THÈSE

Pour obtenir le grade de

Docteur

Délivré par **UNIVERSITE MONTPELLIER 2**

Préparée au sein de l'école doctorale **Sciences Chimiques et Biologiques pour la Santé**

Et de l'unité de recherche **MIVEGEC (Maladies Infectieuses et Vecteurs : Ecologie, Génétique,**

Spécialité : **Entomologie / Parasitologie**

Présentée par **Alexandra Marie**

Identification et validation de nouveaux bio-marqueurs immuno-épidémiologiques pour évaluer l'exposition humaine aux piqûres d'Anophèles, vecteurs de paludisme

Soutenue le 4/04/2014 devant le jury composé de

Mme Valérie Choumet, Chargé de Recherche, Institut Pasteur	Rapporteur
Mr Lionel Almeras, Chargé de Recherche, Aix Marseille Université	Rapporteur
Mme Sylvie Manguin, Directeur de Recherche, IRD	Examinateur
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Mr Remoue Franck, Chargé de Recherche, IRD	Invité
Mme Dorothée Missé, Chargé de Recherche, IRD	Directrice de thèse
Mr Bruno Arcà, Professeur associé, Sapienza Università di Roma	Co-directeur de thèse

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List of Publications

Published

Drame P.M., Poinsignon A., **Marie A**., Noukpo H., Doucoure S., Cornelie S. and Remoue F.: New Salivary Biomarkers of Human Exposure to Malaria Vector Bites. Chapter 23 in Anopheles mosquitoes - New insights into malaria vectors. *Intechbook* 2013.

Marie A., Boissière A., Tchioffo M.T., Awono-Ambéné P.H., Morlais I., Remoue F., Cornelie S.: Evaluation of a real-time quantitative PCR to measure the wild *Plasmodium falciparum* infectivity rate in salivary glands of *Anopheles gambiae*. *Malaria Journal* 2013; 12:224.

Boissière A, Tchioffo MT, Bachar D, Abate L, **Marie A**, Nsango SE, Shahbazkia HR, Awono-Ambene PH, Levashina EA, Christen R, Morlais I. Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *Plos Pathogen* 2012; 8(5):e1002742.

Submitted

Doucoure S., Cornelie S., Drame P.M., **Marie A.**, Elanga Ndille E., Mathieu-Daudé F., Mouchet F., Poinsignon A., Remoue F. Biomarker of Vector Bites : Arthropod Immunogenic Salivary Proteins in Vector-Borne Diseases Control in **Biomarkers in Disease: Methods, Discoveries and Applications. General Methods and their Applications. *Springer*.**

In preparation

Marie A., Hollzmuller P., Rossignol M., Demettre E., Seveno M., Corbel V., Chandre F., Tchioffo M.T., Morlais I., Remoue F. and Cornelie S. Differential expression of salivary proteins between wild *Plasmodium falciparum*-infected and non-infected salivary glands of *Anopheles gambiae*: Toward the identification of biomarkers of infective bites.

Marie A., Roncà R., Poinsignon A., Drame P.M., Cornelie S., Besnard P., Le Mire J., Fortes F., Carnevale P., Remoue F., Arca B. Human Ab response to *Anopheles* cE5 salivary protein: a biomarker of children exposure to *Anopheles gambiae* bites.

Marie A., Drame P.M., Poinsignon A., Noukpo H., Doucoure S., Cornelie S. and Remoue F. Immunoepidemiology for the evaluation of exposure to malaria vector. Chapter in Encyclopedia of Malaria.

Scientific Communications

Oral Communications

Marie A., Poinsignon A., Hollzmuller P., et al.: Development of a biomarker of exposure to *Anopheles* bites: From concept to field applications - Towards of immuno-epidemiological biomarkers for evaluating the human exposure to infective *Anopheles* bites. In: *EVIMalaR Cluster Meeting*. Sète, France. 29-31 October 2013.

Marie A., Hollzmuller P., Rossignol M., et al.: Towards immuno-epidemiological biomarkers for evaluating the human exposure to infective *Anopheles* bites (Symposium: EVIMalaR research highlights in vector and systems biology). In: *6th Pan-African Malaria Conference*. Durban, South Africa. 6-11 October 2013.

Marie A., Cornelie S., Rossignol M., et al.: What modifications in salivary glands are induced by parasites? Differential proteomic study of *Anopheles gambiae* salivary glands uninfected versus infected by wild *Plasmodium falciparum* (Oral section: Vector pathogen interactions). In: *18e conference of the European Society of Vector Ecology.* Montpellier, France. 8-11 October 2012.

Marie A., Cornelie S., Rossignol M., et al.: Differential proteomic study of *Plasmodium falciparum*-infected and non-infected salivary glands of *Anopheles gambiae*, to a biomarker of infective bites (Oral section: Physiology of medical insects). In: *XXIV International Congress of Entomology*. Daegu, Korea. 19-25 August 2012.

Poinsignon A., Drame P.M., **Marie A.** et al.: Vector salivary antigens as epidemiological markers (Symposium: Salivary proteins). In: *XXIV International Congress of Entomology*, Daegu, Korea. 19-25 August 2012.

Poster

Marie A., Ronca R., Drame P.M., et al.: Human antibody response to Anopheles salivary cE5 protein: A serological biomarker of exposure to Anopheles gambiae bites in children (<14 years old) from Angola. In: *Annual General Assembly of MIVEGEC Unit*. Montpellier, France. 12 July 2013.

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Marie A., Cornelie S., Rossignol M., et al.: Differential proteomic study of *Plasmodium falciparum*-infected and non-infected salivary glands of *Anopheles gambiae*, what consequences for the malaria transmission? In: *8th Annual BioMalPar/EVIMalaR Conference*. Heidelberg, Germany. 14-16 May 2012.

Marie A., Cornelie S, Rossignol M., et al: Differential proteomic study of *Plasmodium falciparum*-infected and non-infected salivary glands of *Anopheles gambiae*, to a biomarker of infective bites. In: *Annual Meeting of the French Society for Immunology*. Montpellier, France. 8-10 November 2011.

INTRODUCTION

I. Malaria

Malaria is a vector-borne disease due to a protozoan belonging to the genus *Plasmodium* transmitted by a bite of a female *Anopheles* genus mosquito. This parasite was discovered in the blood of a patient by Alphonse Laveran in 1880 in Algeria. In 1897, Ronald Ross discovered oocysts in the stomachs of female *Anopheles* mosquitoes, suggesting their implication in parasite transmission.

However, this parasite, discovered only in 19th century, has existed for a long time. Several writings report fevers, referring to what was certainly malaria, in China about 2700 BC, in Mesopotamia from 2000 BC, and in Egypt as early as 1570 BC (Cox 2010). Hawass et al, Egyptian and American scientists, demonstrated by DNA analysis that Tutankhamun was infected with malaria when he died (Hawass et al. 2010).

In 2010, the World Health Organization (WHO) reported 219 million malaria cases and 660,000 deaths; 90% of cases are due to *P. falciparum*, the most dangerous parasite responsible for the disease, and 86% of deaths occurred in children. The majority of cases (80%) and deaths (91%) are localized in sub-Saharan Africa (WHO 2012). However, most tropical and subtropical countries are affected by malaria. Nowadays, malaria is endemic in 99 countries, 67 of which are controlling malaria and 32 are following an elimination strategy (Feachem et al. 2010).

