UNIVERSITY OF SÃO PAULO
INSTITUTE OF BIOMEDICAL SCIENCES

MIRIAM HIROMI BORGES

Search of genes playing a role in *Plasmodium* transmission

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Busca por genes que desempenham papel na transmissão do *Plasmodium*

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Search of genes playing a role in *Plasmodium* transmission

Reviewed Version

Thesis presented to the Post-graduation program Biology of Host-Pathogen Interactions at the Institute of Biomedical Sciences of the University of São Paulo, in order to obtain the title of Master of Sciences.

Concentration area: Biology of Host-Pathogen Interactions

Advisor: Daniel Youssef Bargieri

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MIRIAM HIROMI BORGES

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DECLARAÇÃO

Em adendo ao Certificado nº **132/2014/CEUA**, válido até 05/02/2019, e por solicitação do Prof. Dr. **Daniel Youssef Bargieri**, do Departamento de Parasitologia, responsável pela linha de Pesquisa, autorizo a inclusão do(a) aluno(a) **Miriam Hiromi Borges** ao Projeto de Pesquisa ***"Estudos de Genética Molecular de Plasmodium berghe"***, uma vez que se trata de utilização da mesma espécie animal e de métodos experimentais similares ao Projeto.

São Paulo, 06 de setembro de 2017.

Luciane Valéria Sita
Profa. Dra. **Luciane Valéria Sita**
Coordenadora da CEUA-ICB/USP

DEDICATION

Dedico essa dissertação aos meus pais Edimilson e Márcia e à minha irmã Isabela. Vocês são maravilhosos! Obrigada por todo incentivo, palavras de carinho e também por todo apoio que me deram em momentos difíceis. Gu, também agradeço muito por tudo que faz por mim. Obrigada por todo amor e por estar disposto a me ajudar em qualquer circunstância. Aos meus familiares e amigos, obrigada por estarem sempre ao meu lado.

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// RESUMO

BORGES, Miriam Hiromi. **Busca por genes que desempenham papel na transmissão do *Plasmodium***. 2020. 108 f. Dissertação (Mestrado em Biologia da Relação Patógeno-Hospedeiro) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2020.

A malária é uma doença infecciosa que pode ser prevenida, mas se não for corretamente diagnosticada e tratada pode resultar em casos graves e até mesmo em morte. Agravando ainda mais a situação, novos casos de resistência à inseticidas e a antimaláricos vêm surgindo em todo o mundo e por isso a necessidade de eliminar a malária é urgente. Uma boa estratégia para se conseguir a eliminação é o desenvolvimento de vacinas contra qualquer uma das três etapas do ciclo de vida de seu parasito causador (*Plasmodium*). Recentemente, antígenos envolvidos na transmissão do parasito para o mosquito estão sendo propostos como alvo para compor uma vacina bloqueadora da transmissão. Assim, o objetivo desse trabalho é utilizar técnicas de genética reversa e o modelo murino da malária (*Plasmodium berghei*) para estudar um candidato que possa desempenhar papel importante na transmissão da doença. Assim, foi feita uma análise sistemática de genes altamente transcritos em gametócitos maduros de *P. falciparum* na base de dados exclusiva do parasita (PlasmoDB). Ao final, foram encontrados 12 genes sobre os quais ainda não haviam sido reportados estudos na literatura. Um deles, PBANKA_1038800, possui ortólogos em *P. berghei* e em *P. vivax*. A provável proteína obtida a partir desse gene possivelmente está localizada na membrana celular e possui um domínio EGF predito, podendo ser importante para fertilização do gameta, motilidade ou invasão do oocineto ao epitélio do intestino do mosquito. Utilizamos técnicas de genética reversa para estudar a função deste gene na transmissão do parasito. Clones nocautes para o gene PBANKA_1038800 foram genotipados por PCR e fenotipados através de diversos ensaios de fertilização *in vitro*. Esses clones tiveram multiplicação normal no sangue de camundongos e formaram níveis normais de gametócitos circulantes. Ensaios de exflagelação mostraram que o gametócito macho do nocaute era capaz de exflagelar normalmente. Nos ensaios de fertilização *in vitro*, o nocaute não foi capaz de formar zigotos e oocinetos, sugerindo que o gene PBANKA_1038800 desempenha papel importante nos estágios esporogônicos do *Plasmodium* no mosquito.

Palavras-chave: Malária. Transmissão. Genes essenciais. Novos alvos.

ABSTRACT

BORGES, Miriam Hiromi. **Search of genes playing a role in *Plasmodium* transmission**. 2020. 108 f. Master thesis (Biologia da Relação Patógeno-Hospedeiro) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2020.

Malaria is a preventable infectious disease, but if not properly diagnosed and treated it can result in severe cases and even death. Further aggravating the situation, new cases of resistance to insecticides and antimalarials are emerging all over the world and, therefore, the need to eliminate malaria is urgent. A good strategy to achieve elimination is to develop vaccines against any of the three stages in the life cycle of its causative parasite (*Plasmodium*). Recently, antigens involved in the transmission of the parasite to the mosquito are being proposed as a target to compose a transmission blocking vaccine. Thus, the objective of this work is to use reverse genetic techniques and the murine model of malaria (*Plasmodium berghei*) to study a candidate that may play an important role in the transmission of the disease. Thus, a systematic analysis of highly transcribed genes in mature *P. falciparum* gametocytes was performed in the exclusive parasite database (PlasmoDB). The search resulted in 12 genes which had not yet been reported in the literature. One of them, PBANKA_1038800, has orthologs in *P. berghei* and in *P. vivax*. The probable protein obtained from this gene is possibly located on the cell membrane and has a predicted EGF domain, which may be important for gamete fertilization, motility or invasion of the ookinete to the epithelium of mosquito midgut. We used reverse genetic techniques to study the function of this gene in the transmission of the parasite. Knockout clones for the PBANKA_1038800 gene were genotyped by PCR and phenotype through various *in vitro* fertilization assays. These clones had normal multiplication in the blood of mice and formed normal levels of circulating gametocytes. Exflagellation tests showed that the knockout male gametocyte was able to exflagellate normally. In *in vitro* fertilization assays, the knockout was not able to form zygotes and ookinetes, suggesting that the PBANKA_1038800 gene plays an important role in the sporogonic stages of *Plasmodium* in the mosquito.

Keywords: Malaria. Transmission. Essential genes. New targets.

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LIST OF ABREVIATIONS

ACTs	Artemisinin-based combination therapy
AMA1	Apical Membrane Antigen 1
BOD	Bio-Oxygen Demand
BSG	Basigin
CDC	Centers For Disease Control and Prevention
ChAd63	Chimpanzee adenovirus serotype 63
CEUA	*Comissão de Ética no Uso de Animais* (Animal Use Ethics Committee)
CONCEA	*Conselho Nacional de Controle de Experimentação Animal* (National Council for Control of Animal Experimentation)
CQ	Chloroquine
CSP	Circumsporozoite Protein
CTRP	Circumsporozoite and TRAP Related Protein
CyRPA	Cysteine-rich protective antigen
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EC	Exflagellation Center
EGF	Epidermal Growth Factor
gDNA	Genomic Deoxyribonucleic acid
GDP	Gross Domestic Product
GFP	Green fluorescent protein

GHIT	Global Health Innovative Technology
GIMO	Gene Insertion/Marker Out line
GO	Gene Ontology
GPI	Glycosylphosphatidylinositol
HA	Hemagglutinin
HBsAg	Hepatitis Virus B surface antigen
hDHFR	Human Dihydrofolate Reductase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSPGs	Heparan Sulphate Proteoglycans
IFN-γ	Interferon gamma
INTs	Insecticide-treated bed nets
iRBC	Infected Red Blood Cell
IRS	Indoor Residual Spraying
LB Agar	Luria-Bertani Agar
MCS	Multiple Cloning Site
MTRAP	Merozoite Thrombospondin-Related Anonymous Protein
MSP1	Merozoite Surface Protein 1
MVA	Modified Vaccinia Virus ANKARA
MVIP	Malaria Vaccine Implementation Programme
NIH	National Institute of Allergy and Infectious Diseases
nLuc	Nano-Luciferase
Pb1038800^{KO}	*Plasmodium berghei* Ookluc_1038800 knockout
PbANKA	*Plasmodium berghei* ANKA line

PbOokluc	*Plasmodium berghei* Ookluc
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PfEMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein 1
RBCs	Red Blood Cells
RLU	Relative Light Units
RPMI	Roswell Park Memorial Institute 1640 Medium
RH5	Reticulocyte-binding protein homolog 5
RIPR	RH5 interacting protein
RNA	Ribonucleic acid
TBA	Transmission Blocking Antigens
TBV	Transmission Blocking Vaccines
TMH	Transmembrane Helices
TNF-α	Tumor Necrosis Factor Alpha
VLP	Virus-like particles
WHO	World Health Organization

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1 INTRODUCTION

Malaria (*mal'aria* in Italian, meaning "bad air") is an infectious disease that is probably in the world since prehistory (CAMARGO, 1995; COX, 2010). For more than 2500 years, people believed that the "periodic fevers", a classic symptom of the disease, were caused by miasmas raising from marshy places. In 1880 this belief changed after the discovery of the causative malaria agent by Alphonse Laveran, after examining blood from individuals suffering with the malignant fever (COX, 2010; LAVERAN, 1907).

Human malaria is caused by a protozoan from the genus *Plasmodium*, being *P. falciparum* and *P. vivax* the species responsible for the majority of cases in the world. The parasite is transmitted through the bite of an infected female *Anopheles* mosquito. In Brazil, the disease is still endemic in the Amazonian region and the main specie is *P. vivax* (WHO, 2020b). The complex life cycle of this parasite involves two hosts: a vertebrate (human), its intermediate host, and a invertebrate (mosquito), the definitive host. Thus, *Plasmodium* life cycle has three important phases, being one inside the mosquito and the pre-erythrocytic and erythrocytic stages inside the vertebrate host (COX, 2010).

According to the World Health Organization, almost half of the world's population was at risk to contracting malaria in 2018. In the same year, more than 228 million cases were reported and there were an estimated 405.000 deaths cases from malaria. Every year, most of the mild and fatal cases occur in the African Region, affecting people living in poverty and with difficult access to adequate health services. Moreover, among these people are those who develop the most severe form of the disease, including infants, young children and pregnant women (WHO, 2020b).

Malaria is still endemic and represents a great threat in many countries. For this reason, its elimination is urgent. After 2010, eight countries received the "malaria free certificate" from the World Health Organization, among them Algeria, Morocco, Argentina and Paraguay, being two in Africa and two in South America, where *vivax* malaria is difficult to control and to eliminate. Besides that, in 2018, three countries (Iran, Malaysia and Timor-Leste) reported zero indigenous malaria cases for the first time (WHO, 2019).

To the present day, some strategies are used to reduce risk of malaria transmission. In order to prevent mosquito contact with humans, the use of insecticide inside houses and even the use of bed nets treated with insecticides, have been implemented in endemic areas. Also, treatment of infected people plays an important role regarding the prevention of parasites transmission to the mosquito. Generally, these preventives human-mosquito contact methods

help, but are not sufficient. Vaccines would be very important in reducing transmission as well (CARTER; MENDIS, 2002).

Before the genomic era, the search focus to the elimination was on new and more effective formulations for the already known antigens. With development of new technologies it was possible to access information in the area of genomics, proteomics and transcriptomics. Therefore, the focus changed to the discovery of new antigens for the development of novel vaccines, since it became possible the detection of potential vaccine candidates through bioinformatics (DAVIES et al., 2015).

A promising strategy is the creation of vaccines that are able to block *Plasmodium* transmission from the human host to the mosquito. The aim of this form of vaccination is to considerably reduce transmission in endemic areas, requiring the occurrence of an immune response in the human being, but targeting specific antigens of the sexual stage of the parasite (GONÇALVES; HUNZIKER, 2016). Inside vertebrate host, pre-fertilization antigens are expressed in gametocytes, but antibodies against them can only act after gametes egress from erythrocytes (process that occurs inside the mosquito), preventing zygote formation (SAUERWEIN; BOUSEMA, 2015). As this idea is relatively recent, new antigens have to be discovered in order to develop a transmission blocking vaccine.

Recent work carried out with MTRAP, a protein previously thought to play a role during the parasite asexual multiplication, showed the effectiveness of reverse genetic techniques to identify new important antigens for transmission, since the experiment with the MTRAP deficient mutant proved that this protein is dispensable for the parasite during blood stages, but is essential for gametes egress from erythrocytes, and consequently, for *Plasmodium* transmission to the mosquito (BARGIERI et al., 2016). This work confirms the importance of our search strategy for new candidates, where 12 potentially important genes for parasite's transmission were identified. Thus, our main objective was to analyze one of the twelve genes found and check if it is involved in the transmission of *Plasmodium* using reverse genetic techniques.

2 LITERATURE REVIEW

2.1 Malaria: general aspects

2.1.1 Brief history

Malaria is an important parasitic disease that is probably in the world since prehistory, having first appeared in Africa. This ancient disease has always been a serious problem in every part of the world, with exception of polar and subpolar regions, and its extensive spread happened because of human traveling and dispersing across continents (CAMARGO, 1995).

Between 2700 BC to 1570 BC, several reports pointed to possibly presence of malaria in China, Mesopotamia and Egypt due to periodic fevers in those regions (COX, 2010). More certainly, in 400 BC, Hippocrates was the first to describe tertian and quartan fevers in people living in swamps and its association with splenomegaly. There were also reports during ancient Rome history and Middle Ages (CAMARGO, 1995).

The word malaria probably came from the Italian word *mal'aria* ("bad air"). For over 2500 years, people used to believe that the "periodic fevers" were caused by miasmas raising from marshy places. Only from the 17th century, with the discovery of bacteria and its importance as cause of infectious disease, the idea that those fevers were due to miasmas was questioned, and the search for the real cause was intensified (COX, 2010).

In 1880, while working in Algeria (a country in North Africa), a French doctor named Charles Louis Alphonse Laveran examined patients with the malignant fever, and found parasites in their blood. Laveran saw and described different phases of what he discovered, and that now is known as male and female gametocytes, trophozoites and schizonts. At the time, the doctor described the causative agent of malaria and named it "*Oscillaria malarie*". Later, the name was modified to *Plasmodium malariae*. In 1907, Laveran was awarded the Nobel Prize in Physiology or Medicine in recognition of his discoveries about the importance of protozoa in causing diseases (LAVERAN, 1907).

Five years after Laveran's discovery, an Italian doctor named Camillo Golgi, known for his contributions to central nervous system studies, was visiting a colleague in Rome, named Ettore Marchiafava, who showed Golgi some of his observation on malaria. Golgi returned to his city, Pavia, and started to analyze malaria cases in the swamps of Po River. He soon established the temporal coincidence between the regular fever with release of the schizonts and toxins in the bloodstream, describing the cycle in the red blood cells (GARNHAM, 1988).

The presence of *Plasmodium* in the blood was a fact, but how it was transmitted was still a mystery. Only in 1897, the British physician Ronald Ross, using an avian model, demonstrated that hematophagous mosquitoes could transmit the parasites to a non-infected animal, after having bite an infected one, incriminating the insect as the vector of avian malaria. Ross also demonstrated *Plasmodium* life-cycle inside the mosquito. His discoveries awarded him the Nobel Prize in 1902 (INSTITUTE OF MEDICINE, 2004; ROSS, 1897).

In 1898, a group of Italian scientists, formed by Giovanni Grassi, Giuseppe Bastianelli and Amico Bignami demonstrated that *Plasmodium* could be transmitted to humans through the bite of the *Anopheles* mosquito (MANGUIN; CARNEVALE; MOUCHET, 2008).

All of these findings were of great importance, especially on the discovery of an efficient treatment against malaria. From the end of the 15th century until the discovery of quinine in the middle of the 19th century, the European population suffered great losses due to the high mortality caused by infectious diseases, including malaria. The implementation of quinine in the fight against this disease proved to be very efficient, since at the end of the 19th century, as malaria mortality declined progressively until today with its eradication in Europe and most part of North America, with exception of Mexico (CARTER; MENDIS, 2002).

The mostly accepted theory on the arrival of *Plasmodium falciparum* in America is that it dated from the middle of the 16th century until beginning of the 18th century, when the disease was still endemic in Europe. During this time, South America was colonized by Portugal and Spain. Colonists traveled for months on ships, crossing the entire Atlantic Ocean, and it is very likely that they carried with them the most virulent human malaria parasite. Besides that, the transatlantic African slave trade also contributed to the introduction of this parasite in many South America countries (GRIFFING et al., 2015; RODRIGUES et al., 2018).

While the theory on the arrival of *P. falciparum* in America is well accepted, the origin of *P. vivax* in the New World is still controversial. One hypothesis is that the parasite has entered the continent with migrants from Southern Asia and Western Pacific, even before colonization (CARTER; MENDIS, 2002; RODRIGUES et al., 2018). However, it is possible that it happened after the arrival of European and African peoples. Once in America, the parasite found a receptive *Anopheles* mosquito, fact that contributed to its natural spread, making malaria an endemic disease (CARTER; MENDIS, 2002).

In Brazil, the first records of malaria cases after the arrival and settle of the Portuguese, occurred in 1587, among the Tupinambá indians. Only at the end of the19th century, malaria became a severe problem. During that period, the collection of latex from rubber trees represented an important economic activity in Brazil. This fact caused the migration of workers

from the Ceará state (Northeast of Brazil) to the Amazon Region (North of Brazil), the main producer of rubber. As they were immunologically susceptible to malaria, almost all of them acquired the disease and many died of it (DEANE, 1986).

2.1.2 Main species and Plasmodium life cycle

Malaria is an infectious disease caused by a protozoan belonging to the *Apicomplexa* phylum (composed of eukaryotic protozoa of mandatory intracellular life) and to the genus *Plasmodium*, capable of invading and multiplying within erythrocytes and hepatocytes. As well as the majority of apicomplexans parasites, *Plasmodium* develops inside a parasitophorous vacuole and its proliferation depends on invasion of a host cell, where replication occurs until the host cell is lysed. The different stages of development of *Plasmodium* parasites have different shapes, sizes and functions. Only sporozoites and merozoites have an apical complex formed by rhoptries and micronemes, which are organelles involved in the cell invasion process (MORRISSETTE; SIBLEY, 2002).

Plasmodium parasites can infect a wide variety of vertebrates, including reptiles, birds and humans, and it is transmitted to the host through the bite of an infected female *Anopheles* mosquito, which uses host blood for egg production (COX, 2010).

All over the world there are more than 400 *Anopheles* species, of which about 30 are important vectors for malaria transmission (WHO, 2020b). From those, around 18 are prevalent in South America and five in Brazil, being *Anopheles darlingi* responsible for the majority of malaria transmission in the Amazon region, but other species as *An. Albitarsis*, *An. Aquasalis*, *An. cruzzi* and *An. bellator* can also spread the disease, especially in the extra-Amazonian region (CARLOS et al., 2019; WHO, 2019). In Africa, where the majority of malaria cases occur, the main vector responsible for *Plasmodium* transmission is the *An. gambiae* (MILES et al., 2017).

Human malaria is caused by five *Plasmodium* species:

a) *Plasmodium malariae*, which was described in 1880 by Laveran, is responsible for the “quartan fever”, as a fever episode occurs every 72h. This period corresponds to when merozoites exits the host erythrocyte (preferably old cells). Due to this long period of multiplication, parasitemia is usually low in patients infected with *P. malariae* (COLLINS; JEFFERY, 2007);

b) *Plasmodium vivax* was described by Grassi and Feletti in 1890 and is responsible for the “tertian fever”, as the erythrocytic cycle repeats approximately every 48h. Parasitemia in patients infected with *P. vivax* is relatively low, because it prefers to infect reticulocytes rather

than erythrocytes. Another important characteristic is the ability to develop hypnozoites, a dormant form of the parasite that can occur during the liver stage. This is the specie prevalent in South America and Asia, but not limited to these areas (ADAMS; MUELLER, 2017);

c) *Plasmodium falciparum* was described by William Welch in 1897 (SINGLETON, 2018). As well as the *P. vivax*, its erythrocytic cycle lasts 48h. This specie is responsible for the majority of deaths due to malaria, especially in sub-Saharan Africa. The severity is due to the fact that the mature form of the parasite can bind to the endothelial cells of blood micro vessels, causing localized obstruction, mainly in the brain, leading to cerebral malaria (MEIBALAN; MARTI, 2017; SMITH et al., 2013);

d) *Plasmodium ovale* was described by John Stephens in 1922 and before that, this specie was considered by many investigators as a variant of *P. vivax* due to the similar morphology. Besides that, the erythrocytic cycle lasts 48h and the parasite can also develop hypnozoites. Its transmission is limited to sub-Saharan Africa and Southeast Asia (COLLINS; JEFFERY, 2005; COX, 2010);

e) *Plasmodium knowlesi* was described in the early 1930s by Sinton and Mulligan. In nature, it is found in pig-tailed and long-tailed macaques. Its transmission is limited to Southeast Asia, and the recognition that this parasite could infect humans occurred only after 2004, when an outbreak of knowlesi malaria occurred in Malaysia. Moreover, probably several cases have been misdiagnosed, because it was only after the development of new molecular diagnostic techniques that was possible to distinguish morphologically *P. knowlesi* from *P. malariae*. Among all the five species that infect humans, *P. knowlesi* has the shortest erythrocytic cycle, lasting 24h (SINGH; DANESHVAR, 2013).

The duration of the erythrocytic cycle vary according to the specie of the parasite, being 24h in infections with *P. knowlesi*, 48h with *P. vivax*, *P. falciparum* and *P. ovale* and 72h with *P. malariae*. It is during this cycle that the main symptoms (fever, chills, sweat, etc.) appear. A fever peak can occur after the rupture of the red blood cells (RBCs) and release of merozoites in the bloodstream, resulting in cyclic fever episodes occurring every time the parasite completes one erythrocytic cycle. However, in mixed infections (more than one specie of the parasite infecting the host) or in the beginning of the disease, this fever pattern may not occur, making the correct diagnosis more difficult (SINGH; DANESHVAR, 2013).

Plasmodium life cycle is similar among the five species that affect humans. In order to develop, the parasite must infect two hosts: a vertebrate (human), its intermediate host, and an invertebrate (mosquito), the definitive host. Thus, *Plasmodium* life cycle (Figure 1) can be divided in two phases (COX, 2010):

a) inside human host: where the asexual proliferation (schizogony) occurs. Comprehends two stages:

- pre-erythrocytic stage: when *Anopheles* is infected with *Plasmodium* parasites, it carries infective sporozoites in the salivary glands. While doing the blood meal, saliva is deposited in order to prevent coagulation, and sporozoites are injected into the host skin (MEIBALAN; MARTI, 2017; MÉNARD et al., 2013). The parasite uses gliding (circular movements) to move, a portion (around 25%) reaches the peripheral circulation, and most of them migrate through the bloodstream until reaches the liver. Once there, the parasite invades hepatocytes through the binding of a surface protein of the sporozoite, the circumsporozoite protein (CSP), to the liver heparan sulphate proteoglycans (HSPGs), a receptor present on the plasma membrane of liver cells (FREVERT et al., 1993; MÉNARD et al., 2013). Following hepatocyte invasion, sporozoites start to grow and multiply inside the parasitophorous vacuole (MÉNARD et al., 2013). Inside these cells, the sporozoite develop into a liver schizont, and through asexually replication (schizogony), form thousands of merozoites. During *P. vivax* and *P. ovale* development, instead of engaging in differentiation, some sporozoites form hypnozoites, the dormant stage that remains in the liver for a non-determined period of time (can be days, months or years) before developing into merozoites. With the completion of the differentiation in the host hepatocytes (exoerythrocytic schizogony), merozoites are released, reaching peripheral bloodstream (NILSSON et al., 2015);

- erythrocytic stage: once in the bloodstream, hepatic merozoites invade circulating RBCs, initiating the asexual erythrocytic stage (blood cycle). It is during this multiplication cycle that the ring form develop into replicative schizonts, which develop into invasive merozoites that rupture the RBCs, being released in the circulation. Once released, merozoites invade new cells and start a new asexual replication round. This process of invasion and re-invasion can last from 24h to 72h, depending on the species of the parasite. (NILSSON et al., 2015; SINGH; DANESHVAR, 2013). During the blood cycle, a small portion of merozoites transform into sexual progeny (male and female gametocytes) instead of multiplying. Gametocytes continue to circulate until ingested by a mosquito through blood meal (MEIBALAN; MARTI, 2017). During *P. falciparum* infection, a portion of merozoites enter the extravascular space of the bone marrow and progress to a process named gametocytogenesis, which comprehends five stages (I-V) of gametocytes maturation. Once completely mature, the gametocytes return to the peripheral circulation, until they are taken up by a mosquito (NILSSON et al., 2015).

b) inside mosquito vector: where the sexual reproduction (sporogony) takes place.

