

CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
DEPARTMENT OF BIOCHEMICAL SCIENCES
&
UNIVERSITY OF NAVARRA
FACULTY OF PHARMACY
DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY
INSTITUTE OF TROPICAL HEALTH

**Molecular cloning of *YinP* gene from *Leishmania major* using two red fluorescent pXG-mCherry plasmids.
Valuable tools for gene expression location.**

Master thesis

Supervisor: Prof. Ing. Vladimír Wsól, Ph.D.
Tutor: Prof. Paul Nguewa, Ph.D.

Pamplona and Hradec Králové,
October 2015 - August 2016

Kateřina Musilová

KARLOVA UNIVERZITA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
KATEDRA BIOCHEMICKÝCH VĚD
&
UNIVERSIDAD DE NAVARRA
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INSTITUTO DE SALUD TROPICAL

**Molekulární klonování genu *YinP* z *Leishmania major*
za použití dvou červených fluorescenčních
pXG-mCherry plasmidů.
Cenné nástroje pro lokalizaci genové exprese.**

Diplomová práce

Vedoucí diplomové práce: Prof. Ing. Vladimír Wsól, Ph.D.

Tutor: Prof. Paul Nguewa, Ph.D.

Pamplona a Hradec Králové,
říjen 2015 - srpen 2016

Kateřina Musilová

DECLARATION

"I declare that this thesis is my original author's work. All sources of information, which have been used, are named in list of literature and quoted properly. This thesis has been used to obtain neither the identical nor a different degree."

PROHLÁŠENÍ

„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.“

In Hradec Králové, 22 August 2016

V Hradci Králové, 22. srpna 2016

.....
signature of candidate
podpis diplomanta

Firstly, I would like to express my gratitude to my supervisor Prof. Paul Nguewa, PhD. for the useful comments, remarks and guidance through the process of this master thesis.

Furthermore I would like to thank Miriam Algarabel for introducing me to the topic and methods as well for the support on the way.

Also, I like to thank the colleagues in the lab, especially Andrés Vacas, Connar Leeming and Pepe Peña, who have willingly helped me many times during my research. My thanks also belong to Martin Miláček for the provided technical assistance.

I would also like to acknowledge Prof. Ing. Vladimír Wsól, PhD. for being my supervisor in the Czech republic.

Finally, I would like to express my gratitude to my loved ones, who have supported me throughout entire studies. My deepest appreciation belongs to Tomáš Dostál, because this thesis would never been written without his continuous encouragement.

ABSTRACT

Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Biochemical Sciences

Candidate: Kateřina Musilová

Supervisor: Prof. Ing. Vladimír Wsól, Ph.D.

Tutor: Prof. Paul Nguewa, Ph.D.

Title of diploma thesis: Molecular cloning of *YinP* gene from *Leishmania major* using two red fluorescent pXG-mCherry plasmids. Valuable tools for gene expression location.

In the 21st century, leishmaniasis remains a major health problem in numerous developing countries. Around 2 million cases of leishmaniasis are reported every year and estimated mortality is over 20,000 deaths annually. Antileishmanial drugs are often unaffordable for affected people and display severe toxic side effects. Potent human vaccines are not available. This, together with increasing resistance, is a reason why new effective, safe, and affordable medicines are greatly needed.

Leishmaniasis is caused by *Leishmania* species. These parasites are transmitted by phlebotomine sand flies, which also provide to the leishmania environment necessary for their development into infective forms. The process of transformation into a stage infective for vertebrate hosts is called metacyclogenesis. Nowadays, genes, enzymes, and proteins possibly exhibiting a function in the metacyclogenesis are extensively examined. One of the genes suggested to play a role during the development of the *Leishmania* infective stage is *YinP*.

The main objective of this study was to reveal where *YinP* gene is expressed in the leishmanial cell. Two plasmids, pXG-mCherry12-*YinP* and pXG-mCherry34-*YinP*, were constructed to contribute to finding. In these vectors, *YinP* gen was inserted directly next to the gen for fluorescent protein (mCherry), to generate fluorescent fusion proteins expressed in the parasites. The created plasmids were introduced in *Leishmania major* parasites by electroporation.

Fluorescent microscopy disclosed that red fluorescence of mCherry fused with *YinP* was localized only in a part of nucleus. The parasites transformed with pXG-mCherry plasmids without *YinP* inserted displayed red fluorescence in the entire cell. Therefore, our results showed that *YinP* protein is expressed in the nucleus.

ABSTRAKT

Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra biochemických věd

Kandidát: Kateřina Musilová

Školitel: Prof. Ing. Vladimír Wsól, Ph.D.

Tutor: Prof. Paul Nguewa, Ph.D.

Název diplomové práce: Molekulární klonování genu *YinP* z *Leishmania major* za použití dvou červených fluorescenčních pXG-mCherry plasmidů. Cenné nástroje pro lokalizaci genové exprese.

Ve 21. století zůstává leishmanióza závažným zdravotním problémem v mnoha rozvojových zemích. Každoročně je hlášeno více než 2 miliony případů a odhadovaná mortalita je více než 20 000 úmrtí každý rok. Léky, které se proti leishmanióze v současnosti používají, vykazují závažné nežádoucí účinky a nakažení lidé si je často nemohou dovolit. Účinné humánní vakcíny zatím nejsou dostupné. Z těchto důvodů, spolu s narůstající rezistencí, jsou nové účinné, bezpečné a dostupné léky proti leishmanióze velice potřeba.

Původcem leishmaniózy jsou zástupci rodu *Leishmania*. Tito parazité jsou přenášeni pouštní muškou rodu *Phlebotomine*, které zároveň leishmáním poskytují prostředí nezbytné pro jejich vyžívání do infekčních forem. Proces přeměny do stádia infekčního pro savčí hostitele je nazýván metacyklogeneze. V současné době jsou intenzivně zkoumány geny, enzymy a proteiny, které by mohly hrát podstatnou roli během metacyklogeneze. Jedním z genů, který je pravděpodobně významný během vývoje infekčního stádia leishmanií je *YinP*.

Hlavním cílem této studie bylo odhalit, kde v buňce leishmanií je *YinP* exprimován. Za tímto účelem byly připraveny dva plasmidy: pXG-mCherry12-*YinP* a pXG-mCherry34-*YinP*. V těchto vektorech byl gen pro *YinP* vložen bezprostředně vedle genu pro fluorescenční protein (mCherry), aby po expresi v parazitární buňce vznikl fúzní fluorescenční protein. Vytvořené plasmidy byly do *Leishmania major* transfekovány pomocí elektroporace.

Následně fluorescenční mikroskopie odhalila, že červená fluorescence mCherry spojeného s *YinP* proteinem byla lokalizována pouze v části jádra, zatímco parazité transformovaní pXG-mCherry plasmidy bez vloženého genu *YinP* vykazovaly červenou fluorescenci v celé buňce. Naše výsledky tedy ukazují, že *YinP* protein je exprimován jako protein jaderný.

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1. INTRODUCTION

This project was realized as a part of a wider scientific project whose results were not published in time of writing this thesis. For this reason, some of information were confidential and because of that it was not possible to reveal the real identity and the sequence of the gene of interest examined during this project. In the text, the gene (and protein) is always mentioned under the name *YinP*.

Although a public attention is traditionally focused on the most common or in media promoted health problems (like cardiovascular diseases, cancer, HIV, diabetes mellitus and genetic disorders), there exist diseases which stand little bit out of the center of interest but which affect considerable amount of people and which have a great impact on their lives and health. Among these can be included a group of tropical neglected diseases (NTD), as defined by World Health Organization (WHO). NTDs comprise various diseases that share several main characteristics. They influence primarily poor populations in developing countries, where there is not appropriate access to clean water, health care and medicines. Some of these diseases are vector-borne and they are all bonded to a tropical and subtropical climate. There is a huge need to improve prevention, diagnostic procedures and treatment of NTDs.

Leishmaniasis is one of the NTDs. It is caused by protozoan parasites *Leishmania spp.* and transmitted by female sand flies. There are more forms of leishmaniasis that can be distinguished accordingly to their clinical manifestation and gravity. The severity varies from mild form, causing skin ulcers and scars, to damage of inner organs such as spleen and liver, which is the most dangerous form that is mainly lethal if untreated.

Around 1.5 million new cases of leishmaniasis and 25,000 deaths occur annually. Effective human vaccines are not available and interventions used to reduce amount of vectors and reservoir animals are not sufficient enough. There are a few drugs to treat this disease, unfortunately they are often not affordable for affected people and they display severe adverse effects. This, together with increasing occurrence of drug-resistant parasites, are reasons why new antileishmanial drugs are urgently needed. A research focused on a better understanding of protozoan organism and possible novel therapeutic targets seems to be essential. If we deepen our knowledge about protozoan parasites and improve comprehension of their biology, we can, step by step, get closer to the invention of new effective, safe and affordable medicines to treat leishmaniasis.

This project was realized during Erasmus+ programme at the Institute of Tropical Health at University of Navarra (ISTUN) under supervision of Dr. Nguewa, in the group of Molecular Parasitology, Therapeutic Targets and Drug Discovery.

Part of the research conducted under ISTUN is focused on leishmaniasis and its

causative agent *Leishmania major*. Different possible novel therapeutic targets are investigated and characterized to provide information needed for innovative and better antileishmanial drug development. This group recently found a novel gene called "*YinP*". It may play a critical role during metacyclogenesis driving to the development of the most infective parasite stage.

The main aim of this work was to localize *YinP* expression in the leishmania cells.

2. THEORETICAL PART

2.1 Leishmaniasis and leishmania

The Neglected Tropical Diseases (NTD), as defined by World Health Organization (WHO), nowadays counts almost 20 member diseases. The term "tropical" in the group's title implies an area, where these diseases are endemic - they occur mainly in tropical and subtropical climate. The word "neglected" refers to the fact that these diseases are not in the center of public and scientific attention - they seldom influence people in developed countries and there are more visible health issues such as malaria, tuberculosis or HIV.

Leishmaniasis is one of the neglected tropical diseases. It is caused by protozoan parasites from *Leishmania* spp. and transmitted by sand flies. In fact, more than only one distinct disease, leishmaniasis are a complex group of diseases caused by around 20 different species of *Leishmania* genus, that produce clinical symptoms ranging from simple self-healing skin ulcers to destruction of mouth and nasopharyngeal cavities with the neighbouring tissues to life-threatening systemic disease (Hepburn, 2003; L. J. Roberts et al., 2000).

2.1.1 Brief history

Leishmaniasis occurs in both the Old World (Africa, Asia, Europe) and the New World (North and South America). Usually, the Old World leishmaniasis is considered to have older history and its origin can be most likely settled in East Africa. However, it is not sure whether leishmaniasis was present in the New World before European invasion or not (Tuon et al., 2008; Aversi-Ferreira et al., 2014).

The first descriptions of a disease with features of cutaneous leishmaniasis can be traced to the 7th century BC, but due to archaeological findings, figures and statues, people already suffered from this disease around 2,000 BC or even 4,000 BC. The first mentions of a visceral form of leishmaniasis (also called kala-azar) are much recent. This form was probably noticed for the first time in the 1820s in India (Cox, 2002).

Although symptoms of visceral leishmaniasis were observed formerly, they were at first misinterpreted as a quinine-resistant malaria till 1903, when Sir W. B. Leishman, a Scottish pathologist, described oval formations, so far unknown parasites, in a spleen from a patient who died of kala-azar. At approximately the same time, Irish medical officer Ch. Donovan observed the same parasites in a blood and spleen tissue of kala-azar infected boy. Conflict over discovery rights was solved by Sir R. Ross, who created the genus *Leishmania* and gave the parasite binomial name *Leishmania donovani* (Cox, 2002; Hervaldt, 1999).

At the contrary to the discovery of the causative agents of visceral leishmaniasis, there still exist doubts about detection of parasites responsible for cutaneous form of leishmaniasis. Perhaps we will never know whether it was P.F. Borovsky, D. Cunningham or J. H. Wright who first described leishmanial parasites from skin sores (Cox, 2002).

2.1.2 *Leishmania* spp. taxonomy

Around 20 various species from genus *Leishmania* infect human and cause leishmaniasis. (Aversi-Ferreira et al., 2014; Hervaltdt, 1999)

A taxonomic classification of *Leishmania* spp. is complicated due to stormy changes in en eukaryotic taxonomy which started around 1990, when were emphasized phylogenetic inter-connections among species. At the moment, the system is not completely stable yet and the classification can be seen from different points of view.

Acoording to the classical system *Leishmania* spp. are parasites from genus *Leishmania*, family Trypanosomatidae, order Kinetoplastida, class Zoomastigopohra, subphylum Mastigophora, phylum Sarcomastigophora, subkingdom Protozoa, kingdom Protista. ("Mastigophora", 2003)

As consistent with the modern perspective, *Leishmania* spp. can be classed in genus *Leishmania*, family Trypanosomatidae, order Trypanosomatida, class Kinetoplastida, phylum Euglenozoa, supergroup Excavata, domain Eucaryota (Votýpka et al., 2013; Hughes & Piontkivska, 2003).

Leishmania spp. are assigned to two different subgenera: *Lesihmania* and *Viannia*. This division is based on the parasites' behavior in vector and primary host. Nevertheless, some authors also mention the third subgenus named *Sauroleishmania*, which is composed only of species from the Old World (Momen & Cupolillo, 2000). This subgenus does not comprise species infecting human or other mammals, as hosts of *Sauroleishmania* are reptiles (Bates, 2007). Though, *Sauroleishmania* can be found as a distinct genus excluded from *Leishmania* (Bañuls et al., 2007).

The related species inside the subgenus can be aggregated in so-called species complexes (Lopes et al., 2010; Bañuls et al., 2007).

At the beginning, species classification stood on geographical and microscopy characteristics and clinical manifestation. Later biochemical and immunological aspects assumed the main role. All single species are not always morphologically distinguishable, but minor or greater differences can be revealed by molecular biology techniques and iso-enzyme analysis (Lopes et al., 2010; Bañuls et al., 2007).

Regardless, the existence and independence of some species is still under discussion. Above all, *Leishmania chagasi* is a persisting source of discrepancy among scientists' opinions. Sometimes it is considered to be identical to *L. infantum*, in some sources is

treated like *L. infantum* subspecies and some authors separate these two as equivalent species (Dantas-Torres, 2006; WHO, 2010).

2.1.2.1 *Leishmania* subgenus

Species included in this genus can be found in both the Old World and the New World and they are transmitted by sand flies of the genus *Phlebotomus* (Lopes et al., 2010). These include the *L. donovani* complex: *L. donovani*, *L. infantum* and *L. chagasi*; the *L. mexicana* complex: *L. mexicana*, *L. amazonensis*; *L. venezuelensis*; *L. tropica*; *L. major*, *L. aethiopica*, *L. pifanoi*, etc. (Bañuls et al., 2007; Dantas-Torres, 2006).

2.1.2.2 *Viannia* subgenus

Viannia contains only species of the New World, which are transmitted by insects from the genus *Lutzomyia*, that is a different genus of the same Phlebotominae subfamily as the sand flies which spread leishmaniasis in the Old World (Sharma & Singh, 2008). Among the most important members of this subgenus belong *L. braziliensis*, *L. peruviana*, *L. colombiense*, *L. panamensis* and *L. guayanaensis* (Carreira et al., 2014; Bates, 2007).

2.1.3 Main forms of leishmaniasis

Three main clinical forms of leishmaniasis can be distinguished - visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL). A mucosal manifestation of leishmaniasis together with other subordinate forms such as diffuse cutaneous leishmaniasis (DCL), disseminated leishmaniasis and post-kala-azar dermal leishmaniasis (PKDL) is usually related to immune status of a patient (Vélez et al., 2015; Croft et al., 2006).

Individual species are usually connected largely with only one type of the disease, but it is not a strict rule (Figure 1). The type of leishmaniasis, which develops in a patient, depends mainly on the particular species of infective agent from *Leishmania* spp. and on an immune response of the patient (Aversí-Ferreira et al., 2014).

2.1.3.1 Visceral leishmaniasis

Visceral leishmaniasis is the most dangerous form of the disease which damages inner organs, parasites infect liver, spleen, lymph nodes and bone marrow. This form is mainly lethal if untreated and it can be deadly even with a treatment. VL is also known as black fever, DumDum fever or most frequently under Indian name kala-azar. Among its symptoms belong fever, weight loss, anemia, weakness, splenomegaly and hepatomegaly (L. J. Roberts et al., 2000). VL is usually caused by *L. donovani*, *L. infantum* and *L. chagasi* (Marcili et al., 2014; Palumbo, 2010; Aversí-Ferreira et al., 2014; González et al., 2015).

2.1.3.2 Cutaneous leishmaniasis

Cutaneous leishmaniasis is the most prevalent form (causing up to 75 % of all cases of leishmaniasis), which is by far not as severe as VL. There are many local names used for CL, e.g. oriental sore, balkh sore, chiclero ulcer or tropical ulcer. It usually presents as skin ulcers or sores localized at sandfly's bite site. They can be painless but, especially in case of secondary bacterial infection, they can hurt. Ordinarily the ulcers heal spontaneously after several months leaving atrophic scars behind. Despite the tendency to the spontaneous resolution, it has significant social, psychological impact (as a stigmatization, social exclusion) and financial consequences (Kassi et al., 2008). Among agents responsible for CL belong mostly *L. major*, *L. tropica* and *L. aethiopica* in the Old World and *L. mexicana*, *L. amazonensis* and *L. venezuelensis* in the New world (Amato et al., 2007; WHO, 2010; Hervaltdt, 1999; Kassi et al., 2008).

2.1.3.3 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis is a rare but very grisly form which can develop as a consequence of CL. It can appear months or years after primary cutaneous infection as a new reactivated metastatic ulceration. It affects in devastating way mucosal membranes and cartilages of nose, mouth and throat cavities and it induces disfiguration of face and it can lead to connected respiratory or alimentary disorders. However, MCL evolves in less than 5 % of patients who went through CL (Amato et al., 2007; L. J. Roberts et al., 2000). *L. braziliensis* is the major causative species of MCL, but cases with *L. amazonensis*, *L. guayanensis*, *L. aethiopica* and *L. panamensis* were reported (Palumbo, 2010; Averssi-Ferreira et al., 2014).

2.1.3.4 Diffuse cutaneous leishmaniasis

DCL is sometimes distinguished as a separate form of leishmaniasis but it can be included under CL as its uncommon subform (Lopes et al., 2010; L. J. Roberts et al., 2000). In contrast to CL, where ulcers develop at the place of infection transmission, skin lesions produced during DCL are non-ulcerative, disseminated and do not heal spontaneously. It occurs, above all, in patients with immunodeficiency disorders (WHO, 2008). Ordinarily, DCL is caused by *L. amazonensis*, *L. pifanoi*, *L. aethiopica* or *L. mexicana* (Hervaltdt, 1999; Carreira et al., 2014; Stark & Vidyashankar, 2014). Diffuse cutaneous leishmaniasis can be confused with disseminated leishmaniasis, but in DCL are not affected mucosal membranes and heavy parasite infiltration in skin lesions is typical. (Vélez et al., 2015) 90 % of all DCL cases is localized in Peru, Bolivia and Brazil (Lopes et al., 2010).

2.1.3.5 Post-kala-azar dermal leishmaniasis

PKDL is not well understood phenomenon caused by *L. donovani* (and very exceptionally by *L. infantum*) in patients after successful treatment for visceral leishmaniasis

(WHO, 2013). Its manifestation is restricted to skin lesions of macular, papular or nodular type. Face and trunk are typically involved. It is endemic in two main regions, namely South Asia (India) and East Africa (Sudan), but there are distinctions between the disease in those two areas (Mukhopadhyay et al., 2014). The African version occurs in up to 50% patients shortly after treatment and has tendency to self-healing, whereas Indian PKDL develop in around 5-10% usually 2 - 3 years after VL cure and requires therapy (WHO, 2013; McGwire & Satoskar, 2014). The mortality due to PKDL is not high, however socioeconomic impact is extensive. (Stark & Vidyashankar, 2014). Moreover, it is supposed that PKDL patients play important role as reservoirs for the disease transmission. (Hepburn, 2003) More factors, such as genetic susceptibility, history of UV skin damage and antimonial treatment, are believed to participate in the development of PKDL (Mukhopadhyay et al., 2014).

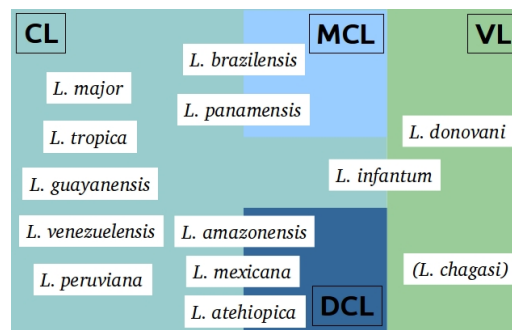


Figure 1: Main clinical forms of leishmaniasis and their causative agents

CL = cutaneous form, MCL = mucocutaneous form, DCL = diffuse cutaneous form, VL = visceral form. The most predominant causes of CL are *L. major*, *L. tropica* and *L. aethiopica*. The main species bounded to MCL is *L. braziliensis*. The clinical manifestation of the *L. infantum* infection can vary (VL occurs in West Africa and Americas, whereas CL is more frequent in Mediterranean region). Only chosen species are shown. *L. chagasi* is by some authors considered to be identical with *L. infantum*, other authors exclude it as a separate species. Based on Sundar and Chakravarty (2015); Hervaltdt (1999); Aversi-Ferreira et al. (2014); Stark and Vidyashankar (2014).