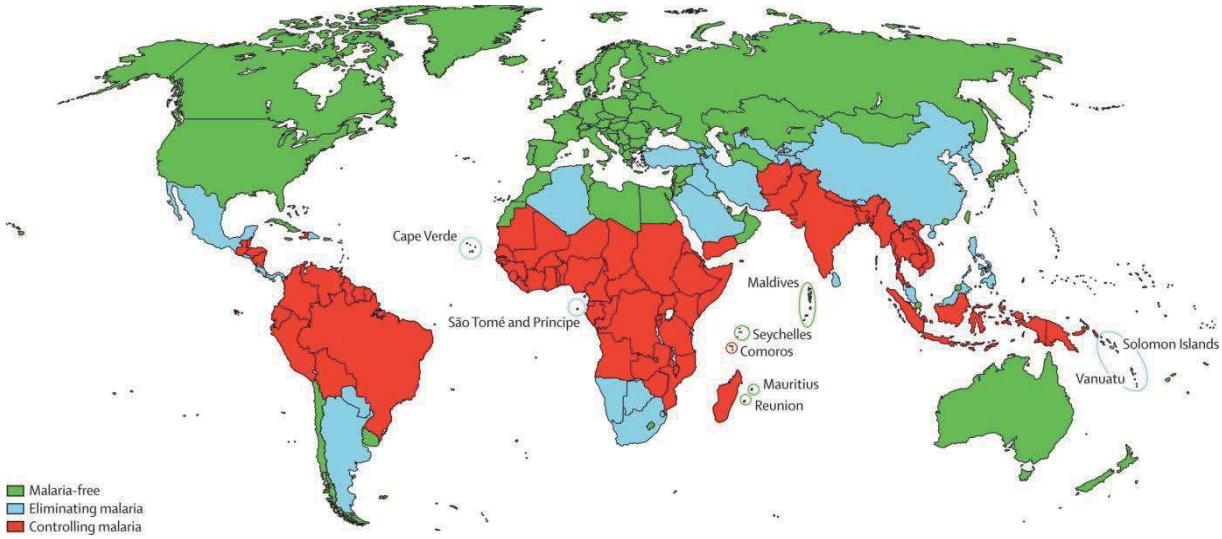

Figure 1. Categorization of countries as malaria-free, eliminating malaria or controlling malaria, 2010. (Feachem et al, 2010).

II. The Plasmodia

The *Plasmodium* is a protozoan parasite belonging to the phylum Apicomplexa, order Haemosporidia, family Plasmodiidae and genus *Plasmodium*. There are more than 100 species of *Plasmodium*, which can infect many animals such as reptiles, birds, and various mammals. However, only five species are known to infect humans:

- *Plasmodium falciparum* (Welch 1898), described by Celli and Marchiafava in 1889–1890, is the most virulent species causing the severe form of malaria and possibly death. It is largely present in sub-Saharan Africa, Asia, and South America (Manguin et al. 2008). In Africa, this species is predominant and causes 95% of malaria cases (WHO 2012).

- *Plasmodium malariae*, discovered by Laveran in 1880 and described by Golgi in 1886, is the only malaria parasite that has a quartan cycle (three-day cycle). Its distribution coincides with that of *P. falciparum*, and in Africa mixed infections with these two species are observed (Collins and Jeffery 2007). However, this species represents only 2–3% of malaria infections. One characteristic is that the parasite can cause long-lasting chronic infection if untreated (Igweh 2012).

- *Plasmodium vivax*, described by Golgi in 1886, is the most frequent and widely distributed cause of recurring (Benign tertian) malaria due to its dormant liver stages (hypnozoites). It is particularly present in Asia, Latin America, and in some parts of Africa (Igweh 2012).

- *Plasmodium ovale*, discovered by Stephens in 1922, is not frequent and is essentially found in West Africa and in the islands of the western Pacific (Manguin et al. 2008).

- *Plasmodium knolewsi* is typically found in Southeast Asia in macaques and has recently been recognized as the fifth *Plasmodium* species to cause malaria in humans (Singh et al. 2004, Subbarao 2011).

1. *Plasmodium falciparum* life cycle

The life cycle of *P. falciparum* is complex and needs two strict hosts: the human, the intermediate host, where asexual reproduction of the parasite takes place, and the female *Anopheles* mosquito, the definitive host, where sexual reproduction occurs (Figure 2).

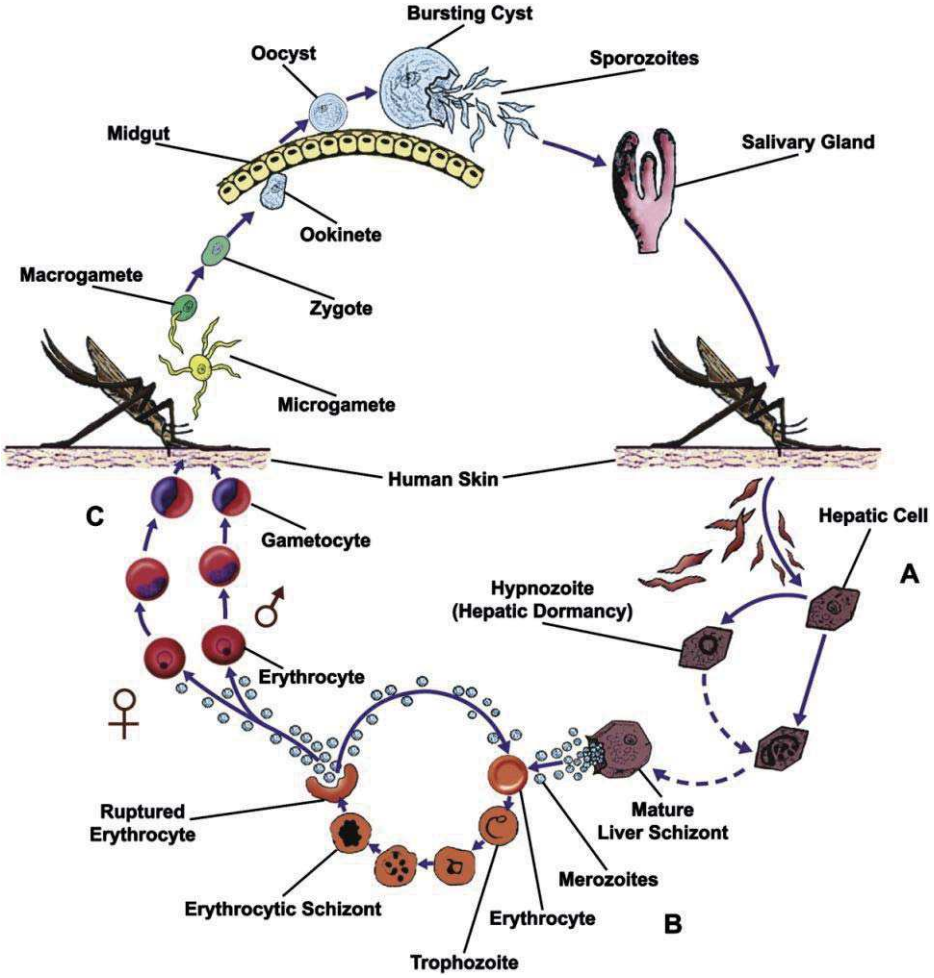

Figure 2. Life cycle of *Plasmodium falciparum*. (Lopez et al, 2010).