When another *Anopheles* mosquito bites an infected person, it ingests, along with the blood meal, female and male gametocytes, which rapidly undergo activation under environmental stimuli (such as lower temperature, presence of the mosquito-derived molecule xanthurenic acid and extracellular pH about 8), forming a single macrogamete (female gamete) and eight flagellated microgametes after male gametocyte division (exflagellation). This maturation process (gametogenesis) occurs inside the mosquito midgut (BENNINK; KIESOW; PRADEL, 2016; NILSSON et al., 2015). Gametes fertilization originates the zygote, the only diploid stage of the parasite. The zygote undergoes meiosis, originating the motile ookinete that invades the midgut epithelium and forms an oocyst under the basal lamina of the gut lining, in which the parasite replicates creating thousands of sporozoites. After oocyst rupture, the sporozoites are released and enter the hemolymph. Infective sporozoites use their motility to migrate to the salivary glands to be released into human with the next *Anopheles* blood meal on another host (MÉNARD et al., 2013).

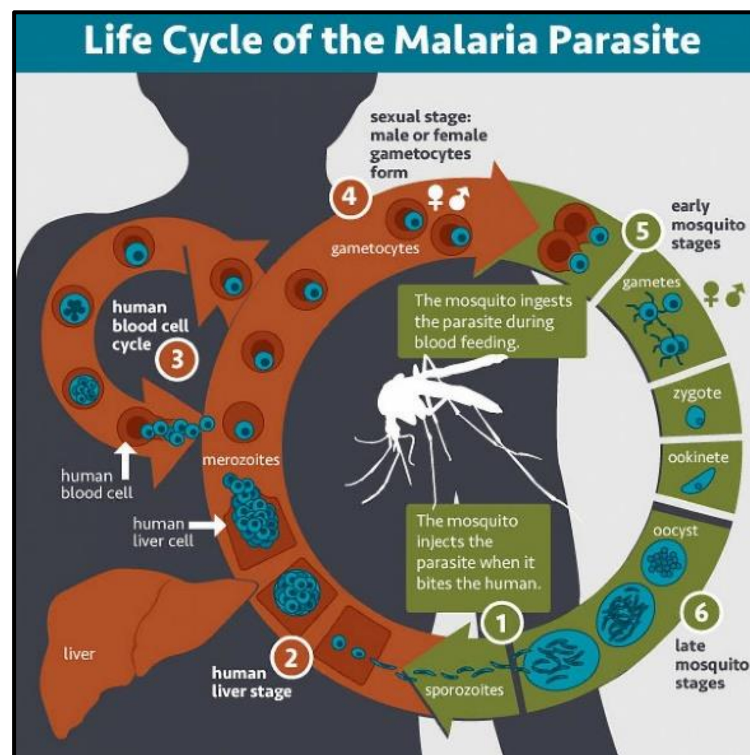


Figure 1 - Malaria life cycle. (1) The infected female *Anopheles* mosquito injects sporozoites in the human skin. (2) The parasite migrates to the peripheral circulation, reaching the liver and invading hepatocytes. Inside the human liver cell, schizonts are formed and after asexual replication, merozoites are formed. (3) Merozoites are released, reaching the bloodstream, where they invade red blood cells, initiating the replication cycle. (4) Part of these merozoites instead of multiplying, develop into male and female gametocytes. (5) Mosquito ingests gametocytes during blood feeding and inside its midgut they activate, forming both male and female gametes, which fertilize forming the zygote. The zygote develops into the motile ookinete. (6) Ookinete invades the epithelium of the mosquito midgut forming the oocyst. Inside it, hundreds of sporozoites are formed, and when released, this sporozoites invades the salivary glands of *Anopheles* mosquito. Source: (adapted from NIH, 2016).

2.1.3 Pathogenesis

The progress of malaria disease can vary according to the host's immunological conditions. Aspects related to the parasite are also important, such as which specie is infecting and how long is its incubation period. The incubation period corresponds to the time between the mosquito bite and the appearance of first malaria symptoms. In general, this period varies from 7 to 40 days and usually *P. falciparum* infections presents the shorter incubation period (9 to 14 days), while *P. malariae* infections presents the longer (18 to 40 days) (CDC, 2019).

Plasmodium vivax parasites have a long incubation time, that can vary from 12 days to several months, due to the fact that during the pre-erythrocytic stage a portion of sporozoites can remain dormant in the liver. This latent parasite (hypnozoites) can start development months after mosquito inoculation in the human host. This ability to relapse gives an important advantage to *P. vivax* concerning the control and elimination of the disease (ADAMS; MUELLER, 2017; PRICE et al., 2007). Besides that, the long incubation time makes the control of the disease more difficult, because the infective form for *Anopheles* mosquitos, the gametocytes, are already being produced even before the appearance of first symptoms. Thus, the infected human host can transmit the parasite to the vector, before the correct malaria diagnostic and treatment.

Pathogenesis refers to how a disease develop and how the organism reacts to it. The pathogenesis of human malaria can be asymptomatic, and despite being preventable and treatable, the disease, that normally is uncomplicated, can become severe. The main characteristic of each are:

a) Asymptomatic malaria is characterized by absence of clinical symptoms in an infected person. It is important regarding transmission, because infection persists for a long time without treatment, harboring gametocytes that can infect *Anopheles* mosquitoes (ZHAO et al., 2018);

b) Uncomplicated malaria is characterized by clinical symptoms as high fever, chills, sweats, headaches, nausea, and general malaise (CDC, 2019). As these symptoms are non-specific, uncomplicated malaria is hard to distinguish from other diseases that have similar clinical manifestation, which makes correct diagnosis and treatment more difficult. (PRICE et al., 2007). These manifestations can be synchronized with repeated cycles of parasite growth inside host RBCs (MILNER, 2018). During this erythrocytic stage of the parasite's life cycle, merozoites multiply inside the RBCs, accumulating toxic wastes such as hemozoin pigment and glycosylphosphatidylinositol (GPI). Once the host cell membrane integrity is disrupted, the

toxic substances are released in the bloodstream, stimulating immune responses to produce cytokines that influence the pathophysiology of the disease (MAWSON, 2013);

c) Severe infection is, in general, a consequence of an exaggerated immune response to untreated infection. In particular cases, such as infected pregnant women, international travelers (going to endemic malaria areas, such as sub-Saharan Africa, Americas and Asia), infant and young children from endemic areas of sub-Saharan Africa, the disease can progress to "severe malaria", and its complications include severe anemia, cerebral malaria, respiratory distress, kidney failure, cardiovascular collapse and even death (CDC, 2019; MILLER et al., 2013; WHO, 2020a).

An important virulence factor of *P. falciparum* malaria parasite is its capacity to modify the surface of the host infected RBCs, exposing adhesive proteins called Erythrocyte Membrane Protein 1 (PfEMP1), encoded by the *var* gene family (MILNER, 2018). These proteins are involved in the cytoadhesion process, binding to receptors present on the surface of microvascular endothelial cells. This process is important for the parasite to evade immune responses and splenic clearance mechanisms, since the mature-staged infected RBCs are removed from the bloodstream and sequestered in vital organs (MILNER, 2018; SMITH et al., 2013). For the host, the consequences are the sequestration and endothelial disfunction in organs such as brain (cerebral malaria), lungs (respiratory distress) and placenta (placental malaria) (CDC, 2019; SMITH et al., 2013). Until relatively recently, malaria caused by *P. vivax* was considered benign and responsible for causing mild clinical symptoms, but diverse studies showed that this parasite can induce cytoadherence, progressing to severe form, mainly in malaria vivax endemic areas (CARVALHO et al., 2010; PRICE et al., 2007).

2.2 The malaria problem

2.2.1 Malaria in the world

With the arrival of Europeans and West Africans to America in the mid-18th century, malaria was officially present in all Regions of the world. However, from the end of the 19th century and mid-20th century, the number of malaria deaths declined until its extinction in temperate zones such as North America and Europe (CARTER; MENDIS, 2002). This successful elimination was due to the antimalaria efforts (i.e. use of quinine) and the local climate patterns. *Plasmodium* parasites need specific conditions to continue its development inside the mosquito host. With the decrease in temperature, the time needed to complete development increases. That is why transmission is more difficult below 18°C. Besides that,

many *Anopheles* mosquito species stop biting at very low temperature. All these facts explain the small transmissibility in countries within temperate zones. To illustrate this situation (malaria elimination in temperate zones), it is possible to see the global distribution of *Plasmodium* transmission risk from 1946 to 1994 (Figure 2), a period of intense antimalaria efforts (SACHS; MALANEY, 2002).

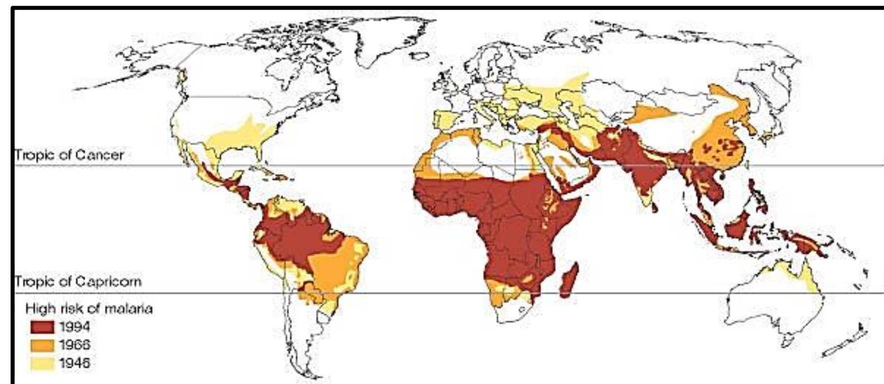


Figure 2 - Pattern of malaria transmission worldwide in 1946, 1966 and 1994. From 1946 (yellow) to 1994 (red) there was an expressive change in the global pattern of malaria transmission, since the disease started to concentrate in the tropical zones. This pattern change reflects the intense antimalaria efforts in the temperate zones, the difficulty that the parasite has to complete the life cycle inside the mosquito due to low temperatures in temperate regions and, for the same reason, the decrease in transmission after mosquito suspend biting activity. Source: (adapted from SACHS; MALANEY, 2002).

Globally, an estimated 3,7 billion people in 90 countries are living in endemic malaria areas (Figure 3). According to most recent data from the World Health Organization (WHO), in 2018, there were approximately 228 million cases of malaria worldwide, a decrease of 3 million cases compared to 2017 (WHO, 2019).

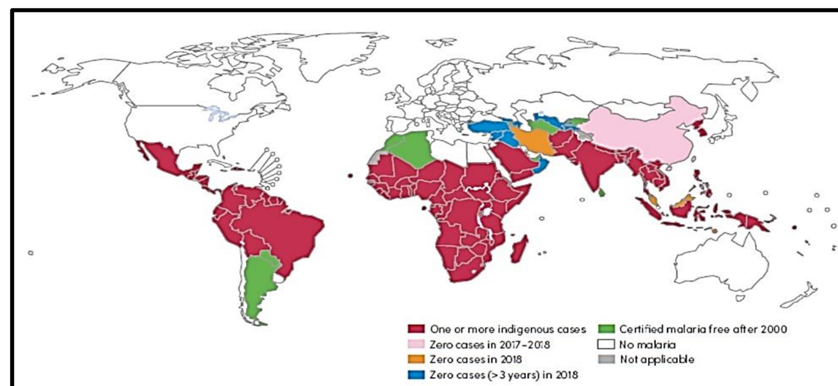


Figure 3 - World malaria cases distribution in 2018. Endemic countries for malaria in 2018 are represented in red. China and El Salvador reported zero indigenous cases in 2017 and 2018 (countries in light pink). In 2018, Iran, Malaysia and Timor-Leste reported zero cases for the first time (countries in orange). Countries with zero indigenous cases for more than three consecutive years are considered as having eliminating the disease (represented in blue). After 2010, eight countries (United Arab Emirates, Turkmenistan, Kyrgyzstan, Sri Lanka, Morocco, Algeria, Argentina and Paraguay) were certified malaria free (countries in green). Source: (adapted from WHO, 2019).

This parasitic disease is widespread in many regions of the world. In 2018, the vast majority of cases (93%) occurred in Africa, with a predominance of *P. falciparum* parasite, coincidently present in the same latitude where the *Anopheles gambiae* vector predominates. The South-East Asia Region was the second most affected, with about 3,4% of the cases, followed by the Eastern Mediterranean Region, with 2,1% and the Americas, with 0,4% of the cases. In the last three regions, the predominant specie is *P. vivax* (WHO, 2019).

The low incidence of *P. vivax* in Africa is related to a specific genetic characteristic. *Plasmodium vivax* infects preferably reticulocytes by attaching to the Duffy receptor. The fact that Duffy-negative blood group is widespread in Africa population, results in a natural protection against it. However, recent data showed that Duffy-negative people can also be infected by *P. vivax*, although the number of infected individuals is very small when comparing to Duffy-positive infected individuals (OLLIARO et al., 2016; RECHT et al., 2017).

Usually, infections caused by *P. falciparum* can be controlled by the host immune system after causing intermittent episodes of fever. However, the disease can be severe fatal in some cases, mainly in young children living in endemic malaria areas of Africa, travelers, pregnant women and individuals with weakened immune system (MILLER et al., 2013; WHO, 2020a). The first prevention method is to avoid mosquito bite, but depending on the malaria risk of the area, travelers should take preventive medicine. Travelers must look for a health service if they show symptoms of malaria (mainly fever) within three months after being exposed to risk areas (WHO, 2020a). In 2018, an estimated 405.000 deaths due to malaria were reported in the whole world. The fatal cases were largely related to infections caused by *P. falciparum* in Sub-Saharan Africa (94% of all malaria deaths worldwide), being children under 5 years old the most affected group, accounting for 67% of all malaria deaths in the world (WHO, 2019).

2.2.2 Malaria in South America

Malaria transmission in South America, especially in the Amazonian region, is a complex process that involves many factors, such as: environmental conditions (i.e. temperature, annual rainfall cycle and vegetation patterns, which contribute to reproduction of *Anopheles* mosquitoes); communities living far from health facilities, making difficult a correct diagnosis and treatment; main local economic activities involving deforestation contributes to workers exposure to the vector; and social behavior, related to incorrect use of vector control methods (i.e. sleep outside bed nets, exposure without use of repellent, especially between dusk and down, the period when *Anopheles* prefers to bite). These factors restrain efficiency of antimalaria interventions (SOLANO-VILLAREAL et al., 2019).

Most of the countries in South America still suffer from the disease, with exception of Argentina and Paraguay, which were certified as malaria free since 2010. Besides that, from the 19 countries that are at risk, with approximately 138 million people exposed to *Plasmodium* and its vector *Anopheles* in 2018, only four concentrated 94% of all malaria cases, mainly in the Amazonian region: Venezuela (51%), Brazil (23%), Colombia (10%) and Peru (6%) (Figure 4). Nevertheless, fatal cases are rare: from the 753.700 cases reported in 2018, less than 0,1% (338 cases) resulted in death (WHO, 2019).

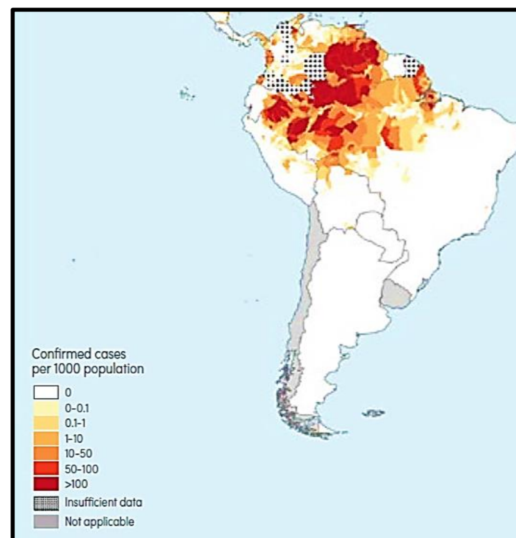


Figure 4 - Malaria cases in South America in 2018. Confirmed cases per 1000 population in South America. The majority of confirmed cases are located in the Amazonian region, mainly in Venezuela (51%), Brazil (23%), Colombia (10%) and Peru (6%). Source: (adapted from WHO, 2019).

In general, the highest burden of *P. vivax* infection is seen throughout South America, Southeast Asia and Eastern Mediterranean Region. In 2018, this specie of the parasite was responsible for 79,50% of all malaria cases in Americas, 20,5% were caused by *P. falciparum* and mixed infections and less than 1% by other *Plasmodium* species (WHO, 2019).

2.2.3 Malaria in Brazil

Infections in Brazil are caused mainly by *P. vivax* parasites (responsible for almost 90% of the reported cases in 2018), but infections caused by *P. falciparum* (around 9% in 2018), mixed infections (less than 1%) and other species (less than 0,01%) are also reported (WHO, 2019). In the Brazilian Amazonian region, *Anopheles darlingi* is the main vector, and in the extra-Amazonian region, *An. aquasalis*, *An. bellator* and *An. cruzzi* are important for autochthonous transmission of *Plasmodium* parasites (CARLOS et al., 2019).

In addition to temperature, precipitation and water level are able to change the seasonality of malaria, since they are factors that can influence the mosquito's ability to proliferate. In

general, the seasonal peak in Brazilian Amazonian region occurs between the wet and dry seasons (MINISTÉRIO DA SAÚDE, 2019a).

From 2010 to 2015, Brazil was the country with the highest number of malaria cases reported in Latin America. However, with the serious crisis in Venezuela, this scenario reverted and this country started to present the highest values of incidence of the disease (in 2018, more than 470.000 cases were reported in Venezuela against 217.900 in Brazil, and 423 deaths against 44 deaths, respectively). About 99% of all malaria cases in Brazil occur in the Brazilian Amazonian region, that is still endemic for malaria, with multiple risk areas (Figure 5). This enormous territory is formed by nine states: Acre (AC), Amazonas (AM), Rondônia (RO), Roraima (RR), Pará (PA), Amapá (AP), Mato Grosso (MT), Tocantins (TO) and Maranhão (MA), being Acre the state with the highest number of malaria cases, with three municipalities (Cruzeiro do Sul, Mâncio Lima and Rodrigues Alves) being a persistent malaria hot-spot (CONN et al., 2018; WHO, 2019).

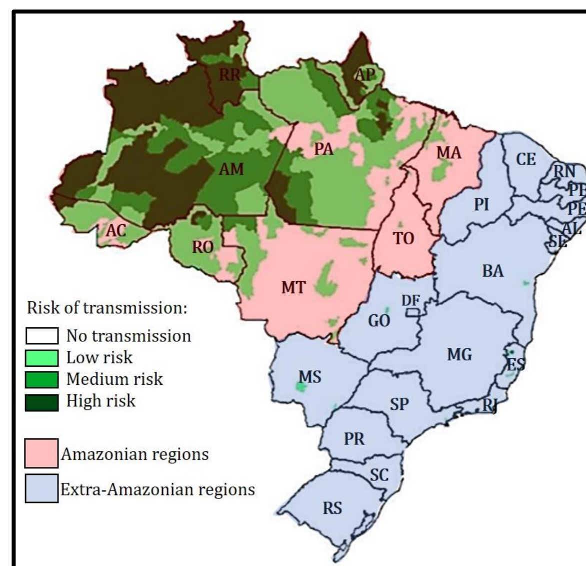


Figure 5 - Malaria transmission risk map, Brazil, 2018. The Brazilian Amazonian region covers an estimated 60% of Brazil’s territory, and it is formed by nine states (red shaded): Acre (AC), Amazonas (AM), Rondônia (RO), Roraima (RR), Pará (PA), Amapá (AP), Mato Grosso (MT), Tocantins (TO) and Maranhão (MA). Areas in dark green represents the highest risk for indigenous malaria transmission, while medium green represents medium risk and light green represents the lowest risk. Areas in white/with no trace of green have no risk for indigenous transmission. States in the extra-Amazonian region (blue) are: Mato Grosso do Sul (MS), Goiás (GO), Piauí (PI), Ceará (CE), Rio Grande do Norte (RN), Paraíba (PB), Pernambuco (PE), Alagoas (AL), Sergipe (SE), Bahia (BA), Minas Gerais (MG), Espírito Santo (ES), Rio de Janeiro (RJ), São Paulo (SP), Paraná (PR), Santa Catarina (SC), Rio Grande do Sul (RS) and the Brazilian Federal District, Distrito Federal (DF). Source: (adapted from MINISTÉRIO DA SAÚDE, 2019b).

For many years, malaria caused by *P. vivax* were considered benign. However, the morbidity and the high incidence of the disease caused by this specie have a huge impact on the population’s health, and cause an important consequence on economic and social development (CARLOS et al., 2019). This parasite is very complicated to control and eliminate in endemic

malaria areas. *P. vivax* causes low parasitemia, which makes its detection difficult, and as a consequence, correct diagnosis and treatment are frequently late, meanwhile gametocytes can be ingested by the mosquito if the patient is bitten again. Besides that, *P. vivax* parasites can produce hypnozoites, the dormant liver form, that can lead to relapse of the disease months later (RECHT et al., 2017).

The Amazonian region in Brazil is not the only one where people are affected by malaria. Imported cases of the disease occurred after several movements of migration and immigration of people leaving endemic areas (i.e. originated from Amazonas, Pará, Rondônia and the African country Angola) in search for a better life condition. All these movements caused the disease to settle also in the extra-Amazon region, which was possible because competent malaria vectors are present in the hole country, especially in the Atlantic rainforest (mainly *An. bellator* and *An. cruzzi*) and the Brazilian Northeastern and Southeastern coasts (mainly *An. aquasalis*) (CARLOS et al., 2019).

Malaria is a disease closely related to poverty, since the mortality estimation is higher in countries with lower Gross Domestic Product (GDP) *per capita*. In Brazil, the vast majority of cases are found in rural or indigenous areas, with a low lethality rate (2/100.000 cases), in contrast to what occurs in non-endemic regions, where the lethality is up to 100 times higher. Usually, the high lethality rates that occur in extra-Amazonian regions are due to the fact that people became infected in endemic areas and then moved to where the disease is not common, a fact that makes it difficult for medical doctors to suspect malaria. As malaria symptoms are non-specific, the disease can be misdiagnosed as another that is more common in the local (i.e. dengue) and treated incorrectly in the first days after the symptoms onset (MINISTÉRIO DA SAÚDE, 2019a; LORENZ et al., 2015). To try to reduce these errors and the mortality rate in non-endemic regions, it is important to alert people to the risk of contracting malaria, better capacitate professionals and develop more efficient diagnostic methods.

2.2.4 Difficulties in malaria control and elimination

Malaria is a preventable, diagnosable and treatable disease. With different innovations and intervention programs, it is possible to control and/or eliminate this parasitic disease, as historically proven.

During the 19th-20th centuries, malaria was widely distributed around the world. In Europe, its transmission was always unstable due to the fact that environmental conditions were not ideal for *Plasmodium* development, although there are mosquitoes capable of transmitting the parasite. However, the disease elimination by human actions was possible. The development

of water treatment methods resulted in reduced mosquito breeding sites. Besides that, improvement in housing conditions also contributed to malaria transmission decrease. Finally, the implementation of elimination programs, drug therapy mainly with quinine and insecticide spraying, lead to the eradication of malaria across Europe, officially in 1975 (PIPERAKI, 2018). In Brazil, several attempts of elimination have been made. During the 1930s, the Northeast region was invaded by *An. gambiae* mosquitoes during shipping traffic between Brazil and Senegal. In the following years, several efforts were made to combat this mosquito and success came in the early 1940s. Despite that, malaria was still a major public health problem in Brazil. For this reason, during the 1950s, a national eradication campaign involving DTT spraying inside houses and use of chloroquine as a preventive method was implemented, leading to the elimination of the disease in the majority of the country (with exception of the Amazon region) in the 1970s (OLIVEIRA-FERREIRA et al., 2010).

Malaria transmission obligatory involves three organisms: the parasite, the mosquito and the human being. Control and elimination can be achieved by developing strategies aimed at them, such as new diagnostic methods and more efficient treatments, new approaches to combat the vector and development of vaccines (WHO, 2019).

Early diagnosis and correct treatment reduce the chance of spreading the disease and also prevent severe cases and death. However, the majority of serious cases occur in localities where people have poor access to health services (WHO, 2019).

There are diverse diagnostic methods that can be employed to detect *Plasmodium*, and the more sensitive are PCR-based detection methods. However, its use is not feasible in the routine of most malaria endemic areas. The most used methods are based on the detection of parasites in a blood sample from the patient, through conventional light microscopy or rapid test. The main problem related to these methods is the fact that *P. vivax* malaria with low parasitemia is frequently undiagnosed, contributing to the spread of the parasite (DAYANAND; ARCHUR; GOWDA, 2018).