2.1.4 Distribution and burden of leishmaniasis

Unfortunately, information collected from many regions is not complete and fully reliable, therefore we have rather rough estimations about the distribution and current epidemiological status than accurate numbers.

Approximately 90 countries on four continents are affected by leishmaniasis. More than 350 million people live in areas where leishmaniasis is endemic and they are potentially exposed to risk of getting infected. Estimated incidence is 0.5 million new cases of visceral leishmaniasis and 1.5 million of cutaneous leishmaniasis every year. VL is with more than 50,000 deaths annually the second most frequent cause of death among parasitic diseases worldwide (behind malaria). Approximated number of disability-adjusted life years lost (DALY) is almost 2.5 million (WHO, 2010). DALY is a tool, which enable

to quantify an impact of a disease on population. It takes to account both the mortality and the morbidity. It can be understood as the sum of lost years of life (due to premature death) and years of healthy life lost due to disability (King, 2015; Spiegel et al., 2008).

The geographical distribution of this disease is determined by the occurrence of sandfly vectors. As they need for their development warm climate with high humidity, a leishmaniasis incidence is bounded to tropical and subtropical areas including South and Middle Americas, North and East Africa, South Europe, Middle East and South Asia (Hervaldt, 1999; Aversi-Ferreira et al., 2014).

There is a significant inconsistency between the prevalence of different forms of leishmaniasis.

In the last 10 years, around 65 % of CL cases were in only six states (in descending order): Algeria, Brazil, Iran, Afghanistan, Syria and Colombia. (Aversi-Ferreira et al., 2014) Around 90 % of VL cases are reported from five countries: India, Bangladesh, Brazil, Sudan and Nepal (Hailu et al., 2005). MCL and DCL are widespread mainly in Brazil, Bolivia and Peru (Lopes et al., 2010; WHO, 2010).

Maps showing regions with the highest incidence of cutaneous and visceral leishmaniasis in 2013 can be seen in **Figures 2 and 3**.

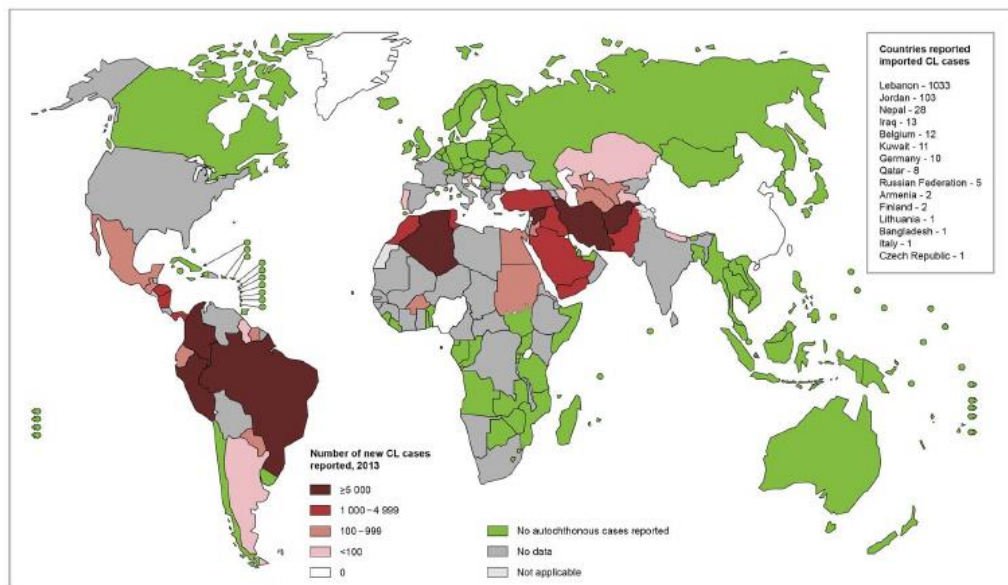


Figure 2: Geographic distribution of cutaneous leishmaniasis in 2013 according to WHO data

Adapted from World Health Organization webpage; modified. (Status of endemicity of cutaneous leishmaniasis, worldwide, 2013. Retrieved 2016/03/28 from http://www.who.int/leishmaniasis/burden/Leishmaniasis_Burden_distribution_VL_CL_2013.pdf).

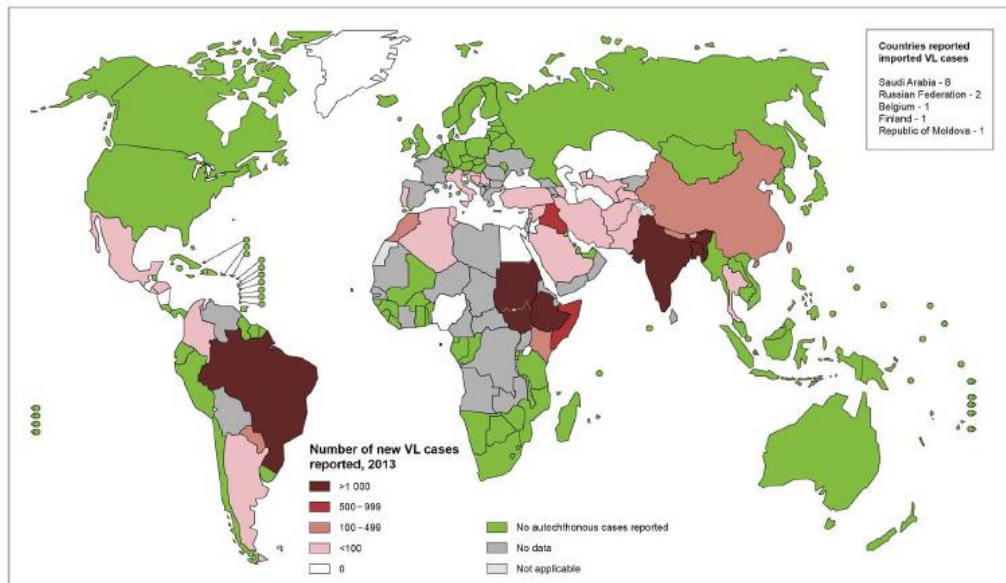


Figure 3: Geographic distribution of visceral leishmaniasis in 2013 according to WHO data

Adapted from World Health Organization webpage; modified. (Status of endemicity of visceral leishmaniasis, worldwide, 2013. Retrieved 2016/03/28 from http://www.who.int/leishmaniasis/burden/Leishmaniasis_Burden_distribution_VL_CL_2013.pdf).

2.1.5 Morphology of leishmania parasites

Leishmania are eukaryotic, single-celled, polarized organisms, which are obligative parasites. During their life cycle, two main morphotypes can be discriminated: amastigote and promastigote form (Kima, 2007). Although these types are quite different, they share numerous features, which are often typical for whole family Trypanosomatidae (**Figure 4**) (Lopes et al., 2010).

Amastigote stage is an intracellular form that is present in vertebrate hosts, in which usually provokes leishmaniasis symptoms. They are round, non-motile with internalized flagellum. In diameter, they usually measure from 3 μm - 7 μm (Lopes et al., 2010). Inside the mammalian host, they are phagocytosed by various immune cells (macrophages, neutrophils, dendritic cells), but they are resistant to microbicidal activities of the immune cells and survive (Prina et al., 2003). Inside the macrophages, they reside inside parasitophorous vacuole (which protects them from acid hydrolysis of the host phagolysosome), where they multiply (till macrophage ruptures and amastigotes are released) (Kima, 2007).

Promastigotes remain as extracellular parasites inside the insect vectors. Their cell is elongated with one attached flagellum, that is fundamental for their movement through the sand fly's body from midgut to foregut and proboscis, to empower them to infect new mammalian hosts. The flagellum can be same length as the cell or even longer

and it comes out from anterior pole (they move in the direction, where is the flagellum placed) (Bates, 2007).

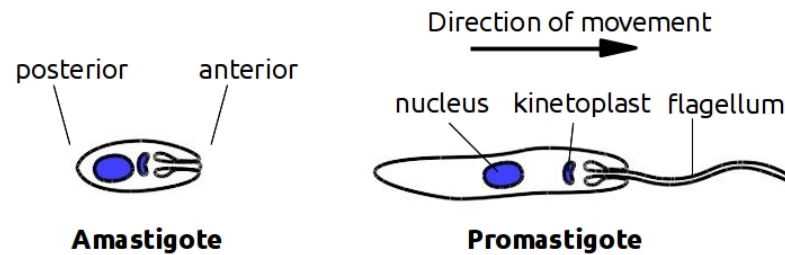


Figure 4: Schematic representation of *Leishmania* spp. amastigote and promastigote stage morphology

Adapted from Wikipedia; modified. (The six main morphologies of trypanosomatids. Retrieved 2016/03/09 from https://upload.wikimedia.org/wikipedia/commons/thumb/2/29/TrypanosomatidMorphologies_PlainSVG.svg/800px-TrypanosomatidMorphologies_PlainSVG.svg.png).

Flagellum

The flagellum is organ of motility which is present in all stages of the parasite's life, although the amastigote form has the flagellum internalized and non-functional. The flagellum consists of microtubules organized to a formation called axoneme. The importance of the flagellum for parasite does not lies only on the motility, but it also participates in leishmania attachment to host membranes and it may play role in intracellular signaling pathways (Moreira et al., 2014; Lopes et al., 2010).

Nucleus

As *Leishmania* are eukaryotic organisms, they have nucleus surrounded by porous membrane. The nucleus is composed form condensed chromatin, nucleoplasm, nucleolus and nuclear membrane (Lopes et al., 2010).

Kinetoplast

Leishmania spp. contain a special structure named kinetoplast. The kinetoplast is an organelle where mitochondrial DNA is concentrated. Kinetoplast DNA (kDNA) consits from circular structures of two types: maxicircles and minicircles. It is placed near the basal body, where the flagellum emerges from. In comparison to nuclear DNA, kDNA exhibits higher content of T-A base pairs and different density (Wheeler et al., 2012; Lopes et al., 2010; Hernández-Montes et al., 2012).

2.1.6 Life cycle of leishmania

Leishmania spp. are characteristic due to their life cycle, that contains three main stages: amastigote, procyclic promastigote and metacyclic promastigote form, but more stages can be identified (**Figure 5**). Individual stages differ in size, shape, motility and infectiousness. For their development, insect hosts are essential (Yao et al., 2010; Lopes et al., 2010; Bates, 2007).

2.1.6.1 Developmental stages of *Leishmania* spp.

Amastigote stage

The amastigote stage is the one present in human (mammal in general) and responsible for the disease manifestation. Amastigotes differentiate from metacyclic promastigotes after they transfer from an infected sand fly vector during its bloodmeal. When metacyclic promastigotes from insect's saliva reach the host, they are phagocytosed by macrophages. Inside the macrophage, metacyclic promastigotes develop into the amastigote stage. Amastigotes are non-motile, their flagellum is internalized. The shape is round and its size is around 5 μm in diameter. The multiplication in macrophages continues and it leads to the immune cell lysis. Amastigotes are released and phagocytosed by additional macrophages and the process repeats (Lopes et al., 2010; Bates, 2007).

Procyclic promastigote stage

When a sand fly feeds on an infected mammalian host it also ingests leishmania with the blood. Inside the sand fly's midgut, parasites transform into extracellular replicative forms - procyclic promastigotes. This development is stimulated by the increase of pH and decrease of temperature. Procyclic promastigotes are weakly motile, possess a flagellum and their cell has an elongated shape of size around 15 μm (Bates, 2007; Lopes et al., 2010).

Nectomonad promastigote stage

After several days of a rapid multiplication in midgut of sand fly, procyclic promastigotes undergo further transformation into a migratory form called nectomonad promastigote stage. This form is the biggest one in the whole cycle, highly motile and migrates, using the flagellum, to an upper midgut (Bates, 2007; Ramalho-Ortigao et al., 2010; Gossage et al., 2003). Nectomonad stage is capable of attaching to the epithelium of the midgut to avoid being excreted when fly defecates (Dostálová & Volf, 2012).

Leptomonad promastigote stage

When the nectomonad parasites reach the stomodeal valve of sand fly, they then attach to the surface and differentiate into leptomonad promastigotes, which continue

dividing. This stage is responsible for the production of promastigote secretory gel (PSG), which creates a kind of plug obstructing sand fly's anterior midgut. Before the sand fly can start feeding again, at least partial egesting of the PSG plug is necessary, and this action facilitate parasites' transmission (Bates, 2007; Ramalho-Ortigao et al., 2010; Gossage et al., 2003).

Haptomonad promastigote stage

A role of haptomonad promastigotes is still uncertain. They probably develop from some of nectomonad and leptomonad promastigotes and stay attached to the stomodeal valve and foregut of the sand fly. They are not infective, but perhaps contribute, with the PSG plug, to transmission of metacyclic promastigote parasites (Bates, 2007).

Metacyclic promastigote stage

At the end, some of the leptomonad promastigotes transform into the form infective for mammalian hosts - metacyclic promastigotes. This form is non-dividing and, in comparison to procyclic promastigotes, shorter (around 8.5 μm long). Their flagellum is longer than the cell. Metacyclic promastigotes are found in the thoracic midgut and proboscis of the sand fly, from which place they are deposited to a new host skin during a vector feeding (Lopes et al., 2010; Bates, 2007).

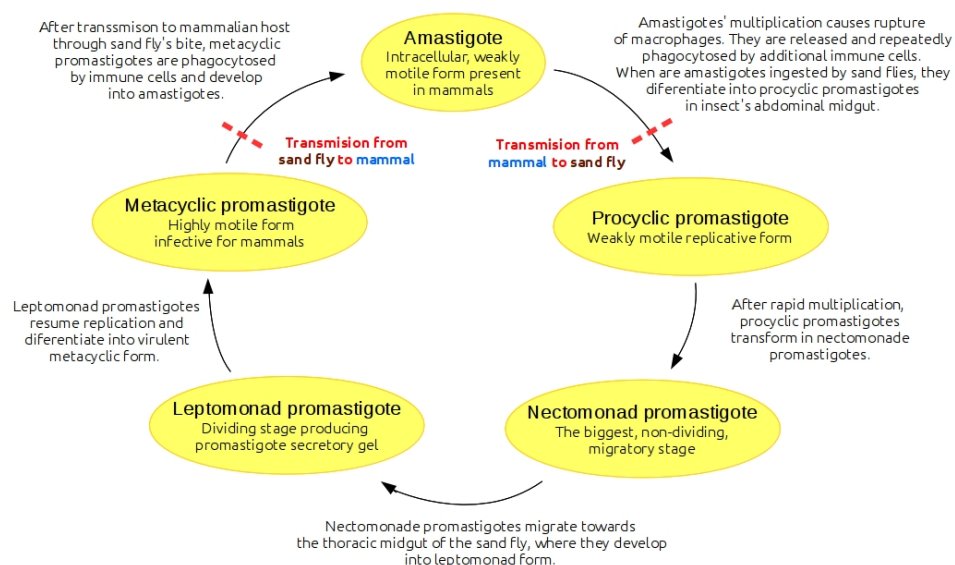


Figure 5: Diagram of life cycle of leishmania parasite

Schematic diagram showing five main stages in the life cycle of leishmania. Based on Ramalho-Ortigao et al. (2010); Lopes et al. (2010); Bates (2007); Sehgal et al. (2012).

2.1.6.2 Metacyclogenesis

All development changes, which leishmania parasites go through inside the insect vector, can be aggregated under the term metacyclogenesis. It includes the whole

transformation from replicative non-infective procyclic stage to metacyclic promastigotes, which are infective for human and animals. Metacyclogenesis is accompanied with number of changes - different protein expression, morphological transformation and biochemical changes (Moreira et al., 2014). The process is complex and fundamental for the development of virulent parasites able to infect mammals (Yao et al., 2010).

2.1.7 Transmission, vectors and reservoir animals

Leishmaniasis can be disseminated as zoonotic (from animals to humans) or anthroponotic (from humans to humans) infection (González et al., 2015).

Although a transmission via insect vector is the absolutely predominant way how leishmaniasis is widespread, other means of transmission were observed and described (Figueiró-Filho et al., 2004). Leishmaniasis can be transmitted through placenta from mother to foetus (congenital or vertical transmission) (Esch & Petersen, 2013). Blood transmission (shared needles, transfusion, organ transplantation) and sexual transmission were also reported. These routes of leishmaniasis transmissions are rare, but they may play considerable role in the areas, where invertebrate hosts are absent (Turchetti et al., 2014).

Seasonality of leishmaniasis closely correlates with changes in populations of vectors and reservoir animals during the year. In relation to climate changes (e.g. temperature, humidity), habitats suitable for vector survival are extending and leishmaniasis endemic areas are widening together with them. (Esch & Petersen, 2013; Antoniou et al., 2013)

2.1.7.1 Phlebotomine sand fly vectors

Only around 10 % of approximately 600 known species of sand flies from the genus *Phlebotomus* are able to distribute leishmania parasites (Sharma & Singh, 2008). However, these invertebrate hosts are not simple carriers, they provide to parasites environment fundamental for their development (Bates, 2007).

Species from two subgenera are responsible for *Leishmania* transmission: *Phlebotomine* in the Old World and *Lutzomyia* only in the New World (Esch & Petersen, 2013). Some species able to transmit leishmania parasites are considered to be permissive vectors (i. e. they support development of multiple *Leishmania* spp.), these are e. g. *Phlebotomine perniciosus*, *P. arabicus*, *Lutzomyia longipalpis*, *Lu. cruzi* and *Lu. evansi* (Antoniou et al., 2013). Conversely, *P. papatasi* and *P. serengeti* are specific vectors competent only for maturation of *L. major* and *L. tropica*, respectively (Volf & Peckova, 2007). This fact probably hangs together with specific molecules involved in attachment of the parasites to the sand fly midgut epithelium (Dostálová & Volf, 2012).

Phlebotomine sand flies are tiny (1.5 - 3 mm in length) and hairy. They are active

principally from dawn to dusk. They are silent and weak fliers, they do not expand far from their habitat. This is a reason, why leishmaniasis occurrence can vary within a narrow area. Only female sand flies suck blood to obtain enough nutrients for their eggs' development (Sharma & Singh, 2008). The transmission itself occurs during insect's feeding, when infected sand fly injects promastigote parasites into a mammal host (Esch & Petersen, 2013).

2.1.7.2 Reservoir animals

Most of *Leishmania* spp. causing leishmaniasis can persist in other mammalian hosts than only human. Infected animals can serve as a source of leishmania parasites for insect vectors and contribute to dissemination of the disease (de Sousa & Day, 2012). Both wild and domesticated animals can be reservoirs of *Leishmania* and the infection can be either symptomatic or latent (Grimaldi et al., 2012). Rodents and canines belong to the most frequent reservoir hosts, but it can be also felines or members of didelphidae (opossums) family (Esch & Petersen, 2013; González et al., 2015).

2.1.8 Possible control strategies to defeat leishmaniasis

There are different tactics, which can be used to restrain leishmaniasis. Among preventative methods can be comprised vector control, reservoir hosts control and vaccination. To minimize impact of leishmaniasis, early diagnosis and appropriate treatment are needed.

2.1.8.1 Vector control

As leishmaniasis is a vector-borne disease, one of the most effective precautions is to avert human-vector contact (Sharma & Singh, 2008). This can be reached through various means and a very important one is the education of people in affected areas about risks and preventative methods (Stockdale & Newton, 2013).

If possible, people in endemic areas should not stay outside during the night, when phlebotomine sand flies are most active searching for blood meal (Sharma & Singh, 2008). While sleeping, people are recommended to use bed nets (simple fine-mesh or insecticide treated) in order to protect themselves from contact with sand flies (Kimutai et al., 2009).

Sand flies are still sensitive to insecticides (although there is an evidence of DDT sand fly resistance) and their application helps to reduce risk of contamination (Stockdale & Newton, 2013). Nonetheless, it is not clear whether there are significant differences in effectiveness between indoor spraying, impregnation of bed nets, curtains, clothes or bedsheets (Kimutai et al., 2009; González et al., 2015). As other measure, surrounding of human habitations can be made unfitting for sand fly living and breeding. This envi-

ronmental interventions include control of reservoir animals and improving of housing standards (Sharma & Singh, 2008).

2.1.8.2 Control of reservoir hosts

Visceral leishmaniasis

There are two main causative agents of VL: *L. donovani* and *L. infantum*. *L. donovani* is predominant especially in the Old World (West Africa, India) and it is considered to be an anthroponotic infection, which means that humans are the main competent reservoirs. The key interventions to reduce incidence are early diagnostics and treatments (de Sousa & Day, 2012).