The cycle starts with the bite of a female *Anopheles* mosquito infected by *P. falciparum*, which transmits the parasite to humans during a blood meal. Sporozoites are injected into the skin (Sinnis and Zavala 2008) and migrate to the liver. Some of the sporozoites go to the draining lymph nodes where they are degraded by the immune system (Amino et al. 2006). The rest invade the hepatic cells and the parasite undergoes intense asexual reproduction to mature into schizonts. Then schizonts rupture and merozoites are released in the bloodstream (Sturm et al. 2006). These steps belong to the exo-erythrocytic cycle (Figure 2.A). After this initial replication in the liver, the parasites undergo asexual replication into erythrocytes (erythrocytic cycle (Figure 2.B)). Merozoites infect red blood cells and transform into

trophozoites (ring stage). Mature trophozoites divide asexually to produce schizonts, which rupture, releasing merozoites that infect healthy red blood cells. Some trophozoites differentiate into female and male gametocytes in the sexual erythrocytic stage (Talman et al. 2004). These gametocytes are ingested during a bite by a mosquito in which they finish the sexual cycle. Asexual forms ingested during the blood meal are digested in the *Anopheles* midgut. The sporogonic cycle begins in the midgut where the male gametocyte undergoes a process of exflagellation, becoming a mobile exflagellated microgametocyte (up to eight flagella). At the same time, female gametocytes differentiate into one macrogametocyte. The development of gametocytes is initiated by environments signals of the midgut such as a drop in temperature (Billker et al. 1997). Fertilization leads to the formation of a zygote 1 h after the blood meal. Rapidly, the zygote transforms into a motile ookinete able to disrupt and cross the peritrophic matrix and the midgut epithelial cells (Vlachou et al. 2004). Between the intestinal epithelium and the basal membrane of the midgut, the ookinete differentiates into an oocyst. After maturation, oocysts burst releasing thousand haploid sporozoites in the hemolymph. Some of them are killed by the immune system and the others (10–20%) are passively carried to the salivary glands (Hillyer et al. 2007). The invasion of salivary glands by the sporozoites is mediated via receptor–ligand interactions (Sultan et al. 1997). Then sporozoites invade epithelial cells with the formation of a transient vacuole and mature in the secretory cavity. The majority of sporozoites remain in these cavities and only a few enter the salivary duct (Frischknecht et al. 2004) and will be transmitted to a new human host during a blood meal. On average, 100 sporozoites are injected by a single infected bite (Medica and Sinnis 2005).

2. Human pathogenesis induced by *P. falciparum*

2.1 Symptomatology

The five species of *Plasmodium* cause variable symptoms, but *P. falciparum* is by far the most dangerous, accounting for the majority of deaths and giving rise to a broad range of pathological manifestations. Only the erythrocytic stage causes symptoms due to the bursting of red blood cells.

The malaria symptomatology also depends on genetic factors (Kwiatkowski 1999) and on the acquired immunity of the infected human. Indeed, malaria mortality tends to be low in areas where transmission is both high and year-round (Trape and Zoulani 1987), and the proportion of severe cases is often high where transmission is unstable and during epidemics. Moreover, the susceptibility of the human host to *P. falciparum* declines with age. In malaria-endemic areas, severe forms are rare after 5 years of age, and from 10 to 15 years of age, the simple forms become increasingly benign. This phenomenon seems to be explained by a progressive acquisition of immunity that is short-lived and partial, referred to "premunition" (Sergent 1950). This immunity reflects the balance between host and parasite and can disappear in 12–24 months in people who leave the endemic area (Deloron and Chougnet 1992). Premunition is maintained by antigenic stimulations due to repeated malaria infections and to exposure to *Anopheles* infective bites (Struik and Riley 2004). The development of this "premunition" immunity is dependent on the epidemiological context, the seasonal, and the intensity of the malaria transmission at the level of the geographical area. In some dry regions with unstable malaria, transmission varies from season to season and year to year, delaying the acquisition of this immunity. This situation is encountered in most of sub-Saharan Africa. However, this immunity enables asymptomatic carriage constituting a parasite reserve that can infect mosquitoes and perpetuate malaria transmission.

Uncomplicated malaria and classic symptoms

The initial manifestations of the disease correspond to the first cycles of erythrocytic development of the parasite. In nonimmune individuals, symptoms appear about 11 days after the infective bite, but this timing can fluctuate between 9 and 30 days. Symptomatology is similar to flu-like symptoms such as fever at 39–40°C, headaches, shivering, sweating, muscle soreness, vomiting, and sometimes gastrointestinal troubles. The classical malaria paroxysm presents three stages: a cold stage, followed by a hot stage with a terminal sweating stage. Fever may be intermittent, every 2 days or continuous. Hepatomegaly and splenomegaly can be observed as well (Bartoloni and Zammarchi 2012). These manifestations are rare in children less than 5 months of age (Bruce-Chwatt 1952), who present parasitemia without clinical symptoms (Kitua et al. 1996). Although the subject may not appear seriously ill, grave complications may develop at any stage. In nonimmune people, children and immune-depressed subjects, *P. falciparum* malaria may progress very rapidly to severe malaria unless appropriate treatment is started, possibly leading to death.

Severe malaria

The complications involve the central nervous system (cerebral malaria), pulmonary system (respiratory failure), renal system (acute renal failure), and/or hematopoietic system (severe anemia).

Cerebral malaria is the leading cause of nontraumatic encephalopathy in the world (Newton et al. 2000). Cerebral malaria includes a high risk of sequelae (epilepsy, language disorders, motor deficit, etc.), especially in children, and elevated mortality ranging from 10 to 30% (Brewster et al. 1990). Cerebral malaria is characterized by a progressive loss of consciousness leading to coma. Without treatment, death can occur within 72 h. Coma appears with consciousness impairment, convulsions, decerebrate rigidity, and opisthotonus (Bartoloni and Zammarchi 2012). Visceral and systemic manifestations can be associated. Hypoglycemia, anemia, pulmonary edema, hepatomegaly, splenomegaly, and renal failure can also be observed.

In hyperendemic regions, where the number of infective bites per human per year is more than 1000, severe anemia is more frequent than cerebral malaria. Anemia is caused by the bursting of infected erythrocytes and a decrease in their production in the bone marrow (Pradhan 2009). An unsuitable immune response could play a role in the occurrence of severe malaria anemia. Cytokines play a role in erythropoiesis and some evidence suggests that cytokines and other mediators of inflammation are implicated in deficient erythropoietin production (Haldar and Mohandas 2009). Moreover, co-infections and nutrient deficiency could exacerbate anemia (Ekvall 2003, Haldar and Mohandas 2009). The treatment against anemia used is blood transfusion, which carries a risk of HIV transmission, particularly in endemic areas (Ekvall 2003).

2.2 Diagnosis

Malaria diagnosis is based on the direct observation of the parasite in human blood. The gold standard methods are the thick blood and thin blood smear (Wongsrichanalai et al. 2007). These methods are labor-intensive and require skilled staff and dedicated equipment. Both tests quantify and identify the *Plasmodium* species, but the best species determination is obtained with the thin blood smear method. However, the thick blood smear method has sensitivity 20–30 times higher than the thin blood smear method and is widely used in the field to evaluate prevalence, parasite density, and the gametocyte index.