Antimalarial medicines are crucial to achieve control and elimination of the disease. The main objective is to eliminate the parasites from the bloodstream by stopping schizogony and discontinuing the development of gametocytes. There is also the possibility of using drugs that seek to eliminate hypnozoites (MINISTÉRIO DA SAÚDE, 2019a). People infected with *P. vivax* usually are treated with chloroquine (CQ), a drug used to remove the parasites from the bloodstream. In order to eliminate hypnozoites, the treatment is done with primaquine. The artemisinin-based combination therapy (ACTs) is the best against infections caused by *P. falciparum* and against *P. vivax*, in places where CQ resistance is reported. Resistance to

antimalarial drugs is a frequent problem and for this reason it is important to search for new medicines and implement them (DAYANAND; ARCHUR; GOWDA, 2018; WHO, 2019).

In order to prevent mosquito contact with humans living in endemic areas, some strategies have been implemented, such as the use of insect repellents, especially between dusk and down (period that mosquitos prefer to bite), indoor residual spraying (IRS) with insecticides and the use of insecticide-treated bed nets (INTs), especially by infants, children under five years old and pregnant women. Also, treatment of infected people plays an important role regarding the prevention of gametocytes transmission to the mosquito (CARTER; MENDIS, 2002). Generally, these preventives human-mosquito contact methods help, but are not sufficient, mainly because mosquito resistance to insecticides have been reported in 73 countries until 2018 (WHO, 2020b).

With all these methods implemented to reduce malaria burden, especially the use of IRS, INTs and ACTs, the number of clinical cases decreased 40% since 2000 (BERG et al., 2019). However, evidences point to increasing *Anopheles* resistance to insecticides and emerging *Plasmodium* resistance to ACTs. In order to continue this reduction and fight resistance, more strategies are needed, such as development of new antimalarial agents and the implementation of effective vaccines (BEESON et al., 2016).

2.3 The importance of vaccination

The use of vaccines is proven to be important in the prevention of several diseases that can cause serious consequences for those infected. An important example of this is the case of smallpox, a deadly human disease caused by a virus, eradicated after an intense vaccination campaign around the world.

The first vaccine was developed by an English doctor called Edward Jenner during his observations on people who got sick after milking cows infected with the “cowpox” (eruptions on the teats of the animals), presenting sores on their hands. He then decided to test the possibility to propagate the disease from the cow to a human and then to another, in order to protect the second person from the disease. In his first experiment, pustule from the wound of the hands of a woman (Sarah Nelmes) who had been infected by the “cowpox” was inoculated in the arm of a boy named Phipps. Some days after the inoculation, Phipps was not presenting any symptoms related to the disease (with exception to the pustule in his arm). Some months later, the boy was inoculated with the variola virus (responsible for causing the smallpox) and did not get sick. Edward Jenner infected more children and they all resisted to the infection. There the first vaccine was created. In 1798 his discoveries were known worldwide, and until

1800, the idea of vaccination was accepted and adopted in many countries. In 1980, after a global campaign of vaccination, the smallpox was declared the first (and only until today) infectious disease eradicated (FENNER et al., 1988).

Following the example of smallpox eradication, many infectious diseases can be prevented with vaccination. Poliomyelitis is another great example of how intense global efforts are important in this process of elimination. With intense vaccination campaign around the world, only three countries (Afghanistan, Nigeria and Pakistan) remain endemic for polio, with only 12 cases reported recently (MINISTÉRIO DA SAÚDE, 2020).

In addition, mass immunization contributed to the control and elimination of four diseases in the Americas: the first to be eradicated was smallpox in 1971, followed by efficient control of poliomyelitis in 1994, rubella in 2015 and measles in 2016 (SECRETARIA DE ESTADO DA SAÚDE, 2016). However, the facts changed in 2019, as, after decreasing adherence to vaccination, several outbreaks of measles began to reappear. This is a good example to show that the best way to achieve eradication or efficient control of the different diseases is by the use of effective vaccines, as its causative agents continue to circulate around the world.

In general, the development of vaccines follows some steps:

a) basic research with identification and validation of the target antigen;

b) preclinical development of vaccine concept, using animal models to test efficacy and security in animal models;

c) three phases of clinical development: phase I is related to tests in humans to prove that the vaccine is secure; phase II has as the main objective to establish the immunogenicity and efficacy in controlled trials, and phase III is implementation in susceptible populations;

d) last step is regarding registration and availability of the vaccine for the population (GHIT FUND, 2019).

2.3.1 Vaccines against malaria

There are still no vaccines against diseases caused by parasites, such as malaria. In general, this is probably due to the fact that these pathogens have complex characteristics that hinder the development of vaccines. In the specific case of *Plasmodium*, vaccine development is hindered by its complex life cycle, the ability to rapidly evade from immune responses and antigenic variation among its surface structures (SACKS, 2014).

In order to prevent the development of *Plasmodium* parasites, antibodies against proteins presented by the pathogen are produced through humoral and cell-mediated responses. During the erythrocytic cycle (rupture of RBCs by merozoites), TNF-α is released, leading to the classic

malaria symptom, fever. Besides that, other cytokines (i.e. IL-10 and IFN-γ) are released and monocytes and neutrophils are activated to complement the response (MILNER, 2018; OUATTARA; LAURENS, 2015). Although the human organism recognizes the antigens, the parasite succeeds in evading the inefficient immune responses and the protection developed is not long lasting (SACKS, 2014). That is why people that are constantly exposed to *Plasmodium* are susceptible to various malaria infections. Even if someone has already contracted the disease, there is no protection against a new infection (CAMARGO, 1995). Nevertheless, observations of adults from endemic areas showed that successive contact with the parasite induce the development of clinical immunity, limiting the intensity of the disease and mortality rates. These findings stimulated the search for an effective malaria vaccine, which together with the use of antimalarials and vector control would help to eliminate the disease (ARÉVALO-HERRERA et al., 2011).

Since 1930 many efforts have been made in order to develop a malaria vaccine. In 1945, Jules Freund published a study in which injection of killed *P. knowlesi* parasites could not produce a good protective immune response in monkeys against the same parasite. However, the addition of adjuvants in combination with different antigens improved the response with a partial protection (FREUND et al., 1945). After this first attempt, many others have emerged. A successful one occurred in 1967 with vaccination in mice with x-irradiated sporozoites of *P. berghei*, the rodent malaria parasite model, injected through the mosquito bite (NUSSENZWEIG et al., 1967). Serum antibodies collected from vaccinated animals showed the ability to neutralize sporozoites (NUSSENZWEIG et al., 1969). This work with x-irradiated sporozoites made possible the first human malaria vaccine trial. Since then, there was an increase in investments in this area to fight this dangerous disease.

Before the genomic era, the search focus to malaria elimination was more effective formulations for the already known antigens. With new technologies discovered, it was possible to access information in the area of genomics, proteomics and transcriptomics. Therefore, the focus changed to discovery of new antigens for the development of novel vaccines, since it became possible the detection of potential vaccine candidates through bioinformatics (DAVIES et al., 2015).

With the complex life cycle of *Plasmodium* parasites, generally vaccines can be developed focusing on sporozoites, the form of the parasite that infects the liver, or on merozoites, the form that is present during the rupture of the blood cells (stage where the symptoms of the disease are present), or even on gametocytes and/or ookinetes, which occur inside the mosquito. Thus, there are three main groups to target for a malaria vaccine candidate

(Figure 6): pre-erythrocytic (prevent infection), erythrocytic/blood-stage (prevent disease) and transmission blocking (interrupt parasite transmission) (OUATTARA; LAURENS, 2015).

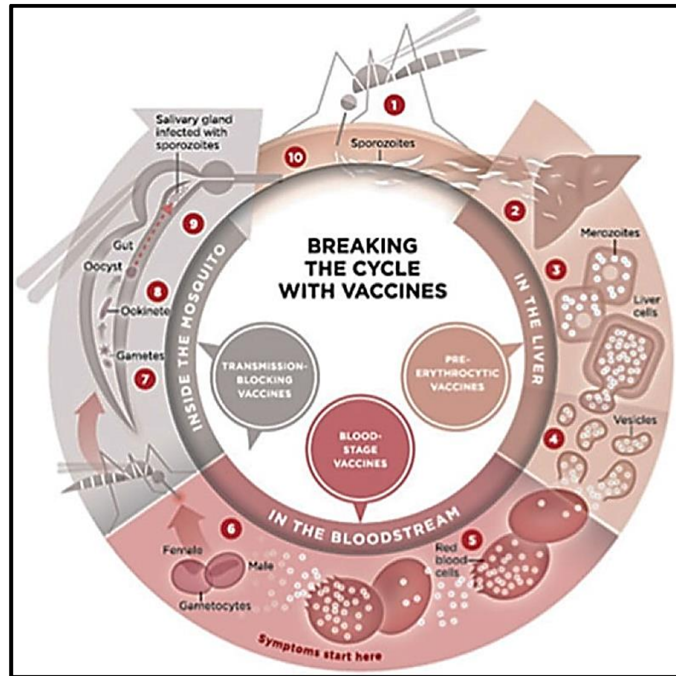


Figure 6 - Malaria life cycle and division of malaria vaccines groups. Malaria vaccines can be divided into three groups. Pre-erythrocytic vaccines are directed to sporozoites antigens in order to prevent the first stage inside the human, which occurs inside the liver. Erythrocytic/blood-stage vaccines are intended to prevent invasion of merozoites to the red blood cells and the symptoms of the disease. Transmission blocking vaccines have as main objective to prevent transmission of the parasite from humans to mosquito, and as a consequence, to interrupt the sporogonic cycle within the *Anopheles* midgut. Source: (adapted from OUATTARA; LAURENS, 2015).

Some of the main antigens/vaccine candidates are exposed on the following table:

Table 1 - Main *Plasmodium* antigens for pre-erythrocytic, erythrocytic and transmission blocking vaccines development.

Vaccine group	Antigen	Antigen description	Vaccine name	Vaccine type	Vaccine objective	References
Pre-erythrocytic	CSP	Circumsporozoite Surface Protein; expressed on the surface of sporozoites of *P. falciparum*	RTS,S/AS01E	Subunit vaccine	Kill the parasite, preventing liver infection	FRIMPONG et al., 2018; WHO, 2015
Erythrocytic	MSP1	Merozoite Surface Protein 1; expressed on merozoite surface	MSP1 vaccines	Subunit vaccines	Block merozoite invasion to RBCs	BEESON et al., 2016

Vaccine group	Antigen	Antigen description	Vaccine name	Vaccine type	Vaccine objective	References
	AMA1	Apical Membrane Antigen 1; expressed on merozoite surface	AMA1 vaccines	Subunit vaccines	Block merozoite invasion to RBCs	BEESON et al., 2016; LEE et al., 2019
	RH5	Reticulocyte-binding protein homolog 5; expressed on the rhoptries of merozoites	RH5 vaccines	Subunit vaccines	Block merozoite invasion to RBCs	BEESON et al., 2016; GALAWAY et al., 2017
Transmission blocking	Pfs48/45	*P. falciparum* proteins expressed by gametocytes and gametes	Pfs48/45 vaccines	Subunit vaccines	Block transmission of parasite to the mosquito	ZHENG et al., 2019
	Pfs230	*P. falciparum* proteins expressed by gametocytes and gametes	Pfs230 vaccines	Subunit vaccines	Block transmission of parasite to the mosquito	EKSI et al., 2006
	Pfs25/Pvs25	*P. falciparum*/*P. vivax* proteins expressed by gametes and ookinetes	Pfs25/Pvs25 vaccines	Subunit vaccines	Block transmission of parasite to the mosquito	MCLEOD et al., 2019; ZHENG et al., 2019

Source: elaborated by the author based on (BEESON et al., 2016; EKSI et al., 2006; FRIMPONG et al., 2018; GALAWAY et al., 2017; LEE et al., 2019; MCLEOD et al., 2019; WHO, 2015; ZHENG et al., 2019;).

2.3.1.1 Pre-erythrocytic vaccines

The main objective of a vaccine against sporozoites and liver stages is to kill the parasite, preventing hepatocyte infection and progression to the symptomatic stage of the disease. Thus, a good malaria pre-erythrocytic vaccine should induce humoral immunity in order to produce antibodies against sporozoites, avoiding its invasion to hepatocytes, and T-cell responses capable of identify and destruct infected hepatocytes (FRIMPONG et al., 2018).

A good reason to search for vaccine candidates at this stage is the fact that infected hepatocytes can present parasite antigens to immune cells. During liver cell invasion, sporozoites use the circumsporozoite surface protein (CSP), its most abundant surface protein, to pass through cells within a parasitophorous vacuole (DUFFY et al., 2012). The CSP is also expressed on the surface of liver stage schizonts (CASARES; BRUMEANO; RICHIE, 2010).

The first malaria vaccine has been in development since 1987 (BERG et al., 2019). This vaccine is based on the CSP of *P. falciparum* (Figure 7), a protein with a molecular size of 58 kDa, composed of 412 amino acids with 37 tetrapeptide repeats and a central domain (highly conserved among strains of *P. falciparum*) containing immunodominant T $CD4^+$ and $CD8^+$ epitopes, termed Th2R and Th3R, respectively (CASARES; BRUMEANO; RICHIE, 2010; OUATTARA; LAURENS, 2015).

The formulation, called RTS,S/AS01E, is a subunit vaccine, made of part of the CSP (central domain repeat) fused to particles of the Hepatitis Virus B surface antigen (HBsAg). This formulation is mixed with an adjuvant system AS01E (liposome based) in order to improve vaccine immunogenicity and promote long term protection (DIDIERLAURENT et al., 2017; OUATTARA; LAURENS, 2015).

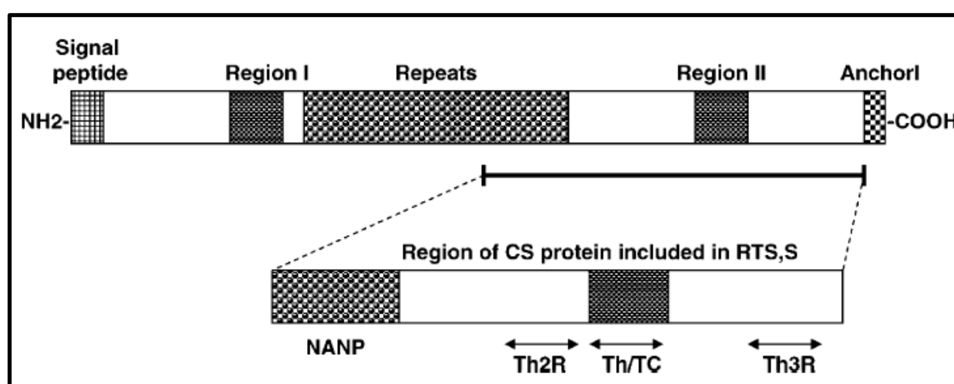


Figure 7 - Schematic representation of the Circumsporozoite Surface Protein (CSP). The CSP has a molecular size of 58 kDa and is composed of 412 amino acids, containing a central repeat domain with approximately 41 repeats of NANP (N, asparagine; A, alanine and P, proline) amino acids sequences, flanked by two non-repetitive regions on each side (Region I and Region II). On the one hand, the N-terminus region is conserved among *P. falciparum* strains. On the other hand, the C-terminus is highly polymorphic. The region of CSP included in the RTS,S vaccine is composed of part of the central domain repeat and immunodominant T $CD4^+$ an $CD8^+$ epitopes (Th2R and Th3R, respectively). Source: (adapted from CASARES; BRUMEANO; RICHIE, 2010).

RTS,S/AS01E phase III clinical test was conducted during five years and concluded in 2014. This trial involved seven sub-Saharan African countries (Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique and Tanzania) and more than 15.000 young children and infants (BERG et al., 2019). Among children aged 5-17 months, four doses of the vaccine reduced clinical malaria by 39% and severe malaria by 32%. The first three doses were given at monthly intervals and the fourth, essential to enhance and extend protection, was given 18 months after the previous dose (DIDIERLAURENT et al.,2017; WHO, 2015). The vaccine also reduced the necessity for blood transfusion due to anemia by 29% (WHO, 2015). These results are far from that expected for an ideal malaria vaccine. However, even a partial protection in endemic areas, especially where many children die, can reach a substantial positive public health impact.

After phase III, the next step for the RTS,S/AS01E vaccine is its implementation as a complementary malaria control method in endemic regions, with the intention to evaluate the impact that it has in public health. The Malaria Vaccine Implementation Programme (MVIP) is the malaria vaccine pilot programme coordinated by the WHO and started in 2019 in Ghana, Malawi and Kenya. The objective is to separate 750.000 children in vaccinated and unvaccinated groups (COELHO et al., 2017). The specific focus of the MVIP is to verify the feasibility of RTS,S/AS01E four doses administration in the vaccinated cluster and vaccine safety when routinely used (BERG et al., 2019).

2.3.1.2 Erythrocytic vaccines

During merozoite invasion of RBCs, a complex process involving multiple proteins and receptor-ligand interactions occurs, with modifications on the parasite surface. Due to this complexity, the identification of potential candidates to compose a vaccine is challenging. However, much progress has been made in recent years, enabling greater understanding of the process of merozoite invasion to RBCs (BEESON et al., 2016).

Research of malaria vaccines has focused in the search of potential erythrocytic stage antigens expressed by merozoites, due to the fact that during this stage successive invasion, multiplication and ruptures of the RBCs occurs, causing the malaria clinical symptoms (ZHENG et al., 2019). For this reason, the main objective is to prevent mild and severe disease by blocking merozoite invasion to the RBCs, reducing parasitemia and transmission (OUATTARA; LAURENS, 2015). Antibodies against merozoites are a good strategy to control blood-stage infection (LEE et al., 2019).

In endemic areas, people exposed frequently to *Plasmodium* and repeated infections are very common. Those in this condition gradually develop natural protection against the parasite, resulting in asymptomatic or mild infections. An erythrocytic vaccine would mimic this naturally-acquired immunity, as it allows exposure to the parasite during pre-erythrocytic stage of the disease (FRIMPONG et al., 2018).

The most important proteins for invasion of merozoites into blood cells are present on the surface or in the rhoptries and micronemes present in the apical region of the parasite. These proteins can be of integral membrane type, GPI-anchored or peripherally associated surface protein (BEESON et al., 2016). During merozoite invasion of RBCs, surface proteins of the parasite can remain in the membrane of erythrocytes, being directly exposed to immune responses produced by T cells and B cells (ZHENG et al., 2019).

Main candidates to compose an erythrocytic vaccine are: Merozoite Surface Protein 1 (MSP1), Apical Membrane Antigen 1 (AMA1) and Reticulocyte-binding protein homolog 5 (RH5) (OUATTARA; LAURENS, 2015; ZHENG et al., 2019).

Merozoite Surface Protein 1 (MSP1) is the most abundant GPI-anchored merozoite surface protein, being important for erythrocyte invasion. Before merozoite invasion, MSP1 (with approximately 195 kDa) is cleaved into four polypeptides fragments of 83 kDa (N-terminal), 30 kDa and 38 kDa (internal fragments) and 42 kDa (GPI-anchored C terminal). During invasion, another cleavage occurs with the last fragment, resulting in one of 19 kDa and another of 33kDa. Its C-terminal portion, $MSP1_{19}$, remains attached to the surface of the merozoite and is carried into the host cell along with the parasite. This portion has its importance, as it is highly conserved among the parasite species (BEESON et al., 2016).

Apical Membrane Antigen 1 (AMA1) is an integral membrane protein (with 88 kDa) expressed in sporozoites during pre-erythrocytic stage, and in order to be relocated to the merozoite surface, this protein is processed in a microneme-secreted 66 kDa, being essential for RBCs invasion (BEESON et al., 2016).

This antigen is considered immunogenic as it enables the production of antibodies, which have been shown important in preventing invasion of host cells (LEE et al., 2019). For this reason, AMA1 proved to be a target for composing an erythrocytic vaccine. A promising strategy is the use of virus-like particles (VLP) vaccine expressing AMA1, since it has already been shown that a codon-optimized VLP vaccination increases the production of B cells, $CD4^+$ and $CD8^+$ T cells responses, reducing parasitemia and increasing survival rate of immunized mice challenge-infected with *P. berghei* (BEESON et al., 2016; LEE et al., 2019).

One of the most important antigens to compose an erythrocytic vaccine is the reticulocyte-binding protein homolog 5 (RH5). The RH5 antigen is found in the rhoptries of merozoites, and is an atypical RH family member, as it is much smaller (45 k Da) (BEESON et al., 2016), does not have a transmembrane region and is essential for parasite growth in blood stage (GALAWAY et al., 2019). Also, RH5 form a functional protein complex, where two other secreted proteins are part: CyRPA (cysteine-rich protective antigen) and RIPR (RH5 interacting protein). The complex RIPR-CyRPA-RH5 is tethered to the merozoite surface protein P113 and binds to host basigin (BSG) present on the erythrocyte surface, by the RH5 N terminus (RH5Nt) (Figure 8) (GALAWAY et al., 2017).

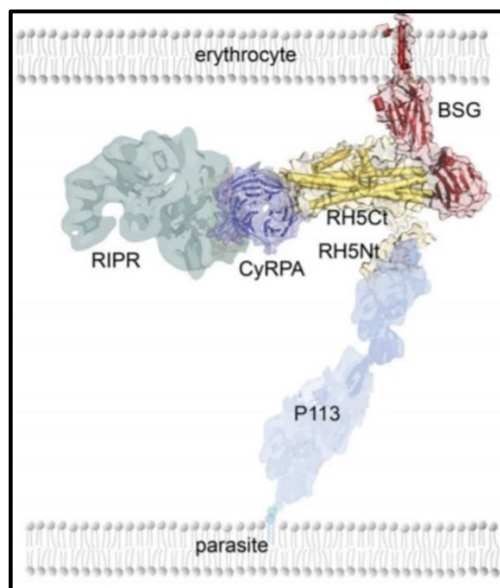


Figure 8 - RH5 invasion complex. RH5 N terminus (RH5Nt) is attached to the merozoite surface protein P113, binding to the erythrocyte surface via interaction with basigin (BSG). The cysteine-rich protective antigen (CyRPA) interacts directly with RH5 and recruits RH5 interacting protein (RIPR) to the complex. Source: (adapted from GALAWAY et al., 2019).

The main obstacle that hinders the development of a vaccine at this stage is the difficult to find antigens that are conserved between different strains of the parasite, and satisfactorily susceptible to antibody neutralization, in other words, antigens that have a meaningful impact on parasite multiplication rate. Studies have shown that in order to prevent merozoite invasion *in vivo*, a high antibody concentration is necessary (COELHO et al., 2017). Nevertheless, phase I clinical trials are underway to test vaccines containing the RH5 antigen. The most promising results until now showed that immunization in humans can safely induce antibodies, B cells and T cells responses against *Plasmodium falciparum* RH5 antigen. Also, this immunization provided high levels of antibodies, exceeding the serum antibody responses obtained by adults naturally exposed to malaria in Africa for many years (PAYNE et al., 2017).

2.3.1.3 Transmission blocking vaccines (TBV)

In the luminal region of the mosquito's intestine, the gametocytes form gametes which originate the zygote, which after 15-25h differentiates into the ookinete, which has motility. The ookinete passes through the epithelium of the mosquito midgut, giving rise to the oocyst that produces thousands of sporozoites, a form that migrates from the midgut to invade the mosquito's salivary gland to be transmitted to the vertebrate host (LI et al., 2004).

Some strategies are studied in order to interrupt parasite transmission from humans to mosquitoes, mainly those based on immune interference of *Plasmodium* development, such as prevent gametes fertilization and zygote formation, ookinete invasion to the epithelium of the

midgut, oocyst maturation and sporozoites migration to the salivary glands (ARÉVALO-HERRERA et al., 2011).

Efficient malaria transmission depends on the availability of infectious gametocytes in the human blood when the *Anopheles* mosquito takes its blood meal. A relatively innovative and promising strategy is the creation of vaccines inducing antibodies that are able to block *Plasmodium* transmission from the human host to the mosquito vectors (GONÇALVES; HUNZIKER, 2016). These antibodies would not protect immunized people from being infected with the parasite, for this reason the transmission blocking vaccines (TBV) are known as "altruistic vaccines" (OUATTARA; LAURENS, 2015). However, TBV could contribute to the establishment of malaria control in areas of intense transmissibility, when used in combination with drug-based therapies and/or with erythrocytic or pre-erythrocytic vaccines. The main objective of the vaccination is to interrupt *Plasmodium* transmission through immunological responses against parasite sexual stages proteins, blocking fertilization, halting reproduction and reducing parasite's infectivity (FRIMPONG et al., 2018; OUATTARA; LAURENS, 2015).

