل غياب أي دراسة حول التعبير عن المورثة *P36/LACK* في النوع *L. tropica* أو حول الدور المحتمل لهذا البروتين في الوقاية من الإصابة بالداء الجلدي هدف البحث إلى:

1. **دراسة التعبير عن المورثة *LACK* في سلالة من طفيلي الليشمانيا المدارية *L. tropica* وذلك في كلا طوري النمو أمامي السوط وداخلي السوط.**
2. **تنسيل مورثة *LACK* ضمن حامل تعبير بروتيني وإنتاج البروتين LACK كبروتين مؤشب تمهيداً لاختبار قدرته على الوقاية من الإصابة بالداء الجلدي في دراسات مستقبلية.**

خطوات العمل والنتائج:

عزل الطفيلي الليشمانيا واستنباته وتنميطه:

تم الحصول على عدة عزلات (خمسة عزلات) من طفيلي الليشمانيا من مرضى الليشمانية الجلدية المراجعين لمشفى الأمراض الجلدية والزهرية في جامعة دمشق، ومن استنبت الطفيلي على أوساط مناسبة. نمطت بعدها هذه العزلات جزئياً باستعمال تقانة PCR بالاعتماد على DNA الجينومي genomic DNA المستفرد منها.

وقد تبيَّن من خلال التنميط أن العزلات المحلية جميعها تعود لنوع اللشمانيا المدارية *L. tropica*.

تضخيم المورثة *P36/LACK* وتنسيلها وسلسلتها:

عزلت المورثة *P36/LACK* بتقانة PCR وباستعمال مرئسات نوعية خاصة بالمورثة LACK-FL-F/R، ومن ثم نسلت ضمن حامل pDrive cloning vector وسلسلت بهدف

تحديد تتالي نكليوتيداتها، وبمقارنة التتالي الناتج مع التتاليات الموجودة في البنك الجيني GenBank لعدد من أنواع اللشمانيا الأخرى تبين وجود نسبة تشابه similarity عالية تصل إلى 98 %.

دراسة التعبير عن المورثة *P36/LACK* في أطوار مختلفة من حياة الطفيلي:

بهدف دراسة التعبير عن المورثة *P36/LACK* على مستوى الانتساخ، عزل RNA الكلي من أطوار مختلفة من دورة حياة الطفيلي؛ طوري النمو والاستتباب للأشكال خارجية السوط، بالإضافة للطفيلي بشكله داخلي السوط؛ ومن ثم اصطناع cDNA باستخدام أنزيم النسخ العكسي، وتبين بتطبيق تقانة RT-PCR أن المورثة *P36/LACK* تعبر عن نفسها في هذه المراحل الثلاثة من حياة الطفيلي. بعد التأكد من انتساخ المورثة صار بالإمكان تنسيلها بهدف إنتاج البروتين الموافق.

التنسيل والتعبير البروتيني عن P36/LACK:

نقلت المورثة *P36/LACK* من حامل التنسيل pDrive إلى حامل التعبير المورثي pRSET باستخدام أنزيمات التقييد *Bam*HI/*Eco*RI وتفاعل ربط ligation reaction. يتميز هذا الحامل باحتوائه على تسلسل من عديد الهيستيدين يسهل عملية تنقية البروتين المؤشب بالاعتماد على تقانة كروماتوغرافيا التآلفية. تم تحوير سلالة من *E. coli* بناتج تفاعل الربط، حرضت بعدها بهدف إنتاج البروتين P36/LACK.

تنقية البروتين واختباره بالتبصيم المناعي:

تمت تنقية البروتين المؤشب P36/LACK (40 kDa) من السيتوبلاسما بتقانة الكروماتوغرافيا التآلفية وباستعمال أعمدة أليفة لعديد الهيستيدين تحوي على شوارد النيكل. اوضح الرحلان الكهربائي على هلامة عديد الأكريلاميد SDS-PAGE نقاوة هذا البروتين . كما تم إجراء اختبار تبصيم مناعي immunoblotting باستعمال أضداد نوعية anti-6×His tag antibody موجهة ضد ذيل عديد الهستدين المدمج بالبروتين المؤشب.

اختبار مناعي للبروتين P36/LACK المؤشب:

اختبرت قدرة مجموعة من أمصال المرضى المصابين باللليشمانيا الجلدية على تعرف البروتين P36/LACK المؤشب باستعمال التبصيم المناعي، حيث تبين وجود أضداد في كل

الأمصال المستعملة تتعرف البروتين P36/LACK المؤشب. بناءً على هذه النتيجة يمكن افتراض إمكانية استخدام هذا البروتين في التشخيص المصلي للمرض إضافة لإمكانية اختبار دوره المناعي ومن ثم البحث في قدرته على الوقاية من داء الليشمانيا الجلدية في دراسات مستقبلية.