Indirect diagnosis is also achieved by serology testing. Rapid Diagnostic Tests (RDTs) detect malaria antigens in a small amount of blood, usually 5–15 μL. The RDT is based on the immunochromatographic assay with monoclonal antibodies directed against the target parasite antigen and impregnated on a test strip (Murray et al. 2008). The test is easy to use, inexpensive, requires no specific skills, and the result is rapidly obtained (about 20 min) and easy to interpret. Generally speaking, the *P. falciparum* diagnostic tests have at least 95% sensitivity. This depends on the antigen and the batch used, which can be damaged by extreme temperature and humidity. The most common RDTs are specific to *P. falciparum* antigens (histidine-rich protein 2 (HRP2) and the lactate dehydrogenase enzyme (LDH)), but a test is available that allows the specific detection of *P. vivax* antigens (LDH). Another RDT based on a conserved sequence of the LDH in all human malaria species can distinguish *P. falciparum* malaria from the other species (Pan/pf/pv). The specific detection of *P. ovale* and *P. malariae* antigens is not sufficiently optimized at this time (with lower sensitivity) (Wongsrichanalai et al. 2007, Murray et al. 2008).

Clinical diagnosis can also be used alone but has poor specificity due to the unspecific symptoms common to other infections (Rogier et al. 2001), which could result in unsuitable treatment and participate in the increase in drug resistance.

Molecular tools have also been developed for identification of all *Plasmodium* species, such as PCR and quantitative PCR. However, these techniques are expensive and are not suitable for field use.

3. Parasite control strategies

3.1 Drug therapy / treatment

The fight against the parasite is based on drug therapies and improvement of medical care access. Since 2001, WHO has recommended chemotherapy based on the combination of existing drugs (e.g., amodiaquine, mefloquine, sulfadoxine, pyrimethamine) with artemisin derivatives (artemisin-based combination therapies or ACTs), for the treatment of uncomplicated *P. falciparum* malaria (WHO 2010). These ACTs act rapidly, have a short life, and are effective against *P. falciparum* multi-chemoresistant. An additional advantage from a public health perspective is that artemisinin derivatives reduce gametocyte carriage and thus

the potential transmission of malaria (Okell et al. 2008). This contributes to malaria control, particularly in areas of low-to-moderate endemicity. However, ACT failures have been highlighted in areas along the Cambodia-(Myanmar)-Thailand border (Wongsrichanalai and Meshnick 2008, Na-Bangchang et al. 2010, Na-Bangchang and Karbwang 2013) and clinical resistance to artemisin, the major efficient component of ACTs, have been demonstrated (Noedl et al. 2008, Noedl et al. 2010). This could be due to the use of unregulated artemisin monotherapy since 1970, increasing drug pressure. It is also recommended to use artemisin or an artemisin derivative with another compound to decrease resistance selection (Dondorp et al. 2010). To prevent the spread of the artemisin resistance, a program was launched in Cambodia and Thailand (Samarasekera 2009). This program uses a multifaceted approach, including early diagnosis and appropriate treatment of malaria, decreasing drug pressure, optimizing insect vector control, targeting the mobile population, strengthening disease management and surveillance systems, and operations research.

3.2 Vaccines

Vaccination is the ultimate way to interrupt the transmission of an infectious disease. Today, no parasitic vaccines against human disease are available. However, several vaccine candidates have been developed against malaria and are now being tested in clinical trials, with various levels of efficacy (Figure 3) (Tongren et al. 2004). All are developed from diverse antigens from different stages of parasite development (exo-erythrocytic, blood, and sexual stages).

The most promising candidate is the RTS,S vaccine, which induces a cellular response and the production of antibodies (Ab) against the CircumSporozoite Protein (CSP), the most predominant surface antigen of the sporozoite stage (exo-erythrocytic stage). This vaccine is also composed of the hepatitis B surface antigen. The RTS,S/AS01 candidate malaria vaccine was tested in a phase 3 trial in seven African countries in children aged from 6 to 12 weeks and from 5 to 17 months. This vaccine reduced clinical episodes of malaria and severe malaria by approximately half during the 12 months after vaccination in children 5–17 months of age (Agnandji et al. 2011). In infants from 6 to 12 weeks, a modest protection (31%) was obtained against both clinical and severe malaria. However, for very young children, this vaccine was coadministered with Expanded Program on Immunization (EPI) vaccines and consequently this coadministration might have resulted in immune interference. Other factors

could also influence the modest efficacy of the RTS,S/AS01 vaccine in infants, who seem to be less susceptible to malaria in the immediate postvaccination period owing to maternally acquired immunity, fetal hemoglobin, and lower exposure. Moreover, the efficacy of this vaccine decreases over time. Consequently, other studies have to be conducted to understand the complex interplay between the intensity of exposure to malaria, the immune response, and vaccine efficacy (Agnandji et al. 2012, Olotu et al. 2013). GlaxoSmithKline Company is set to submit an application for a marketing license with the European Medicines Agency (EMA) in 2014. The new vaccine has the backing of the UN's Geneva-based WHO, which states that it will recommend RTS,S for use starting in 2015, providing it obtains approval (Kelland 2013).

A new vaccine, the PfSPZ vaccine, has been tested in a phase 1 trial and has shown convincing results. It is based on the inoculation of attenuated, aseptic, purified, and cryopreserved *P. falciparum* sporozoites by the intravenous route. It has been demonstrated that this vaccine is safe, well-tolerated, and produces protection against malaria in healthy volunteers (Seder et al. 2013).

A major limitation to developing a vaccine against the blood stage is the considerable antigenic variation of the surface protein of *Plasmodium*. Consequently, it has been shown that the use of a mono-antigen vaccine against the blood stage can induce protection against only parasites carrying the corresponding allele and could be more useful in a multicomponent malaria vaccine (Genton and Corradin 2002). The vaccine candidate FMP2.1/AS02A targeting the apical membrane antigen-1 (AMA-1) and tested in Mali during a phase 3 trial finally showed no significant protection against clinical malaria and seems to have strain-specific efficacy. The use of this antigen associated with another blood antigen could increase its efficacy (Thera et al. 2011).