In order to achieve transmission interruption, the strategy of the TBV is the induction of an immune response against specific antigens of the parasite's sexual stages within the immunized person (GONÇALVES; HUNZIKER, 2016). Inside the vertebrate host, pre-fertilization antigens are expressed in gametocytes, but antibodies against them can only be recognized after gametes egress from erythrocytes (process that occurs inside the mosquito). This fact confers an advantage, since antigens do not undergo selective pressure of the human immune system. During the blood meal in an infected/immunized person, the *Anopheles* mosquito ingests cytokines (i.e. IFN-γ and TNF-α), which affects gametocyte's infectivity, and antibodies that prevent parasite development by binding to surface proteins of gametocytes, gametes, zygotes or ookinetes (ARÉVALO-HERRERA et al., 2011; SAUERWEIN; BOUSEMA, 2015).

The list of antigens being proposed to compose a TBV is short. The main candidates are called transmission blocking antigens (TBA) and the most studied are antigens conserved across species: *P. falciparum* proteins expressed by gametocytes and gametes Pfs48/45 and Pfs230 (synthesized during primary gametocyte development), and protein Pfs25 (and its ortholog Pvs25 in *P. vivax*) expressed on the surface of gametes and ookinetes. These antigens have been expressed as recombinant proteins and formulated in different adjuvants (ARÉVALO-HERRERA et al., 2011; ZHENG et al., 2019).

Pfs48/45 and Pfs230 are surface proteins expressed by gametocytes and gametes that together form a stable complex, bounding to the parasite membrane via GPI anchor, being

essential for gamete fusion (ZHENG et al., 2019). Pfs230 apparently is important for fertilization (EKSI et al., 2006).

In 1998, the Pfs48/45 was expressed as a recombinant protein, presenting high immunogenicity. However, it could not induce transmission blocking immunity during pre-clinical tests, suggesting that the recombinant proteins could not mimic the native epitope conformations (MILEK et al., 1998). More recently, a study performed in area of high transmissibility established that antibodies anti-Pfs48/45 found in more than 600 asymptomatic malaria exposed individuals, are capable of blocking transmission of *P. falciparum* to mosquitoes in a direct membrane feeding assay (DMFA) (STONE et al., 2018).

Pfs25 is a 25 kDa gamete and ookinete surface protein important for survival of the motile form within mosquito midgut, epithelium invasion and maturation of oocyst (MCLEOD et al., 2019; ZHENG et al., 2019). In 2005, a TBV was first tested in Phase I clinical trials, where 30 naïve volunteers were vaccinated by intravascular injection with Pvs25H vaccine, formulated with the adjuvant Alhydrogel. The formulation was safe and immunogenic and antibodies anti-Pvs25 partially blocked *P. vivax* transmission to mosquitoes in the membrane feeding assay. However, the levels of antibodies were not the expected for an effective vaccine (MALKIN et al., 2005). Later in 2008, both Pfs25 and Pvs25 were tested in Phase I clinical trials, being formulated with the adjuvant Montanide ISA 51. In this case, thirty-six volunteers were immunized and transmission blocking immunity was feasible. However, strong systemic adverse events were observed and the trial had to be interrupted (WU et al., 2008). More recently, Pfs25 fused to IMX313 (technology used to obtain homogenous oligomers of the antigen) and expressed in chimpanzee adenovirus serotype 63 (ChAd63) and modified vaccinia virus Ankara (MVA) vectors, led to a meaningfully improved antibody responses during pre-clinical studies (LI et al., 2016).

Evaluating all the tests performed until today with different transmission blocking vaccines, the conclusion is that antibodies against gametocytes, gametes and ookinete surface proteins can reduce mosquito infection. An important aspect that hinders TBV development is the fact that the main protein candidates have a very complex structure, making hard its expression in their proper conformation (ARÉVALO-HERRERA et al., 2011).

The main difficult to develop an effective TBV is to find a high immunogenic vaccine formulation capable of inducing consistently high antibody titers of long duration. Besides, the difficult to find a safe and efficient adjuvant that can boost the immune system is also an obstacle in the search for a good TBV.

Using reverse genetics techniques in *P. berghei*, it was shown that the Merozoite Thrombospondin-Related Anonymous Protein (MTRAP), is not crucial for merozoite invasion of erythrocyte as previously reported. Instead, this protein is important for gametes egress and consequently, MTRAP is involved in *Plasmodium* transmission to mosquitoes. The MTRAP gene was knocked out, and this did not affect blood stage growth, but blocked parasite transmission. Moreover, in this study it was demonstrated that MTRAP is expressed on the surface of *P. berghei* and *P. falciparum* gametocytes and gametes, and thus might represent a new TBA, contributing to the increase in possible antigens to compose a transmission blocking vaccine (BARGIERI et al., 2016).

This work on MTRAP is an example of how reverse genetics can be used to identify new TBA. As the idea of a transmission blocking vaccine is relatively recent, new antigens have to be discovered. Gametocytes proteome includes gametocyte and gamete specific proteins. A systematic analysis on transcriptomes of *Plasmodium* parasites, specifically of genes expressed in gametocytes (form of the parasite transmitted from humans to the mosquito) may lead to the discovery of new transmission antigens.

3 OBJECTIVES AND JUSTIFICATION

Parasites from the genus *Plasmodium* are responsible for thousands of deaths in the world, especially in the African region. For this reason, malaria elimination is urgent, however, no effective control strategy against this disease has been discovered yet.

Before the "genomic era", the main focus of the researches for malaria elimination was new and better formulations against already known antigens. With technological advances in genomics, transcriptomics and proteomics, it was possible to find new potential vaccine candidates and drug targets. Thus, the development of vaccines and drugs based on new antigens have been extensively researched.

Plasmodium vivax parasites cannot be maintained in continuous laboratory cultures. This fact forces researchers to use information obtained through experiments with *P. falciparum* and apply them for the development of vaccines against *P. vivax* (OLLIARO et al., 2016).

Using the systematic analysis, previous results from the laboratory showed that it is possible to identify genes highly transcribed specifically in mature gametocytes, initially in *Plasmodium falciparum*, as this species has a large and more complete database. Gametocyte maturation of *P. falciparum* undergoes five morphologically distinct phases of development: stage I are very immature and have similar appearance as trophozoites; stage II are slightly different from the previous, but is the initial stage when the transcriptional program is characteristic of sexual stages; stage III are the parasites that started elongation; stage IV are characterized by nearly complete elongation, occupying almost the whole RBC cytoplasm; stage V are the mature gametocyte, ready for transmission (NGOTHO et al., 2019).

The *Plasmodium* stage-specific transcriptomes are available in the online genome database "*Plasmodium* Genomic Resources" (PlasmoDB – plasmodb.org). This platform was used in this research. As the objective was to find genes highly expressed in mature gametocytes, the search of genes was made using two data sets based on RNA sequence evidence: *P. falciparum* 3D7 "strand specific transcriptomes of 4 life cycle stages", which covers late trophozoite, schizont, gametocyte II and gametocyte V, and *P. falciparum* 3D7 "transcriptomes of 7 sexual and asexual life stages", which covers ring stage, early trophozoite, late trophozoite, schizont, gametocyte II, gametocyte V and ookinete (LÓPEZ-BARRAGÁN et al., 2011).

The search strategy was based in six steps (Figure 9):

a) step 1: from the first data set ("strand specific transcriptomes of 4 life cycle stages" – 3D7 4Stages RNA), 874 genes were selected for a minimum of 2-fold up-regulation of transcription from schizonts to gametocyte II;

b) step 2: from the previous 874 genes found, a second step performed with the same data set identified 403 genes for a minimum of 2-fold that were up-regulated from gametocyte II to gametocyte V. A combination of results found in both steps was performed, and only 85 genes were present in the first and in the second steps;

c) step 3: in order to validate and increase the stringency of the results, during this third step the second data set ("transcriptomes of 7 sexual and asexual life stages" – 3D7 7Stages RNA) was used. The search identified 3186 genes as up-regulated from gametocyte II to gametocyte V. A combination of results found in step 2 and in step 3 was performed, and 80 genes were present in both data sets of transcriptomes;

d) step 4: from the 80 genes found, a "predicted signal peptide" selection was made. From this selection, thirty-one genes were identified. The importance of this characteristic is due to the fact that genes with a predicted signal peptide can transcribe proteins with signal peptide that enter a secretion pathway, and thus can be possibly found on the cell surface, being a good target for antibodies;

e) step 5: since the intention of this search was to find conserved genes in the main species (*P. falciparum* and *P. vivax*), in this fifth step, only genes with orthologs in *P. vivax* were selected from the 31 previously found, resulting in 29 genes;

f) step 6: from the 29 genes previously found, only one did not have orthologs in *Plasmodium berghei*. This aspect is important, because this work is based on the use of the murine model of malaria.

Thus, from the initial 874 genes, only 28 fulfilled the requirements established.

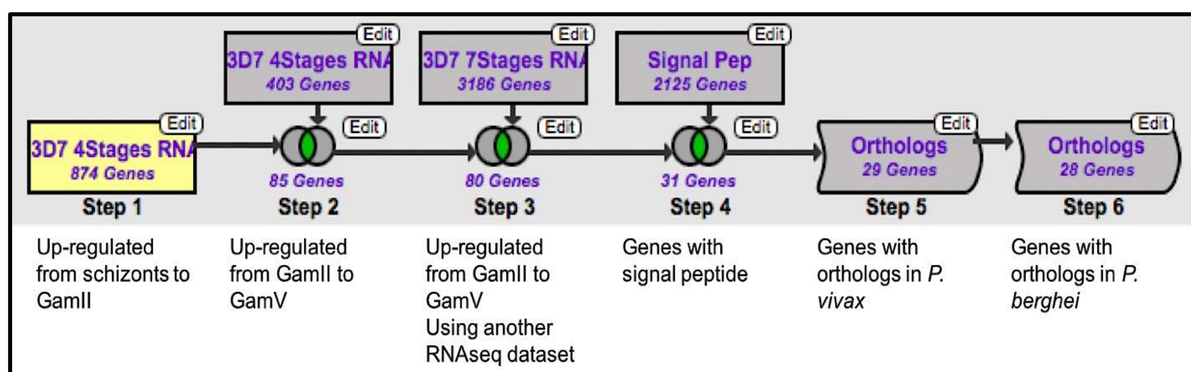


Figure 9 - ***in silico*** **analysis for selection of genes with transcription up-regulated in mature gametocytes.** The platform used in this research was the online genome data base "*Plasmodium* Genomic Resources", PlasmoDB (plasmodb.org), where *Plasmodium* stage-specific transcriptomes are available. The research was performed using

two data sets ("strand specific transcriptomes of 4 life cycle stages" – 3D7 4Stages RNA and "transcriptomes of 7 sexual and asexual life stages" – 3D7 7Stages RNA) from *P. falciparum*. The yellow box represents the results found during Step 1, where the first data set (3D7 4Stages RNA) was used, and 874 genes for a minimum of 2-fold up-regulation of transcription from schizonts to gametocyte II were selected. Step 2, from the previously 874 genes found, only 85 genes were selected for a minimum of 2-fol up-regulation of transcription from gametocyte II to gametocyte V. Step 3, using the second data set (3D7 7Stages RNA), were identified 3186 genes up-regulated from gametocyte II to gametocyte V, and a combination of results found in step 2 and in step 3 was performed, where only 80 genes were present in both data sets of transcriptomes. Step 4, from the 80 genes found, a "predicted signal peptide" selection was made. From this selection, thirty-one genes were identified for presence of putative signal peptides. Step 5, in this step, only genes with orthologs in *P. vivax* were selected from the 31 previously found, resulting in 29 genes. Step 6, from the 29 genes, a selection was made in order to find those with orthologs also in *P. berghei*, resulting in 28 final genes. Source: (adapted from: PlasmoDB)

However, from the 28 genes selected, only 12 had not yet been studied in the literature. These 12 genes are protein coding genes and were ranked based on several criteria, such as the presence of a predicted transmembrane (TM) region, conservation of the amino acid sequence between *P. falciparum* and *P. vivax*, presence of known domains (i.e. EGF-like, found in gene 1; pleckstrin homology, found in gene 2; SUEL-type lectin, found in gene 3) and presence of the Gene Ontology (GO) terms. The list of the selected genes and important characteristics is shown in Table 2:

Table 2 - List of gametocytes specific genes of interest.

Gene number	Gene ID on PlasmoDB			Has SignalP?	Has TMHMM?	Protein length	Important characteristics	GO term name
	P. falciparum	P. vivax	P. berghei					
1	PF3D7_1403200	PVX_086272	PBANKA_1038800	No	No	210 aa	EGF_3 domain-containing protein; coiled coil regions	x
2	PF3D7_1146100	PVX_092905	PBANKA_0902900	Yes	No	175 aa	Signal peptide (membrane or secreted?); Pleckstrin homology domain	x
3	PF3D7_1327100	PVX_116525	PBANKA_1342300.1	Yes	No	291 aa	SUEL-type lectin domain-containing protein	Carbohydrate binding protein
4	PF3D7_1213500	PVX_123365	PBANKA_1429300	Yes	Yes	650 aa	Integral membrane protein GPCR180 putative; Rhodopsin-like GPCR transmembrane domain	G protein-coupled receptor signaling pathway; integral component of membrane
5	PF3D7_0630000	PVX_114600	PBANKA_1128800	Yes	No	279 aa	CPW-WPC family protein	x
6	PF3D7_1103500	PVX_090860	PBANKA_0943400	Yes	No	585 aa	CPW-WPC family protein	x
7	PF3D7_0418800	PVX_090030	PBANKA_0720900	Yes	Yes	265 aa	MOLO1 domain-containing protein, putative	Acetylcholine-gated channel complex; crystalloid
8*	PF3D7_1204400	PVX_084260	PBANKA_0603300	Yes	Yes	349 aa	Name: G37 Sexual stage-specific protein G37	Plasma membrane; cell surface; integral component of membrane
9*	PF3D7_0825700	PVX_089075	PBANKA_0704900	Yes	No	289 aa	Name: CryPH Crystalloid-specific PH domain-containing protein, putative	Cytoplasm; crystalloid
10	PF3D7_0417000	PVX_089940	PBANKA_0719100	Yes	No	281 aa	Uncharacterized protein; possibly TM	x
11	PF3D7_0318000	PVX_095330	PBANKA_0807200	Yes	No	218 aa	Uncharacterized protein; possibly GPI	x
12	PF3D7_1246000	PVX_101190	PBANKA_1459100	No	Yes	511 aa	Conserved Plasmodium protein; TM domain	Integral component of membrane

* from the start of this work until the end, those two genes were studied by different researches. Number 8, named G37 (LIU et al., 2018) and number 9, named CryPH (JENWITHISUK et al., 2018).
Source: elaborated by the author based on information available on PlasmoDB and UniProt.

The main aspects analyzed during the search were: presence or not of SignalP (Signal Peptides – appendix A), presence or not of TMHMM (Transmembrane helices in proteins – appendix B), protein length and presence of GO terms. According to Ashburner et al. (2000, p. 2) "The goal of the [GO] Consortium is to produce a structured, precisely defined, common, controlled vocabulary for describing the roles of genes and gene products in any organism".

The first gene of the list, PBANKA_1038800, is a conserved *P. berghei* gene with syntenic (co-localization of genetic loci on the same chromosome within species) orthologs (genes in different species with the same origin) in *P. falciparum* and *P. vivax*.

The hypothetical protein length comprises 210 amino acids and is predicted to localize to the cell membrane, and to possess coiled coil regions domain and an EGF (Epidermal Growth Factor)-like domain (Figure 10), related to the EGF domain of laminin (extracellular protein). These domains often appear in tandem repeats and are found in the extracellular portion of transmembrane proteins or in secreted proteins (LEBRUN; CARRUTHERS; CESBRON-DELAUW, 2014). EGF domains are usually involved in ligand-receptor interaction and parasite association to the host cell surface, thus mediating important functions (MEISSENER et al., 2002; SOUZA, 2006). Since gamete fertilization, ookinete motility and epithelium midgut invasion depend on multiple protein interactions, the gene PBANKA_1038800 may be involved in one of these processes.

Coiled coil regions were also found in the transcript of the PBANKA_1038800 gene. Coiled coil domain is a type of secondary structure with a heptad repeat pattern of apolar residues and composed of two or more α-helices which interweave to from a cable like structure. These domains have diverse biological function, such as facilitating critical maintenance, repair and replication of genetic material, drive the transportation of material within the cell and assist enzymatic activities (TRUEBESTEIN; LEONARD, 2016).

```
        10         20         30         40         50
MKIVKVIIFL NVYYFFDYVH ELTKNNADLF YALCKYVKLE IAHNNKNKEK
        60         70         80         90        100
SNETNEHILE KIRDGDLIED IKQYTAFDTD INTIVYDKKN NEIKNWAIKR
       110        120        130        140        150
RILEEKDCNL DCGNNGFCVN DYGIEYCSCK YGYSVDINMF KCEENCKINN
       160        170        180        190        200
GGCDPNAECI QLDEKGEEEN NVMKGVRVLC KCKNNNNKYG GYYCPENDHS
       210
YAQHDDYLIF
                                        EGF_3  Coiled coil
```

Figure 10 – PBANKA_1038800 predicted protein sequence, highlighting the EGF and coiled coil domains. The hypothetical protein length comprises 210 aa, and is predicted to possess coiled coil domain between positions 86 – 106 and EGF_3 domain between positions 146 – 194. Source: adapted from UniProt.

The rationale for this work was based on the one for drugs and vaccine development (Figure 11). A good illustration that represents the main steps towards this is supported by the information provided by the Global Health Innovative Technology (GHIT) Fund:

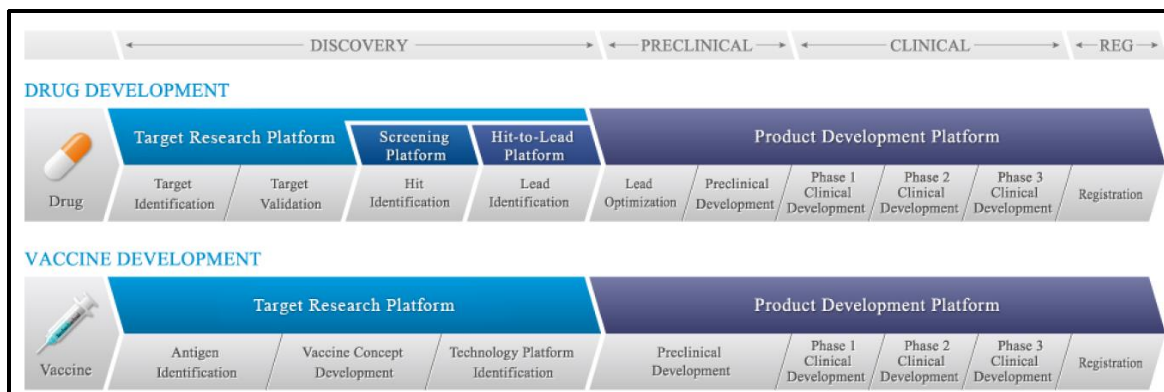


Figure 11 - Steps of drug and vaccine development. The main steps for drug and vaccine development can be divided in four phases: discovery, preclinical, clinical and regulation. Source: adapted from GHIT Fund.

The objectives of this work are aligned with the first steps of drug and vaccine development: target identification and validation.

Based on the justification above, the main objective of this work was to study whether the first gene of the list of genes, PBANKA_1038800, is involved in *Plasmodium* transmission using genetic reverse techniques. The specific objectives were:

1) Transfect plasmid into *Plasmodium berghei* parasites and generate mutants to select a PBANKA_1038800 knockout;
2) Genotyping mutant clones by PCR and select one to perform the next steps;
3) Phenotyping mutants using the *in vitro* fertilization assay to verify if the gene is involved in *Plasmodium* transmission.

4 MATERIAL AND METHODS

4.1 Animals and parasite strain

Four week-old BALB/c mice were used in the experiments. Mice were bred and maintained in the animal facility of the Department of Parasitology at the Institute of Biomedical Sciences, University of São Paulo, under CEUA ICB (132/2014/CEUA) and standards established by the National Council for Control of Animal Experimentation (*Conselho Nacional de Controle de Experimentação Animal* - CONCEA) .

The *P. berghei* ANKA (PbANKA) line, *P. berghei* Ookluc (PbOokluc) (CALIT et al., 2018) and *P. berghei* Ookluc_1038800 knockout ($Pb1038800^{KO}$) are stored in liquid nitrogen and at -80°C. To prepare the vial stocks, 150 µl of blood from infected mice are mixed with 300 µl of Alsever's solution (Sigma-Aldrich, A3551) with 10% glycerol (Sigma-Aldrich, G5516).

For the experiments, animals are infected by intraperitoneal injection of 200 µl of thawed stocks. Blood smears in glass slides (Kasvi, K5-7105-1) are stained with Fast Panoptic (Laborclin, 620529) to follow parasitemia and gametocytemia, which is counted by direct light microscopy with 100X oil immersion objective (Nikon E200).

To perform euthanasia, two techniques are used: in the first one, animals are put inside a Carbon Dioxide (CO_2) chamber, located in the animal facility of the Department of Parasitology at the Institute of Biomedical Sciences. In the second technique, euthanasia is performed with cervical dislocation. Before this procedure, the mice are anesthetized with 2000 µl of a solution of PBS 1X (1250 µl), ketamine (500 µl) and xylazine (250 µl). The solution is injected intraperitoneally.

4.2 Construction of the plasmid for gene knockout ($Pb1038800^{KO}$)

4.2.1 Plasmid used to knockout the gene

The host laboratory has available a plasmid with a multiple cloning site (MCS) flanking a cassette that confers red fluorescence (expressing the mCherry protein) to *Plasmodium berghei* parasites, and a cassette for drug selection (human Dihydrofolate Reductase - hDHFR), conferring pyrimethamine resistance. This plasmid is known as "DCOmCherry" (DCO = Double Crossing Over), with pUC18 background used for cloning (Figure 12).

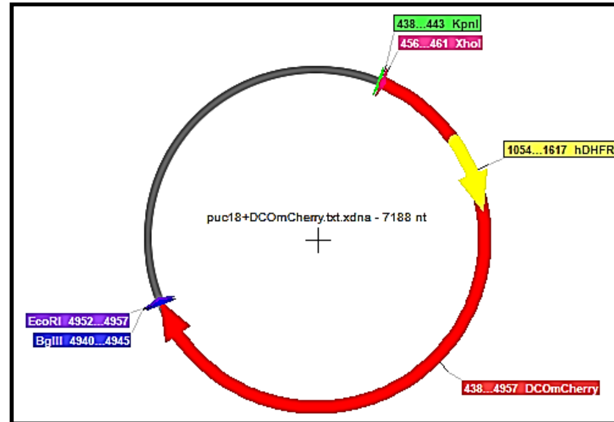


Figure 12 - Plasmid "DCOmCherry" with pUC18 background used for cloning. This plasmid contains a multiple cloning site (with sequences for the enzymes KpnI, XhoI, EcoRI and BglII) flanking a cassette that confers red fluorescence (mCherry) to parasites and a cassette for drug selection (hDHFR), conferring pyrimethamine resistance. Source: author's compilation.

4.2.2 Amplification of the fragments

To generate the plasmid to knockout the target gene, 816 base pairs (bp) of the upstream sequence (Homology Region 5' – HR 5') and 811 bp of the downstream sequence (Homology Region 3' – HR 3') of the gene PBANKA_1038800 from PbANKA strain genomic DNA (gDNA) were PCR amplified following the protocol exemplified on Table 3 and with primers showed on Table 4.

Table 3 - General protocol for a final 25 µl volume of the PCR mixture.

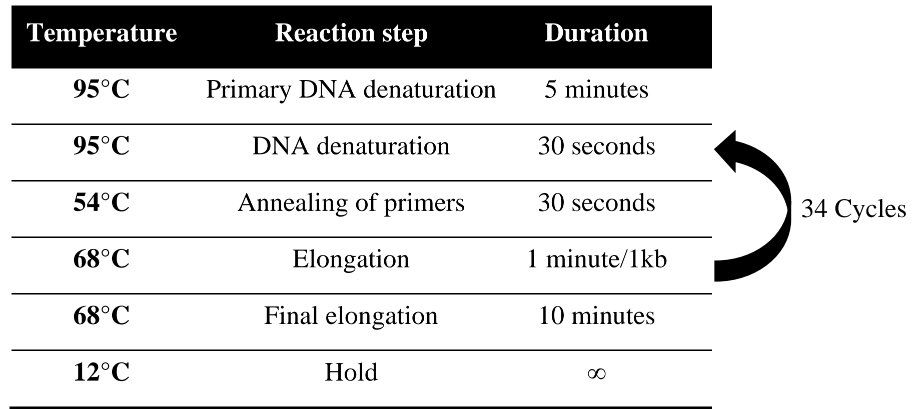
Components	Volumes	Reference
Ultrapure Distilled Water	14,375 µl	10977-023
10x Ex Taq Buffer	2,5 µl	SD0307
dNTP 0,2 mM	2 µl	SD0304
Primer Forward 1 mM	2,5 µl	-
Primer Reverse 1 mM	2,5 µl	-
Takara Ex Taq DNA Polymerase	0,125 µl	RR001C
gDNA PbANKA 50 ng	1 µl	-

Table 4 - Primers designed to amplify sequences from the gene PBANKA_1038800, with the objective to knockout this gene.