In contrast, *L. infantum* parasites are rarely found in the human skin and humans are only dead-end hosts. Because the reservoirs are animals, the infection caused by *L. infantum*, which occurs in the New World and Mediterranean region, is perceived as zoonosis (Grimaldi et al., 2012; Kimutai et al., 2009). The most important reservoir hosts are dogs, but wild and peridomestic animals (such other canine species, opossums, wild felines) can be also considerable (Roque & Jansen, 2014). Although the problem of canine leishmaniasis (CanL) is extensive mostly in Brazil, over 2.5 million dogs infected with *L. infantum* were revealed and zoonotic VL human cases were reported from distant European countries (related probably to dog transportation and climate changes) (de Sousa & Day, 2012; Otranto & Dantas-Torres, 2013).

More different approaches to control CanL are available, but none of them is without disadvantages. The main problems of traditionally performed culling (mostly euthanization) of leishmaniasis seropositive dogs are limited diagnostic sensitivity, low compliance of dog owners, ethical dilemma and insufficient evidence of its effectiveness (Otranto & Dantas-Torres, 2013). Preventive measures, namely vaccination and insecticide-impregnated collars (or topical spot-on administration), demonstrate better results, but high costs and necessity of repeated application hinder these means from field use (Esch & Petersen, 2013; de Sousa & Day, 2012; Otranto & Dantas-Torres, 2013).

Cutaneous leishmaniasis

The majority of CL cases is caused by *L. major* and *L. tropica*. *L. tropica* is ordinarily transmitted from person to person, whereas in leishmaniasis caused by *L. major* reservoir animals play a significant role (Pratlong et al., 2009). The leading species concerned in *L. major* epidemiology cycle are rodents (Esch & Petersen, 2013). Rodent population can be just hardly controlled, but possible methods are e.g. use of poisoned baits or traps, sanitization of area or destroying of rodent's burrows (flooding, deep ploughing) (Kimutai et al., 2009; Sharma & Singh, 2008; WHO, 2008).

2.1.8.3 Vaccination

Today, no human vaccine against leishmaniasis is available. There are more reasons for that such as lack of proper experimental animal models, limited knowledge about complex immune interactions between parasite and hosts and a shortage of money invested to the research focused on neglected diseases (Sundar & Singh, 2014; Kumar & Engwerda, 2014).

People cured of leishmaniasis develop lifelong immunity, which makes the disease a proper candidate to search for prophylactic vaccination. Nevertheless, therapeutic vaccines that could help to health restoration would be valuable too (Kumar & Engwerda, 2014).

Through history various approaches were attempted: phlebotomine vector's salivary proteins and DNA; live virulent, attenuated and killed parasites; recombinant proteins; DNA vaccines, etc (Rezvan & Moafi, 2015). Some of candidates were denied for insufficient immunogenicity, other for being unsafe and additional because of inconsistent results (Duthie et al., 2012).

In Brazil, two vaccines are licensed against canine VL: one based on subunit antigen (fucose mannose ligand antigen) and the other containing A2 recombinant protein of *L. donovani* (Duthie et al., 2012; Sundar & Singh, 2014). One prophylactic vaccine against canine leishmaniasis is authorized by European Medicines Agency. It is composed of purified proteins secreted by *L. infantum* with saponin adjuvant. It was proven, that immune response provoked by this vaccine lasts at least one year (Moreno et al., 2014).

2.1.8.4 Current therapy

When all preventative interventions fail and leishmaniasis infection develops, chemotherapy comes into play. Several drugs to treat leishmaniasis are available. Unfortunately, all of them have certain limitations such as high toxicity, increasing resistance, parenteral administration route, species-selective activity, low safety, etc. (de Menezes et al., 2015). Novel leishmanicidal drugs remain a priority. Several promising candidates with proven antileishmanial activity have been suggested such as amidines, selenium derivatives and others (Fernández-Rubio et al., 2015; Soeiro1 et al., 2013). New chemotherapeutic targets are still searched (Sangshetti et al., 2015).

In case of cutaneous form, a crucial problem is diversity of numerous species causing the infection, because they noteworthy vary in their responsiveness to treatment (Croft & Olliaro, 2011).

Guidelines for the recommended treatments reflect clinical forms of leishmaniasis, species of causative agent, geographical placement, extension of infection symptoms and patient factors (as immune system state) (Sundar & Chakravarty, 2015).

To avoid (or at least delay) development of resistance, shorten therapy duration

and reduce adverse effect severeness, variable combined multidrug protocols were tested (de Menezes et al., 2015). As favourable combinations were observed e. g. combination of miltefosine with paromomycine or with liposomal amphotericin B (Singh et al., 2012; Barrett & Croft, 2012).

Pentavalent antimonials

Two different drugs containing pentavalent antimonium in the structure are now in use: sodium stibogluconate and meglumine antimoniate. They are used in therapy more than a half century and they are first line drugs with the exception of regions with high occurrence of resistant strains. Namely in Bihar state in India, antimonial therapy fails in up to 65% cases, due to resistance, insufficient duration of treatment and other reasons (Sundar & Chakravarty, 2015).

The mechanism of action is not fully clarified, but it is supposed that these molecules work as pro-drugs and antileishmanial activity appears after reduction to trivalent antimony. Injection application is necessary, administration can be intramuscular (**IM**), intravenous (**IV**) or intralesional (Sangshetti et al., 2015).

Their main advantage is low cost and therefore good availability. On the other hand, adverse effects including pancreatitis, cardiotoxicity (with QT interval prolongation), nephrotoxicity and hepatotoxicity are not rare (Frézard et al., 2009). Furthermore, long therapy course (usually 28 - 30 days) and painfulness of daily injections can lead to patient non-compliance (de Menezes et al., 2015).

Amphotericin B deoxycholate

Amphotericin B (AmB) was originated as macrolide polyene antifungal drug (Sangshetti et al., 2015). It interacts with cell membrane of parasites causing its weakening, leakage of monovalent ions and subsequently cell lysis. In areas with extended antimonial resistance, Amphotericin B is a drug of choice (Singh et al., 2012).

AmB is administered IV, usually in a long slow infusion. Its efficacy is extremely high and in the current no resistance is observed. Regrettably, severe toxic effects are very frequent. These are infusion reaction (fever, hypotension, nausea, tachypnoe, etc.), electrolyte imbalance connected with nephrotoxicity and hepatotoxicity. Some of them can be prevented by pre-medication. Monitoring and hospitalization for couple of weeks are needed (de Menezes et al., 2015; Sundar & Chakravarty, 2015).

Lipid formulations of amphotericin B

A few different formulations of amphotericin B were prepared in order to decrease adverse effects. From new lipid-associated formulations (liposomal, colloid suspension and lipid complex) liposomal amphotericin B proved the lowest renal toxicity (Botero et al., 2014).

Side effects exhibited by lipid formulations are significantly decreased. The AmB effectiveness was conserved and pharmacokinetics and bioavailability were improved. The therapy length was reduced (from 30 - 40 days for conventional AmB) to only 3-5 days (de Menezes et al., 2015). In India, single-dose therapy has been shown to cure 95% treated patients with VL (Barrett & Croft, 2012). The only administration route is parenteral through IV infusion, although oral formulation was successfully tested on mice (Wasan et al., 2009). The biggest barrier for wider use of these drugs is their very high price. Moreover, heat instability of lipid AmB formulation is problematic within affected areas (de Menezes et al., 2015).

Miltefosine

In the current, miltefosine, is the only one existing oral therapy against leishmaniasis. Initially it was developed as an anticancer drug. Miltefosine cumulates in parasites and induces apoptosis, however, the precise mechanism of the action is not well characterized, perhaps it is related to inhibition of some metabolic pathways (Dorlo et al., 2012).

Generally, miltefosine is well tolerated, among its side effects belong nausea, vomiting and rarely nephrotoxicity and hepatotoxicity (Dorlo et al., 2012). Teratogenicity of miltefosine was evidenced in mice and its use is prohibited for fertile women without satisfactory contraception (Murray, 2012). Resistance have not be reported from the nature, but risk of the resistance development has to be taken into account, due to its long biological half-life and easy induction of resistance under laboratory conditions. It is effective against *L. donovani*, but *L. braziliensis* and *L. major* seems to be insensitive to it. The most important advantage of miltefosine is doubtless its oral administration. (de Menezes et al., 2015)

Paromomycin

Paromomycin (also known under the name aminosidine or monomycin) is a broad-spectrum aminoglycoside antibiotic and its effect leans in inhibition of proteosynthesis. It was isolated from *Streptomyces rimosus* var. *paromomycinus* (Wiwanitkit, 2012).

For the VL treatment IM administration is used, whereas in case of CL topical application is preferred (Sangshetti et al., 2015). It is relatively cheap drug but its effectiveness vary with geographical distribution (it is not effective in east Africa, whereas for CL treatment in Brazil are results excellent). (Sundar & Chakravarty, 2015) After parenteral administration adverse effects such as ototoxicity, hepatotoxicity and nephrotoxicity can be observed (de Menezes et al., 2015).

Pentamidine

Pentamidine is an antimicrobial drug primarily used to prevent or treat pneu-

mocystis pneumonia. Two different salts are in use: isethionate and methanesulfonate. The mechanism of pentamidine action is not explained properly, but it probably interferes with kinetoplast DNA, mitochondrial function and with protein like ubiquitin (Sangshetti et al., 2015; Nguewa et al., 2005).

As antileishmanial medicine, pentamidine is mainly used in combined protocols (with allopurinol or pentavalent antimonials) against CL in South America, because its species-specific effectiveness and increasing resistance (Croft & Olliario, 2011; Monge-Maillo & Lopéz-Veléz, 2013). Pentamidine is administered IM and its side effects include myalgia, pain at the site of application, headache, pancreatic damage (possibly leading to development of diabetes mellitus), hypotension, tachycardia and electrocardiographic changes (Sangshetti et al., 2015; Oliveira et al., 2011).

Azole antifungal drugs

There is evidence that some of triazole derivatives from azole antifungal drugs have effect against leishmaniasis (Barrett & Croft, 2012). In the Old World, several trials reported activity of fluconazole against *L. major* and itraconazole against *L. tropica* (Croft & Olliario, 2011).

Combined regimens

Till now, there are no available drug combinations to treat leishmaniasis. Nonetheless, rational co-administration of different antileishmanial medicines is being practised in order to dwindle drug dosage (together with toxicity), shorten therapy length and decline selection of insensitive parasites resulting in resistance development (Barrett & Croft, 2012).

The combinations of liposomal amphotericin B with paromomycin or miltefosine were evaluated as highly efficient. Multidrug regimens consisting of miltefosine with paromomycin and paromomycin with sodium stibogluconate have shown similar efficacy to monotherapies but were appraised as beneficial due to shorter therapy course and milder adverse effects (de Menezes et al., 2015).

Several co-administration regimens of antileishmanial agents with drugs from various ATC categories were compared to conventional protocols. Combination of antimonials with pentoxifylline resulted in significantly shorter and more effective treatment of MCL and CL (Sundar & Chakravarty, 2015; Barrett & Croft, 2012). As well, pentamidine administered in combination with allopurinol showed a positive effect (Singh et al., 2012; Monge-Maillo & Lopéz-Veléz, 2013). Different approach in order to increase effectiveness of antileishmanial treatment is combination with immunomodulators to contribute to immune response and accelerate self-cure of cutaneous leishmaniasis. Classical antiprotozoan drugs together with e.g. imiquimod or BCG vaccine were tested and demonstrated higher cure rates (Barrett & Croft, 2012).

2.2 *YinP*

Recently, many genes related to the metacyclogenesis process of *Leishmania* were discovered. They were analysed in order to validate them as possible novel therapeutic targets. One of the genes investigated in the ISTUN was *YinP*. *YinP* is strongly conserved gene observed in many species from yeast to human, which may be involved in processes connected with cell proliferation (Algarabel-Olona et al., 2015).

Firstly, *YinP* structure was analysed using in silico methods and its structure was then predicted (**Figure 6**). According to the anticipated secondary and tertiary structures, a nuclear localization signal (NLS) was discovered and the subcellular localization of *YinP* in *Leishmania* spp. was presumed as nuclear (Algarabel-Olona et al., 2015).

The presence of an oncogenic domain in the gene was revealed, supporting the idea, that the gene can be involved in cell differentiation. Experiments performed in the ISTUN showed that the expression of *YinP* was considerably higher in the infective metacyclic stage of *Leishmania major* parasites than in procyclic stage (Algarabel-Olona et al., 2015).

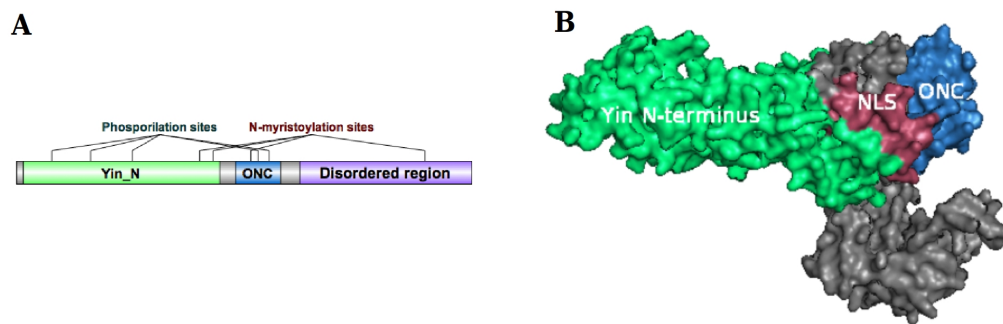


Figure 6: Predicted secondary and tertiary structure of the *YinP* gene

(A) Secondary structure prediction with phosphorylation and N-myristoylation sites illustrated. Three domains are anticipated to be localized in *YinP*: oncogenic (ONC), N-Terminal (*Yin_N*) and disordered region. (B) Tertiary structure prediction showing domains and nuclear localization signal (NLS). Adapted from Algarabel-Olona et al. (2015)

2.3 Fluorescent proteins as molecular biology tools

Simply, fluorescent proteins (FPs) are substances able to glow in specific color under UV light. These are proteins able to absorb electromagnetic energy of specific wavelength and emit light of different frequency (Day & Davidson, 2009).

They consist from protein shell structure and chromophore, which is proper emitting component. FPs are usually made up from ≈ 230 amino acids organized in a shape called β -barrel scaffold. Chromophore is formed from residues of three amino acids (which are part of the protein) by posttranslational modifications and it is localized in the

center of the compact β -barrel structure (Stepanenko et al., 2013; Kremers et al., 2011).

Usage of FPs is extremely wide comprising labeling at different biological levels from organisms to tissues, cells, organelles to single proteins or nucleic acids. They also enable to examine localization, function and interactions of various bio-constituents, perform drug screenings and much more (Chudakov et al., 2010).

As insinuated, protein localization is only one of uncountable number of applications, for which can be FPs employed. Creating in-frame fusion of a protein of interest with suitable FP is effective technique of protein localization. N-terminal or C-terminal fusion proteins can be created to avoid influence of FP on posttranslation modifications and subsequent impact on gene expression localization (Crivat & Taraska, 2012).

Probably the most know and best examined FP is green fluorescent protein (GFP) originally isolated in early 1960s from the jellyfish *Aequorea victoria* (Kremers et al., 2011). However nowadays, wide palette of fluorescent proteins with different attributes is available to scientists providing them powerful tools for various research fields (Day & Davidson, 2009). To select an appropriate fluorescent protein for employment for a certain purpose, it is necessary to consider several FP characteristics (Chudakov et al., 2010).

Brightness determines needed dose of excitation light to provide a sufficient fluorescent signal. Percentage of EGFP (enhanced derivative of GFP) brightness is commonly used for brightness quantification. Time required for a chromophore maturation (post-transaltion modifcaion, process through which FP becomes fluorescent) is measured as a maturation rate. For some applications fast maturation is essential. Other of the key factors of FPs characterization is photostability, which depends on many factors, both intrinsic and extrinsic. It should be also taken in account, that pH range can influence fluorescence brightness. Blue and red variants are usually less sensitive to pH changes then green and yellow FPs. Normally, fluorescent brightness increases with increasing pH (Chudakov et al., 2010). Tendency to form oligomers or aggregates can be, in some of utilizations, limiting factor of fluorescent protein use. In subcellular localization of a protein expression, forming of FP oligomers can interfere with normal function and localization of the protein of interest. On the other hand, dimeric or tetrameric structure can be sometimes advantage (Campbell et al., 2002; Chudakov et al., 2010).

2.3.1 Use of fluorescent proteins in *Leishmania* spp. investigation

During last twenty years, molecular biology techniques and knowledge about *Leishmania* have made noticeable progress. Different transgenic *Leishmania* species were successfully engineered expressing both episomal and integrated genes for fluorescent proteins. It was proven that FPs can be advantageously used for protein tagging, drug and vaccines testing or study of disease progression (Rocha et al., 2013).

Gene for GFP was cloned into episomal pXG vector and used to obtain green flu-

orescent *L. donovani* and *L. major*. After C-terminal fusion with gene for lipophosphoglycan, its function was conserved and expression was due to fluorescence localized in small region of the parasite cell (Ha et al., 1996).

L. major, *L. infantum* and *L. tarentolae* strains stably expressing GFP were prepared using pLEXSY vector resulting in integration of GFP gene in the specific locus in the parasite chromosome. Green fluorescence was observed in both promastigote and amastigote stage and transfected parasites were successfully used for *in vivo* imaging (Bolhassani et al., 2011; Kamau et al., 2001).

Fluorescent *L. amasonensis* parasites stably expressing both RFP (red fluorescent protein) and GFP were developed and red version was used for fluorimetric drug screening (Rocha et al., 2013).

Red fluorescent *L. major* parasites of both types of expression (episomal and constitutive) were also achieved. *In vitro* studies showed that mCherry fluorescence is proportional to number of parasites. It was validated that transgenic parasites emitting red fluorescence can be employed for *in vivo* studies (Calvo-Álvarez et al., 2012; Vacchina & Morales, 2014).

2.3.2 mCherry

mCherry is a member of mFruit fluorescent protein series, which was derived through extensive modifications from DsRed FP originally isolated from sea anemone *Discosoma striata* from Anthozoa class. Letter “m” in name refers to monomeric structure of these proteins (Day & Davidson, 2009).

mCherry is, besides mStrawberry and mOrange, the most promising member of the mFruit group. Its emission peak lays at 610 nm and excitation maximum is presented at 587 nm (Chudakov et al., 2010). Important advantages of mCherry are high photostability and tissue penetration and the fact that it does not require any posttranslation modifications. mCherry maturation is quick and enable real-time analysis (Vacchina & Morales, 2014). Although, its intrinsic brightness level is only $\approx 50\%$ of EGFP, it is sufficient for use, while low brightness with higher photostability can lead in better signal-to-noise ratio (Chudakov et al., 2010).

The structure of mCherry protein is usually described as β -barrel scaffold which consists from 11 β -sheets (**Figure 7**). Both termini (amino and carboxy) head out form compact scaffold and are available for fusion with other proteins. α -helix with chromophore are placed in the center of the barrel (Day & Davidson, 2009).

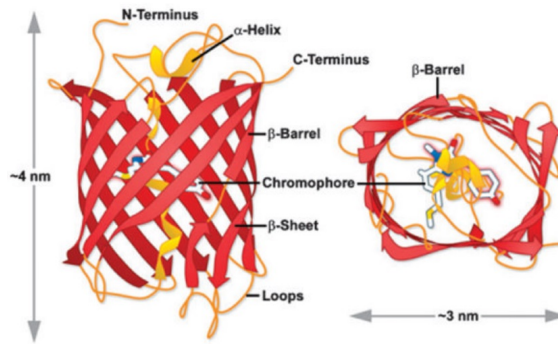


Figure 7: mCherry structure

β -barrel scaffold structure of mCherry consists from 11 β -sheets as in all FPs, but shows more elliptical symmetry than GFP derivatives. Both termini (amino and carboxy) head out from compact scaffold and are available for fusion with other proteins. α -helix with chromophore are placed in the center of the barrel. Adapted from Day and Davidson (2009), modified.

2.3.3 pXG-mCherry plasmids

One investigation project of the group of Molecular Parasitology, Therapeutic Targets and Drug Discovery of Institute of Tropical Health (ISTUN) under the University of Navarra is focused on designing novel molecular biology tools, which enable to study leishmania parasites and leishmaniasis. In this group, two pXG-mCherry plasmids were constructed (**Figure 8**).

pXG-hyg vector was used as a mainstay to which DNA inserts were ligated. These inserts contain mCherry sequences fitted with various restriction enzyme sites at the ends. pXG-mCherry12 plasmid is suitable for creation of N-terminal fluorescent fusion proteins whereas pXG-mCherry34 was designed to provide C-terminal fusion proteins (Vacas et al., 2015).

Both plasmids were electroporated into *Leishmania major* parasites and mCherry expression was proven with fluorescent microscopy. It was also demonstrated that mCherry fluorescence of transformed parasites was proportional to the number of parasites, pXG-mCherry plasmids can be therefore used as a tool for fluorometric quantification (Vacas et al., 2015).