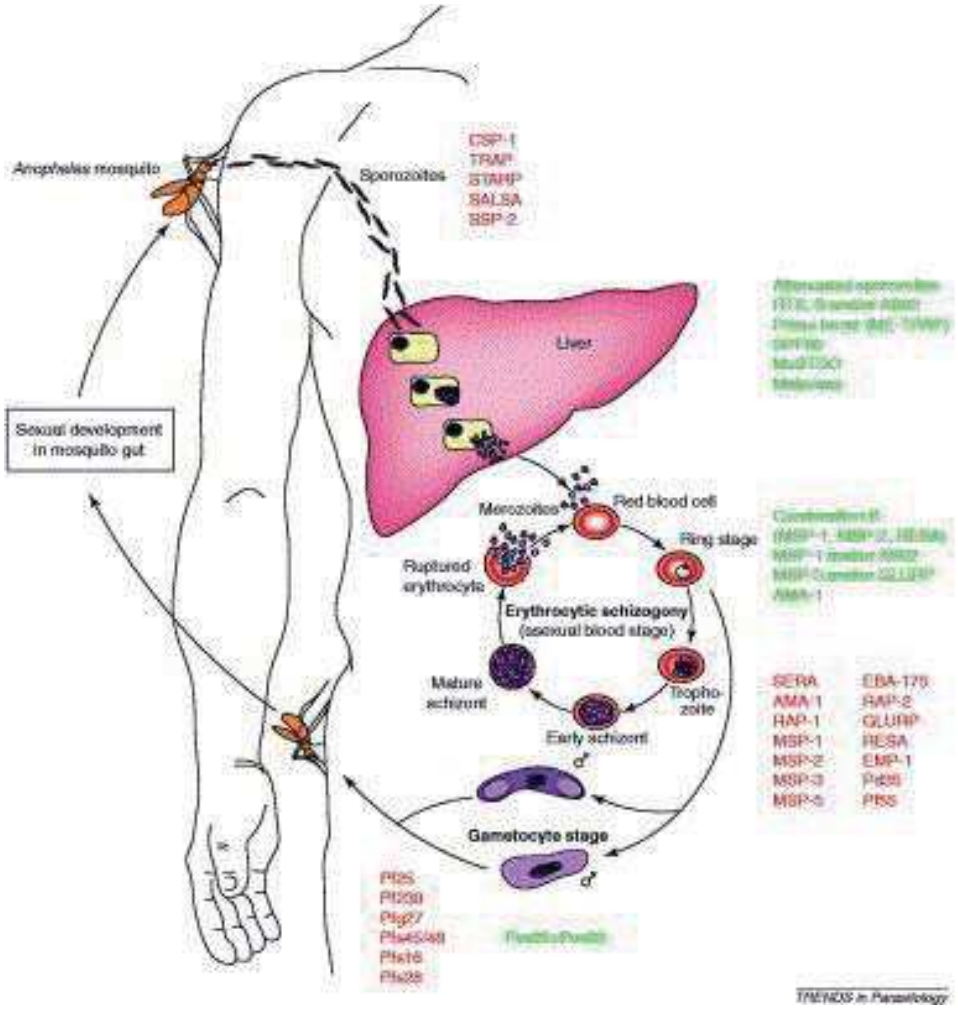

Figure 3. Antigens developed for malaria vaccines.
Life cycle of *Plasmodium falciparum* showing individual antigens that are being, or have been, evaluated as vaccine candidates (in red), and vaccine constructs that are currently being, or have been, evaluated in clinical trials (in green), for each stage of the life cycle. Abbreviations: AMA, apical membrane antigen; CSP, circumsporozoite surface protein; EBA, erythrocyte-binding antigen; EMP, erythrocyte membrane protein; GLURP, glutamate-rich protein; ME–TRAP, multiple epitope–thrombospondin-related adhesive protein; MSP, merozoite surface protein; *Pf, Plasmodium falciparum* protein; *Pv, Plasmodium vivax* protein; RAP, rhoptry-associated protein; RESA, ring-infected erythrocyte surface antigen; SALSA, sporozoite- and liver-stage antigen; SERA, serine-repeat antigen; SPf66, synthetic *P. falciparum* 66; SSP, sporozoite surface protein; STARP, sporozoite threonine- and asparagine-rich protein; TRAP, thrombospondin-related adhesive protein. (Tongren et al, 2004)

III. The *Anopheles* vector

The vector, by definition, is a hematophagous arthropod that provides the active transmission of a pathogen from one vertebrate to another vertebrate, after pathogen multiplication and/or transformation in its organism.

Two other concepts are essential concerning vectors and vector-borne disease transmission: vectorial competence and vectorial capacity.

Vectorial competence (Dye 1992, Lord et al. 1996) refers to the ability of the vector to infect itself after an infected bloodmeal, to provide the development of the pathogen, and to transmit it to a vertebrate. Vectorial competence measures the level of pathogen/vector coadaptation and depends essentially on genetic factors.

Vectorial capacity expresses the transmission potential of a vector population. It depends on factors bound to vectors, pathogens, and the environment: the density of the vector population, the frequency of vector–host contact, and the survival of the vector. The concept of vectorial capacity was translated mathematically by Garret-Jones (1964) from parameters defined by McDonald (1957).

Plasmodium vectors are dipterous insects belonging to the *Culicidae* family and *Anophelinae* subfamily. A total of 484 *Anopheles* species have been identified (Harbach 2004), 68 of which are considered as vectors of human *Plasmodium* in the world. In sub-Saharan Africa, five species account for the major vectors of *Plasmodium*: *An. gambiae s.s.*, *An. arabiensis*, *An. funestus*, *An. nili s.l.*, and *An. moucheti* and possess a high vectorial capacity (Manguin et al. 2008). Moreover, approximately 20 other species are secondary vectors and each species prefers a specific biotope.

Only female *Anopheles* are hematophagous and are active at night (from sunset to sunrise), and bite principally between 11 p.m. and 3 a.m. However, some species prefer to bite in the early evening or early morning. As is true for all aspects of *Anopheles* behavior, the time of biting varies from population to population (or even from individual to individual), from one site to another, and in different seasons.

The human *Plasmodium* vector species are considered to be anthropophilic (feeding on humans) as opposed to those that are zoophilic (feeding on animals). Some species are strictly

anthropophilic (*An. gambiae s.s.*) and others could be both in absence of humans (*An. melas* for example).

1. Anopheline life cycle

Anopheline mosquitoes are holometabolous, meaning that they undergo complete metamorphosis with four different stages in their life cycle (Figure 4). The first three stages (eggs, larvae, pupae) are referred to as pre-adult stages and are exclusively aquatic. The last stage, the adult or imago stage, is an air stage.

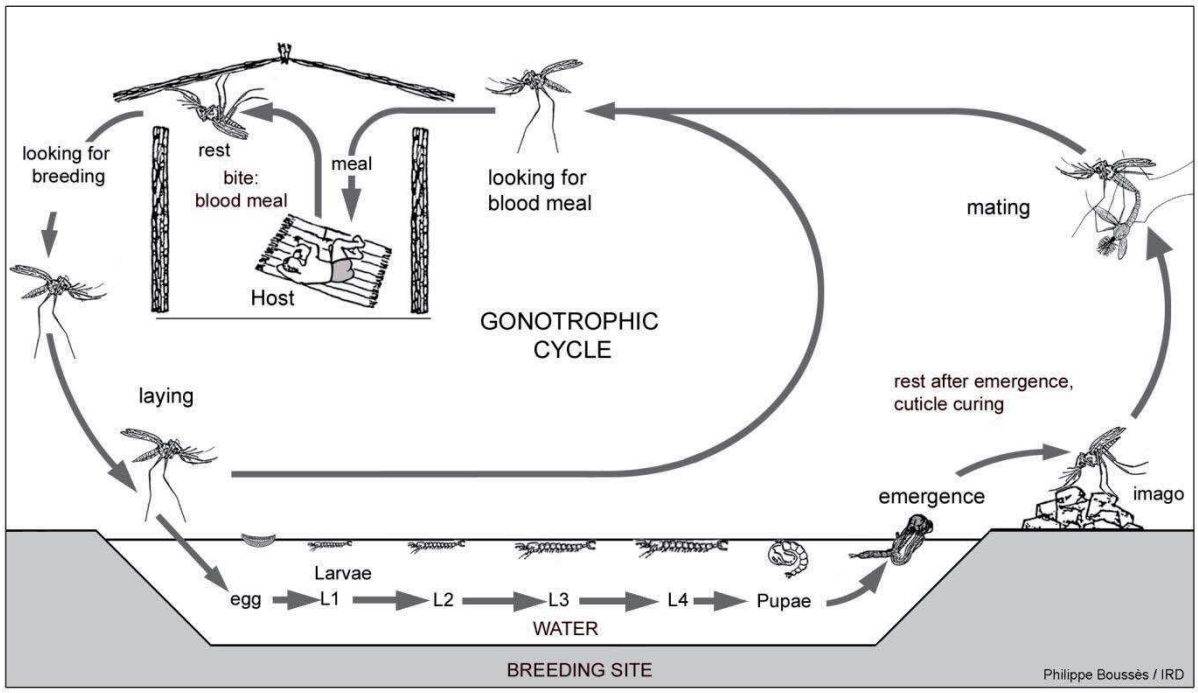

Figure 4. *Anopheles* life cycle. © Philippe Boussès.