Primers	Sequence 5' – 3'	Restriction site underlined	Tm (°C)
5'Fw_Pb1038800KO	GGGGGTACCTCACGCATTCTTATTGATTTG	KpnI	54
5'Rv_Pb1038800KO	CCCCTCGAGTGTTAATTCATGCACATAGTC	XhoI	56
3'Fw_Pb1038800KO	GGGAGATCTATGCTCAGCATGATGATTAC	BglII	56
3'Rv_Pb1038800KO	GGGGAATTCAATATGCACGCACGATATAC	EcoRI	56

PCR was carried out and put inside the PCR Thermal Cycler (T100 Thermal Cycler, BioRad). The cycling parameters used are specified in the following table:

Table 5 - PCR cycling program.



Temperature	Reaction step	Duration
95°C	Primary DNA denaturation	5 minutes
95°C	DNA denaturation	30 seconds
54°C	Annealing of primers	30 seconds
68°C	Elongation	1 minute/1kb
68°C	Final elongation	10 minutes
12°C	Hold	∞

34 Cycles

PCR product can be detected via electrophoresis by applying 3 µl to a 1% agarose gel stained with ethidium bromide.

4.2.3 Plasmid construction

Both amplified sequences (HR 5’ and HR 3’) were cloned first in TOPO TA plasmid (Invitrogen, 45-0641) and the ligation transformed in *E. coli* DH5-Alpha strain and cultivated in LB Agar plates with ampicillin. Colonies were selected and plasmid DNA isolation was made with NucleoSpin Plasmid kit (Macherey-Nagel, 740588.250).

Inserts HR 5’ and HR 3’ were removed from TOPO plasmid and cloned in a digested and dephosphorylated DCOmCherry plasmid, upstream hDHFR (with KpnI and XhoI restriction enzymes) and downstream mCherry (with BglII and EcoRI restriction enzymes), respectively (Figure 13A).

First ligation (DCOmCherry + insert HR 3’) was made according to Table 6, transformed in *E. coli* DH5-Alpha strain and cultivated in LB Agar plates with ampicillin. Colonies were selected for plasmid DNA isolation with NucleoSpin Plasmid kit, and the presence of insert is confirmed by digestion with restriction enzymes. Second ligation (DCOmCherry + insert HR 3’ + HR 5’) followed the same procedures as the first ligation.

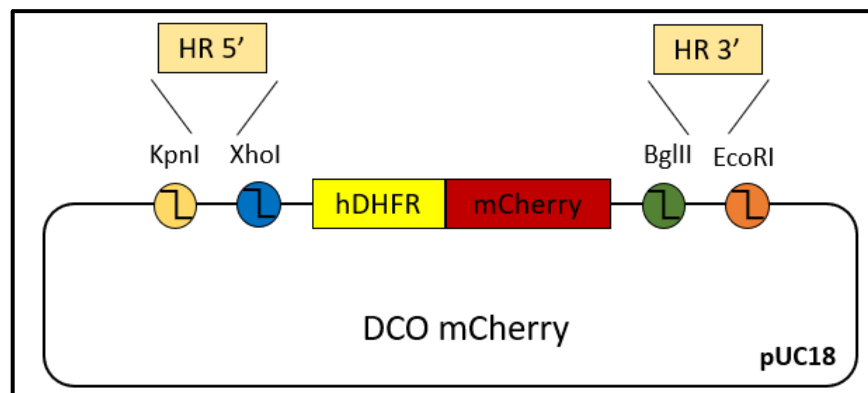
Table 6 - Ligation DCOmCherry + insert HR 3’.

DCOmCherry + insert HR 3’		
Components	**Volumes**	**Reference**
Ultrapure water	14 µl	10977-023

10X T4 DNA Ligase Buffer	2,0 µl	EL0011
T4 DNA Ligase	1,0 µl	EL0011
Plasmid (50 ng)	2,0 µl	-
Insert (14, 1 ng)	1,0 µl	-

The targeting sequence was linearized and removed from the plasmid with restriction enzymes KpnI and EcoRI and 2 µg were used for transfection. The theory is that the homology regions drives recombination by double crossing over, replacing the coding sequence of the gene of interest with the fluorescence and drug selection cassettes (Figure 13B).

A – Construction of the target plasmid



B – DCO recombination

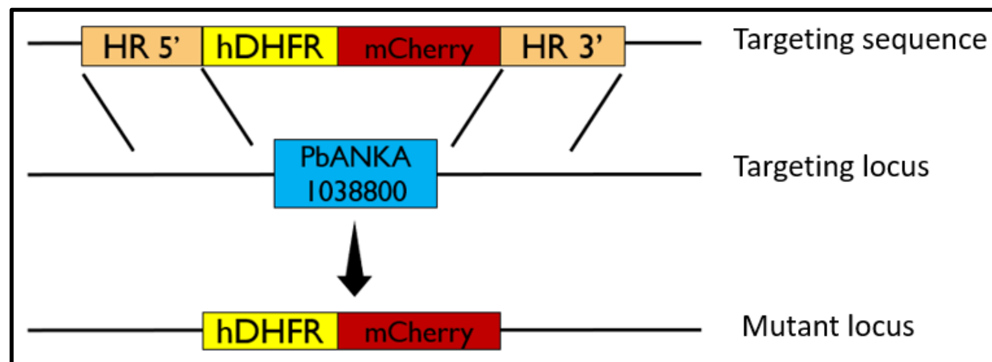


Figure 13 - Scheme showing the strategy for plasmid construction and DCO recombination. (A) Construction of the target plasmid. DCOmCherry plasmid has a pUC18 background and is used for cloning. This plasmid has a cassette for drug selection (hDHFR) and a fluorescent cassette (mCherry), flanking two cloning regions. Homology Region 5' (HR 5') were cloned upstream hDHFR, using KpnI and XhoI restriction enzymes, and Homology Region 3' (HR 3') were cloned downstream mCherry, using BglII and EcoRI restriction enzymes. (B) Double Crossing Over strategy. The sequence flanking the cassettes were removed after digestion with KpnI and EcoRI, and transfected in PbOokluc parasites. Both homology regions make it possible to delete the PbANKA_1038800 gene and replace it with two cassettes (hDHFR and mCherry). Source: author's compilation.

4.3 Transfection and mutant selection

4.3.1 P. berghei line used for transfection (PbOokluc)

The target sequence was transfected into schizonts of a new transgenic line of *P. berghei* named Ookluc (PbOokluc), derived from the *P. berghei* ANKA recombinant Gene Insertion/Marker Out (GIMO) line. PbOokluc was generated (Figure 14) in the host laboratory, and is a reporter of zygote and ookinete conversion by specific nano-Luciferase (nLuc) expression at these stages, under the control of an ookinete specific promoter (Circumsporozoite and TRAP Related Protein - CTRP).

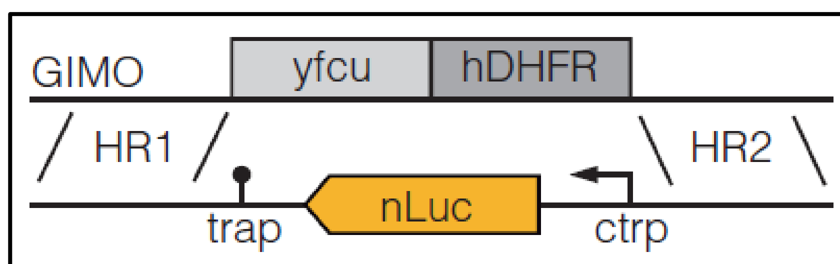


Figure 14 - Strategy for generating the Ookluc line. The coding sequence of nanoluciferase (nLuc) was cloned upstream the 3' UTR of the Thrombospondin-Related Adhesive Protein (TRAP) and downstream the Circumsporozoite and TRAP Related Protein (CTRP) promoter, into the plasmid pL0043. This plasmid contains the homology regions (HR1 and HR2) for integration in GIMO locus. Source: (adapted from CALIT et al., 2018).

yfcu: resistant marker yeast cytosine deaminase/uridyl phosphoribosyl transferase

hDHFR: resistant marker human dihydrofolate reductase

The PbOokluc has low and insignificant luciferase activity in blood stages. However, when the parasite is put into ookinete medium and incubation at 21°C (conversion assay), the luciferase activity increases over time, being compatible with the time for zygote formation (6 hours after incubation, when the specific promotor becomes active), and reaching a maximum of activity after 24 hours, when ookinetes are formed.

4.3.2 Synchronization medium preparation

Transfection followed the protocol available (JANSE; RAMESAR; WATERS, 2006). For transfection and knockout selection, six BALB/c mice were infected with PbOokluc. Synchronization medium have to be prepared one day before transfection. When parasitemia reached 5% approximately, blood was collected by cardiac puncture in heparinized syringes (heparin sodium salt from Sigma-Aldrich, H3393) and added to a sterile synchronization medium composed of RPMI 1640 supplemented with Glutamax (Thermo Scientific, 61870036), HEPES (Thermo Fisher Scientific, 15630080), Neomycin (Sigma-Aldrich, N1142) and Fetal Bovine Serum (Thermo Fisher Scientific, SH30071.03).

The synchronization medium was filtered with a vacuum filter/storage bottle system (Corning Incorporated, 431097) which was pumped under the hood. In order to eliminate

leucocytes and plasma, the mixture of medium and blood was put in a 50 ml centrifuge tube (Corning Incorporated, 430829) and centrifuged (Centrifuge 5804 R, Eppendorf) for 10 minutes and 450 g at room temperature. Then, the pellet was resuspended with 35 ml of complete medium and incubated in incubator tri-gas (37°C, 5% CO_2 and 10% O_2), under agitation (90 rpm – KS 130 basic, IKA) for approximately 16 hours.

4.3.3 Transfection and selection of PbOokluc parasites

After the mentioned period of 16 hours, a centrifugation step was performed using Nycodenz 55% (from a 30% stock), being possible to separate red blood cells infected by more mature forms of the parasite, such as the asexual schizonts, from the lighter ones by means of a density gradient.

Approximately 2 µg of the target sequence were introduced into the synchronized parasites (schizonts) by electroporation using the Amaxa® Nucleofactor electroporator, set at program U33 and using the human T cell Nucleofactor kit (Lonza, VPA-1002).

Transfected schizonts were injected into a tail vein of two naïve BALB/c mice (parental mice) and 24h after transfection the positive selection of mutant parasites started by treatment of mice with pyrimethamine (Sigma-Aldrich, 46706) in the drinking water (70 mg/liter) during seven days.

4.3.4 Transfer of selected parasites to naïve mice

Blood of both infected mice (parental population) was collected and diluted in 200 µl of PBS and injected intraperitoneally in other four naïve mice (two for each parental mice), known as transfer population. Approximately 24h after the procedure, the transfer population received the treatment with pyrimethamine in the drinking water for selection of mutants.

When parasitemia was ≥ 3%, all six mice were anesthetized and euthanized by cervical dislocation, and their blood collected for storage, with a small portion used to extract gDNA with DNeasy Blood & Tissue Kit (QIAGEN, 69506). The obtained gDNA is used to genotype parasites.

4.4 Genotyping mutant clones by PCR

4.4.1 Genotyping strategy

Genotyping clones by PCR is a strategy used to verify if the knockout of the gene was successful. With this technique it is possible to compare a specific sequence of a gene in two

situations: in the control group (where the sequence is unaltered) and in the mutant group (where the sequence is modified). For this project, the entire coding sequence of the gene PbANKA_1038800 was removed (knocked out) and replaced by two selection cassettes (hDHFR+mCherry).

For the PCR genotyping (Figure 15), one primer was designed to amplify the region upstream the coding sequence of the gene (P1) and one primer was designed to amplify the region downstream the coding sequence of the gene (P6). Those primers were used in both reactions, that is, for the control and mutant. The other four primers were designed to amplify part of the coding sequence (specific for the control – P2 and P4) and part of the modified sequence (specific for the mutant – P3 and P5).

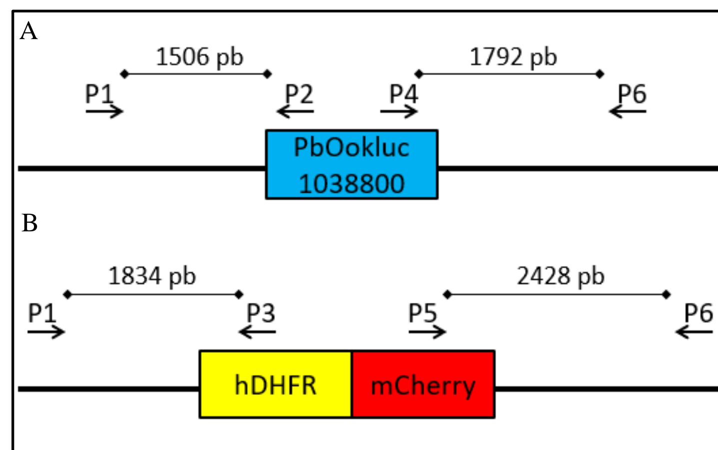


Figure 15 - Scheme representing control and mutant regions that were amplified for PCR genotyping. (A) Two pairs of primers were used to specifically amplify the control PbOokluc_1038800 locus: P1 and P2 (generating a 1506 bp fragment) and P4 and P6 (1792 bp). (B) Two pairs of primers were used to specifically amplify the mutant locus, where the gene sequence is expected to have been replaced by the two hDHFR + mCherry selection cassettes: P1 and P3 (1834 bp) and P5 and P6 (2428 bp). Source: author's compilation.

4.4.2 Genotyping by PCR

Selected clones were genotyped by PCR using PbOokluc gDNA, as control, and gDNA obtained from the six mice used for transfection, as the mutant. Primers were designed to amplify the control strain PbOokluc_1038800 locus (P1+P2 and P4+P6) and the mutant Pb1038800^{KO} locus (P1+P3 and P5+P6). Primers used are showed on Table 7.

Table 7 - Primers designed to genotype control and mutant locus, to confirm the presence of both hDHFR and mCherry selection cassettes in the mutant parasites.

Target	Primers	Sequence 5' – 3'	Tm (°C)
Control/Mutant	5'Fw_P1	GCAGGAGGACCTTATTCG	56
Control	5'Rv_P2	CTATTCCATAGTCGTTTACAC	58

Mutant	5'Rv_P3	CACAGCGACGATGCAGTT	56
Control	3'Fw_P4	GGAGGATGTGATCCAAATG	56
Mutant	3'Rw_P5	GCATGGACGAGCTGTACA	56
Control/Mutant	3'Rv_P6	TCGTGCTCGAAGTCATCC	56

4.5 Cloning of parasites

To obtain a single parasite clone, limiting dilution was performed. For that, two BALB/c mice were infected (donor population) with 200 µl of thawed stocks from the transfer population. Two days after infection, one mice presented 0,27% of parasitemia (that is approximately 21,6 x 10^6 infected red blood cells per milliliter of blood – iRBC/ml).

To perform cloning, blood from the donor was diluted in PBS 1X to 5 iRBC/ml. Thus, 100 µl of this solution is the same as 0,5 iRBC. Twenty BALB/c mice were infected intravenously with 100 µl of the solution, so one in two animals are expected to receive a single parasite.

After approximately seven to ten days of infection, parasitemia of infected animals was determined and euthanasia was performed for blood collection and preparation of vial stocks. The gDNA was extracted for genotyping.

4.6 Growth curves (parasitemia and gametocytemia)

In order to assess whether the knockout of the PBANKA_1038800 gene was capable of affecting parasite development during the blood cycle, two BALB/c mice were infected with control PbOokluc and two with mutant Pb1038800^{KO}. When parasitemia reached approximately 0,1%, blood was collected from infected animals and diluted in PBS. From the dilution, approximately 0, 001% of infected red blood cells were intravenously transferred to other ten mice,.

Parasitemia and gametocitemia of the ten mice were followed during eight days. For that, blood from all infected animals were collected every day during the period of the study and approximately at the same hour each day. A drop of blood from each mice was used to perform blood smears in glass slides, which were stained with fast panoptic to follow parasitemia and gametocytemia.

After eight days, the results were collected and it was possible to construct two graphs: one with the results from the parasitemia and other with the results from the gametocytemia.

At day eight, all ten mice were anesthetized and euthanized by cervical dislocation. Blood from mutants were collected and stored at -80ºC.

4.7 Verifying viability of male gametocyte

4.7.1 Exflagellation assay theory

Inside the human organism, gametes cannot egress, because there are some necessary conditions for this occurrence, such as the presence of the xanthurenic acid, basic pH (8.3) and 21°C, conditions found in the midgut of *Anopheles* mosquitoes.

Inside a mosquito midgut, gametes egress from the host erythrocytes. The female gametocyte forms a single gamete (macrogamete) and male gametocyte undergoes three mitotic divisions, producing eight flagellated microgametes that perform a microtubule-based movement to leave the host cell, a process known as exflagellation. After the egress, microgametes bind macrogametes and also erythrocytes around, forming a cell cluster or in other words, an exflagellation center (EC) (Figure 16).

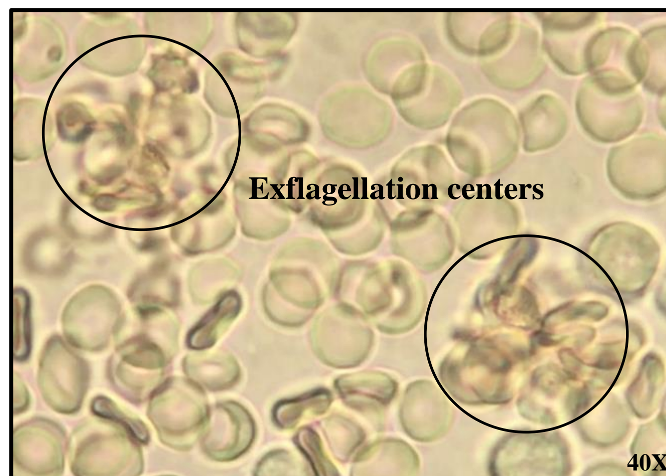


Figure 16 - Exflagellation centers. The exflagellation centers are formed by microgametes attached to macrogametes and erythrocytes. Those were observed during an exflagellation assay made with blood collected from mice infected with control PbOokluc. Source: author's compilation.

The main objective of the exflagellation assay in to verify the viability of male gametocytes, that is to say, verify if they can feel the necessary stimulation that make possible their egress and gamete exflagellation, essential for the fertilization.

4.7.2 Exflagellation assay

To perform the exflagellation assay, 4 µl of blood from infected mice (with PbOokluc or Pb1038800^{KO}) are added to 16 µl of ookinete medium, composed of RPMI 1640 (Thermo Scientific, 61870) with 0,025 M HEPES (Thermo Scientific, 15630080), penicillin-streptomycin-neomycin (Sigma Aldrich, P4083), xanthurenic acid 100 M (Sigma Aldrich, D120804) at a pH of 8,3 and hypoxanthine 50 mg/L (Sigma Aldrich, H9636).

The mixture of infected blood and ookinete medium is put on top of a glass slide, covered by a coverslip. It is then incubated at 21°C, and after 15 minutes it is possible to see the process of exflagellation of the male gametocyte and the exflagellation centers under the microscopy (40X), which can be counted. The quantities found in 20 fields in the control can be compared with those founded in the mutant, when gametocytemia is similar (maximum 0,4% of difference).

4.8 Phenotype mutants using *in vitro* fertilization assay

4.8.1 Conversion assay preparation

To test if gene knockout affected the development of parasite during sexual stages (i.e. gametes fertilization, zygote and ookinete formation), BALB/c mice were infected with PbOokluc or Pb1038800^{KO} parasites, and when gametocytemia reached more than 0,4% the conversion assay was performed as described (RAMAKRISHNAN et al., 2013).

Parasitized blood with high gametocytemia (≥ 0,4%) was obtained by cardiac puncture in heparinized syringes and diluted (1:40 or 1:20), at room temperature, in ookinete medium, which consisted of RPMI 1640 (Thermo Scientific, 61870) with 0.025M HEPES (Thermo Scientific, 15630080), hypoxanthine 50 mg/l (Sigma-Aldrich, H9636), penicillin-streptomycin-neomycin (Sigma-Aldrich, P4083) and xanthurenic acid 100 µM (Sigma-Aldrich, D120804) at a pH of 8.3.

The test is done in triplicate, so dilution is put inside 0.6 ml microtubes and maintained at 21°C in a BOD incubator (FANEM, 347F) for 24 hours, or for some experiments, also at 1h, 2h, 4h and 6h, to evaluate zygote formation. After each time, tubes are stored at 4°C until the reading.

4.8.2 Conversion assay reading

The dilution is transferred to a 96-well plate and 1 volume of the substrate/lysis buffer (Nano-Glo luciferase assay system; Promega) or substrate/ookinete medium (instead of lysis buffer so the cells can be counted by blood smears) is added and after 10 minutes of incubation at 37°C (Series II 3110 Water-Jacketed CO_2, Thermo Scientific), the luciferase activity is determined by measuring the Relative Light Units (RLU) using a microplate luminometer reader (SpectraMax i3; Molecular Devices).

When using ookinete medium, after reading the plate, the mixture of the dilution, substrate and ookinete medium is collected and spin to separate blood from other components.

Two microliters of blood are spread on a glass slide and stained with fast panoptic. Zygotes and ookinetes can then be seen and/or counted under light microscopy with 100X oil immerse objective.

4.8.3 The importance of PbOokluc on phenotyping parasites

Using PbOokluc provides a faster and more precise way to evaluate ookinete and/or zygote formation. It is also possible to phenotype even before parasite cloning. As this recombinant parasite is a reporter of zygote and ookinete conversion by specific nano-Luciferase (nLuc) expression at these stages, when PbOokluc is put into conversion assay, the luciferase activity increases over time, being compatible with the time for zygote formation (6 hours after incubation, when the specific promotor becomes active), and reaching a maximum of activity after 24 hours, when ookinetes are formed (CALIT et al., 2018).

4.9 Statistical Analysis

All statistical analysis were made with GraphPad Prism 8.02. A P value less than 0,05 was considered statically significant. Growth curves, exflagellation and conversion assays (only clone C1) were analyzed by Welch's t test. Exflagellation and conversion assays testing 3 clones (C1, D1 and D2) were analyzed by Welch's ANOVA test and Dunnett's T3 multiple comparisons test.

5 RESULTS

5.1 Generation of the plasmid and transfection

5.1.1 Plasmid for gene PBANKA_1038800 knockout

Genomic DNA from PbANKA strain was used to amplify 816 bp of the upstream sequence (HR 5') of the gene PBANKA_1038800 and 811 bp of the downstream sequence (HR 3') of the same gene.

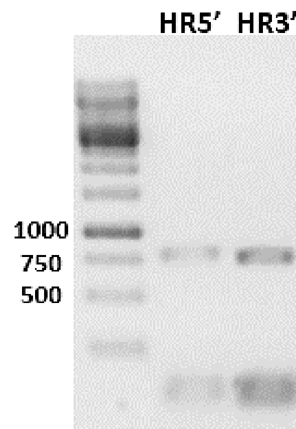


Figure 17 - Electrophoresis showing the amplified PCR fragments from the upstream sequence (HR 5') and downstream sequence (HR 3') of the gene PBANKA_1038800. Primers were designed to amplify the HR 5' sequence, generating a fragment of 816 bp, and the HR 3' sequence, generating a fragment of 811 bp. Source: (author's compilation, 2017).

PCR fragments were cloned into DCOmCherry, creating the plasmid for gene PBANKA_1038800 knockout. The band corresponding to the target sequence (containing approximately 6105 bp) was removed by restriction enzymes (KpnI and EcoRI) (Figure 18) and transfected into PbOokluc schizonts by electroporation.