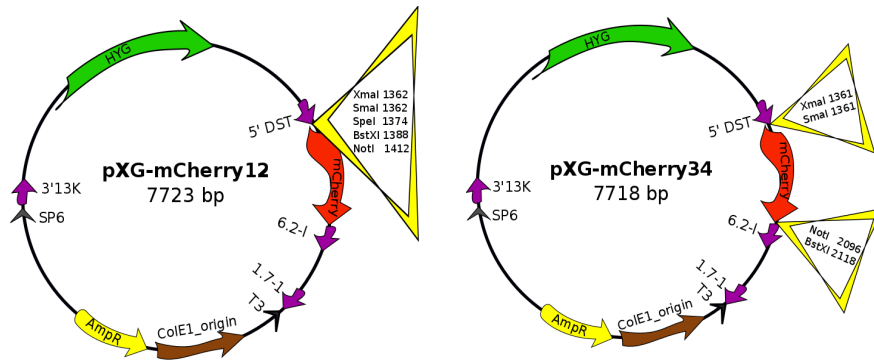


Figure 8: Structure of pXG-mCherry12 and pXG-mCherry34 plasmids

pXG-mCherry12 was designed to create N-terminal fusion fluorescent proteins whereas pXG-mCherry34 produces C-terminal fusion proteins. Plasmids were created in the group of Molecular Parasitology, Therapeutic Targets and Drug Discovery of ISTUN. Adapted from Vacas et al. (2015).

3. THESIS GOALS

The aims of this thesis were:

- to construct pXG-mCherry12-*YinP* plasmid designed to create N-terminal fusion fluorescent protein;
- to construct pXG-mCherry34-*YinP* plasmid designed to create C-terminal fusion fluorescent protein;
- to localize the expression of YinP protein in *Leishmania major* cells through transfection technique using the constructed plasmids.

4. EXPERIMENTAL PART

4.1 Material

4.1.1 Chemicals

European bacteriological agar - Laboratorios CONDA, Spain

0.1% BSA - Takara Bio Inc., Japan

0.1% Triton X-100 - Takara Bio Inc., Japan

Ethidium bromide solution 10 mg/ml in H₂O - SIGMA, United States

Ethylendiaminetetraacetic Acid Disodium Salt 2-hydrate (**EDTA**) - PanReac, Spain

Boric Acid (for analysis) - AppliChem PanReac ITW Companies, Germany

Tris (Tris[hydroxymethyl]aminomethane) ultrapure - Affymatrix, United States

dNTP Mix 40mM (10mM each)- Bioline, Germany

MgCl₂ Solution, 25 mM - Applied Biosystems, United States

Orange G (Sodium salt, Dye content approx. 96%) - Sigma-Aldrich, Germany

1Kb Plus DNA Ladder - InvitrogenTM, United States

DAPI I Counterstain - Abbott Molecular Inc., United States

Ampicillin sodium salt - Sigma-Aldrich, Spain

Gentamicin sulfate salt - Sigma-Aldrich, Spain

Kanamycin sulfate from *Streptomyces kanamyceticus* - Sigma-Aldrich, Spain

Hygromycin B Gold (100 mg/ml) - InvivoGen, France

Potassium chloride (KCl), purissimum - PanReac, Spain

Calcium chloride dihydrate (CaCl₂), ≥ 99 % - Sigma-Aldrich, United States

di-Potassium Hydrogen Phosphate (K₂HPO₄), anhydrous - Panreac quimica, Spain

Natriumhydrogencarbonat zur Analyse (NaHCO₃) - Merck KGaA, Germany

Magnesium chloride (MgCl₂), anhydrous, 99% - Alfa Aesar, Germany

HEPES sodium salt ≥ 99.5 % (titration) - Sigma-Aldrich, United States

Hemin, from bovine, ≥ 90 % - Sigma-Aldrich, China

Fetal bovine serum (**FBS**), qualified, E.U.-approved, South America origin - GibcoTM

Adenine suitable for cell culture, BioReagent - Sigma-Aldrich, China

Biotin ≥ 99 % (TLC), lyophilized powder - Sigma-Aldrich, Spain

6-Biopterine ≥ 97 % - Sigma-Aldrich, Spain

Triethanolamine PA - PanReac, European Union

2-Propanol, pharma grade - AppliChem PanReac ITW Companies, Spain
Isoton[®] II Diluent - Beckman Coulter, Inc., Spain
Alcohol etílico absoluto - Productos Oppac S.A., Spain
Glycerol (RFE, USP, BE, Ph. Eur.)- PanReac Quimica, Spain
Formaldehyde 3.7-4.0 % buffered to pH=7 and stabilized with methanol for clinical diagnosis - AppliChem PanReac ITW Companies, Spain
Hydrochloric Acid 37 % (USP-NF, BP, Ph. Eur.) - Panreac, European Union
H₂O

4.1.2 Enzymes

AmpliTaq[®] DNA Polymerase - Applied Biosystems, United States
NotI - Takara Bio Inc., Japan
Antarctic Phosphatase 5,000 U/ml
T4 DNA Ligase 1U/μl - Invitrogen[™], United States
EcoRI - Takara Bio Inc., Japan

4.1.3 Buffers

10X PCR Buffer II - Applied Biosystems, United States
TBE 1X - prepared in the laboratory (**Section 4.2.6**)
10XH Universal buffer - Takara. Bio Inc., Japan
10X Loading buffer - Takara Bio Inc., Japan
Loading buffer with Orange G - prepared in the laboratory (**Section 4.2.6**)
5X T4 DNA Ligase Buffer - Invitrogen[™], United States
10X Antarctic Phos Reaction Buffer - New England BioLabs Inc., United States
Cytomix buffer - prepared in the laboratory (**Section 4.2.16**)
PBS pH 7.2 (1x), Phosphate Buffered Saline - Gibco[™], United Kingdom

4.1.4 Media

Agarose D1 medium EEO - Laboratorios CONDA, Spain
Bouillon trypticase soja (TSB-D) - bioMérieux SA, France
S.O.C. Medium 10 ml - Thermo Fisher Scientific, United States
(composition: 2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose)

Medium 199 - SIGMA, United States

(Modified, with Earle's salts, without L-glutamine, sodium bicarbonate, and phenol red, powder, suitable for cell culture)

M199* 1X - prepared in the laboratory (**Section 4.2.14**)

4.1.5 Kits

QIAquick[®] Gel Extraction Kit - QIAGEN, Germany

QIAprep[®] Spin Miniprep Kit - QIAGEN, Germany

TOPO TA Cloning[®] Kit, with pCR[®] 2.1-TOPO Vector - Invitrogen[™], United States

4.1.6 Primers

All primers used during this project were made by SIGMA company accordingly to required sequences. Their parameters are given in **Table 1**.

Table 1: Primers used during the research and their specification

| Primer's name | Sequence | |
|---------------|---------------------------------|----------------------|
| | GC content | Melting temperature* |
| YinP Fw | 5'-GCGGCCGCTATTNNNNNNNNNNNNNNNN | |
| | 53.3 % | 63.8 °C |
| YinP12 Rv | 5'-GCGGCCGCNNNNNNNNNNNNNNNN | |
| | 71.4 % | 68.2 °C |
| YinP34 Rv | 5'-GCGGCCGCNNNNNNNNNNNNNNNN | |
| | 62.5 % | 66.4 °C |
| T3 | 5'- ATTAACCCTCACTAAAGGGA | |
| | 40.0 % | 50.3 °C |
| IST39 | 5'-CCC GCCGACATCCCCGACTA | |
| | 70.0 % | 63.9 °C |
| IST40 | 5'-GGGTCACGGTCACCACGCC | |
| | 73.7 % | 63.9 °C |
| IST7 | 5'-NNNNNNNNNNNNNNNNNNNNNNNN | |
| | 47.6 % | 56.5 °C |
| IST9 | 5'-NNNNNNNNNNNNNNNNNNNNNNNN | |
| | 57.1 % | 60.0 °C |
| YinP-Leish Fw | 5'-NNNNNNNNNNNNNNNNNNNNNNNN | |
| | 55 % | 56.1 °C |
| YinP-Leish Rv | 5'-NNNNNNNNNNNNNNNNNNNNNNNN | |
| | 55.0 % | 57.7 °C |

*Data about melting temperature obtained by OligoAnalyzer 3.1 - Integrated DNA Technologies (<https://eu.idtdna.com/calc/analyzer>).

4.1.7 Biological material

competent bacteria *E. coli* (strain Sbt14)

E. coli bacteria (strain Sbt14) containing pXG-hyg-mCherry12 plasmids

E. coli bacteria (strain Sbt14) containing pXG-hyg-mCherry34 plasmids

E. coli bacteria (strain TOP10) containing pXG-hyg-*YinP* plasmid

Leishmania major promastigotes (Lv39c5) - kindly provided by Manuel Soto (Madrid)

4.1.8 Laboratory instruments

Disposable

Sterile eppendorf tubes (0.5 ml, 1.5 ml and 2.0 ml size), sterile falcon tubes (15 ml and 50 ml), MicroAmp[®] Reaction Tubes without Cap (0.2 ml), MicroAmp[®] 8-Cap Strips, plastic Petri dishes, sterile L shaped plastic spreaders, sterile plastic inoculation loops, sterile wooden toothpicks, sterile pipette tips, sterile surgical blades, cryovials, Corning[®] bottle-top vacuum filter systems (0.22 µm, PES, sterile), Parafilm[®] M, Gene Pulser[™] cuvettes (0.4 cm), Accuvette ST (25 ml vials and caps), 0.22 µm Millex[®] GP Filter units, sterile plastic syringes, microscope slides, cover slips, latex and nitrile gloves.

Nondisposable

Automatic pipettes, PIPETBOY acu, pyrex glassware (media bottles, beakers, Erlenmeyer flasks, conical flasks) of different volumes, graduated cylinders, electrophoresis apparatus (chambers, combs and gel trays) of various sizes, spatulas, test tube trays, magnetic stir bars.

4.1.9 Laboratory equipment

| | |
|----------------------|---|
| Thermocycler | GeneAmp [®] PCR System 2400 - Perkin Elmer |
| Thermomixer | Thermomixer comfort - Eppendorf |
| Shaker | Aquatron - Infors HT Stuart orbital shaker SSL1 - Bibby Scientific Limited |
| Vortex mixer | Raex top - Heidolph Vortex mixer 2x ³ - VELP Scientifica |
| Tube rotator | Small tube rotator - JP Selecta |
| Magnetic stirrer | Asincro 379 - JP Selecta |
| Thermostatic bath | IDL-AG20 - Indelab |
| Thermostatic cabinet | Incubator TC 135 S - AQUALYTIC |
| Microcentrifuge | Mini centrifuge C-1200 - Labnet International, Inc. |
| Benchtop centrifuge | Micro Centaur Sanyo - MSE |

| | |
|--------------------------------|--|
| | Allegra X-12R Benchtop Centrifuge - Beckman Coulter (rotor SX4750) |
| Power supply | PowerPack 300 - Bio-Rad |
| Scales | Pasica 1216 M - Sartorius Digital Analytical Balance Scale 125A SCS - Precisa |
| pH meter | micropH 2001 - Crison |
| Gel imaging system | Universal Hood II - Bio-Rad |
| Spectrophotometr | NanoDrop 1000 Spectrophotometer - Thermo Fisher Scientific |
| UV Transilluminator | UV 995551 - Vilber Lourmat |
| Microscope | Eclipse TS100 - Nikon Instruments Axioskop 50 - Zeiss |
| Particle counter | Z1 Coulter [®] Particle counter - Beckman Coulter |
| Electroporation system | Gene Pulser [®] II - Bio-Rad |
| Microwave | CMC 30 DCS - Candy |
| Vacuum pump | Vacuum pump - Millipore |
| Safety cabinet | BIO II A (series 254510309)- Telstar BIO II A (series 2359) - Telstar |
| Autoclave | Autoclave (model 4004372) - JP Selecta |
| Systems for water purification | Ultramatic GR - Wasserlab Ecomatic - Wasserlab |

4.1.10 Software

Quantity One Version 4.6.3 1-D Analysis software by Bio-Rad Laboratories, Inc.

NanoDrop ND-1000 Version 3.1.2 by Coleman Technologies, Inc.

ApE - A plasmid Editor v2.0.49

NIS Elements D - Imaging software by Laboratory Imaging, Ltd.

4.2 Methods

Introductory comments

Autoclaving conditions used during this project were 120 °C, 20 min, 100 kPa.

During the project two grades of water were used: analytical grade water Type II produced by Ecomatic system which was additionally autoclaved - in text symbolized by "H₂O (TII)"; ultrapure water Type I produced by Ultramatic GR system - in text symbolized by "H₂O (TI)".

4.2.1 Preparation of antibiotic aliquots

Two different antibiotics were used during this project to produce selective agar medium - kanamycin and ampicillin. Gentamicin was used to protect M199 medium (for leishmania cultivation) against bacterial contamination. In all cases, the procedure of preparation was the same with one distinction - weight of the antibiotics and therefore final concentrations were different.

Ampicillin

1.000 g of ampicillin was weighted on an analytical scales and it was poured into a 15 ml falcon tube. Inside a safety cabinet 10.0 ml H₂O (TII) was added using plastic pipette. Mixture was vortexed thoroughly till complete dissolution. Then it was filtered through 0.22 µm filter by syringe into a clean falcon tube. From the filtered solution were prepared 500 µl aliquots of the concentration 100 mg/ml in eppendorf tubes and they were stored at -20 °C.

Kanamycin

0.500 g of kanamycin was weighted. 500 µl aliquots of the concentration 50 mg/ml were prepared.

Gentamicin

0.400 g of gentamicin was weighted. 500 µl aliquots of the concentration 40 mg/ml were prepared.

4.2.2 Preparation of bacterial agar plates

Agar plates are ordinary used for culture bacteria, parasites or yeasts. In general, an agar plate is a Petri dish containing agar with nutrients for microorganisms.

It is possible to add an antibiotic as a selection tool. Only organisms which are resistant to the antibiotic can grow on such plates and the fact can be used e.g. to select

bacteria or parasites that were successfully transformed with plasmids carrying gene for an antibiotic resistance.

For one batch of plates was into a media glass bottle (of 1000 ml volume) weighed 7.50 g European bacteriological agar and 15.00 g Bouillon tryptic soja (TSB-D). 500 ml of H₂O (TI) was added and bottle was autoclaved. After autoclaving, bottle with agar medium was kept in thermostatic water bath at 55 °C till the time of preparation of plates.

Bottle with agar medium was taken out from the bath and it was let at RT to cool down to approximately 40 °C. Inside a safety cabinet one 500 µl aliquot of antibiotic solution was added into agar medium and the whole volume was mixed by swirling. The final concentration of antibiotics was 0.2 mg/ml for ampicillin or 0.1 mg/ml for kanamycin.

Inside the safety cabin, agar medium was poured into Petri dishes to half fill them. Then it was let for around 10 min to get solid. After that, agar plates were closed with lids and kept at 4 °C.

4.2.3 Seeding bacteria from long term glycerol stocks

Small mass of bacteria from long term glycerol stock was dissolved in 5.0 ml TSB-D medium and 6,5 µl of ampicillin solution (100 mg/ml) in 50 ml falcon tube. The tube was placed for 6 hours in 37 °C water bath and shaken at 150 rpm.

150 µl of the solution was pipetted on ampicillin agar plate and spread over the entire surface by L shaped stick. Plate was incubated at 37 °C for 14 hours.

4.2.4 Plasmid extraction

Plasmid extraction was performed by using QIAprep[®] Spin Miniprep Kit.

Principle of the method

During the procedure bacterial cell-wall is desintegrated and DNA is denaturated. Then, samples are neutralized. In this step, plasmid DNA renaturates whereas genomic DNA is too long to return in a double stranded form properly. The lysates are cleared by centrifugation and applied into QIAprep Spin Column with silica membrane. The plasmid DNA absorbs to the membrane while impurities are washed away. In the end the purified plasmid DNA is eluted in water or an elution buffer (QIAGEN, 2015a).

Procedure comments

In Buffer P1 was added RNase A (provided by producer) and in Buffer PE was added 24 ml ethanol 96% according to manufacturers instructions. After adding RNase in Buffer P1, it was stored at 4 °C.

Centrifuging was always performed at 13,000 rpm.

Protocol

- In a safety cabinet, all bacteria mass from an agar plate was harvested by L shaped plastic spreader and transferred in 1.5 ml eppendorf tube with 250 µl Buffer P1.
- 400 µl Buffer P2 was added and sample was alternately vortexed vigorously and shaken by hand for 4 min.
- 450 µl Buffer N3 was added and sample was stirred by vortex mixer again.
- After that, tube was centrifuged by table-top centrifuge for 10 min at to form a compact pellete consisting of cellular rests and genomic DNA.
- A supernatant containing plasmid DNA was carefully transferred into a QIAprep 2.0 Spin columne by decanting and it was centrifuged for 1 min.
- A flow-through was discarded and sample was washed by adding 750 µl Buffer PE and centrifuging for 1 min. Then flow-through was discarded and sample was centrifuged for additional 5 min to remove residual wash buffer.
- The collection tube of QIAspin prep column was thrown away and the column was placed in a clean eppendorf tube. 30 µl of H₂O (TII) was poured on a membrane by pipetting to elute the plasmid DNA. The sample was centrifuged for 1 min.
- After that, the flowed eluate was employed for the second elution to increase concentration of the plasmid DNA in the final product.
- A concentration of obtained DNA was measured by NanoDrop. 1.5 µl of a sample was used for a measuring.

4.2.5 Polymerase chain reaction

Principle of the method

Polymerase chain reaction (PCR) is a molecular biology method which is used for in vitro amplification of selected segment of the DNA or RNA. The method is based on an ability of DNA polymerase to create a complementary chain to a single stranded DNA template. The procedure consists of three steps, which are repeated (usually 20 - 40 times).

Firstly is a template DNA exposed to high temperature (around 95 °C). This step causes denaturation of the double stranded DNA, hydrogen bonds between complementary nitrogenous bases are broken and DNA is separated in two single strands.

After that is the temperature decreased to enable annealing of primers (oligonucleotides specially designed for any segment of DNA we want to amplify). One of primers is complementary to the beginning part of DNA section intended to be amplified and the other one to its end.

The third phase consist in elongation of primers by Taq polymerase. It is a thermostable enzyme, whose big advantage is that it is not destructed during denaturation step. Optimal temperature of Taq polymerase activity is 72 °C (Schochetman et al., 1988; Ehrlich, 1989).

Procedure

Primers were under sterile conditions dissolved in H₂O (TII) to prepare 0.1 mM stock solutions, according to the manufacturers instructions. These were then diluted to obtain working solutions of 10 μ M.

50 μ l dNTP Mix 40mM was pipetted in an eppendorf tube and 950 μ l H₂O (TII) was added to create 2mM dNTPs working solution.

A general composition of PCR reaction is given in **Table 2**. A volume of each component was multiplied by number of prepared samples. All reagents, except of Taq polymerase and template DNA, were transferred by pipetting into a sterile eppendorf tube and mixed properly on vortex. 1 μ l of template DNA (or H₂O (TII) in case of negative control) was pipetted inside PCR micro reaction tubes. Taq was added to pre prepared reaction mixture. The final mixture was mixed gently by pipetting and 19 μ l of it was transferred in each micro tube. Tubes were closed with micro caps.

Samples were placed in thermocycler and programme was set depending on used primers and length of amplified region of DNA (**Table 3**).

Table 2: *The composition of PCR reaction mixture for final volume 20 ml*

| Component | Volume [ml] |
|-----------------------------|-------------|
| DNA template | 1.0 |
| PCR buffer 10X | 2.0 |
| dNTPs 2 mM | 2.0 |
| forward primer 10 μ M | 2.0 |
| reverse primer 10 μ M | 2.0 |
| MgCl ₂ 25 mM | 1.0 |
| Taq polymerase 5 U/ μ l | 0.2 |
| H ₂ O (TII) | 9.8 |

Table 3: *The general thermo cycler conditions set for PCR reaction*

| Name of step | Temperature | Duration [min] |
|----------------------|-------------|----------------|
| Initial denaturation | 95 °C | 5:00 |
| Denaturation | 95 °C | 0:30 |
| Annealing | 64 °C* | 0:45 |
| Elongation | 72 °C | 2:10* |
| Terminal elongation | 72 °C | 7:00 |

Initial denaturation and terminal elongation go through only once whereas denaturation, annealing and elongation steps are repeated 30 times.

* these conditions were employed most frequently; if different temperature or time were used, it is explicitly mentioned in the text.

4.2.6 Agarose gel electrophoresis

Principle of the method

Gel electrophoresis is a lab procedure that can be used for visualization and purification of DNA, RNA or proteins. DNA and RNA molecules are separated due to their length, proteins additionally according to their charge. Process is based on moving of DNA fragments, whose backbones are negatively charged, in electric field in direction of positively charged electrode. In the matrix of agarose gel shorter fragments are migrating faster whereas longer fragments are not able to move so quickly (Stellwagen & Stellwagen, 2009).