1.1 Aquatic stage

The female lays about 100 individual fertilized eggs on the surface of the water. Eggs are equipped with floats and hatch 24-48 h after laying (depending on the temperature) to give rise to a single larva that assumes a position parallel to the surface of the water (Figure 5). Larvae feed on detritus at the surface, growing and molting four times. The last molting gives rise to the pupa

Figure 5. *An. gambiae* larvae ©Michel Dukhan

stage. During this stage, the pupa does not eat but undergoes massive morphological remodeling leading to the metamorphosis and emergence of the adult.
The duration of larva life depends on the species as well as the temperature. In tropical areas, the aquatic stage lasts 1–3 weeks. The adult development is greatest between 28°C and 34°C, although adult emergence was highest between 22°C and 26°C (Bayoh and Lindsay 2003).

1.2 Imago stage

As soon as they emerge, adults rest outside the water while its cuticle hardens, their wings are deployed and the reproductive parts of the male become functional. First, adults take a sugar meal to have energy for flight and reproduction. Females mate only once, whereas males mate several times during their lifetime (Clements 1992). However, multiple inseminations for females have been observed (Tripet et al. 2003). Mating takes place in flight in a male swarm at twilight.

Figure 6. Mating between a female *An. gambiae* (at the top of the image) and a male *An. gambiae* (at the bottom of the image) ©IRD

The female stores sperm in a special receptacle called the spermatheca from which spermatozoa are released each time she lays eggs. Just after mating (Figure 6), the female seeks a vertebrate to take a blood meal. This meal provides her with the proteins necessary for oocyte maturation. When the oocytes are mature, they are fertilized with stored sperm and become an egg. Females take a blood meal every 2 or 3 days. The biological cycle starting with the blood meal, blood digestion, oocyte maturation, finding a place to lay eggs, laying, and finally the quest for a new host, is called the gonotrophic cycle (Figure 4). In tropical and subtropical areas, depending on the species, this cycle can last 48–72 h. The number of gonotrophic cycles makes it possible to estimate the age of the female and consequently the life expectancy and the likelihood that it will become infectious (Figure 7). After a *P. falciparum*-infected meal, *An. gambiae* become infectious after about 14 days (this could be modified by environmental conditions such as temperature). Consequently, a female will be infectious after several blood meals and thus several gonotrophic cycles. For example, the

first blood meal is infectious, the time for the parasite to reach the salivary glands is 14 days; the female takes a blood meal every 3 days, so it can take four blood meals before to become infectious. This means that old females (several gonotrophic cycles) are more likely to be infected and more dangerous for humans. In general, the life span of a female is about 3 weeks, allowing her to provide five to eight gonotrophic cycles, whereas the male lives about 1 week.

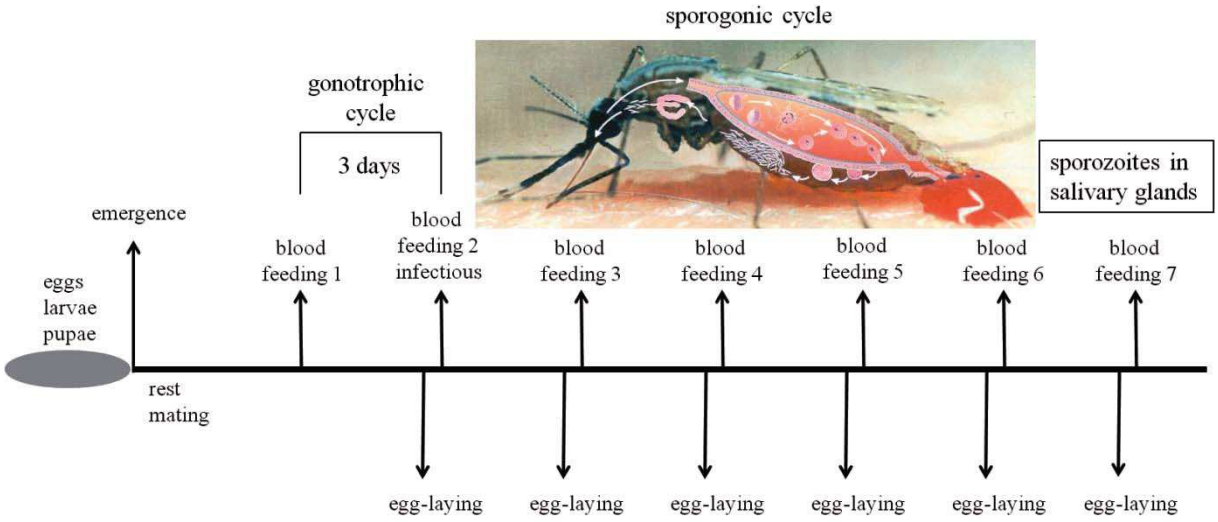

Figure 7. Life expectancy and infectious status.

2. *Anopheles* malaria vectors

2.1. *Anopheles gambiae* sensu lato (s.l.) complex

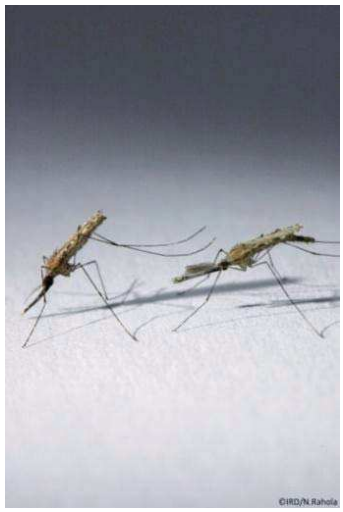

Figure 8. Female and male *An. gambiae* s.s.
©Nil Rahola

The term "species complex" refers to sibling species that are morphologically similar and can only differentiate using infertility as well as cytogenetic and molecular tools. Each sibling species possesses an ecologic and behavioral pattern and a distinct degree of efficiency as a vector.

The *An. gambiae* complex is composed of seven species: *An. gambiae sensu strict* (*s.s.*) Giles 1902 (Figure 8); *An. arabiensis* Patton 1905; *An. melas* Theobald 1903; *An. merus* Dönitz 1902; *An. bwambae* White 1995; *An. quadriannulatus* A Theobald 1911; and *An. quadriannulatus B* Hunt 1998. *An. gambiae s.s.* and *An. arabiensis* are the two major malaria vectors and the last three species are zoophilic; consequently they are not considered to be a human *Plasmodium* vector. These seven species differ in biology, geographical distribution (Figure 9), ecology, and genetic features (Ayala and Coluzzi 2005).

The first report of the existence of different strains of *An. gambiae* was shown by Davidson and Jackson in the middle of the 20th century. They discovered that the crossing experiments between *An. gambiae* Giles from different localities could give sterile males. It was first thought that this sterility was associated with resistance and not until further strains of *An. gambiae* were acquired was this shown not to be the case (Davidson and Jackson 1962).