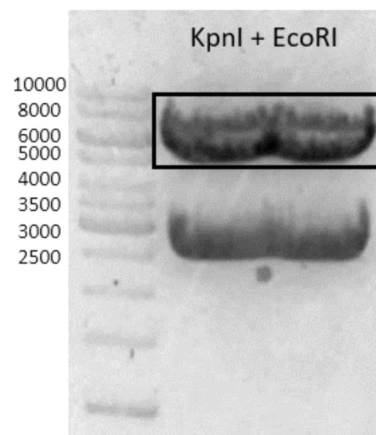


Figure 18 - Electrophoresis of the final plasmid (DCOmCherry+HR5'+HR3') of gene PBANKA_1038800^{KO}, after digestion with restriction enzymes KnpI and EcoRI. The digestion originated two bands: the tallest with 6105 bp, being the one used for transfection and what was left of the plasmid, the lowest with 2687 bp. Source: (author's compilation, 2017).

5.1.2 Transfection and mutant parasites selection

On the first day after transfection, blood from both mice was collected to verify if there were parasites in the blood, that is, if the intravenous injection was done correctly. Parasitemia of mice number one was 0,2% and of mice number two was 0,3%. Thus, both were infected.

For selection of mutant parasites (those with gene PBANKA_1038800 knocked out), the animals were treated with pyrimethamine in the drinking water. After ingested, pyrimethamine competes with dihydrofolate and inhibits the dihydrofolate reductase (DHFR). This enzyme is responsible for the reduction of dihydrofolate to tetrahydrofolate, precursor of RNA and DNA. This way, pyrimethamine causes the reduction of nucleic acid synthesis in parasites of the control group, but not in mutants, as they have the cassette that confers resistance to this drug.

5.1.3 Transfer of selected parasites to naïve mice

Five days after transfection (i.e. four days after start of the treatment), parasitemia was zero. However, two days later, it was possible to observe the presence of some parasites. These results confirm that the majority of the parasites did not survive the treatment.

Seven days after transfection, blood from the parental mice was collected and transferred to four BALB/c mice (transfer population). On the next day, water from both groups (parental and transfer) was replaced: parental mice received common water, and transfer mice received water with pyrimethamine, for selection of mutants. Four days after starting the treatment (i.e. five days after transference and 12 days after the transfection), parasitemia of all animals was ≥3% (Table 8). All mice were anesthetized, euthanized and blood was collected to stock and extraction of gDNA.

Table 8 – Parasitemia of parental and transfer population after transfection and positive selection of mutant parasites.

Population		Parasitemia
Parental population	Pp1	12%
	Pp2	16%
Transfer Population	Tp1	4,9%
	Tp2	3,3%
	Tp3	4,2%
	Tp4	4,4%

Genomic DNA obtained from all six mice was used to genotype the mutants. Blood from Tp3 was used to phenotype and clone.

5.2 Genotype of mutants

5.2.1 PCR of parental and transfer populations

Genotype of mutants was determined by PCR (Figure 19). For that, primers were designed to amplify specifically the control locus (primers P1+P2 and P4+P6) and mutant locus (primers P1+P3 and P5+P6). Two different genomic DNA were used: gDNA from PbOokluc (control) and gDNA obtained from the blood of the six mice used for transfection (two parental and four from the transfer population).

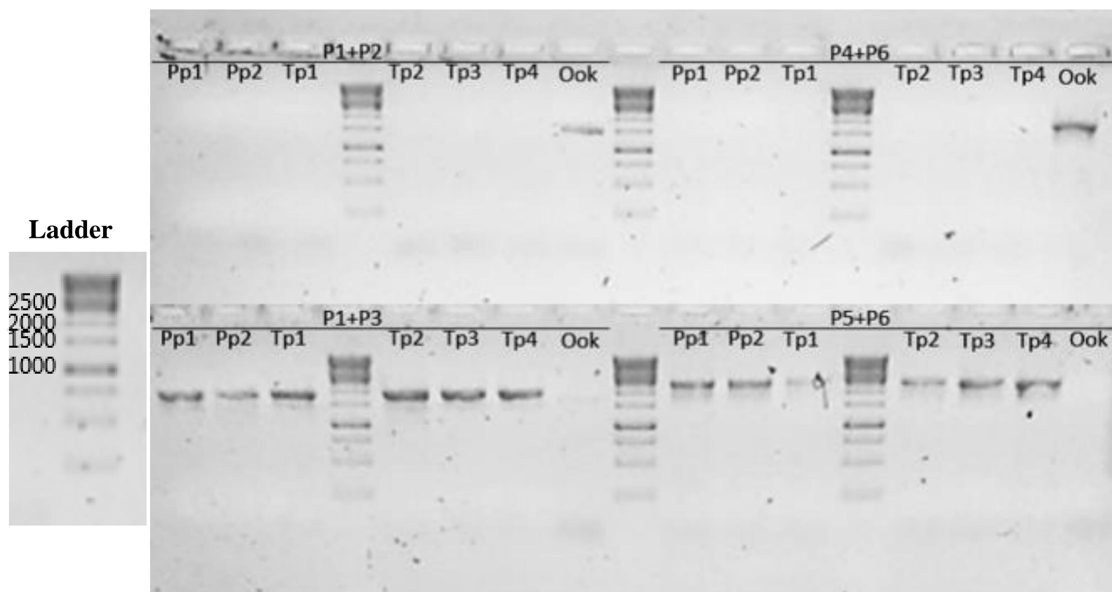


Figure 19 - Genotype of parental and transfer population (From Pb1038800^{KO} transfection). After transfection, blood was collected from all six animals and gDNA was extracted. Genomic DNA from each population and from PbOokluc was used to genotype the parasites by PCR. Primers P1+P2 or P4+P6 (specific to amplify control locus) or P1+P3 or P5+P6 (specific to amplify mutant locus) were used in the reactions. Control was amplified with primers P1+P2 and P4+P6, generating fragments of 1506 bp and 1792 bp, respectively (gel above). Mutant was amplified with primers P1+P3 and P5+P6, generating fragments of 1834 bp and 2428 bp, respectively (gel below). Source: (author’s compilation, 2018).

Ook: Control (PbOokluc) Pp: Parental population Tp: Transfer population

5.2.2 PCR of Transfer population 3

Experiments results confirmed the presence of mutant parasites (Pb1038800^{KO}) in the blood of parental and transfer populations, beyond absence of the wild type locus. PCR was repeated using only gDNA from Tp3 (Figure 20) to confirm these results, because blood from this population would be used on the next experiments, including cloning of the parasite.

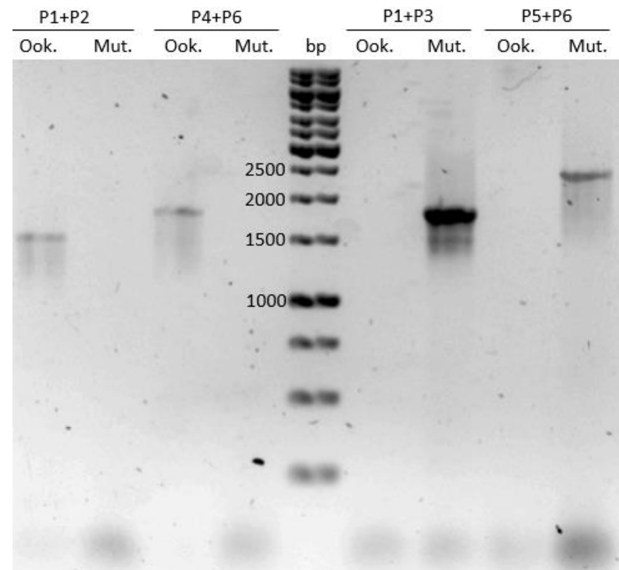


Figure 20 - Genotype of mutant population (Tp3) before cloning of the parasites. PCR was repeated using gDNA of PbOokluc strain as control and gDNA obtained from mice number 3 of transfer population (Tp3). Primers P1+P2, P4+P6 (specific to amplify control locus), P1+P3 and P5+P6 (specific to amplify mutant locus) were used in reactions containing gDNA of PbOokluc strain and gDNA of Tp3. Control was amplified with primers P1+P2 and P4+P6, generating fragments of 1506 bp and 1792 bp, respectively. Mutant was amplified only with primers P1+P3 and P5+P6, generating fragments of 1834 bp and 2428 bp, respectively. Source: (author's compilation, 2018).

Ook.: Control (PbOokluc) Mut.: Mutant (Pb1038800^{KO})

5.3 Cloning of mutant parasites

5.3.1 Cloning of the parasites

After the results from the genotype and the first conversion assay (Figure 12), blood from mice number three of transfer population (Tp3) was used to clone the parasites. Twenty mice were infected and separated in four groups (A, B, C and D) with 5 animals in each group. Nine days after blood injection, only eight (A3, A4, B3, C1, C3, D1, D2 and D5) had parasites in their blood (Table 9).

Table 9 – Infected mice and presence (+) or absence (-) of parasites in blood.

	1	2	3	4	5
A	-	-	+	+	-
B	-	-	+	-	-
C	+	-	+	-	-
D	+	+	-	-	+

Parasitemia was determined (Table 10) and blood from infected mice was stored in liquid nitrogen.

Table 10 – Parasitemia of infected mice after cloning.

Mice	A3	A4	B3	C1	C3	D1	D2	D5
Parasitemia	5,28%	4,14%	1,70%	3,60%	6,93%	3,13%	3,20%	4,45%

5.3.2 Genotype of mutant clones

The eight animals that had parasites were euthanized and their blood collected for gDNA extraction to perform PCR and confirm the presence of mutant parasites (Figure 21).

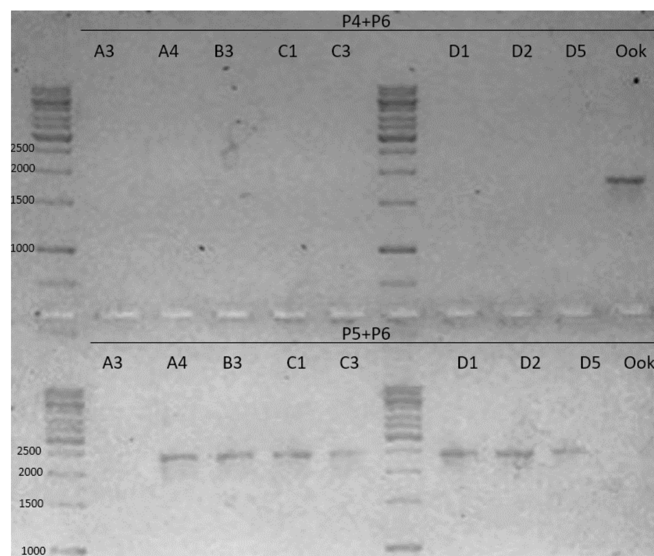


Figure 21 - Genotype of mutant clones. PCR was performed using gDNA of PbOokluc strain as control and gDNA obtained from all clones (A3, A4, B3, C1, C3, D1, D2 and D5). Primers P4+P6 (specific to amplify control locus) and P5+P6 (specific to amplify mutant locus) were used in the reactions. Control was amplified only with primers P4+P6, generating fragment of 1792 bp. Mutants (except clone A3) were amplified only with primers P5+P6, generating fragments of 2428 bp. Source: (author's compilation, 2018).

Ook: Control (PbOokluc)

5.3.3 PCR of Clone C1

Clone C1 was then utilized in all experiments (growth curves, conversion assay and exflagellation assay). PCR was repeated using only gDNA from clone C1 (Figure 22). Primers P4+P6 (designed to amplify only the control, wild type, locus) and P5+P6 (specific to amplify the mutant locus) were used in reactions containing gDNA of PbOokluc strain (control) and gDNA of clone C1.

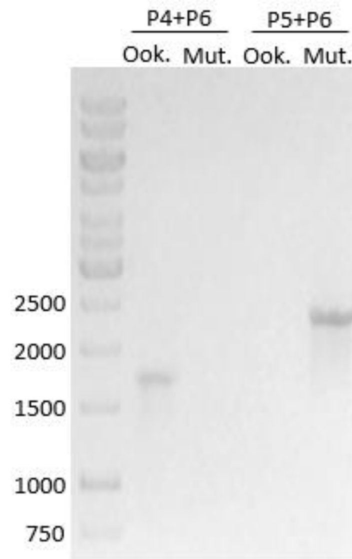


Figure 22 - Genotyping after cloning. After cloning, genotyping was made with the same primers used for genotype before cloning: P4+P6 (specific to amplify control locus) and P5+P6 (specific to amplify mutant locus). Results showed that only control was amplified with primers P4+P6, generating 1792 bp fragment. Besides, reaction with primers P5+P6 generated a 2428 bp fragment, amplifying only the mutant gDNA. Source: (author's compilation, 2018).

Ook.: Control (PbOokluc) Mut.: Mutant (Pb1038800^{KO})

The genotype of mutant clone C1 indicated a positive result, as the fragment that corresponds to the mutant locus was amplified when using primers designed to amplify part of the downstream sequence of the PBANKA_1038800 gene (P6) and part of the mCherry cassette (P5). However, the amplification did not occur when using gDNA from C1 and primers P4 and P6 (wild type locus), which means that only mutant parasites (i.e. with gene PBANKA_1038800 knocked out) were present in the population.

5.4 Growth curves

5.4.1 Mice infection and determination of parasitemia and gametocytemia

The results from the genotypes and the first phenotype (Figure 25) done before cloning, exhibited positive results. The next step was to check if the knockout of the gene PBANKA_1038800 was affecting parasite development during blood cycle. For that, parasitemia and gametocytemia of mice infected with 0,001% of parasitemia of control PbOokluc or mutant Pb1038800^{KO} were followed during eight days.

Blood from all infected mice were collected from the first day after infection, but on day one, parasitemia and gametocytemia were zero. From the second day, parasitemia was determined in all mice, but gametocytes were not detectable. From the third day onwards, parasitemia increased and it was also possible to count gametocytes in both groups.

5.4.2 *Evaluation of Pb1038800^{KO} during blood stages*

With the results obtained after eight days following parasitemia and gametocytemia in mice infected with PbOokluc and in mice infected with Pb1038800^{KO}, being five per group, it was possible to construct two graphs: one with average parasitemia values of each group and the other with average gametocytemia values obtained during the study period (Figure 23).

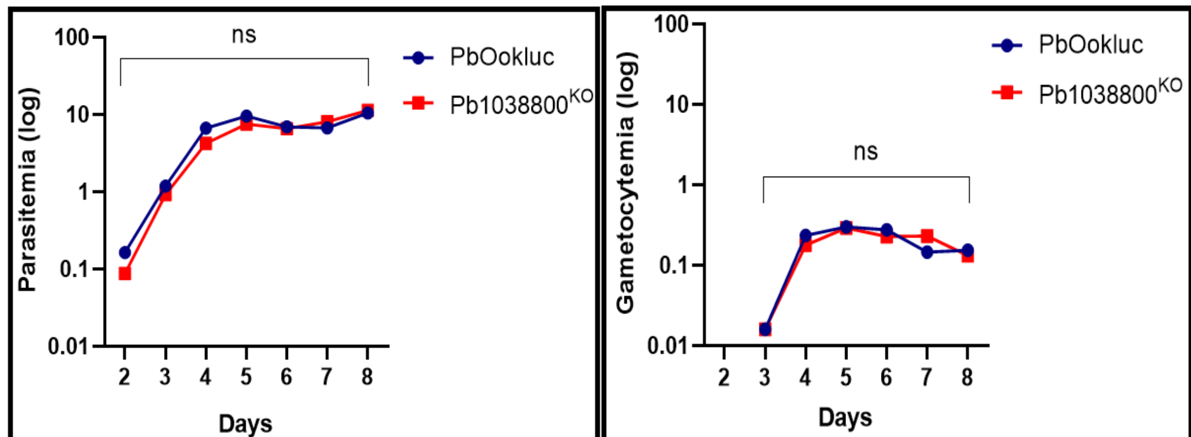


Figure 23 – Growth curves comparing parasitemia and gametocytemia between animals infected with the control parasite (PbOokluc) and the mutant (Pb1038800^{KO}) over a period of eight days. Gene knockout had no impact on blood stage parasite development, as both mutant parasitemia and gametocytemia were very similar to control during the eight days of the experiment. P values of parasitemia (0,8399) and gametocytemia (0,9097) indicates that there is no significant difference between control and mutant results. Source: (author's compilation, 2018).

Analysis made with *GraphPad Prism 8.02 – Welch's t test*

With the results of the parasitemia and gametocytemia curves it is possible to affirm that the knockout clones for the gene PBANKA_1038800 had a normal multiplication during blood stages and also allowed the normal formation of circulating gametocytes (Figure 24).

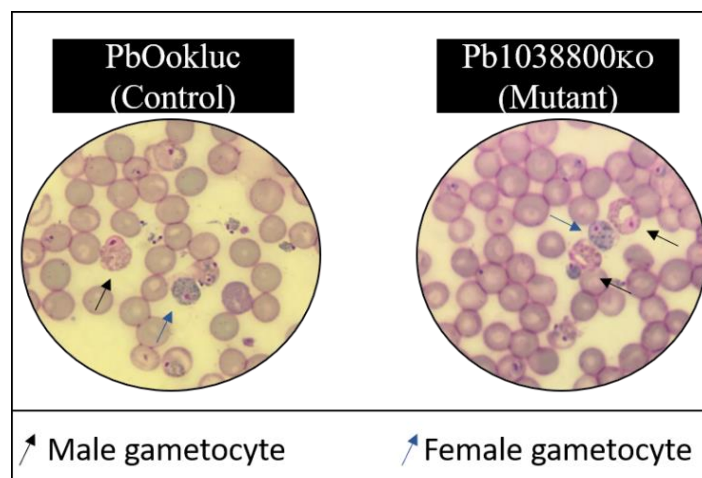


Figure 24 - Parasites found in blood smears of BALB/c mice infected with PbOokluc (control) and Pb1038800^{KO} (mutant), stained with fast panoptic. Male and female gametocytes (indicated with black arrow

and blue arrow, respectively) were found in both cases. Source: (author's compilation, 2018).

All the results indicate that the gene PBANKA_1038800 is not essential for asexual multiplication during blood stages. This was expected, as the mentioned gene was selected through a search strategy that identified genes highly transcribed specifically in mature gametocytes. Even with the knockout, mutant parasites were capable of multiplying and generate gametocytes, which are the form of the parasite ingested by the mosquito that can develop inside its midgut and complete the life cycle, that is to say, perpetuate the disease.

5.5 Exflagellation and *in vitro* fertilization assays

5.5.1 Conversion assay before cloning (Tp3)

After evaluating the importance of the gene during blood stages, it is essential to do the same with the parasite's stages in the mosquito. To estimate in which point of the parasite's life cycle the knockout of the gene PBANKA_1038800 was affecting, the exflagellation and conversion assays were performed, as it allows to verify viability of male gametocytes and parasite development from gametes fertilization to ookinete formation.

The first conversion assay (Figure 25) was performed before cloning. For that, blood from PbOokluc strain (control) and from Tp3 mice (transfer population, from transfection) was used to infect intraperitoneally two BALB/c mice per group. Four days after infection, gametocytemia of control and mutant was 0,6%. The assay was made, and after 24 hours of incubation at 21°C, the dilution was mixed with the lysis buffer and nLuc substrate. An incubation at 37°C was made, and the luciferase activity was determined by measuring the Relative Light Units (RLU), using a microplate luminometer reader (Figure 25).

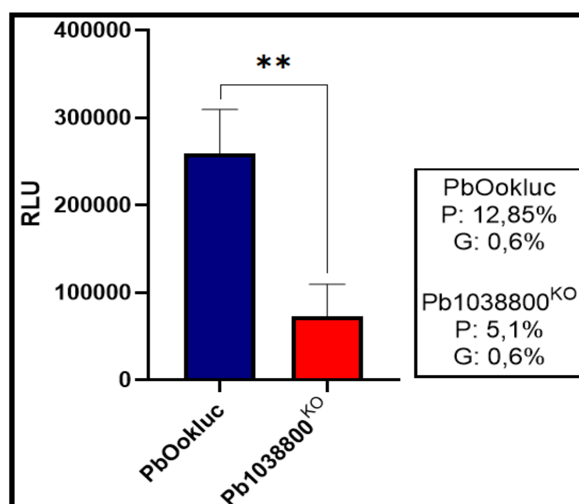


Figure 25 - First conversion assay done before cloning. This assay was made using PbOokluc as the control and blood from mice number 3 from the transfer population (Tp3) as the mutant (Pb1038800^{KO}). The results showed that there was a decrease in the formation of ookinetes, since the Relative Light Units (RLU) are equivalent to the amount of formed ookinetes. P value (**0,0084) indicates that there is a significant difference between the columns for control and mutant results. Source: (author's compilation, 2018).

P= Parasitemia; G= Gametocytemia RLU: Relative Light Units

Analysis made with *GraphPad Prism 8.02 – Welch's t test*

This assay, performed before cloning, already showed a reduction in ookinete formation, since the value of RLU, which are equivalent to the quantity of ookinetes formed, was lower in the mutant when compared to the control. After these positive results, cloning, genotyping (Figure 22) and growth curves (Figure 23) were made.

5.5.2 First conversion assay after cloning (Clone C1)

The first conversion assay performed after cloning was made using blood from control PbOokluc and mutant clone C1. Two BALB/c mice for each group were intraperitoneally infected. Four days after infection, gametocytemia of control was 0,8% and mutant was 0,5%. After 24 hours of incubation at 21°C, the dilution was mixed with the lysis buffer and nLuc substrate, the plate was incubated at 37°C and luciferase activity was determined (Figure 26).

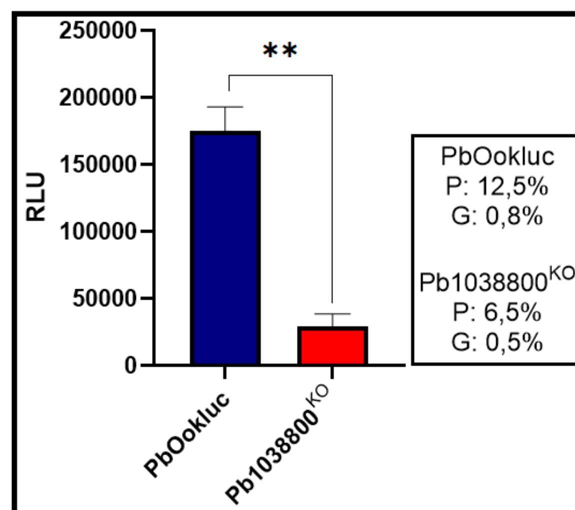


Figure 26 - Phenotype of mutant clone C1. First conversion assay done after cloning, using PbOokluc as the control and Pb1038800^{KO} (clone C1) as the mutant. The results corroborated with the first phenotype (before cloning), as there was a decrease in the formation of ookinetes, since the Relative Light Units (RLU) are equivalent to the amount of formed ookinetes. P value (**0,0011) indicates that there is a significant difference between the columns for control and mutant results. Source: (author's compilation, 2018).

P= Parasitemia; G= Gametocytemia RLU: Relative Light Units

Analysis made with *GraphPad Prism 8.02 – Welch's t test*

The results from the first conversion assay performed after cloning showed that the formation of ookinetes on clone C1 was impaired, because when compared to the control, where it is expected a maximum ookinetes formation after 24 hours of incubation at 21°C, the luciferase activity was low (decrease in the Relative Light Units), meaning that there were less ookinetes formed in the mutant group (infected with Pb1038800^{KO}) than in the control group (infected with PbOokluc).

5.5.3 Exflagellation assay

Phenotype verified after first conversion assay made with clone C1 (Figure 26) showed that the gene PBANKA_1038800 is important for the development of the parasites inside the mosquito. However, only with this assay it was not possible to determine exactly where the knockout was affecting parasite's development, if it was during gametes egress, zygote or ookinete formation.

Inside the midgut, gametocytes egress from the host erythrocyte, forming macrogametes (female) and microgametes (male). Male gametes bind to macrogametes and erythrocytes around, forming exflagellation centers (EC), which can be counted under the microscope and the quantities from the mutant group can be compared with the control group. The best condition to do this is where the gametocytemia of animals from both groups are very similar. The exflagellation assay objective is to verify male gametocyte viability, that is, if male gametocyte is able to egress from erythrocyte and exflagellate.

5.5.4 First exflagellation assay after cloning (Clone C1)

All exflagellation assays were performed after cloning. The first was made with blood from mice infected with PbOokluc (P= 9,57% and G= 0,60%) and with clone C1 of mutant Pb1038800^{KO} (P= 11,22% and G= 0,52%). Four microliters of blood were mixed with 16 µl of ookinete medium on top of a glass slide. After 15 minutes of incubation at 21°C, it was possible to observe around two ECs per 40X microscopy field in both groups (Figure 27).