For visualising of DNA can be used for example ethidium bromide, SYBR Green I or for example GelRed. These compounds are intercalating agents which bind to DNA and work like a fluorescent tag, so the DNA-dye-complex is visible in ultraviolet (UV) or blue light (Borst, 2005).

Procedure

54.0 g Tris, 27.5 g boric acid and 3.72 g EDTA disodium salt was dissolved in 500 ml H₂O (TI) and autoclaved to provide TBE 10x stock solution. TBE 1X was prepared by diluting 1 l of TBE 10X by 9 l of H₂O (TI).

Marker ladder (**Figure 32**) was prepared from 100 µl 1Kb Plus DNA ladder, 200 µl 10X Loading buffer and 700 µl TBE 1X. All ingredients were pipetted in the same eppendorf tube and vortexed.

Loading buffer was prepared by dissolving 3.0 mg Orange G in 6 ml glycerol mixed with 14 ml H₂O (TI).

Ethidium bromide bath was prepared by adding 100 µl ethidium bromide solution in 500 ml of water in a glass container covered with aluminium foil (to protect it from light) and mixed gently.

Agarose gel of 1% concentration was used if not said different.

The opposite opened sites of gel track were covered by a paper tape, a comb was placed in the tray.

Appropriate amount of agarose medium D1 was measured on balances and transferred into an Erlenmeyer flask. Corresponding volume of TBE buffer 1X was added (e.g. 0.35 g agarose medium D1 and 35 ml TBE 1X). Erlenmeyer flask was placed in a microwave for 2-3 min, until the agarose was completely dissolved. After 30 - 40 s was heating in microwave disrupted and solution was swirled. It was repeated each 15 s to avoid boiling of medium. After its entire dissolution, the agarose solution was slowly poured (to avoid bubbles) into a gel tray with placed comb. Gel was let at room temperature (RT) for 15 min to get solid. Comb and tape were removed and gel in the tray was

placed in a gel chamber filled with TBE 1X. Surface of gel has to be completely submerged in TBE buffer.

Usually, 6 μ l loading buffer (containing Orange G as a tracking dye and glycerol to increase a density of sample) were added to every sample. The content of the tube was mixed by pipetting and 12 μ l of it was carefully loaded in a well. 5 μ l marker ladder was loaded in a well to indicate size of separated fragments. The lid of chamber was closed, and chamber was attached to power supply. Voltage was set at 110V.

The power source was turned off and electrodes were disconnected after dye line almost reached the end of the gel. The gel was sunk into a ethidium bromide bath for 20 min. It was shortly washed in a water bath and image of gel was made using Universal Hood II machine. In cases when bands were poorly visible, images were processed using Laplacian edge detection algorithm. (*Laplacian edge detection*, n.d.) If needed, DNA bands of interest were excised from the gel afterwards.

4.2.7 Restriction endonuclease digestion

Restriction endonucleases (REs) are enzymes which were originally isolated from bacteria to which they serve as a protection against heterogenous DNA (Bickle & Krüger, 1993). REs cut DNA by catalyzing a cleavage of phosphodiester bond in nucleic acids. Several different types and subtypes of restriction endonuclease can be distinguished according to the structure, specificity of cleavage sites and other parameters (Pingoud & Jeltsch, 2001).

REs type II are most frequently used as a valuable molecular biology tool (Bickle & Krüger, 1993). They usually consist of two identical subunits. They recognise short sequence of 4 - 8 nucleotides which is ordinarily palindromic (symmetric) and they cut DNA within or very close to it (Pingoud & Jeltsch, 2001). Some of the enzymes digest both DNA strands at the same site, so fragments with blunt ends are created. Other REs have their restriction sites in complementary strands a few nucleotides shifted, therefore after they use DNA fragments with cohesive ("sticky") ends originated (Goodsell, 2002).

EcoRI

EcoRI was discovered in *Escherichia coli* RY13. Its recognition site is 6 bp long and after cutting it creates 4 nucleotide cohesive ends with 5' overhangs after cutting (**Figure 9**). (R. J. Roberts, 1980)

General composition of the *EcoRI* mixture is given in **Table 4**. Digestion was performed at 37 °C.



Figure 9: Recognition sequence and cleavage site of *EcoRI*

Adapted from NEB websites (<https://www.neb.com/products/r0101-ecori>).

Table 4: The composition of *EcoRI* digestion mixture

| Component | Amount |
|------------------------|-------------|
| <i>EcoRI</i> | 1.0 µl |
| 10XH Universal buffer | 2.0 µl |
| plasmid | * |
| H ₂ O (TII) | up to 20 µl |

* used volume depends on the concentration of employed DNA, particular data are always given in the text

NotI

NotI RE was originally isolated from bacteria *Nocardia otitidis-caviarum*. It recognises sequence of 8 nucleotides and after its cleavage sticky single stranded 5' overhangs remain (**Figure 10**) (Samuleson et al., 2006).

General composition of reaction mixture is given in **Table 5**. The reaction proceeded at 37 °C.



Figure 10: Recognition sequence and cleavage site of *NotI*

Adapted from NEB websites (<https://www.neb.com/products/r0189-noti>).

Table 5: The composition of *NotI* digestion mixture

| Component | Amount |
|------------------------|-------------|
| <i>NotI</i> | 1.0 µl |
| 10XH Universal buffer | 2.0 µl |
| 0.1% BSA | 2.0 µl |
| 0.1% Triton X-100 | 2.0 µl |
| plasmid | * |
| H ₂ O (TII) | up to 20 µl |

* used volume depends on the concentration of employed DNA, particular data are always given in the text

4.2.8 TOPO ligation

TOPO TA Cloning Kit with pCR2.1-TOPO Vector was used.

Principle of the method

TOPO ligation takes advantage of fact that Taq polymerase shows template independent activity - after adding of the last nucleotide complementary to a template single strand Taq adds one adenine at the 3'-end of PCR product. The technique is based on use of DNA topoisomerase I, which has restriction and also ligase activity. It recognises specific sequence and cuts DNA on specific site leaving at the 5'-end one unpaired thymine (Invitrogen, 2016).

pCR2.1 vector is provided in linearized form with Topoisomerase I recognition sites at both ends and Topoisomerase covalently bonded to them. At RT in presence of required salts, Topoisomerase I cleave ends of vector, insert hybridize on the basis of complementary pairing with vector and Topoisomerase I ligates DNA backbone by phosphodiesteric bound (Invitrogen, 2016).

Apart from the cloning site, pCR2.1 vector contain also genes for ampicillin and kanamycin resistance to allow selection of bacteria successfully transformed with originated plasmid. Number of diverse recognition sites for REs is placed on both sides of the cloning site (**Figure 11**).

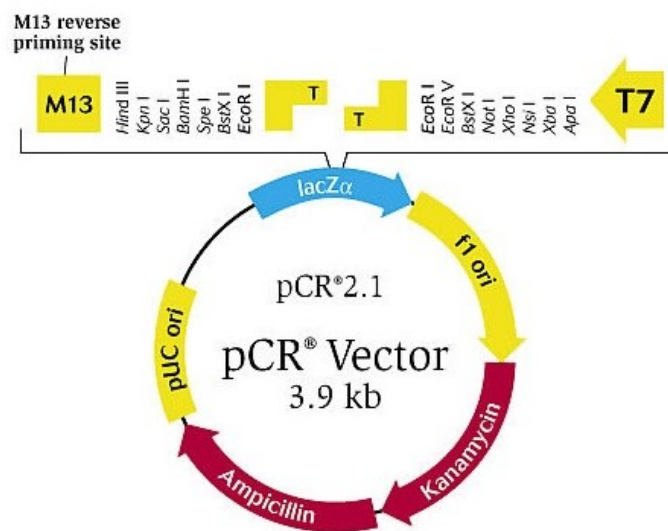


Figure 11: Structure of pCR2.1 TOPO cloning vector

Adapted from Thermofisher websites, modified (<https://www.thermofisher.com/order/catalog/product/K450002>)

Protocol

4 μ l of fresh PCR product of amplification reaction with target insert was pipetted

in an eppendorf tube. 1 μ l of pCR2.1 vector and 1 μ l of salt solution (provided in the kit) was added. The mixture was gently mixed by pipetting and it was let for 15 min at RT to create pCR2.1-insert hybrid plasmids. The product was used for bacterial transformation.

4.2.9 Transformation of bacteria

Competent bacteria were taken from -80°C and immediately placed on ice. 50 μ l of bacteria was transferred into a eppendorf tube by pipetting and placed back on ice. Plasmid DNA was added and without any mixing tube was let for 20 min on ice. After that, it was for 45 s placed in a thermomixer preheated to 42°C . Then, tube was put back on ice. 250 μ l S.O.C. medium was added and the tube was set in a tube rotator for 1.5 hour at 37°C .

50 μ l and rest of volume was pipetted on agar plate (with selective antibiotic added) and the liquid was spread over the surface by L shaped plastic spreader. Plates were incubated for 16 hours at 37°C .

4.2.10 Preparation of template DNA for PCR from bacteria

Separated colonies

A little mass of each colony was, in a safety cabinet, transferred by sterile wooden stick into an eppendorf tube containing 100 μ l H_2O (TII). The tubes were for 25 min placed in a thermomixer warmed up to 100°C . The liquid was consequently used as a template DNA for a PCR.

Bacterial pools

The procedure was same as for separated colonies, only volume of H_2O (TII) in an eppendorf tube was 150 μ l H_2O (TII) and in each tube were placed samples from four different bacterial colonies.

4.2.11 Band gel extraction

Band gel extraction was performed by using QIAquick[®] Gel Extraction Kit.

Principle of the method

The method of DNA extraction from an agarose gel is based on different ability of a silica membrane to bind DNA in high-salt and low-salt buffers. In high-salt buffers, DNA is firmly adsorbed on the membrane that enables DNA purification. Whereas by low-salt buffers (or water) DNA can be eluted from the membrane (QIAGEN, 2015b).

Procedure comments

Centrifuging was always performed at 13,000 rpm.

Protocol

- An empty 2.0 ml eppendorf tube was weighted.
- DNA fragment was excised by a surgical blade from agarose gel using a UV transilluminator and placed into the tube.
- The gel slice in the tube was weighted and weight of it was calculated using the following equation:

$$W_{\text{gel}} = W_{\text{gel in tube}} - W_{\text{empty tube.}}$$

- Three volumes of Buffer QG to one volume of gel was added. 100 mg of a gel corresponds to 100 μl .
- The tube was incubated in a thermomixer at 50, °C for 10 min. Every 2 - 3 min was gel mixed by vortexing to facilitate gel dissolving.
- After the complete dissolution one gel volume of isopropanol was added. Whole mixture was stirred by the vortex mixer.
- The solution was pipetted into a QIAquick spin column placed in a collecting tube and the tube was centrifuged for 1 min. (If the volume was bigger than 800 μl , was loaded and spinned by parts in the same tube.)
- The flow-through was discarded and 750 μl Buffer PE was pipetted into the column. It was centrifuged for 1 min.
- Then flow-through was discarded and sample was centrifuged for additional 5 min to remove residual wash buffer.
- The column was placed in a clean eppendorf tube. 20 μl of H₂O (TII) was poured on a membrane by pipetting to elute the DNA. The sample was centrifuged for 1 min.
- After that, the flowed eluate was employed for the second elution to increase concentration of the DNA in the final product.
- A concentration of obtained DNA was measured by NanoDrop. 1.5 μl of a sample was used for measuring.

4.2.12 Dephosphorylation

Dephosphorylation was used to avoid self-recircularization of the pXG-mCherry plasmids digested by *NotI* during the ligation process. Antarctic phosphatase was used to catalyze removal of 5' phosphate from DNA fragments.

4.0 μl product of plasmid digestion (\approx 400 ng of plasmid) was dephosphorylated (Table 6). The reaction mixture was incubated at 37 °C for 4 hours. Then the enzyme was inactivated by a temperature increase to 65 °C for 20 min.

Table 6: *The composition of the dephosphorylation reaction mixture*

| Component | Amount |
|-------------------------------|------------------|
| Antarctic phosphatase | 1.0 μ l |
| buffer | 2.0 μ l |
| <i>NotI</i> digestion product | 4.0 μ l |
| H ₂ O (TII) | up to 10 μ l |

4.2.13 Ligation

Then ligation reaction was used to fuse *YinP* inserts with target leishmanial pXG-mCherry plasmids. Amount of insert was calculated by ligation calculator due to size and amount of plasmids and size of inserts (*Ligation calculator*, 2006). 1 : 3 vector : insert ratio was used. T4 DNA ligase enzyme was used to catalyze formation of covalent bonds between 5' phosphate of insert and 3' hydroxyl of vector. The incubation time was 20 hours and the incubation temperature was 14 °C (**Table 7**).

Table 7: *The composition of the ligation reaction of YinP inserts with pXG-mCherry plasmids*

| Component | Amount |
|------------------------|------------------|
| T4 DNA ligase | 2.0 μ l |
| buffer | 5.0 μ l |
| vector | 150 ng |
| insert | 119 ng |
| H ₂ O (TII) | up to 20 μ l |

4.2.14 Preparation of M199 medium

For cultivation of *Leishmania* parasites was used M199 1x medium supplied with several additional nutrients.

Preparation of stock solutions

- **FBS:** FBS was warmed up to 65 °C for 1 hour to inactivate complement.
- **HEPES 1 M:** 47.66 g HEPES was dissolved in H₂O (TII) and filled up to 200 ml. pH was adjusted to 7.4 with HCl.
- **HEPES 50 mM:** 650.7 mg HEPES was weighted, dissolved in H₂O (TII) and filled up to 50 ml.
- **Adenine 10 mM in 50 mM HEPES:** 67.6 mg adenine was dissolved in HEPES 50 mM and filled up to 50 ml. pH was adjusted to 7.4 with HCl and filtered through 0.22 μ m

filter.

- **Hemine 0.25 % in 50 % triethanolamine:** 12.5 ml triethanolamine and 12.5 ml H₂O (TII) was mixed. 62.5 mg hemine was dissolved in the solution, the volume was filled up to 25 ml.
- **Biotine 0.1 % in 95 % ethanol:** 23.75 ml ethanol and 1.25ml H₂O (TII) was mixed. 25 mg biotine was dissolved in the prepared solution.
- **Biopterine 0.25 mg/ml:** To 5 mg biopterine was added 20 ml H₂O (TII). The mixture was heated and stirred till the complete dissolution and the volume was adjusted to 20 ml.

Preparation of M199 1x

The whole content of M199 medium bottle was poured into a beaker and 400 ml H₂O (TII) was added. 0.35 g NaHCO₃ was wighted and added into the beaker and it was placed on a magnetic stirrer. After complete dissolution, pH was adjusted to 6.8. The solution was transfused to 1 l graduated bottle. All addition reactants, in form of prepared stock solutions, were added by pipetting, amounts are given in **Table 8**. The volume was adjusted to 1.0 l with H₂O (TII). The medium was filtered using bottle-top vacuum system and stored at 4 °C.

Table 8: *Volumes of reactants used for the preparation M199* 1X medium for final volume 1 l*

| Reagent | Volume [ml] |
|--------------------------------|-------------|
| FBS 10% | 100.0 |
| HEPES 1 M (pH = 7.4) | 40.0 |
| adenine 10 mM (in 50 mM HEPES) | 10.0 |
| HEMIN | 2.0 |
| biotin | 1.0 |
| biopterine | 4.0 |
| gentamicin | 1.0 |

4.2.15 Counting of parasites

Principle of the method

In order to determine parasite concentration in cultures was used a Beckman Coulter particle counter. Its technology is based on measuring changes in impedance caused by particle passing through aperture between two electrodes. The pulse is proportional to particle volume. When the volume of electrolyte (with suspended particles) passing through the aperture is known, the concentration of the particles in the sample can be established (Beckman Coulter, 2016).

Procedure

10 μl of culture was diluted in 10 ml isotone solution and concentration of parasites was measured with particle counter. Detection of particles with diameter bigger than 3 μm was set. The concentration of parasites in the culture was counted using the following equation:

$$c = N \cdot 1,000$$

where c stands for concentration of parasites; N stands for number displayed by counter machine and 1,000 is dilution factor.

4.2.16 Preparation of cytomix buffer

Composition of cytomix buffer used for electroporation was based on on nature intracellular ionic balance in order to increase survival of electroporated cells (van den Hoff et al., 1992).

The composition of the cytomix buffer is given in **Table 9**. All powders were weighed and poured into a beaker. 450 ml of H_2O (TII) was added and the mixture was stirred with magnetic stirrer till complete dissolution of solid compounds. pH was adjusted to 7.6 and the volume of the solution was filled up to 500 ml. At the end, buffer was filtered through 0.22 μm filter and stored at 4 °C.

Table 9: *The composition of cytomix buffer for 500 ml in H_2O (TI) (pH = 7.6)*

| Reagent | Weight | Final concentration |
|--------------------------|---------|---------------------|
| KCl | 4.470 g | 120 mM |
| CaCl_2 | 0.110 g | 0.15 mM |
| K_2HPO_4 | 0.870 g | 10 mM |
| HEPES | 3.250 g | 25 mM |
| EDTA | 0.372 g | 2 mM |
| MgCl_2 | 0.240 g | 5 mM |

4.2.17 Electroporation

Principle of the method

Electroporation is one of molecular biology techniques which can be used for transfection of nucleic acids into eukaryotic cells. Besides electroporation, there are more methods such as calcium phosphate precipitation, microinjections, sonoporation, lipofection, viral transduction and others that can be used for cell transfection (Potter, 1988).

During electroporation, cells are exposed to a short electrical pulse, which causes an increase of cell membrane permeability and make cells susceptible to introduction of

extracellular DNA (Kim & Eberwine, 2010).

Procedure

First, volume of culture needed for electroporation had to be calculated. For each electroporation was used 100×10^6 parasites. Concentration of parasites was measured by particle counter and necessary volume of culture was counted using the following equation:

$$V = \frac{A}{c}$$

where V stands for needed volume of culture, A stands for total number of parasites required for electroporation and c stands for concentration of parasites.

Calculated volume of culture was transferred to 50 ml falcon tube and centrifuged at 3000 rpm and 26 °C for 10 min. Supernatant was discarded and parasite pellet was resuspended in 30 ml of cytomix buffer. The tube with leishmania was centrifuged again using the same conditions as previous time. Then supernatant was discarded and pellet was resuspended in cytomix. Volume of cytomix depended on number of electroporation performed, it was 100 µl for one electroporation sample. 100 µl of the parasite suspension was pipetted in each electroporation cuvette. ≈ 20 µg of extracted plasmid (or water in case of negative controls) was added. Cuvette was placed on ice. 500 µl of cytomix was added and sample was placed back on ice.

For each plasmid were prepared two cuvettes. For one capacitance of electroporator was set to 25 µF and for the another 50 µF capacitance was set. For all samples, voltage 1.5 kV was used. Cuvette's surface was carefully dried by paper and placed in the electroporator between electrodes. Two pulses were given to the cuvette with 10 s distance. Time constants (which are supposed to be between 0.5 - 0.8) were written down. After that, cuvette was placed back on ice.

As much as possible of cuvette's content (without debris from the top of liquid) was transferred to incubation flask with 10 ml of M199* 1x medium without selective antibiotic. A liquid from the both samples with the particular plasmid (25 µF and 50 µF) were transferred to the same flask. Flasks were incubated for 5 hours at 26 °C, shaking at 70 rpm to let parasites recover. Afterwards, 50 µl hygromycin was added into cultures (final concentration 5 µl/ml) to select successfully transformed parasites.

4.2.18 DAPI staining

Principle of the method

DAPI is a fluorescent stain which is commonly used for visualizing nucleic acids. It passes through cell membrane of both dead and also living cells. It binds to A-T rich regions of DNA inside the minor groove (Wheeler et al., 2012).

Procedure

1 % solution of formaldehyde was prepared.

A concentration of parasites in culture was measured by particle counter and volume containing 50×10^6 parasites was transferred to 15 ml falcon tube. The tube was centrifuged for 10 min at 3,000 rpm at 4 °C. Supernatant was discarded and pellet was resuspended in 1 ml PBS and transferred to an eppendorf tube. 10 µl DAPI was added and the tube covered in aluminum foil was placed at 4 °C for 30 min. Afterwards, the tube was centrifuged (10,000 rpm, RT, 5 min). The supernatant was discarded, the pellet was resuspended in 100 µl formaldehyd 1 % and the sample was left for 5 min at RT. 1 ml PBS was added and other centrifugation ((10,000 rpm, RT, 5 min) was performed. The supernatant was discarded and the pellet was once more resuspended in 1 ml PBS and centrifuged. The supernatant was discarded and the pellet was resuspended in 100 µl of PBS mixed with glycerol (40 µl PBS and 60 µl glycerol).

5. RESULTS AND DISCUSSION

5.1 Construction of plasmids

5.1.1 Extraction and checking of pXG-mCherry plasmids

Bacteria from long term glycerol stock containing plasmids pXG-mCherry12 and pXG-mCherry34 (**Figure 12**) were seeded on agar plates with ampicillin like a lawn and incubated at 37 °C for 14 hours. After that, plasmid extraction was performed and concentration and purity of extracted plasmids were measured (**Table 10**).