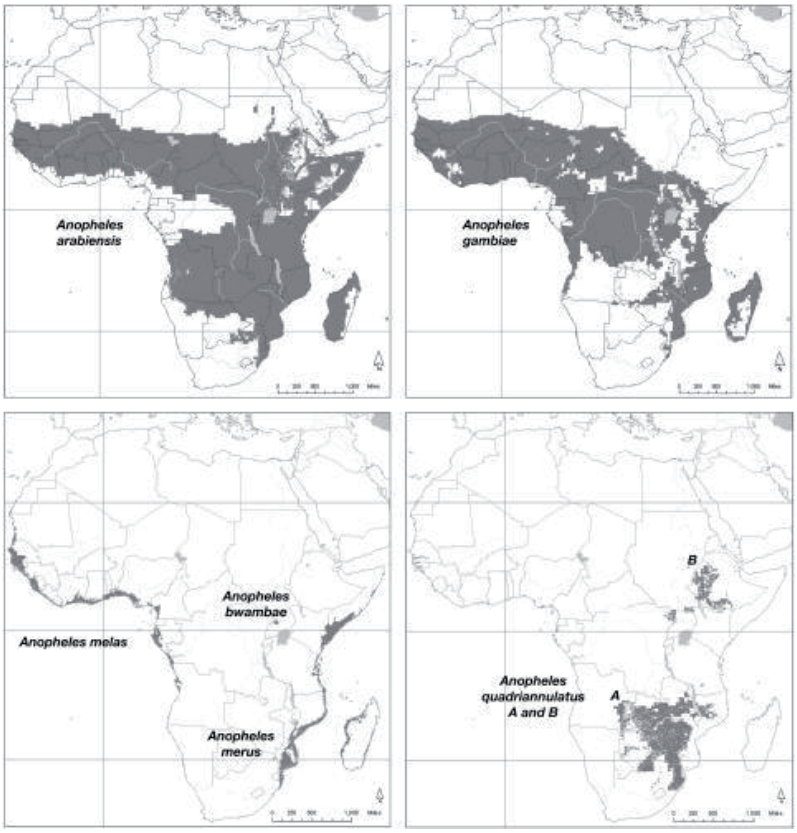

Figure 9. Distribution of species of *An. gambiae* complex. (Ayala and Coluzzi, 2005)

Nowadays, the most reliable methods to identify the different species of the complex are cytogenetic analysis. This is based on the study of polytene chromosomes in the salivary glands of larval stage 4 and/or the nurse cells of the ovarian follicles, but also molecular tools are being developed through the study of the high degree of polymorphism in the Intergenic Spacer (IGS) in ribosomal DNA (rDNA) genes (Scott et al. 1993).

Regarding their ecology, the five species of the complex considered as *Plasmodium* vectors (*An. gambiae s.s.*, *An. arabiensis*, *An. quadriannulatus* A and B, and *An. bwambae*), including the two main malaria vectors, have their larval stages in freshwater with rainwater pools in sunny spots without vegetation, potholes or footprints, and anthropophilic larval habitats such as irrigation ditches, dams, and excavated trenches (Figure 10) (Manguin et al. 2008).

Figure 10. Larval freshwater habitats of An. gambiae s.s. in Youndé, Cameroon.
(photograph by A. Marie, A. Boissière and L. Abate)

An. gambiae s.s. and *An. arabiensis* have a wide distribution throughout the African continent. *An. gambiae s.s.* is well adapted to forest areas and humid savannahs, whereas *An. arabiensis* is concentrated in sites with less rainfall such as the drier savannah areas (Coetzee et al. 2000). However, the geographical distribution of these two species greatly overlaps, and it is frequent to find them in sympatry (Figure 9).

An. gambiae s.s. is considered the most efficient malaria vector with a sporozoite index generally greater than 3%, reaching 10% (Mouchet et al. 1998). The sporozoite index of *An. arabiensis* could be more than 1% (Senegalese Sahel) but can be much lower (Ralisoa Randrianasolo and Coluzzi 1987).

For *An. melas* and *An. merus*, the larval stage takes place in brackish water in full sunshine such as salt marsh grass and mangrove areas (Manguin et al. 2008). *An. melas* is found in the coastal area of West Africa, from Senegal to Angola, and *An. merus* on the coast of East Africa (Figure 8). These two vectors are considered as secondary vectors.

2.2. Anopheles funestus

An. funestus belongs to the Funestus group and is widely distributed throughout the Afrotropical region except in the very dry areas in the northern, southern, and eastern parts of the continent and is highly localized in the great forests. It can also colonize rice paddies as well as swamps where vegetation is high and dense (Figure 11). In general, larval development occurs in permanent and semi-permanent breeding sites (Hamon et al. 1955). Larvae can be found in great numbers at the end of the rainy season and at the beginning of the dry season where they may prolong the action of *An. gambiae* (Mala et al. 2011).

Figure 11. Larval habitats of *An. funestus* in Burkina Faso. © Anna Cohuet

An. funestus is considered an efficient malaria vector with a sporozoite index that can exceed 5% (Cohuet et al. 2004). It can be associated with stable or unstable malaria, depending on the local epidemiological context.

3. Vector control strategies

The goal of vector control strategies is no longer to eradicate vector populations but rather to reduce their densities under an epidemiologically tolerable threshold to decrease malaria transmission sustainably and consequently associated morbidity and mortality. Each stage of *Anopheline* mosquitoes could be a potential target to lower human–vector contact and *Anopheles* density. The first step is to identify the target species, their ecology (larval biotopes), and their behavior (endo- or exophagic, endo- or exophilic) in the given area where vector control will be implemented.

3.1 The reduction of mosquito density

The reduction of vector density is obtained by larva or adult control.

Larvae can be controlled mechanically to destroy breeding sites, using biological control, chemical larvicide to reduce the productivity of breeding sites (Utzinger et al. 2001, Killeen et al. 2002b).

Physical techniques are the destruction of larval habitats by modifying the environment. These modifications are intended to be permanent, such as drainage of swamps, marshes, and creeks, or improvement of water collection with closed pipes, but these need regular verifications to ensure efficacy (Birley 1991, Phillips 1993). The modification of human habitat and behavior such as building houses far away from breeding sites and better management of trash is an important way to avoid human–vector contact. These methods have shown some success in the eradication and/or control of the *Anopheline* populations in Europe (de Zulueta 1998), Brazil (Killeen et al. 2002a), and Egypt (Shousha 1948). Nevertheless, changing human habits is the greatest challenge of vector control.

Biological control is another method using larvivorous fish (*Oreochromus spirulus* Günther, 1984; *Poecilia reticulate* Peters, 1859; *Gambusia affinis* Baird and Girard, 1853) (Walton 2007). A significant decrease of *Anopheles* species in India was obtained with this method (Das and Prasad 1991, Prasad et al. 1993) leading to a reduction of malaria cases (Ghosh et al. 2005, Singh et al. 2006). However, these fish are generally omnivores and feed on all larvae they find, causing damage to the non-target fauna. The bacterial insect pathogens such as *Bacillus thurigiensis var. israelensis* H14 (Bti) and *Bacillus sphaericus* are also used to reduce larval populations (Fillinger et al. 2003). They have the advantage of being safe for humans and for non-target organisms including the vast majority of insects, invertebrates, and vertebrates except for certain species of *Nematocera* (WHO 1999, Lacey 2007). These bacteria produce lethal toxins for larvae. The low persistence of these toxins in water needs regular and repeated treatments of breeding sites, which is operationally difficult (Carnevale et al. 2009). The same limitations are observed with Spinosad, which is a bacterial toxin and growth regulator (Pyriproxifen, Diflubenzuron, Novaluron), the efficacy of which is also difficult to evaluate in the field due to the low mortality of mosquitoes. Chemical insecticides of the organophosphate family (Chlorpyrifos, Fenthion, Temephos, etc.) are also used against larvae (WHO 2006).