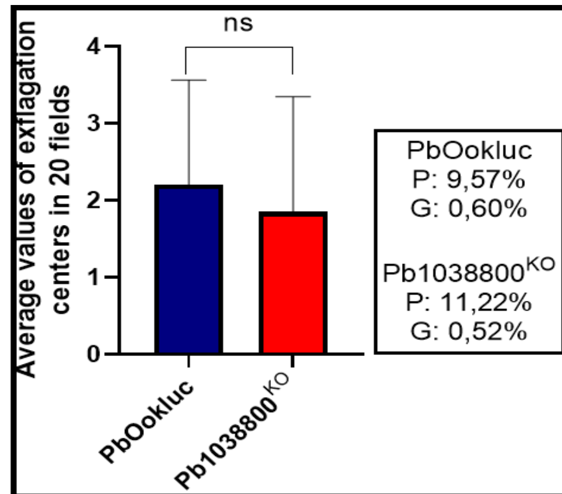


Figure 27 - Exflagellation assay using PbOokluc as the control and Pb1038800^{KO} (clone C1) as the mutant, both with similar gametocytemias. The average values of exflagellation centers were calculated in 20 fields. Mutant was capable to perform exflagellation normally. P value (0,4439) indicates that there is no significant difference between the columns for control and mutant results. This test was made before the fertilization assay comparing two times of incubation (6h and 24h). Source: (author's compilation, 2018).

P= Parasitemia; G= Gametocytemia Analysis made with *GraphPad Prism 8.02 – Welch's t test*

This test was made before the fertilization assay that compares two different times of incubation: 6h, compatible with time for zygote formation and 24h, time necessary for ookinete formation (Figure 28).

The results showed that there was no change in exflagellation pattern, that is, the knockout of PBANKA_1038800 gene did not affect the viability of male gametocytes. Therefore, the parasites were capable of activating and forming microgametes, which formed the exflagellation centers.

5.5.5 Conversion assay with 6h and 24h of incubation (Clone C1)

After exflagellation, conversion assay was performed using two times of incubation: 6h, to evaluate zygote formation and at 24h, to confirm ookinete reduction.

Blood from BALB/c mice infected with PbOokluc and clone C1 from Pb1038800^{KO} was used. Four days after infection, gametocytemia of control was 0,60% and mutant was 0,52%. After 6 hours and 24 hours of incubation at 21°C, the dilution was mixed with ookinete medium with the nLuc substrate, instead of lysis buffer, with the objective to observe if there were zygotes and ookinetes in the mutant group. The samples were incubated at 37°C for 10 minutes, and luciferase activity was determined (Figure 28).

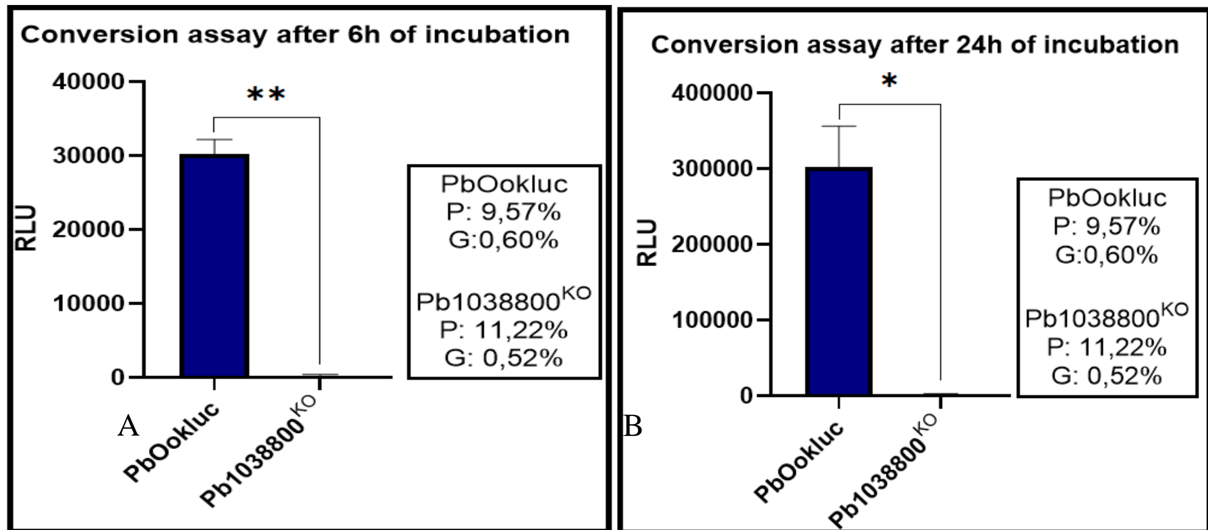


Figure 28 - Phenotyping done with two different times (6h and 24h) of incubation at 21°C. This assay was performed using PbOokluc as the control and Pb1038800^{KO} (clone C1) as the mutant. (A) Results indicates that there was a reduction in the RLU before 6 hours of incubation, which is the time necessary for zygote formation. P value (**0,0013) proves that there is a relevant difference between the groups (control and knockout) analyzed. (B) Results showed that there was a decrease in ookinetes formation in the mutant group (Pb1038800^{KO}) after 24h of incubation. P value (*0,0103) indicates that there is a significant difference between the columns for control and mutant results. Source: (author's compilation, 2018).

P= Parasitemia; G= Gametocytemia RLU: Relative Light Units

Analysis made with *GraphPad Prism 8.02 – Welch's t test*

After reading the plate, the mixture of blood and ookinete medium was collected from each sample and used to detect the presence of zygotes and ookinetes, to confirm the results obtained after the conversion assay. Both forms of the parasite were found in the blood of control group, but none in mutant group. These results suggests that with the knockout of the gene PBANKA_1038800, parasites were not capable of forming ookinetes and zygotes.

5.5.6 Evaluation of male gametocytes viability (Clone C1)

A new exflagellation assay was made with blood from mice infected with PbOokluc (P= 11,02% and G= 0,73%) and with clone C1 from Pb1038800^{KO} (P= 13,95% and G= 0,89%). Four microliters of blood were mixed with 16 µl of ookinete medium on top of a glass slide and covered with coverslip.

After 15 minutes of incubation at 21°C, it was possible to observe an average of 4,5 ECs per 40X microscopy field in both groups (Figure 29).

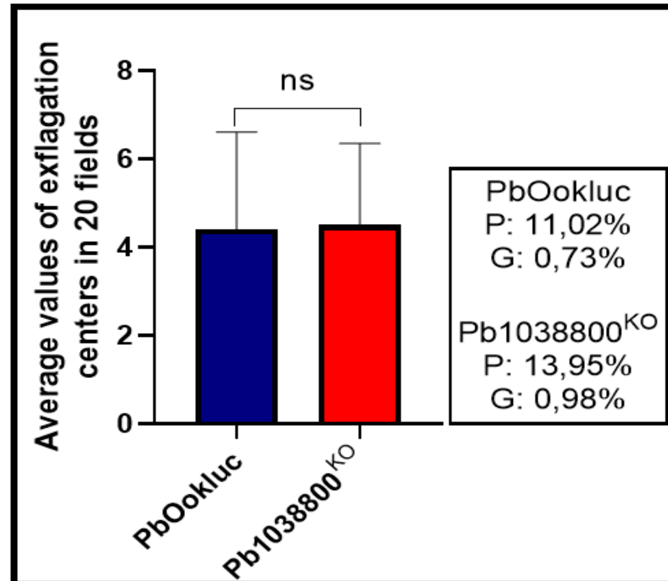


Figure 29 - Exflagellation assay, using PbOokluc as the control and Pb1038800^{KO} (clone C1) as the mutant, both with similar gametocytemias. The average values of exflagellation centers were counted in 20 fields. Results showed that the mutant was capable to perform exflagellation normally. P value (0,8775) indicates that there is no significant difference between the columns for control and mutant results. This test was made before the fertilization assay comparing five times of incubation (1h, 2h, 4h, 6h and 24h). Source: (author's compilation, 2019).

P= Parasitemia; G= Gametocytemia Analysis made with *GraphPad Prism 8.02 – Welch's t test*

Again, with similar gametocytemia of control and mutant, the results of this exflagellation assay, done before conversion assay comparing five times of incubation, showed that the knockout did not affected the viability of male gametocytes.

5.5.7 Conversion assay with 1h, 2h, 4h, 6h and 24h of incubation (Clone C1)

To test at which point before ookinete formation the knockout of gene PBANKA_1038800 affected, the conversion assay was performed in five different times of incubation, compatible with the first moments of gametes fertilization (1h, 2h), zygote formation and maturation (4h, 6h) and ookinete formation (24h).

BALB/c mice were infected with PbOokluc and Pb1038800^{KO} (clone C1). Gametocytemia of control was 0,73% and of the mutant was 0,98%, four days after infection. After each time of incubation at 21°C, the dilution was mixed with ookinete medium. Samples were incubated at 37°C for 10 minutes, and luciferase activity was determined (Figure 30).

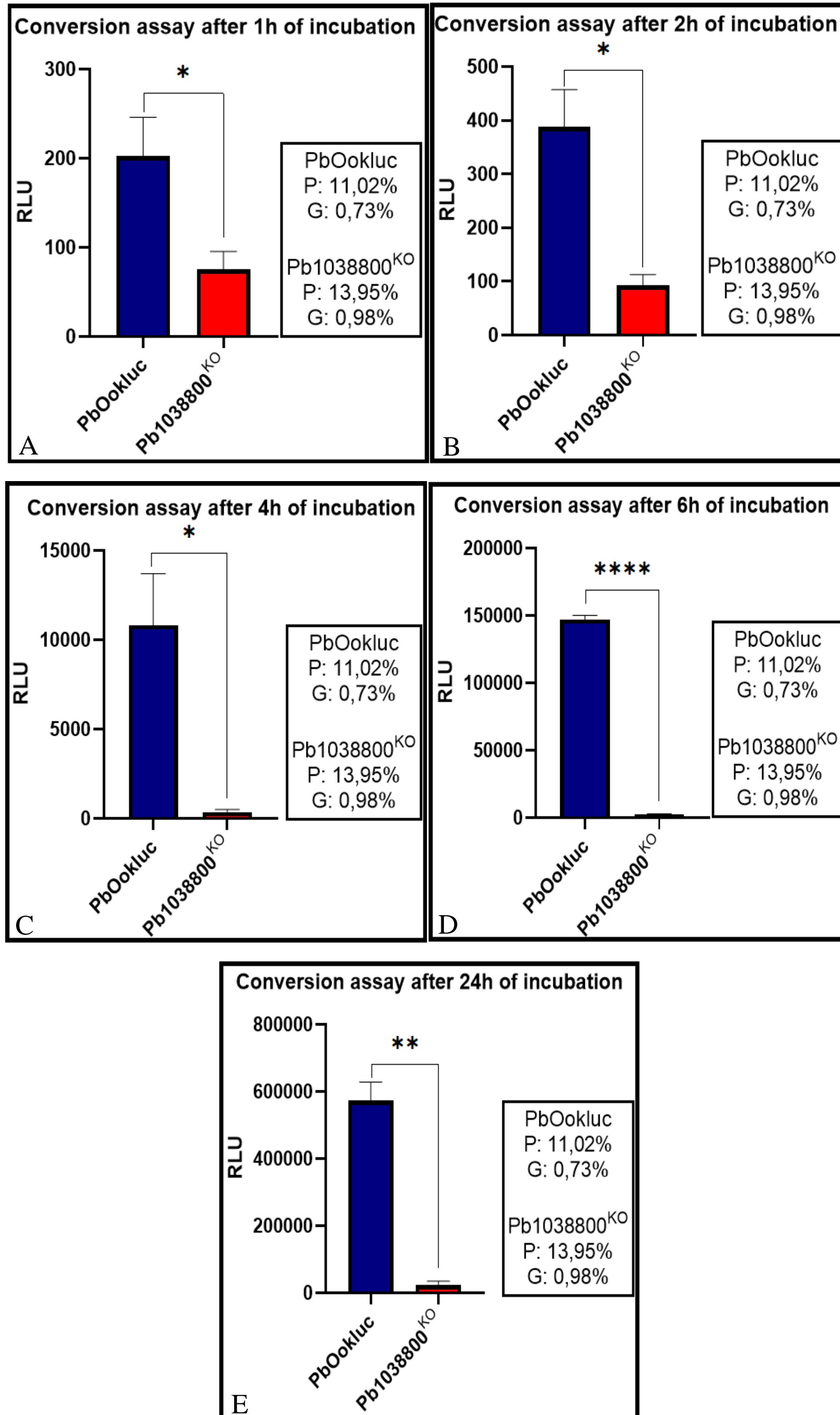


Figure 30 - Phenotyping done with five different times of incubation at 21°C. The test was performed using PbOokluc as the control and Pb1038800^{KO} (clone C1) as the mutant. (A) and (B) Since the first hours of incubation (corresponding to the gametes fertilization), luciferase activity was significantly reduced in the mutant. P value of (A) and (B) (*0,0226 and *0,0127, respectively) corroborates with this fact, since there is a difference between control and knockout results. (C) and (D) After 4 hours of incubation, zygote formation is occurring and after 6 hours, the zygote is mature. Results showed that knockout affected its formation, since there is a reduction in the luciferase activity. P value of (C) and (D) (*0,046 and ****<0,0001) indicates that there is a significant difference

between control and mutant results. (E) After 24 hours of incubation, there was an enormous decrease in ookinetes formation in the mutant group (Pb1038800^{KO}). P value (**0,0023) indicates that there is an important difference between the columns for control and mutant results. Source: (author's compilation, 2019).

P= Parasitemia; G= Gametocytemia RLU: Relative Light Units

Analysis made with *GraphPad Prism 8.02 – Welch's t test*

Phenotype of mutant clone C1 exhibited an interesting result. Luciferase activity was reduced in the mutant (Pb1038800^{KO}), when compared to the control (PbOokluc), since the first hour of conversion to the last (24h). After reading the plate, the mixture of blood and ookinete medium was collected from each sample and used to detect the presence of zygotes and ookinetes. Both forms of the parasite were found only in the blood of control group. It suggests that the gene PBANKA_1038800 is important for the parasite zygote formation, as the assay evidenced that gametes fertilization occurred less in the mutant than in control group. This fact indicates that the development of the parasite was impaired due to knockout of the mentioned gene since gametes fertilization.

5.5.8 Evaluation of male gametocytes viability (Clones C1, D1 and D2)

To confirm the results found in the assays made with clone C1, the last exflagellation and conversion assays were made with three clones: C1, D1 and D2. It was performed with blood from mice infected with PbOokluc (P: 4,92% and G: 0,40%), Pb1038800^{KO} clone C1 (P: 4,46% and G: 0,49%), Pb1038800^{KO} clone D1 (P: 4,02% and G: 0,77%) and Pb1038800^{KO} clone D2 (P: 4,38% and G: 0,41%). Blood was mixed with ookinete medium and after 15 minutes of incubation at 21°C, it was possible to count around 3 ECs per 40X microscopy field in all groups (Figure 31).

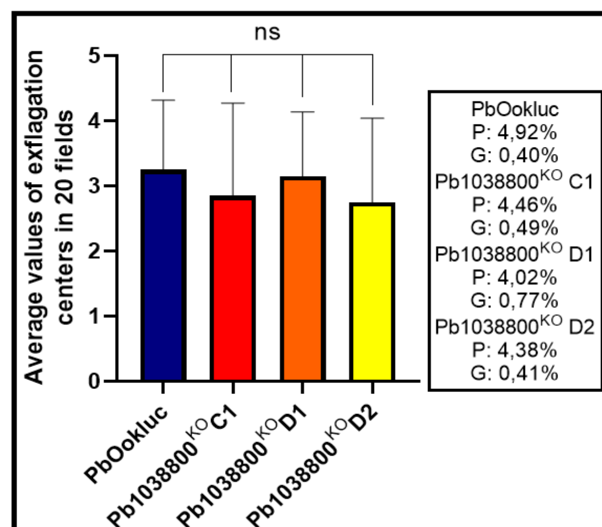


Figure 31 - Exflagellation assay, using PbOokluc as the control and Pb1038800^{KO} (clones C1, D1 and D2) as the mutant clones. The average values of exflagellation centers were counted in 20 fields. Results showed that all mutant clones were capable to perform exflagellation normally. P value (0,5188) from "Welch's ANOVA test" indicates that there is no significant difference between the results of all three mutants when comparing to the control. Comparing each mutant clone with control (Dunnett's T3 multiple comparisons test), P values are: 0,6263 (PbOokluc vs Pb1038800^{KO} C1); 0,9803 (PbOokluc vs Pb1038800^{KO} D1) and 0,4119 (PbOokluc vs Pb1038800^{KO} D2). This assay was performed before the phenotype comparing five times of incubation (1h, 2h, 4h, 6h and 24h). Source: (author's compilation, 2019).

P= Parasitemia; G= Gametocytemia

Analysis made with *GraphPad Prism 8.02 – Welch's ANOVA test and Dunnett's T3 multiple comparisons test*

These results corroborated with the previous assays, as it showed that the knockout did not affect exflagellation of male gametocyte, even in different clones.

With diverse repetitions of the exflagellation assay, and using three of the mutant clones, it is possible to conclude that the gene PBANKA_1038800 is not important for the parasite during this process, as the mutant can exflagellate as much as the control.

5.5.9 Conversion assay with 1h, 2h, 4h, 6h and 24h of incubation (Clones C1, D1 and D2)

Conversion assays were performed with clones C1, D1 and D2 and in five different times of incubation (1h, 2h, 4h, 6h and 24h). This test is important to validate the results in the mutant, regardless of which clone is tested.

To perform this assay, BALB/c mice were infected with PbOokluc and Pb1038800^{KO} (clones C1, D1 and D2). Four days after infection, gametocytemia was determined and in the control it was 0,40%, in clone C1 it was 0,49%, in clone D1 0,77% and in clone D2 0,41%. After each time of incubation at 21°C, the dilution was mixed with ookinete medium with nLuc substrate. Samples were incubated at 37°C for 10 minutes, and luciferase activity was determined (Figure 32).

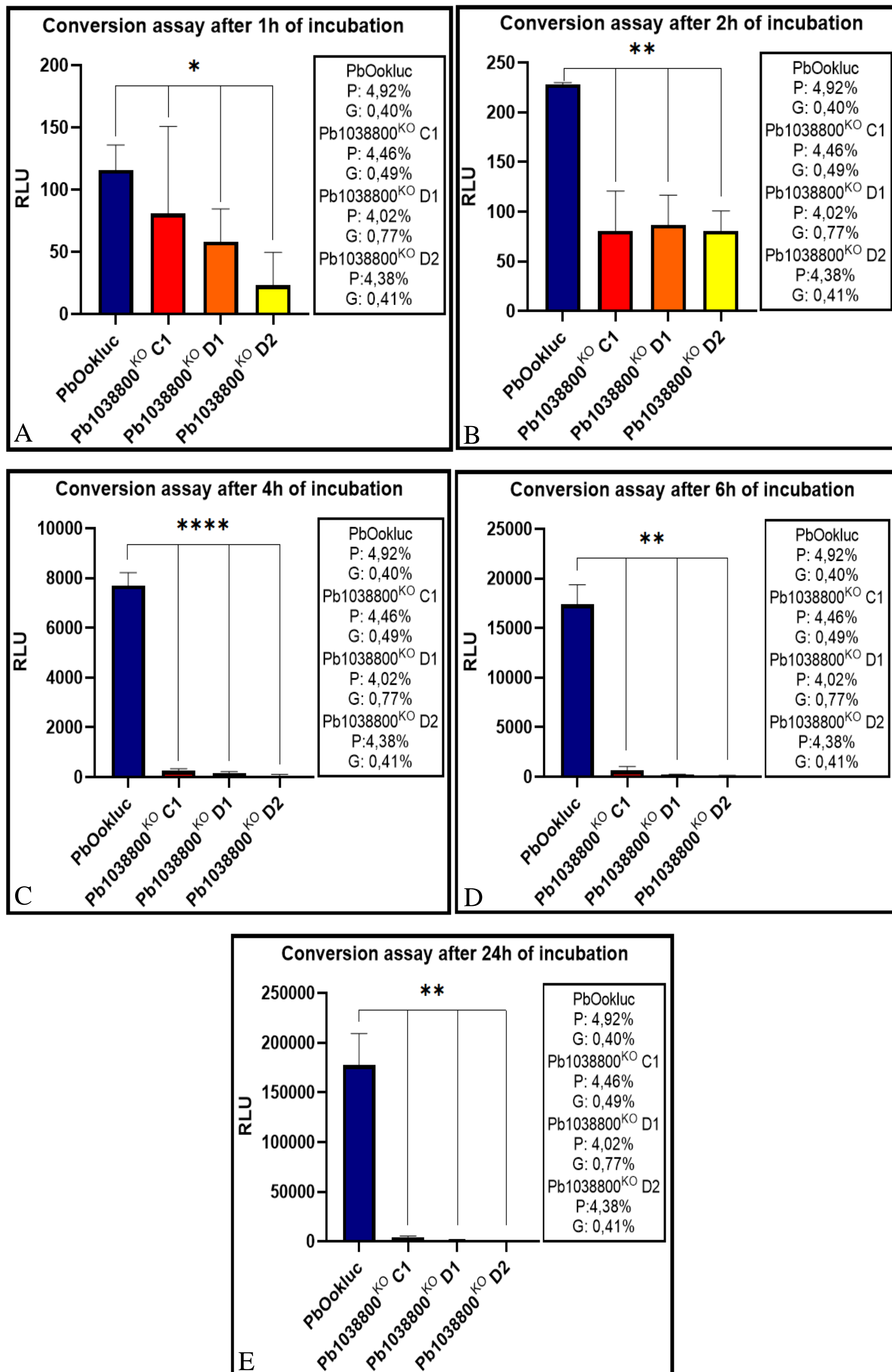


Figure 32 - Phenotyping of three mutant (Pb1038800^{KO}) clones C1, D1 and D2, incubated at 21°C during five different times (1h, 2h, 4h, 6h and 24h). (A) The first hour of incubation (corresponding to the gametes fertilization), showed a decrease in luciferase activity in the mutant. P value of (A) (*0,0477) corroborates with this fact, since there is a difference between control and knockout results. (B) Results of the assay with 2h of incubation showed a significant difference between the mutant clones and the control and P value was 0,0019. (C) and (D) Zygote is forming after 4 hours of incubation, and is mature after 6 hours. Knockout affected this parasite development step, since there is a reduction in the luciferase activity. P values of (C) and (D) (****<0,0001 and

0,0012) proves that there is a significant difference between control and mutant results. (E) 24 hours of incubation showed a decrease in ookinetes formation in all three mutant clones. P value (0,0060) indicates that there is an important difference between the columns for control and mutant results. Comparing each mutant clones with the control, P values were: PbOokluc vs Pb1038800^{KO} C1 (A) 0,7657, (B) 0,0448, (C) 0,0027, (D) 0,0070 and (E) 0,0212. PbOokluc vs Pb1038800^{KO} D1 (A) 0,0947, (B) 0,0277, (C) 0,0030, (D) 0,0086 and (E) 0,0209. PbOokluc vs Pb1038800^{KO} D2 (A) 0,0226, (B) 0,0113, (C) 0,0028, (D) 0,0086 and (E) 0,0206. Source: (author's compilation, 2019).

P= Parasitemia; G= Gametocytemia RLU: Relative Light Units

Analysis made with *GraphPad Prism 8.02 – Welch's ANOVA test and Dunnett's T3 multiple comparisons test*

The last conversion assay, performed with three different mutant clones (C1, D1 and D2) and in five times of incubation (1h, 2h, 4h, 6h and 24h), showed a decrease in the luciferase activity in all mutant clones since the first hour. Results from the other four next assays proved that the knockout of the gene PBANKA_1038800 affected zygote formation (after 4h and 6h of incubation) and ookinete formation (after 24h of incubation at 21°C).

6 DISCUSSION

Effective malaria control is based on interventions like indoor residual insecticidal spraying, use of bed nets treated with insecticides and access to efficient treatment (OUATTARA; LAURENS, 2015). A new malaria vaccine (RTS,S/AS01E) against pre-erythrocytic stage parasites is being implemented in endemic areas to serve as a complementary method for malaria control (COELHO et al., 2017). A novel and promising strategy is the development of transmission blocking vaccines (TBV), inducing antibodies capable of preventing parasite development inside the mosquito vectors by binding to surface proteins of gametocytes, zygotes or ookinetes. Inside vertebrate host, pre-fertilization antigens are expressed in gametocytes, but antibodies against them can only act after gametes egress from erythrocytes (process that occurs inside the mosquito), preventing zygote formation (GONÇALVES; HUNZIKER, 2016; SAUERWEIN; BOUSEMA, 2015). The list of antigens being proposed to compose a TBV is short, with only a few being clinically tested. For this reason, our main objective was to test a gene highly expressed in mature gametocytes in order to verify if it is involved in *Plasmodium* transmission.