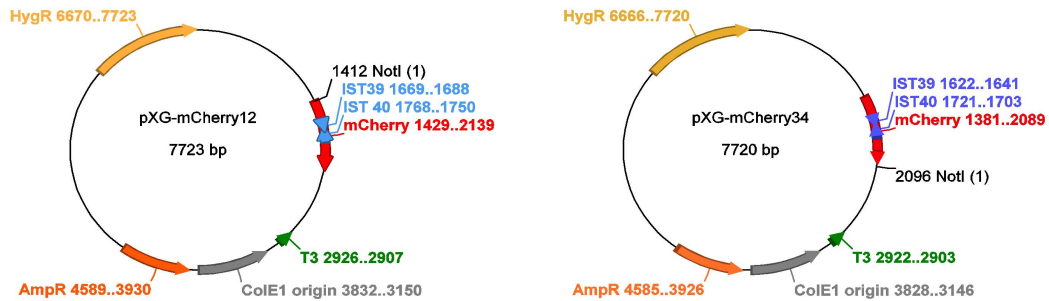


Figure 12: Structure of pXG-mCherry12 and pXG-mCherry34 plasmids

Table 10: Concentration and purity parameters of extracted pXG-mCherry plasmids measured with NanoDrop spectrophotometer

| plasmid | Concentration | 260:280 ratio | 260:230 ratio |
|---------------|---------------|---------------|---------------|
| pXG-mCherry12 | 892.3 ng/μl | 1.92 | 2.07 |
| pXG-mCherry34 | 552.9 ng/μl | 1.87 | 1.78 |

Presence and direction of mCherry in both obtained plasmids were checked by PCR. IST39 + T3 primers were used to confirm correct direction and IST40 + T3 primers to negate opposite direction of mCherry in these plasmids. Elongation time was 1:40 min. Subsequent agarose gel electrophoresis was performed.

Bands of ≈ 1260 bp for pXG-mCherry12 and ≈ 1310 bp for pXG-mCherry34 (using IST39 and T3 primers) confirmed the correct direction of mCherry in the extracted plasmids (**Figure 13**).

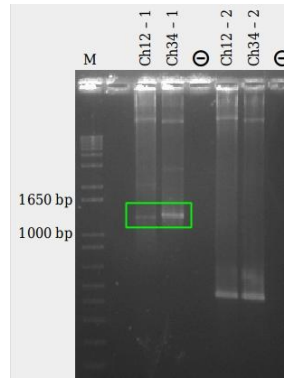


Figure 13: Agarose gel electrophoresis of PCR products to confirm insert (*mCherry*) orientation in the *pXG-mCherry* plasmids

Ch12-1 and **Ch34-1** are PCR products obtained using IST39+T3 primers. **Ch12-2** and **Ch34-2** are products of PCR performed with IST40+T3 primers. **M** is marker ladder. (-) are negative controls. Ampli-cons of 1258 bp for Ch12-1 and 1313 bp for Ch34-1 confirmed *mCherry* presence in the correct direction in isolated *pXG-mCherry*12/34 plasmids.

5.1.2 Amplification of *YinP* insert

pXG-hyg-YinP plasmid (**Figure 14**) was extracted from bacteria and quantified (**Table 11**). Its structure was checked by *EcoRI* digestion. Agarose gel electrophoresis was used to divide plasmid fragments after digestion due to their length. Structure of *pXG-hyg-YinP* plasmid was confirmed (**Figure 15**) and it was subsequently used as template DNA for PCR amplification of *YinP*. *pXG-hyg-YinP* plasmid was later also used as DNA template of positive controls for *YinP* presence in certain PCR reactions.

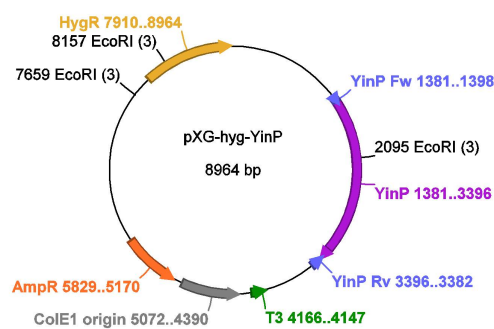


Figure 14: Structure of *pXG-hyg-YinP* plasmid

YinP was amplified by PCR to be consequently inserted into a commercial pCR2.1-TOPO vector. For this purpose, primers YinP Fw, YinP12 Rv and YinP34 Rv were specially designed based on cDNA sequence deposited in the TriTrypDB. In all these primers was inserted *NotI* restriction site (GC|GGCCGC). The reading frame was conserved. For the *pXG-mCherry12-YinP* construction, was necessary to skip terminal codon (TAA) because the aim was to create a N-terminal fusion protein. In the forward primer was also included a *Pichia* Kozak consensus (ANNATGG).

Table 11: Concentration and purity parameters of extracted pXG-hyg-YinP plasmids measured with NanoDrop spectrophotometer

| plasmid | Concentration | 260:280 ratio | 260:230 ratio |
|----------------|---------------|---------------|---------------|
| pXG-hyg-YinP 1 | 415.2 ng/μl | 2.03 | 2.18 |
| pXG-hyg-YinP 2 | 370.4 ng/μl | 2.10 | 2.11 |

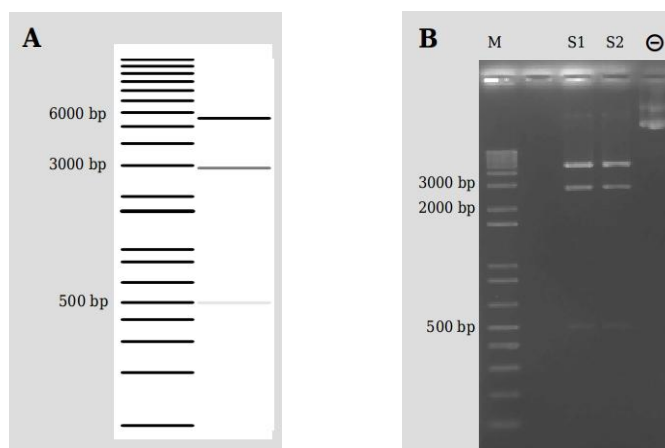


Figure 15: Agarose gel electrophoresis to compare the enzyme restriction map with the size of fragments from pXG-hyg-YinP plasmids digested by EcoRI

(A) Theoretical restriction map of pXG-hyg-YinP after digestion by EcoRI endonuclease. Bands of 5564 bp (62%), 2902 bp (32%) and 498 bp (6%) were expected. (B) Agarose gel electrophoresis of reaction products after EcoRI digestion of pXG-hyg-YinP plasmid. M is marker ladder. S1 and S2 are plasmids after digestion. (-) is negative control where 600 ng of undigested plasmid was used.

1 μl pXG-hyg-YinP plasmid was used as a template DNA for the PCR. Annealing temperature was 64 °C. Primers YinP Fw + YinP12 Rv were used to prepare YinP-12 insert intended to be introduced into pXG-mCherry12 plasmid. For the pXG-mCherry34 construction, YinP-34 insert was amplified using YinP Fw + YinP34 Rv primers.

Before next step, the process was checked by electrophoresis. 6 μl of PCR products was mixed with 6 μl of loading buffer on parafilm and loaded in a gel. Bands of 2000 bp size indicated the presence of YinP (Figure 16). Amplification worked properly.

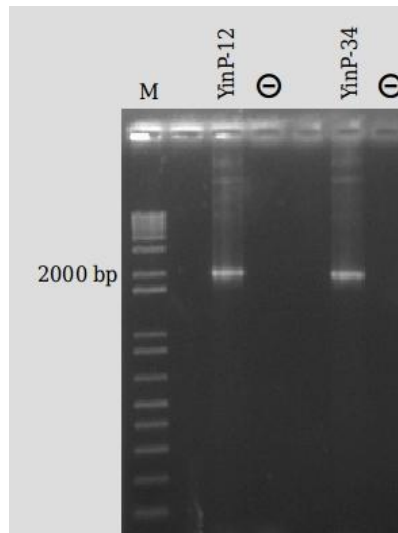


Figure 16: Agarose gel electrophoresis of PCR products to check *YinP* amplification before ligation with pCR2.1-TOPO vector

YinP-12, YinP-34 are PCR products. **M** is marker ladder. (-) are negative controls. Amplicons of 2000 bp size for both samples were expected. PCR reaction worked properly.

5.1.3 Creation of pCR2.1-*YinP*-12 and pCR2.1-*YinP*-34 plasmids

4 μ l of fresh PCR products from previous step were used for TOPO ligation to create pCR2.1-*YinP*-12 and pCR2.1-*YinP*-34 hybrid plasmids (**Figure 17**).

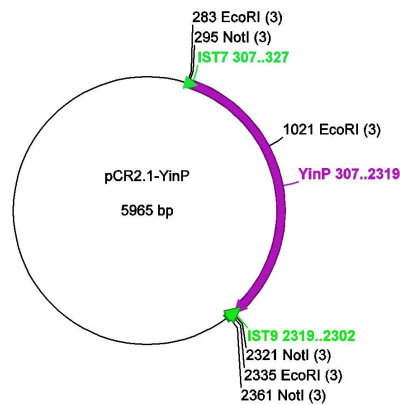


Figure 17: Structure of constructed pCR2.1-*YinP* plasmid

Constructions with *YinP*-12 and with *YinP*-34 differentiate only in three nucleotides (stop codon from *YinP* was skipped when designing *YinP*12 Rv primer).

2 μ l of ligation products were employed as plasmid DNA for bacteria transformation.

Kanamycin agar plates were chosen to seed transformed bacteria. Use of agar plates with kanamycin was in this step crucial, because pXG-hyg-*YinP* plasmid (used as

template for *YinP* amplification) also contains gene for ampicillin resistance. Therefore if ampicillin agar plates had been used, two types of transformed bacteria would have grown - those containing pCR2.1-*YinP* plasmid but also those which had incorporated original pXG-hyg-*YinP* plasmid from PCR reaction mixture.

From bacterial colonies which grew, 8 of each type was randomly selected and separately inoculated on a new kanamycin agar plates for further screening. The plates were then incubated at 37 °C for 16 hours.

From bacteria, template DNA for screening PCR (using IST7 + IST9 primers) was prepared. Agarose gel electrophoresis was performed (**Figure 18**). Bands of 2000 bp size indicated a presence of *YinP*. 7 colonies (all except C 12-5) for pCR2.1-*YinP*-12 construction and 4 colonies (C 34-2, -3, -5 and -7) for pCR2.1-*YinP*-34 construction were *YinP* positive.

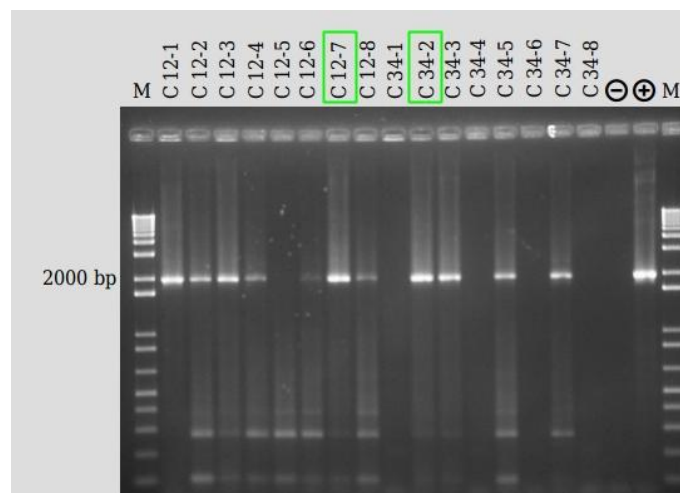


Figure 18: Agarose gel electrophoresis of PCR products from screening PCR to select successfully transformed bacteria

Samples C 12-1 - C 34-8 are PCR products of the screening PCR reaction with DNA templates prepared from colonies transformed by pCR2.1-*YinP*-12/34 plasmids. **M** is marker ladder. (-) is negative control, (+) is positive control. The expected amplicons of 2000 bp indicated *YinP* presence in bacterial colonies. 11 colonies clearly harboured the newly created plasmids containing *YinP* insert. Colonies C 12-7 and C 34-2 were chosen to continue with.

One colony for *YinP*-12 (C 12-7) and one colony for *YinP*-34 (C 34-2) which were *YinP* positive according to the performed PCR were reseeded on ampicillin agar plates. This time, small part of each colony was taken by wooden stick and resuspended in 100 µl TSB-D medium in an eppendorf tube. 50 µl of the solution was poured on an agar plate and spread over the entire agar surface using L shaped stick to produce a bacterial lawn. The plates were let for a few minutes to get dry and incubated at 37 °C for 14 hours.

After the incubation, plasmids were extracted (**Table 12**) and subsequently checked for presence of *YinP* by PCR and by *EcoRI* endonuclease digestion. Structure of created pCR2.1-*YinP*-12/34 plasmids was confirmed (**Figure 19**).

Table 12: Concentration and purity parameters of extracted pCR2.1-YinP-12/34 plasmids measured with NanoDrop spectrophotometer

| plasmid | Concentration | 260:280 ratio | 260:230 ratio |
|----------------|---------------|---------------|---------------|
| pCR2.1-YinP-12 | 732.9 ng/μl | 1.85 | 2.12 |
| pCR2.1-YinP-34 | 724.5 ng/μl | 1.81 | 2.10 |

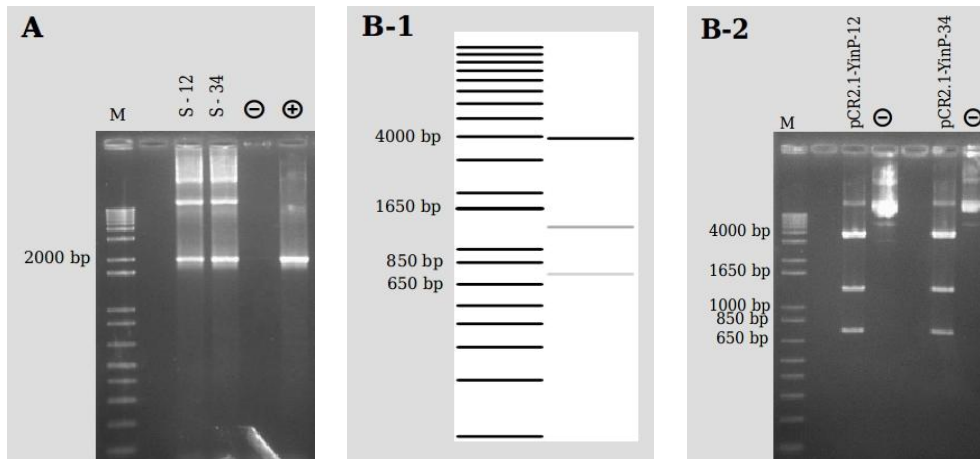


Figure 19: Confirmation of the structure of extracted pCR2.1-YinP-12/34 plasmids and check of YinP presence

(A) Samples S - 12 and S - 34 are PCR products from reaction where pCR2.1-YinP-12/34 plasmids were used as DNA template. M is marker ladder. (-) is negative control. (+) is positive control (pXG-hyg-YinP plasmid used as DNA template). Amplicons of 2000 bp were expected to confirm YinP presence in extracted plasmids. (B) Expected restriction map of pCR2.1-YinP12/34 plasmids. Bands of 3913 bp (66%), 1315 bp (22%) and 737 bp (12%) were expected. (C) Obtained restriction map after agarose gel electrophoresis of *Eco*RI digested pCR2.1-YinP-12/34 plasmids. M is marker ladder. (-) are negative controls (undigested plasmids). 1 μg of plasmid (digested or undigested) was loaded.

5.1.4 Preparation YinP with *Not*I ends

4 μg of each extracted plasmid was digested by *Not*I enzyme. The reaction was incubated at 37 °C for 6 hours. After 3 hours of incubation, 1 μl of fresh enzyme was added.

Whole volumes of these reactions were mixed with 6 μl of electrophoresis loading buffer and agarose gel electrophoresis was performed. As a negative control was loaded 6 μl undigested plasmids (650 ng/μl) also mixed with 6 μl of loading buffer (Figure 20).

YinP with *Not*I ends (bands of 2000 bp size) was excised from the gel and band gel extraction was performed (Table 13). After band extraction 1 μl of the liquid was used as a template DNA for PCR reaction (IST7 + IST9 primers) to check inserts before they were used for ligation with pXG-mCherry plasmids. Presence of *YinP* was confirmed in both samples (Figure 21).

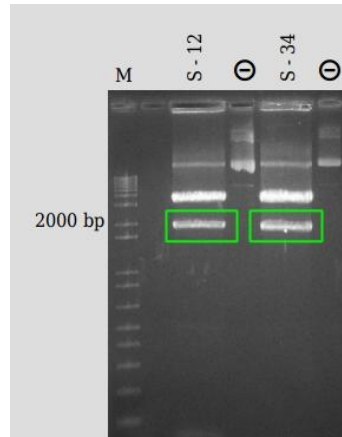


Figure 20: Agarose gel electrophoresis of *NotI* digested pCR2.1-*YinP* plasmids

Into doublewells **S - 12** and **S - 34** were loaded whole reaction volumes from *NotI* digestion of pCR2.1-*YinP*-12 and pCR2.1-*YinP*-34 plasmids. (-) are negative controls (650 ng of undigested plasmids). Bands of 2000 bp size containing *YinP* inserts with *NotI* ends were extracted from the gel.

Table 13: Concentration and purity parameters of extracted *YinP* inserts after *NotI* digestion measured with NanoDrop spectrophotometer

| plasmid | Concentration | 260:280 ratio | 260:230 ratio |
|----------------|---------------|---------------|---------------|
| <i>YinP</i> 12 | 27.0 ng/μl | 1.90 | 0.13 |
| <i>YinP</i> 34 | 29.4 ng/μl | 1.84 | 0.11 |

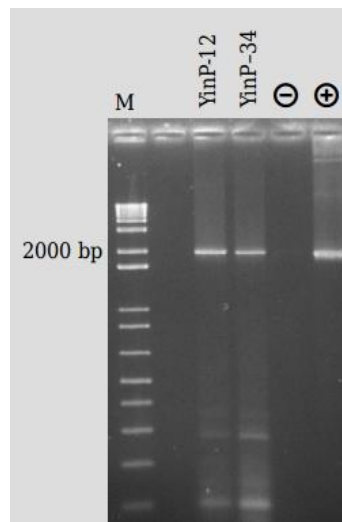


Figure 21: Agarose gel electrophoresis of PCR products to check *NotI* digested *YinP* inserts extracted from an agarose gel

Samples **YinP-12** and **YinP-34** are products of PCR reaction performed to check inserts extracted from the gel. (-) is negative control. (+) is positive control (pXG-hyg-*YinP* plasmid used). **M** is marker ladder. Bands of 2000 bp confirmed the *YinP* presence in samples.

5.1.5 Creation of two novel plasmids: pXG-mCherry12-*YinP* and pXG-mCherry34-*YinP*

pXG-mCherry plasmids extracted from bacteria were digested using *NotI* enzyme. 2.0 µg of each plasmid was digested. The reaction lasted for 48 hours, at 24 hours 1 µl of fresh enzyme was added. After 48 hours, the temperature was increased for 20 min to 80 °C to cause enzyme inactivation.

The digested plasmids were dephosphorylated. Then ligation reaction was performed to fuse together *YinP* inserts and pXG-mCherry plasmids to create object pXG-mCherry12/34-*YinP* plasmids (Figure 22). 150 ng of each *NotI* digested and dephosphorylated plasmid was used.

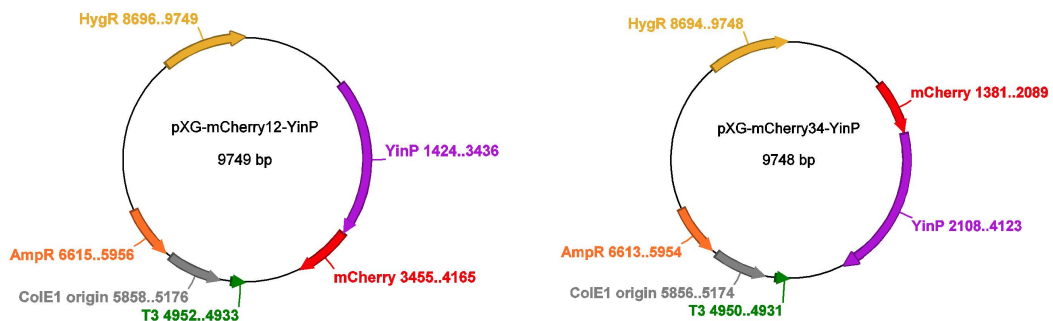


Figure 22: pXG-mCherry12-*YinP* and pXG-mCherry34-*YinP* plasmids

10 µl of ligation products was employed for bacterial transformation. Transformed bacteria were seeded on ampicillin agar plates.

16 colonies of each type were randomly chosen and separately inoculated on new ampicillin agar plates. *YinP* presence was tested in further screening in the next step. The plates were incubated at 37 °C for 16 hours.

Four DNA template pools were prepared for both types of bacteria and the liquid was consequently used for a screening PCR (IST7 + IST9 primers were used). Agarose gel electrophoresis was performed. A band of 2000 bp size indicated the presence of *YinP*, which was proven in all pools (Figure 23).

DNA templates of colony pools were used for another PCR to check direction of *YinP* inside the pXG-mCherry-*YinP* plasmids. Primers IST40 + *YinP*-Leish Fw were used to detect pools with colonies containing pXG-mCherry12-*YinP* with *YinP* in the correct direction. IST39 + *YinP*-Leish Rv were used for the same purpose for pXG-mCherry34-*YinP* pools. Elongation time was 2:30 min. Agarose gel electrophoresis was performed. Bands of ≈ 920 bp indicated positive samples for mCherry12 construction - all 4 pools were shown as positive. Bands of ≈ 2100 bp indicated positive samples for mCherry34 construction - 2 pools were shown as positive (Figure 24).

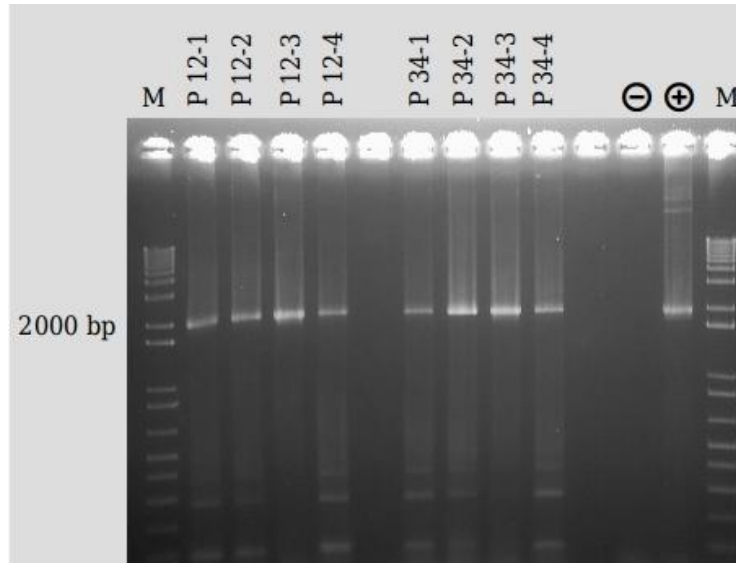


Figure 23: Agarose electrophoresis to identify bacterial pools containing successfully transformed bacterial colonies

Samples P 12-1 - P 34-4 are PCR products. M is marker ladder. (-) is negative control. (+) is positive control (pXG-hyg-*YinP* plasmid was used as template DNA). Bands of 2000bp size indicated presence *YinP* gene. All pools shown as *YinP* positive.

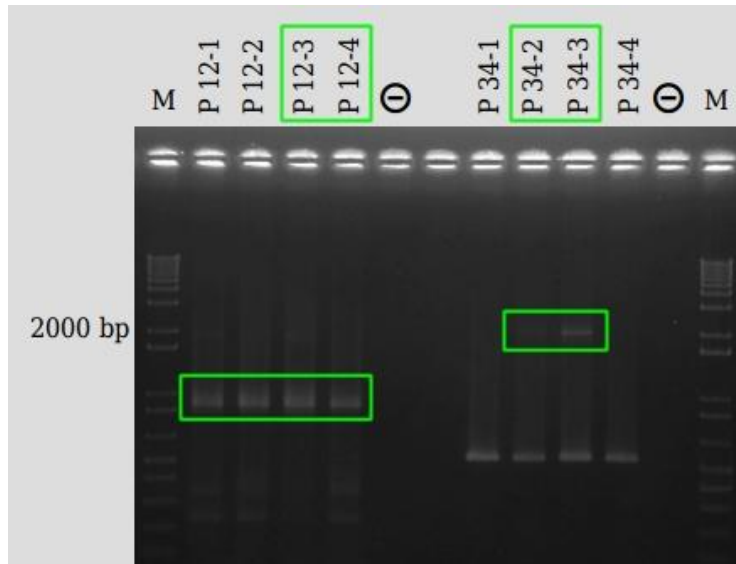


Figure 24: Agarose gel electrophoresis to identify bacterial pools containing bacterial colonies that have incorporated pXG-mCherry-*YinP* plasmids with *YinP* in the correct direction

Samples P 12-1 - P 34-4 are PCR products. M is marker ladder. (-) is negative control. Bands of 920bp size indicated presence of *YinP* gene in the correct direction in pXG-mCherry12-*YinP* plasmids. Bands of 2080bp indicated presence of *YinP* in the correct direction in pXG-mCherry34-*YinP* plasmids. Pools P 12-1, P 12-2, P 12-3, P 12-4, P 34-2 and P 34-3 were detected as containing the expected plasmids. For next steps pools P 12-3, P 12-4, P 34-2 and P 34-3 were chosen.

Separated DNA templates were prepared from all bacterial colonies which were included in the pools P 12-3, P 12-4, P 34-2 and P 34-3 (which appeared positive for *YinP* presence in the correct direction). With these DNA templates were performed another PCR to identify particular positive colonies. The same conditions and primers as with pools were used. Results were checked by agarose gel electrophoresis. Four colonies for pXG-mCherry12 construction (C 12-1, C 12-3, C 12-6 and C 12-8) and two colonies for pXG-mCherry34 construction (C 34-2 and C 34-5) were detected as containing pXG-mCherry-*YinP* plasmids with *YinP* in the correct direction (Figure 25).

All of them were seeded like a lawn on ampicillin agar plates and incubated at 37 °C for 14 hours. Plasmid extraction was performed (Table 14).

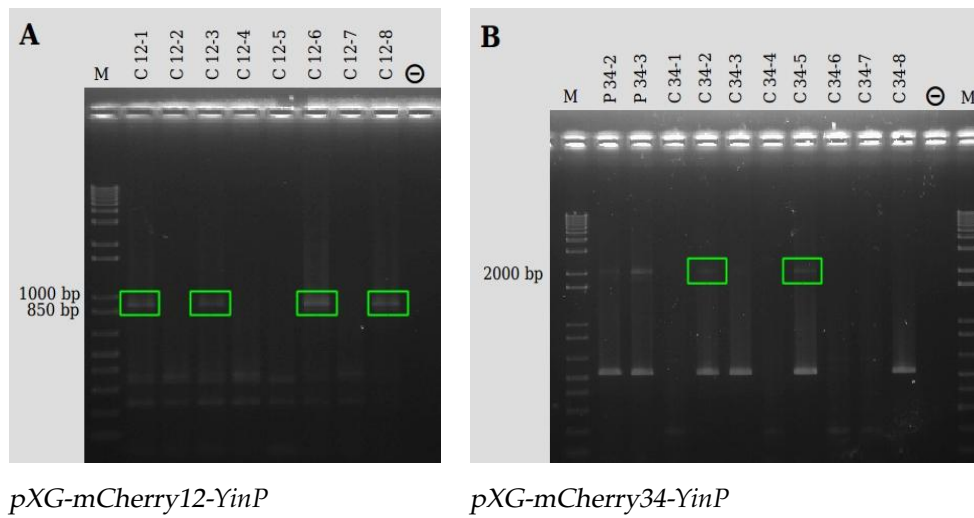


Figure 25: Agarose electrophoresis to identify individual bacterial colonies containing pXG-mCherry-*YinP* plasmids with *YinP* in the correct direction

M is marker ladder. (-) is negative control. (A) Bands of 920 bp size indicated presence of *YinP* gene in the correct direction in pXG-mCherry12-*YinP* plasmids. Samples C 12-1, C 12-3, C 12-6 and P 12-9 were detected as containing the expected plasmids. (B) Bands of 2080 bp indicated presence of *YinP* in the correct direction in pXG-mCherry34-*YinP* plasmids. Samples C 34-3 and C 34-5 were detected as containing the expected plasmids.

Table 14: Concentration and purity parameters of extracted pXG-mCherry12/34-*YinP* plasmids measured with NanoDrop spectrophotometer

| plasmid | Concentration | 260:280 ratio | 260:230 ratio |
|------------------------------|---------------|---------------|---------------|
| pXG-mCherry12- <i>YinP</i> 1 | 708.7 ng/μl | 1.88 | 2.01 |
| pXG-mCherry12- <i>YinP</i> 3 | 407.2 ng/μl | 2.08 | 2.08 |
| pXG-mCherry12- <i>YinP</i> 6 | 344.3 ng/μl | 2.06 | 2.04 |
| pXG-mCherry12- <i>YinP</i> 8 | 1,043.3 ng/μl | 2.02 | 2.21 |
| pXG-mCherry34- <i>YinP</i> 2 | 290.1 ng/μl | 2.13 | 1.77 |
| pXG-mCherry34- <i>YinP</i> 5 | 190.6 ng/μl | 2.10 | 1.76 |

Extracted plasmids were once more tested by PCR. Three different pairs of primers were used - one to confirm *YinP* presence, the second to confirm *YinP* presence in the correct direction and the third to exclude *YinP* insert in the opposite direction (Table 15 and 16). Elongation time was 2:30 min. Results were checked by agarose gel electrophoresis. The structure of all plasmids was shown as correct (Figure 26).

Table 15: Primers used to determine by PCR the direction of *YinP* in pXG-mCherry12-*YinP* plasmids for individual colonies

| | YinP presence | Correct direction | Opposite direction |
|----------------|---------------|-------------------|--------------------|
| Forward primer | IST7 | IST40 | IST40 |
| Reverse primer | IST9 | YinP-Leish Fw | YinP-Leish Rv |

Table 16: Primers used to determine by PCR the direction of *YinP* in pXG-mCherry34-*YinP* plasmids for individual colonies

| | YinP presence | Correct direction | Opposite direction |
|----------------|---------------|-------------------|--------------------|
| Forward primer | IST7 | IST39 | IST39 |
| Reverse primer | IST9 | YinP-Leish Rv | YinP-Leish Fw |

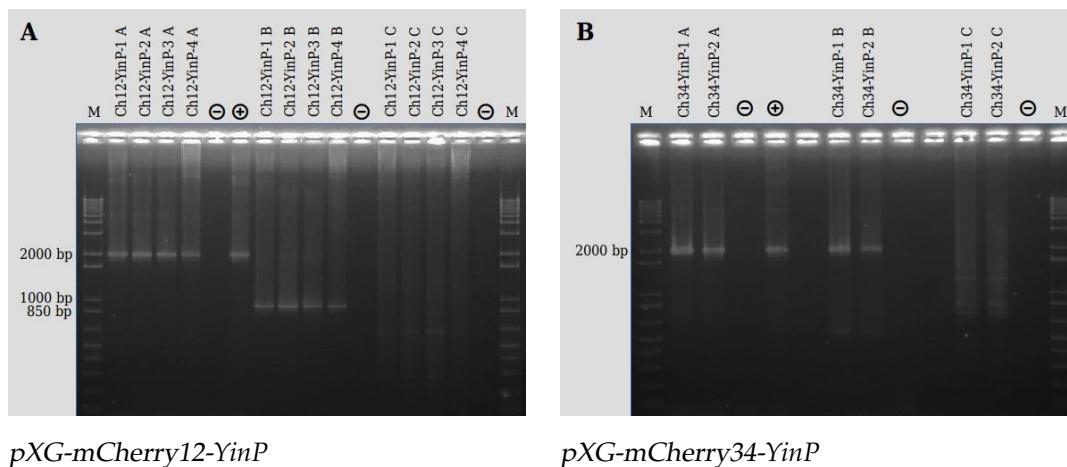


Figure 26: Agarose electrophoresis of PCR products to confirm *YinP* presence and direction in the extracted pXG-mCherry-*YinP* plasmids

M is marker ladder, (-) is negative control, (+) is positive control. (A) 2000 bp bands in the first group of samples [A] confirmed the presence of *YinP*. 920 bp bands in the second group of samples [B] confirmed the *YinP* presence in the correct direction. Absence of 1950 bp bands in the third group of samples [C] confirmed that the *YinP* insert was not in the opposite direction. (B) 2000 bp bands in the first group of samples [A] confirmed the presence of *YinP*. 2080 bp bands in the second group of samples [B] confirmed the *YinP* presence in the correct direction. Absence of 1400 bp bands in the third group of samples [C] confirmed that the *YinP* insert was not in the opposite direction.

In addition, structure of two plasmids for both constructions was also confirmed by *EcoRI* digestion and subsequent agarose gel electrophoresis. The digestion reaction was incubated at 37°C for 1 hour. By comparison of expected and obtained bands, the correct structure was proven for all digested plasmids (**Figure 27**).

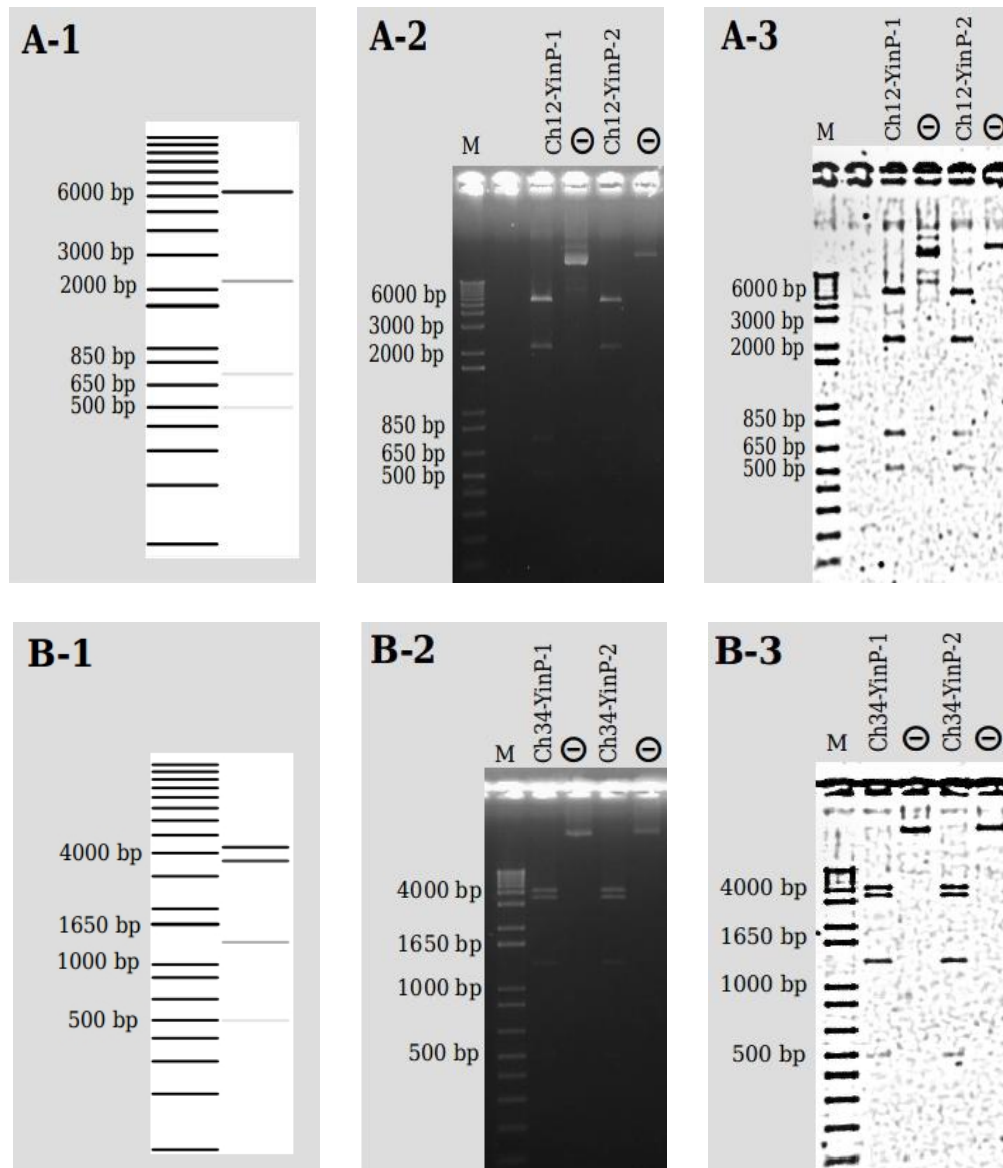


Figure 27: Agarose electrophoresis of PCR products to confirm *YinP* presence and direction in the extracted *pXG-mCherry-YinP* plasmids

(**A-1**) Expected bands after *EcoRI* digestion of *pXG-mCherry12-YinP* plasmid: 6307 bp (65%), 2205 bp (23%), 739 bp (8%) and 498 bp (5%). (From ApE) (**A-2**) Agarose gel electrophoresis after *EcoRI* digestion of *pXG-mCherry12-YinP* plasmids confirming their structure. (**A-3**) The same picture as in A-2 processed by Laplacian edge detection algorithm. (**B-1**) Expected bands after *EcoRI* digestion of *pXG-mCherry34-YinP* plasmid: 4302 bp (44%), 3692 bp (37%), 1319 bp (17%) and 498 bp (5%). (**B-2**) Agarose gel electrophoresis after *EcoRI* digestion of *pXG-mCherry34-YinP* plasmids confirming their structure. (**B-3**) The same picture as in B-2 processed by Laplacian edge detection algorithm.

5.1.6 Verification of plasmid sequence

Two plasmids for both constructions, which were proven from previous steps as being correct, were sent for sequencing to Unidad de Secuenciación de ADN de Universidad de Navarra. 700bp long DNA regions containing mCherry-*YinP* connection were sequenced.

Received sequences were compared to a theoretical nucleotide order by A plasmid editor. Insertion of *YinP* gene in an immediate proximity of mCherry gene was ascertained in all four samples.

In both pXG-mCherry12-*YinP* plasmids one single-nucleotide mutation (transversion) was detected in the connecting section between *YinP* and mCherry. This mutation resulted in serine insertion instead of cysteine. With regard to the fact that this area was designed only to connect both genes without taking into consideration included amino acids, this mutation should have no influence on a function of the constructed plasmids.

5.1.7 Summary

Both target pXG-mCherry12-*YinP* and pXG-mCherry34-*YinP* plasmids were constructed. The whole procedure of their preparation (**Figure 28**) can be briefly summarized in the following steps:

1. *YinP* was amplified by PCR, during the reaction *NotI* restriction sites were inserted in both ends of *YinP*.
2. The *YinP* insert was cloned into a pCR2.1 TOPO vector.
3. *YinP* was cleaved out from the vector by *NotI* enzyme.
4. pXG-mCherry plasmid was digested using the same enzyme to create complementary cohesive ends.
5. After digestion, pXG-mCherry plasmid was desphosphorylated to prevent its self-recircularization.
6. Ligation was performed to insert *YinP* into pXG-mCherry plasmid.

The correct location and direction of *YinP* inside the constructed plasmids were confirmed by three different methods: PCR, restriction enzyme analysis and DNA sequencing.

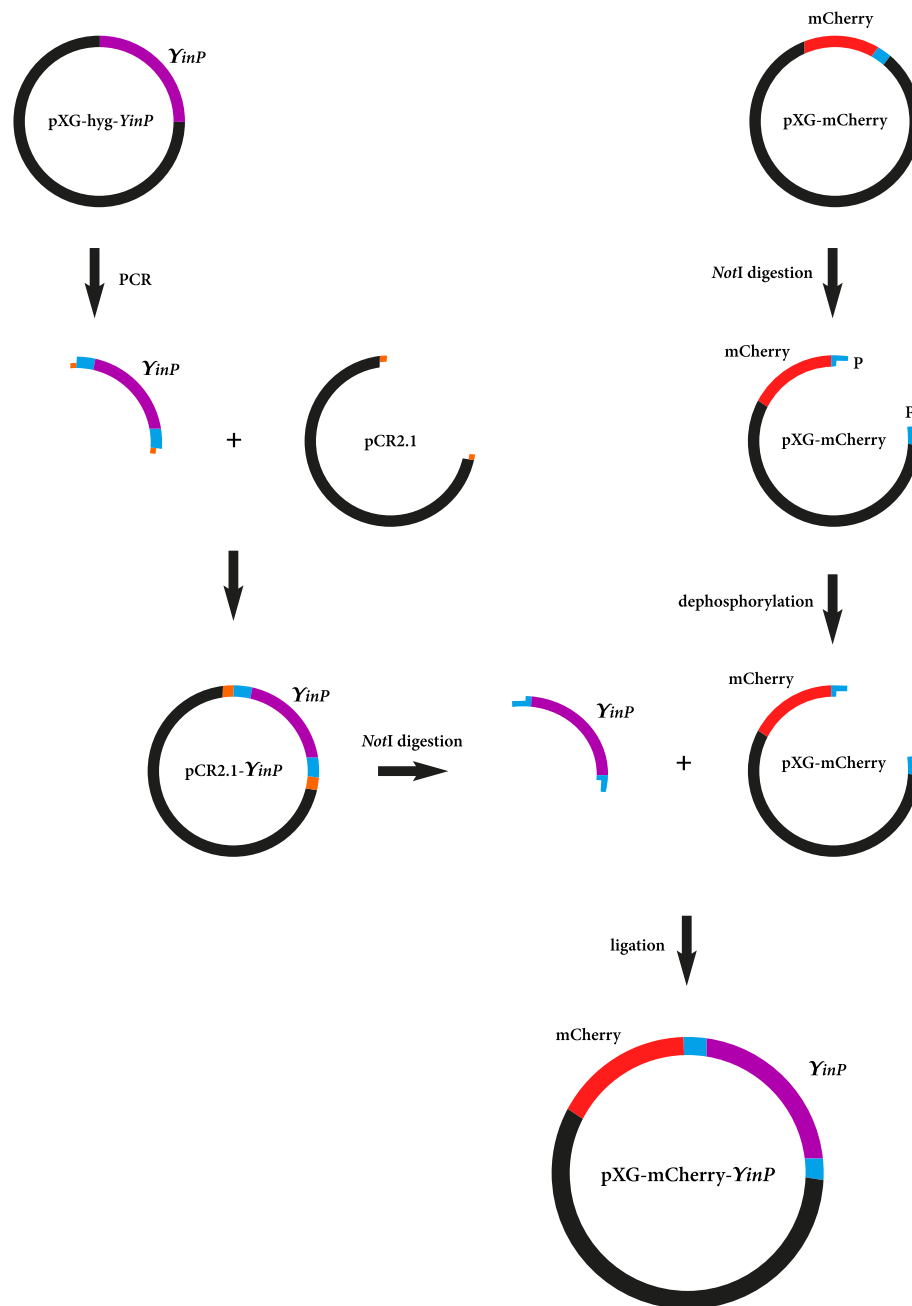


Figure 28: Procedure diagram of pXG-mCherry-YinP plasmids construction

Single main steps of plasmid construction shown with pXG-mCherry12 plasmid. In the left part of picture, PCR amplification of the *YinP* gene, its cloning inside pCR2.1 vector and *NotI* digestion is illustrated. In the right part of picture, pXG-mCherry plasmid digestion and dephosphorylation can be seen. The last portrayed reaction is the ligation of *YinP* gene with pCG-mCherry plasmid.