The *Plasmodium* parasites are complex microorganisms capable of expressing thousands of different proteins according to its life stage. This fact difficult the development of an effective vaccine, as the production of diverse proteins challenges identification of promising antigens which can induce good protective immune response. However, the available *Plasmodium* genome sequence, transcriptome and proteome data bases, allied with bioinformatic tools, facilitated the search of potential vaccine candidates (DAVIES et al., 2015). As an example of the efficiency of this method, the search for upregulated genes encoding antigenic proteins in *Neisseria meningitidis* transcriptomes, led to the identification of vaccine candidates, which were posteriorly included in the human meningococcal vaccine (GRIFANTINI et al., 2002). In an *in silico* search strategy for new gene candidates playing a role in *Plasmodium* transmission, 12 potentially important genes for parasite's transmission were identified using a *P. falciparum* transcriptome database. One of the 12 genes found, PF3D7_1403200, has orthologs in *P. vivax* (PVX_086272) and in *P. berghei* (PBANKA_1038800). This is important, as we were searching for genes that were conserved between the species of *P. falciparum* and *P. vivax*, and we also used the murine model *P. berghei* in the experiments.

In this work we generated a PBANKA_1038800 knockout line in the *P. berghei* Ookluc background (CALIT et al., 2018). Comparing control and mutant growth curves, it was possible to conclude that the knockout was not affecting the development of the parasite during blood

stages, since asexual multiplication and gametocytogenesis were not affected. Exflagellation assays showed that the PBANKA_1038800 knockout did not affect male gametocyte viability. However, conversion assays showed that PBANKA_1038800 knockout affects normal ookinete formation *in vitro*, and further analysis pointed to a deficiency in fertilization.

The results obtained in this work point to the fact that the gene PBANKA_1038800 is important for the continuation of the parasite cycle within the mosquito, indicating that the protein obtained from this gene may be a good target to compose a malaria transmission blocking vaccine. *In silico* analysis indicated that this protein is probably located on the cell membrane, having a predicted EGF-like domain related to the extracellular portion of transmembrane proteins or in secreted proteins (LEBRUN; CARRUTHERS; CESBRON-DELAUW, 2014). EGF-like domain function may be related to interactions between ligand and receptor, indicating the putative importance of the protein in essential stages for the continuation of infection inside the vector, such as fertilization of gametes.

To move forward, it is important to evaluate which step of the sexual parasite life cycle the knockout is affecting. As we demonstrated that there is an important impact from the first two hours of incubation in the conversion assay (compatible with gamete fertilization), it is likely that the knockout of PANKA_1038800 has a negative effect on the fertilization. Since exflagellation assays showed that mutant microgametes formation, it is also possible that the gene may be important for female gametocyte activation, that is, for macrogametes to form and egress the host cell. Fertilization depends on interactions between proteins expressed on the surface of gametes to enable initial binding of male and female gametes (BENNINK; KIESOW; PRADEL, 2016). If the protein obtained from the gene PBANKA_1038800 is important during this process, the gene knockout could be impairing zygote formation. These hypotheses (role in female gametocyte activation or gamete fertilization) can explain the reduction on mutant zygote and ookinete formation observed in the conversion assays.

In order to test the hypothesis that the protein encoded by the PBANKA_1038800 gene is important for female gametocytes, protein localization in gametocytes will be important. To do that, a good strategy is to insert a tag in the C-terminal region of the protein. The main utilized markers for protein localization and studies of gene expression are the green fluorescent protein (GFP) and the hemagglutinin (HA) epitope. The generation of a mutant locus can be done with a single cross over strategy, where the targeting sequence is constructed, containing the homology region to the 3’ end of the PBANKA_1038800 gene, followed by the tag sequence (HA or GFP), a heterologous 3’ UTR and the selection cassette. Fluorescence (GFP) or immunofluorescence assays using specific antibodies (in this case against HA), can be done

to verify if the protein encoded by the PBANKA_1038800 is expressed specifically in female gametocytes (BARGIERI et al., 2016). In order to localize the protein properly, the gene function cannot be altered, so the tagged parasites should be able to develop from ookinetes.

In addition to localizing the protein, an analysis of the ability of specific antibodies produced against this protein to reduce fertilization will be important. The success of an effective TBV depends on the induction of high functional antibody titers capable of blocking parasite transmission from the human host to the mosquito vector, or antibodies which can impair parasite development inside the invertebrate host (PONNUDURAI et al., 1987).

Our results showing an essential role of PBANKA_1038800 for *P. berghei* fertilization and formation of zygotes warrants future studies to determine the transmission blocking potential of vaccines using this antigen.

7 CONCLUSION

With the results obtained, it is possible to affirm that the knockout of the gene PBANKA_1038800 do not have an impact on the development of the parasites during the blood stages, since the multiplication and formation of circulating gametocytes occurred in a similar way to the control, nor did it affect the viability of the male gametocytes. However, the gene is important for the parasite in some stage between gametes fertilization and development of ookinetes. This fact is endorsed by results found through the conversion assay, since the mutant was not able to convert into zygote and ookinete.

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APPENDIX A – SIGNAL PEPTIDES PREDICTION

The SignalP-5.0 server (http://www.cbs.dtu.dk/services/SignalP/) is a tool useful for prediction of signal peptides in proteins from Gram-positive bacteria, Gram-negative bacteria Archaea and Eukarya. This server can discriminate between three types of signal peptides: Sec/SPI (secretory signal peptides), Sec/SPII (lipoprotein signal peptides) and Tat/SPI (translocation signal peptides). Besides, if the result of the prediction is "Other", the probability indicates that the sequence does not have a signal peptide. The probability of the presence of a cleavage site (CS) is also evaluated.

Figure A-1 – PBANKA_1038800. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,0474/Protein type: Other - Likelihood: 0,9526. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

Figure A-2 – PBANKA_0902900. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,9975/Protein type: Other - Likelihood: 0,0025/Cleavage site between positions 21 and 22: SNA-EK – Likelihood: 0,9627. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

Figure A-3 – PBANKA_1342300. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,8248/Protein type: Other - Likelihood: 0,1752/Cleavage site between positions 23 and 24: IKC-LY – Likelihood: 0,4564. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

Figure A-4 – PBANKA_1429300. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,878/Protein type: Other - Likelihood: 0,122/Cleavage site between positions 24 and 25: VES-KV – Likelihood: 0,8654. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

Figure A-5 – PBANKA_1128800. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,9799/Protein type: Other - Likelihood: 0,0201/Cleavage site between positions 23 and 24: SNC-YE – Likelihood: 0,9501. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

Figure A-6 – PBANKA_0943400. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,8804/Protein type: Other - Likelihood: 0,1196/Cleavage site between positions 19 and 20: IIG-LK – Likelihood: 0,7689. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

Figure A-7 – PBANKA_0720900. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,941/Protein type: Other - Likelihood: 0,059/Cleavage site between positions 22 and 23: VLA-KI – Likelihood: 0,7049. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

Figure A-8 – PBANKA_0603300. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,6194/Protein type: Other - Likelihood: 0,3806/Cleavage site between positions 22 and 23: VDC-VT – Likelihood: 0,2926. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

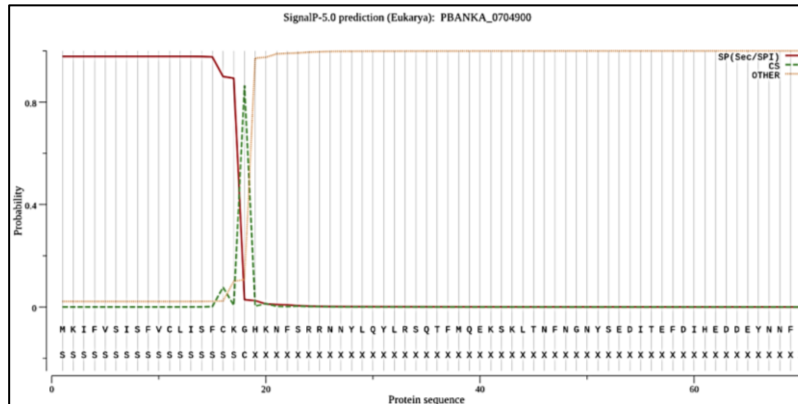

Figure A-9 – PBANKA_0704900. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,9781/Protein type: Other - Likelihood: 0,0219/Cleavage site between positions 18 and 19: CKG-HK – Likelihood: 0,8650. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

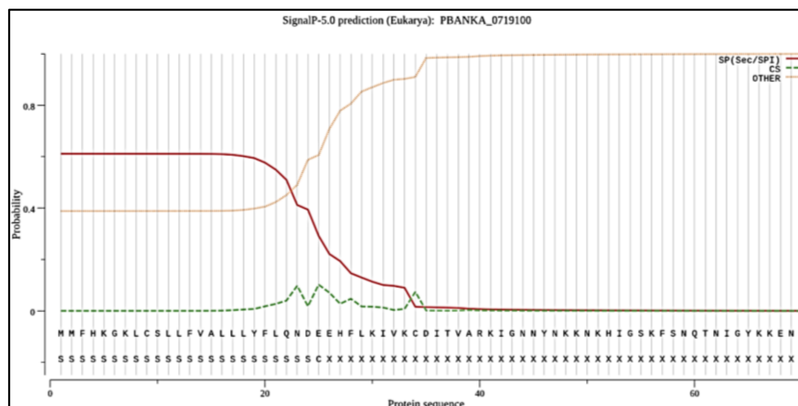

Figure A-10 – PBANKA_0719100. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,6121/Protein type: Other - Likelihood: 0,3879/Cleavage site between positions 25 and 26: NDE-EH – Likelihood: 0,1019. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

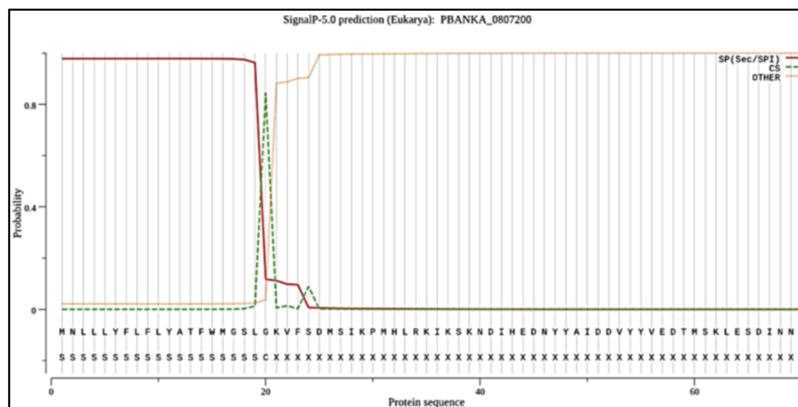

Figure A-11 – PBANKA_0807200. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,9783/Protein type: Other - Likelihood: 0,0217/Cleavage site between positions 20 and 21: SLG-KV – Likelihood: 0,8453. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

Figure A-12 – PBANKA_1459100. Protein type: Signal Peptide (Sec/SPI) - Likelihood: 0,069/Protein type: Other - Likelihood: 0,9931. Source: SignalP-5.0 Server (ARMENTEROS et al., 2019).

APPENDIX B – PREDICTION OF TRANSMEMBRANE HELICES IN PROTEINS

The TMHMM 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) is a program for prediction of transmembrane helices (TMH) in proteins. The program gives statistics and information related to the location of the predicted transmembrane helices and the predicted location of the intervening loop regions.

Exp number of AAs in TMHs: expected number of amino acids in transmembrane helices. Result > 18 = transmembrane protein/have a signal peptide.

Exp number, first 60 AAs: expected number of amino acids in transmembrane helices in the first 60 amino acids of the protein.

Total prob of N-in: total probability that the N-term is on the cytoplasmic side of the membrane.

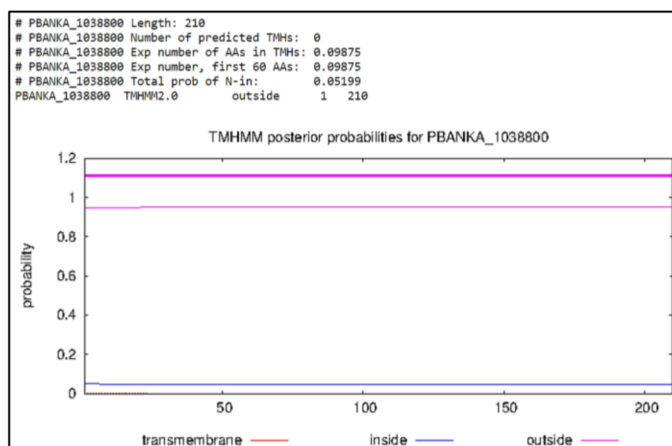

Figure B-1 – PBANKA_1038800. The protein does not have transmembrane helices. Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

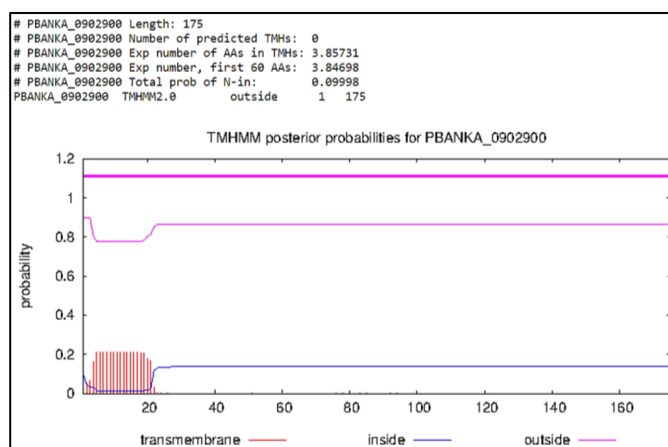

Figure B-2 – PBANKA_0902900. The protein does not have transmembrane helices. Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

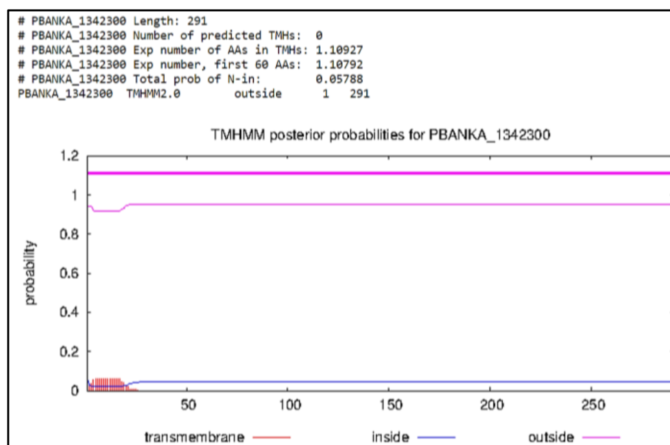

Figure B-3 – PBANKA_1342300. The protein does not have transmembrane helices. Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

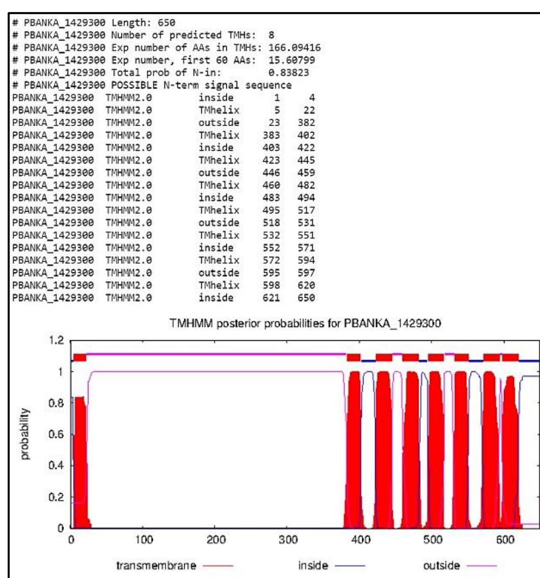

Figure B-4 – PBANKA_1429300. The prediction is that the protein have 8 transmembrane helices. High probability (0,83823) that the N-term is on the cytoplasmic side of the membrane Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

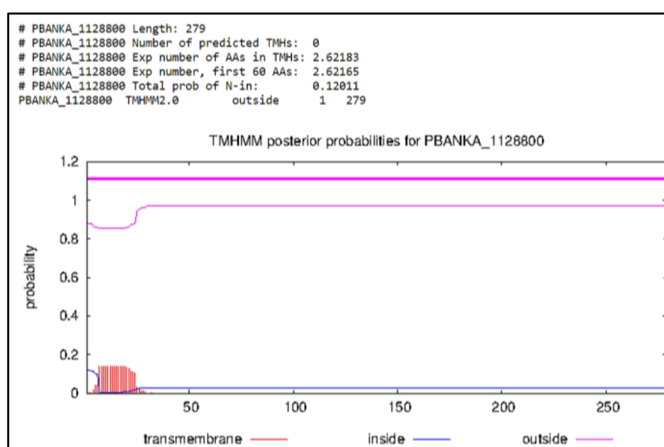

Figure B-5 – PBANKA_1128800. The protein does not have transmembrane helices. Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

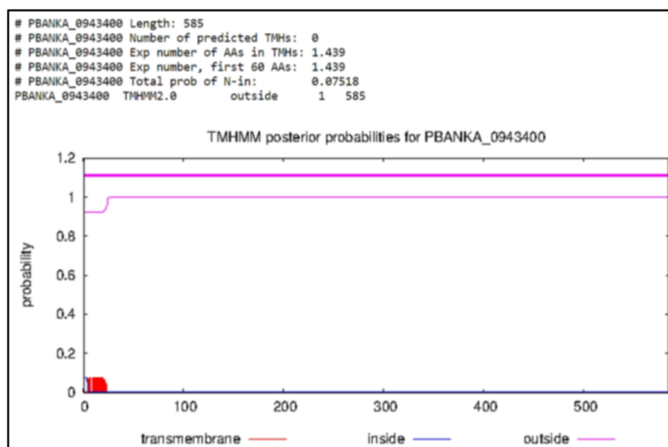

Figure B-6 – PBANKA_0943400 The protein does not have transmembrane helices. Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

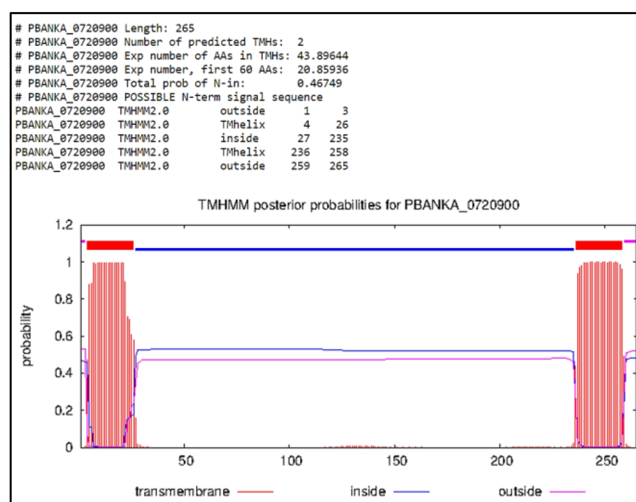

Figure B-7 – PBANKA_0709900. The prediction is that the protein have 2 transmembrane helices. High probability of 0,46749 that the N-term is on the cytoplasmic side of the membrane Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

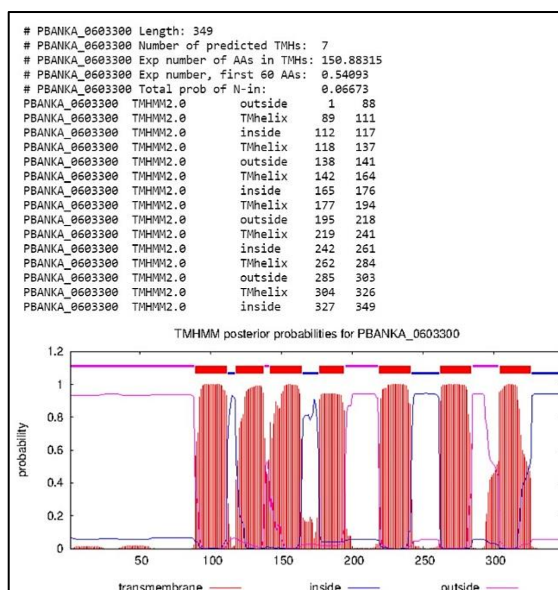

Figure B-8 – PBANKA_0603300. The prediction is that the protein have 8 transmembrane helices. High probability (0,83823) that the N-term is on the cytoplasmic side of the membrane Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

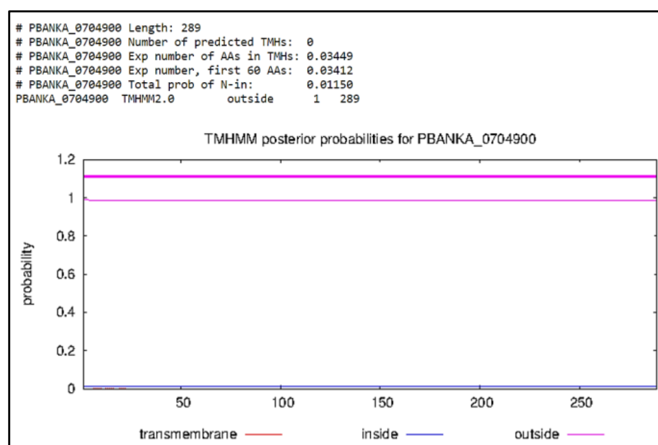

Figure B-9 – PBANKA_0704900. The protein does not have transmembrane helices. Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

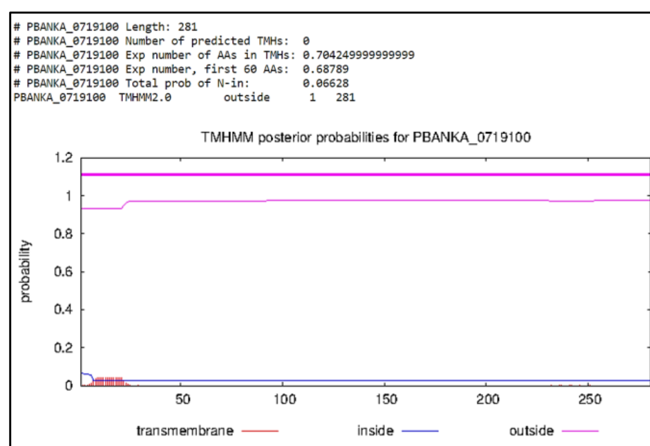

Figure B-10 – PBANKA_0719100. The protein does not have transmembrane helices. Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

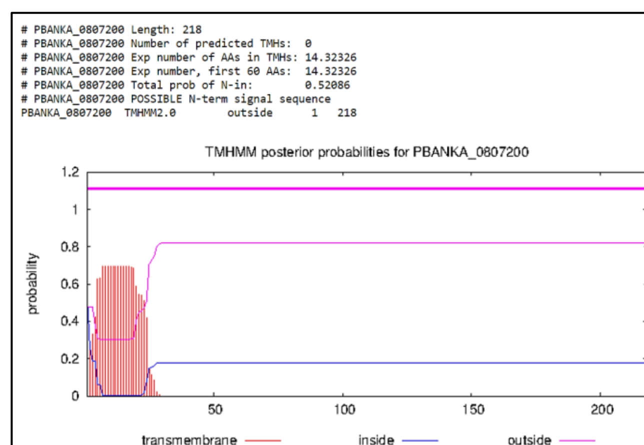

Figure B-11 – PBANKA_0807200. The protein does not have transmembrane helices. Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).

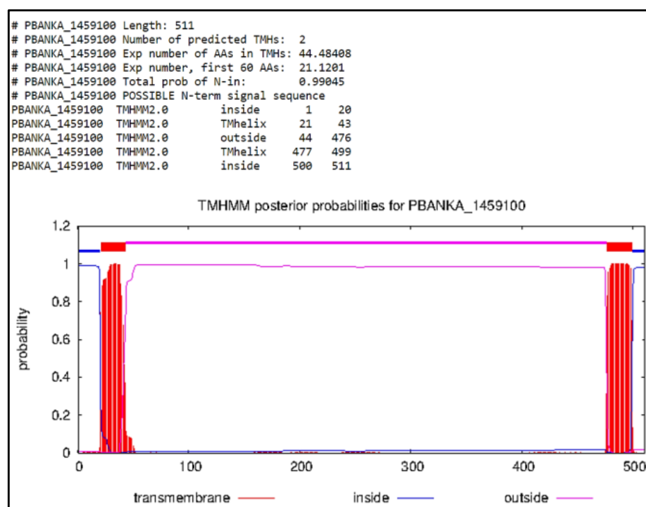

Figure B-12 – PBANKA_1459100. The prediction is that the protein have 2 transmembrane helices. High probability (0,99045) that the N-term is on the cytoplasmic side of the membrane Source: TMHMM 2.0 (MÖLLER; CRONING; APWEILER, 2002).