5.2 Gene expression location in leishmania

Four different plasmids were used and transfected into leishmania parasites by electroporation: pXG-mCherry12, pXG-mCherry34, pXG-mCherry12-*YinP* and pXG-mCherry34-*YinP*. Two separated double-pulse electroporations were performed with all of them: one with 25 μF capacitance and the other with 50 μF capacitance. Time constants were in most cases close to the interval 0.5 - 0.8, which is supposed to be ideal (**Table 17**).

Table 17: Time constants achieved during electroporation

| Plasmid | Capacitance | T ₁ | T ₂ |
|----------------------------|------------------|----------------|----------------|
| pXG-mCherry12 | 25 μF | 0.48 | 0.42 |
| | 50 μF | 0.82 | 0.84 |
| pXG-mCherry34 | 25 μF | 0.48 | 0.44 |
| | 50 μF | 0.82 | 1.00 |
| pXG-mCherry12- <i>YinP</i> | 25 μF | 0.48 | 0.42 |
| | 50 μF | 0.86 | 0.82 |
| pXG-mCherry34- <i>YinP</i> | 25 μF | 0.46 | 0.42 |
| | 50 μF | 0.84 | 0.76 |

After 5 hours of recovery, parasites were cultivated in medium with addition of hygromycin to select successfully transformed leishmania. When cultures reached the sufficient number of parasites, they were fixed, stained with DAPI, and subsequently observed under fluorescent microscopy.

To show appearance of *Leishmania major* promastigotes and location of nucleus and kinetoplast, wild-type parasites were also cultivated and prepared for microscopy. Blue fluorescence emitted by DAPI bounded to DNA was observed. A smaller brighter blue spot close to the flagellum is a kinetoplast and the other bigger blue spot, approximately in the middle of the leishmanial cell, is a nucleus (**Figure 29**).

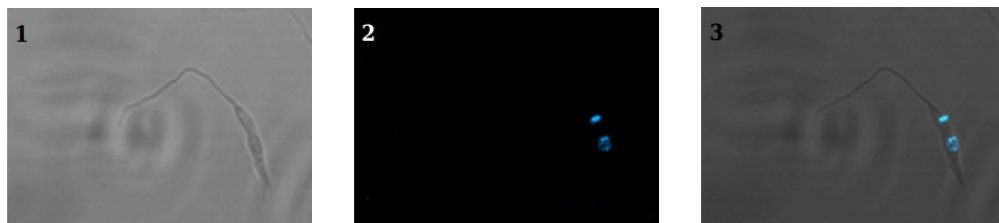


Figure 29: Wild-type *Leishmania major* promastigote after DAPI staining

In all three photos is shown the same wild-type *Leishmania major* promastigote under different microscopy conditions. (1) bright field microscopy. (2) fluorescence microscopy. (3) merge microscopy (bright field and fluorescence).

Blue fluorescence is emitted by DAPI bounded to DNA - nucleus (bigger blue spot, approximately in the middle of the cell) and kinetoplast (smaller, brighter blue spot close to the flagellum).

Fluorescence of mCherry protein in parasites expressing pXG-mCherry12 and pXG-mCherry34 was spread in the entire cell without any specific localization (**Figure 30 [A]** and **Figure 31 [A]**).

On the contrary, the red fluorescence of mCherry fused with YinP protein was observed only inside a blue fluorescent area within the leishmanial cells, e.i. in the nuclei of the examined parasites (**Figure 30 [B, C]** and **Figure 31 [B, C]**). The nuclear localization of the red fluorescence produced by mCherry was observed in both cases of electroporated parasites, it means those expressing pXG-mCherry12-YinP and pXG-mCherry34-YinP. That should exclude a possibility that mCherry binding to N-terminal or C-terminal of YinP protein has any influence on its expression location.

The difference in red fluorescence localization between parasites expressing the original pXG-mCherry plasmids (with no inserted gene) and the new constructed pXG-mCherry-YinP plasmids is clearly visible.

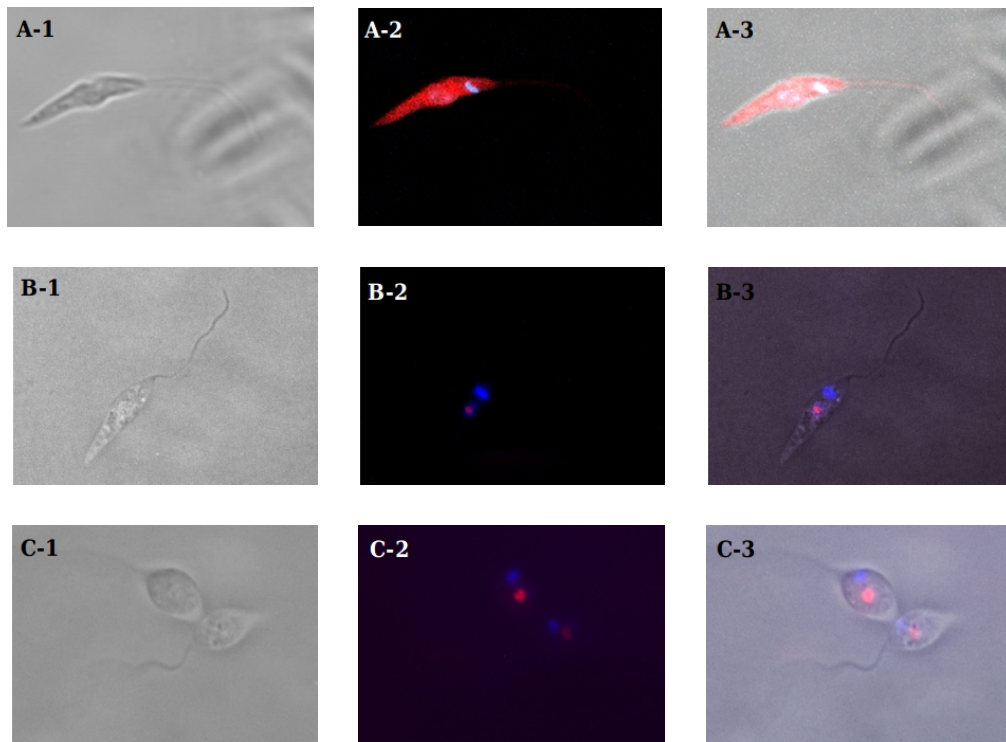


Figure 30: Fluorescent images of *Leishmania major* promastigotes transfected with pXG-mCherry12 or pXG-mCherry12-YinP plasmids after DAPI staining

Blue fluorescence is emitted by DAPI bounded to DNA in the nucleus (bigger spot, harder to see, in the middle of the cell) and kinetoplast (brighter, in the anterior part of the cell close to the flagellum). Red fluorescence is emitted by mCherry. **(A)** *Leishmania major* promastigote expressing pXG-mCherry12 plasmid under bright field [1], fluorescence [2] and merge [3] microscopy. **(B, C)** *Leishmania major* promastigotes expressing pXG-mCherry12-YinP plasmids under bright field [1], fluorescence [2] and merge [3] microscopy. In these cases, red fluorescence emitted by mCherry is localized only in the areas of the nuclei.

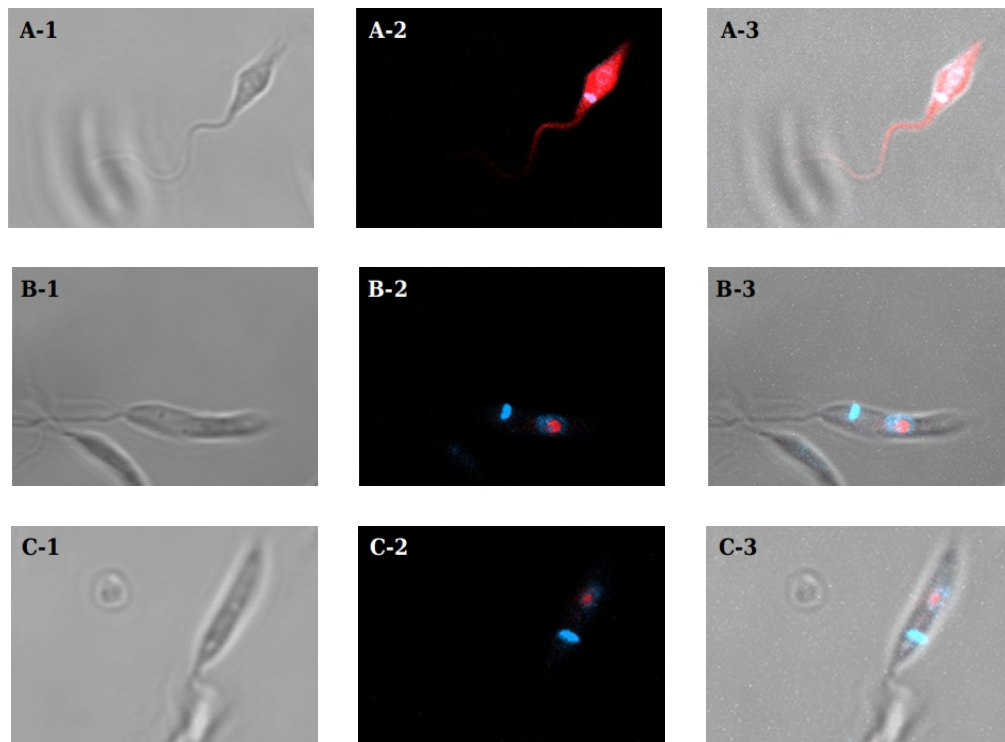


Figure 31: Fluorescent images of *Leishmania major* promastigotes transfected with pXG-mCherry12 or pXG-mCherry12-YinP plasmids after DAPI staining

Blue fluorescence is emitted by DAPI bounded to DNA in the nucleus (in the middle of the cell) and kinetoplast (brighter, in the anterior part of the cell close to the flagellum). Red fluorescence is emitted by mCherry. **(A)** *Leishmania major* promastigote expressing pXG-mCherry34 plasmid under bright field [1], fluorescence [2] and merge [3] microscopy. **(B, C)** *Leishmania major* promastigotes expressing pXG-mCherry34-YinP plasmids under bright field [1], fluorescence [2] and merge [3] microscopy. In these cases, red fluorescence emitted by mCherry is localized only in the areas of the nuclei.

Therefore our results showed that the YinP protein is expressed in the nucleus, actually only in a part of the nucleus. This detected nuclear localization of the YinP protein corresponds positively with our presumption based on in silico studies and the predicted tertiary structure of the protein (Algarabel-Olona et al., 2015). Further experiments need to be performed, to analyse whether such an expression is related to a nucleolus.

If YinP protein will show itself up as a promising target in a new anti-leishmanial therapy, any potential molecule targeted against it will most probably have to be able to penetrate into the leishmania nucleus.

6. CONCLUSION

All objectives of this master thesis were successfully achieved.

Both target plasmids (pXG-mCherry12-*YinP* and pXG-mCherry34-*YinP*) containing *YinP* gene in the very proximity of the gene encoding fluorescent mCherry protein were constructed. Their structures were confirmed by PCR, restriction analysis and the connecting region was also sequenced.

Subsequently, these plasmids were used for a transfection of *Leishmania major* via electroporation. Parasites were cultivated in medium with antibiotics added to select those individuals able to express inserted plasmids. After incubation, leishmania cells were stained with DAPI to visualize their nucleus and kinetoplast under fluorescent microscopy.

Fluorescence of mCherry fused with YinP protein was observed in the nucleus, whereas when pXG-mCherry plasmid without *YinP* gene was expressed, fluorescence was spread in the entire cell. The nuclear localization of the red fluorescence produced by mCherry was observed in both cases of electroporated parasites, i.e. those expressing pXG-mCherry12-*YinP* and pXG-mCherry34-*YinP*. That should exclude a possibility that mCherry binding to N-terminal or C-terminal of YinP protein has any influence on its expression location.

To conclude, YinP protein in *Leishmania major* is expressed in the nucleus, which was established by cell localization of fluorescent fusion proteins. These proteins were synthesised by parasites on the basis of the two novel constructed plasmids containing *YinP* gene: pXG-mCherry12-*YinP* and pXG-mCherry34-*YinP*.

ABBREVIATIONS

| | |
|--------------|---|
| ATC | anatomical therapeutic chemical (drug classification) |
| BC | before Christ |
| CanL | canine leishmaniasis |
| CL | cutaneous leishmaniasis |
| DALY | disability-adjusted life year |
| DAPI | 4',6-diamidino-2-phenylindole |
| DCL | diffuse cutaneous leishmaniasis |
| EDTA | ethylenediaminetetraacetic acid |
| EGFP | enhanced green fluorescent protein |
| FBS | fetal bovine serum |
| FP | fluorescent protein |
| GFP | green fluorescent protein |
| HEPES | 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid |
| IM | intramuscular |
| ISTUN | Instituto de Salud Tropical Universidad de Navarra |
| IV | intravenous |
| kDNA | kinetoplastid DNA |
| MCL | mucocutaneous leishmaniasis |
| NLS | nuclear localization signal |
| NTD | neglected tropical disease |
| PCR | polymerase chain reaction |
| PKDL | post kala-azar dermal leishmaniasis |
| PSG | promastigote secretory gel |
| RE | restriction endonuclease |
| RFP | red fluorescent protein |
| rpm | revolutions per minute |
| RT | room temperature |
| Tris | tris[hydroxymethyl]aminomethane |
| TSB-D | Bouillon tryptic soja |
| UV | ultraviolet |
| VL | visceral leishmaniasis |
| WHO | World Health Organization |

Nucleotides:

| | |
|----------|----------|
| A | adenine |
| C | cytosine |
| G | guanine |

T thymine
N nucleotide (A/C/G/T)

In the figures:

M marker ladder (1 Kb Plus DNA ladder)
- negative control
+ positive control

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APPENDICES

A 1 Kb Plus DNA ladder

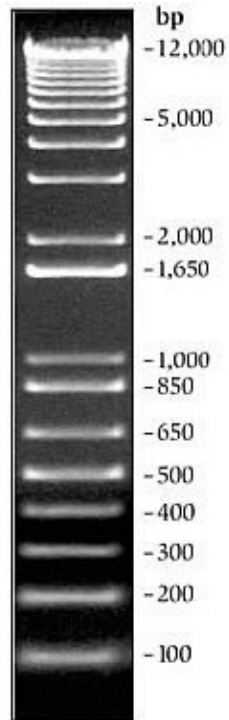


Figure 32: *1 Kb Plus DNA ladder*

0.9 μ l, 0.9% agarose gel, stained with ethidium bromide. Adapted from ThermoFisher Scientific websites, modified. (<https://www.thermofisher.com/order/catalog/product/10787018>)

B Extended pXG-mCherry-YinP plasmids' maps

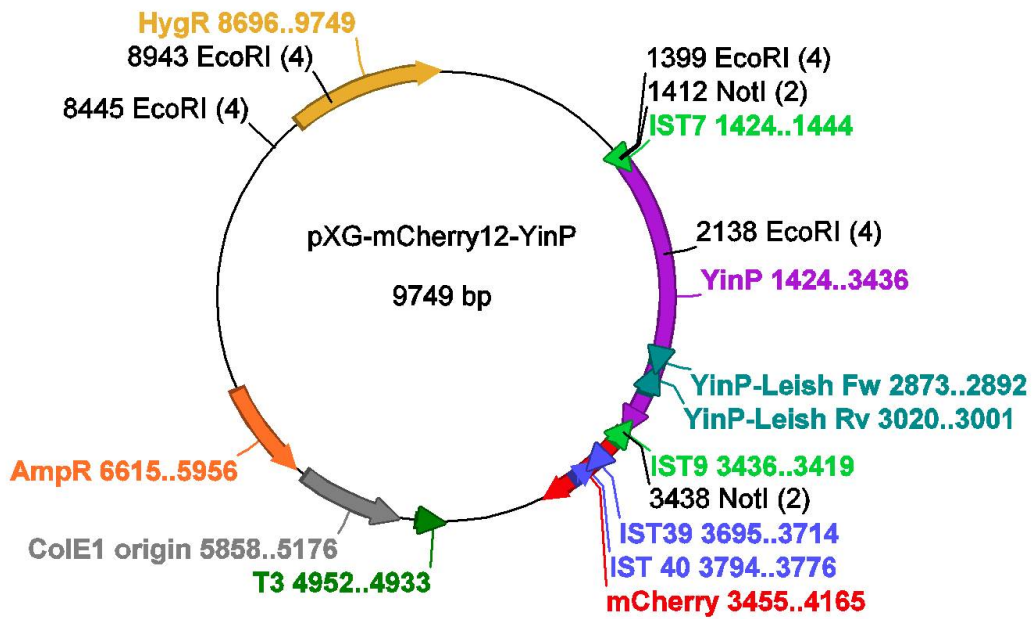


Figure 33: *pXG-mCherry12-YinP* plasmid with all used primers and REs digestion sites illustrated

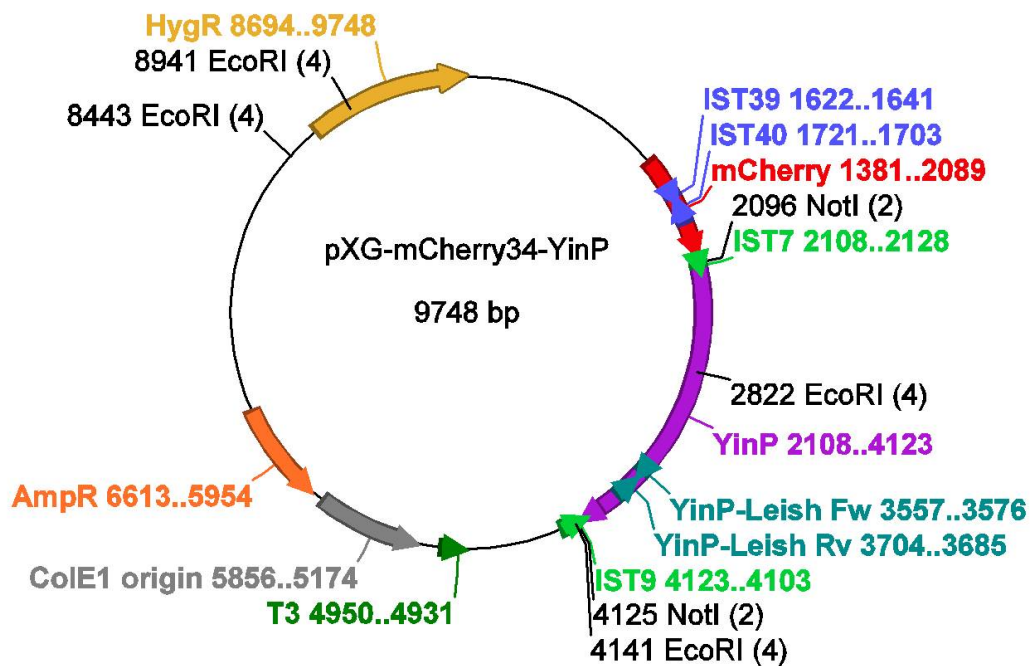


Figure 34: *pXG-mCherry34-YinP* plasmid with all used primers and REs digestion sites illustrated