However, controlling larvae populations is a complex task. Efficacy depends on breeding site access, number, size, and permanence. Moreover, *An. gambiae* is able to grow in various habitats from paddy fields to bovine footprints. Consequently, only follow-up over several years and strong support from the local population can make this strategy a success (WHO 2006).

The other major control strategies target adult mosquitoes by decreasing density as well as longevity, which can be determinant in malaria transmission. As explained in section III.1.2., the old female *Anopheles* are the most dangerous because they become infectious 14 days after an infected blood meal. Consequently, the reduction of the mosquito life span decreases the probability that it will infect a human. The two major methods recommended by the WHO are insecticide-treated nets (ITNs) and indoor residual spraying (IRS).

The only insecticide family used on ITNs belongs to the pyrethroid family, safe for humans (WHO 2006). They have several effects: i) a repellent effect at long distances to limit the number of *Anopheles* entering homes, ii) an irritant effect at short distances, causing mosquitoes to exit dwellings, iii) a knock-down effect if the mosquito touches the ITNs, and iv) a possible lethal effect. Most products remain active for 6–12 months and/or resist three washings, requiring retreatment thereafter. Today, nets are treated with insecticide incorporated into the fibers resisting multiple washes (over 20 washings) and remaining active for 3–5 years depending of the fiber type, called long-lasting insecticide-treated nets (LLINs) (Figure 12) (Guillet et al. 2001).

Figure 12. Insecticide-treated nets in a house in Mayotte. © Frederic Jourdain

The density of adult *Anopheles* mosquitoes can also be reduced using IRSs (Figure 13) or insecticide-treated plastic sheeting (ITPS), but they act only on endophilic and endophagic mosquitoes such as *An. gambiae* and *An. funestus*. Indeed, the anopheline mosquitoes bite during the night in the house (endophagy) and remain inside (endophily) on the wall for blood digestion. These behaviors allow IRS and ITPS to act as a repellent and kill the mosquitoes, thus disrupting malaria transmission. The insecticide families used are organochlorines (DDT), organophosphates (malathion, fenitrothion, etc.), pyrethroid (alphacypermethrin, deltamethrin, lambda-cyhalothrin), and carbamate (bendiocarb, propoxur) (WHO 2006).

Currently, WHO recommends the combined use of ITNs and IRS (WHO 2013) even though many studies have not shown a real advantage to associating them (Nyarango et al. 2006, Protopopoff et al. 2008, Yakob et al. 2011, Corbel et al. 2012).

Figure 13. Indoor residual spraying in a house in Mayotte. © Vincent Robert

3.2 Reduction of human–vector contact

The most efficient method to reduce anopheles–human contact is the bednet (with or without insecticide) used during the night during the *Anopheles* activity period, but these nets are effective only if they remain undamaged (Clarke et al. 2001, Mwangi et al. 2003) (Figure 12).

Another physical barrier is the use of fencing or netting in windows or doors to avoid mosquitoes entering houses. This method requires fences and screens to be undamaged and all openings closed (Lindsay et al. 2003).

Chemical repellents (DEET, IR 35/35, KBR 3023) applied on the skin (Rowland et al. 2004a, Rowland et al. 2004b) or clothes can be used except in children before 30 months of age. However, this method is not adapted to populations living in endemic areas and is preferentially used by travelers and military personnel. This strategy also has a limited impact in the prevention of malaria because repellents are more widely used during the day than at night when *Anopheles* bites.

3.3 Insecticides and resistance

Insecticides used in vector control against malaria belong to four families of chemical compounds (organophosphates, organochlorines, carbamates, and pyrethroids). DDT (dichlorodiphenyltrichloroethane), belonging to the organochlorine family, is the most famous insecticide and was the first to be used in the 1950s. It contributed greatly to the eradication of malaria in Europe (Bruce-Chwatt 1977) and North America (Andrews et al. 1954). However, its uncontrolled use, particularly in agriculture, was rapidly stopped due to its toxicity causing a great deal of damage to the environment. After advice to stop the use of DDT in the 1980s, in 2006 the WHO recommended its use for IRS under high surveillance (WHO 2011).

However, resistance to these insecticide families has appeared. DDT resistance was first documented in 1948 in houseflies (Lindquist and Wilson 1948) and the first resistance in anophelines was observed in Greece in *An. sachavori* in 1953 (Livadas and Georgopoulos 1953). Today, resistances of other insecticide families have appeared. Mosquitoes have developed different resistance mechanisms depending on the insecticide family.

Two types of resistance exist, allowing the insects to avoid contact with insecticides and the physiological resistance whose modes of action are as follows (Figure 14): i) reduction in insecticide penetration by modification of the cuticle or intestinal membrane, ii) increase in excretion and/or insecticide detoxification, and iii) modification of the insecticide target.

Each mechanism is controlled by at least one gene whose mutation can induce resistance. In *Anopheles*, the most important mechanisms are target modification and metabolic resistance.

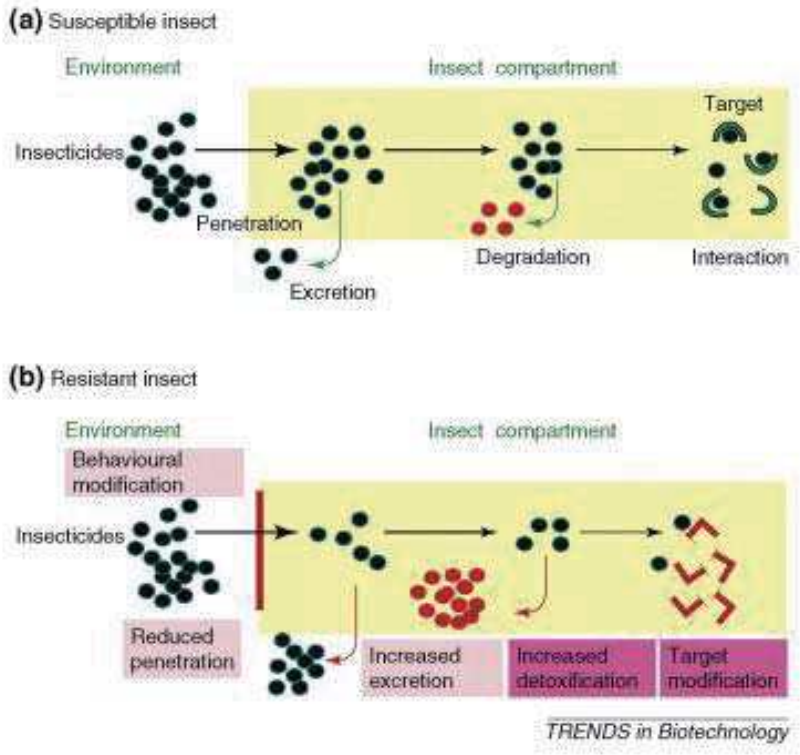

Figure 14. Diagram of mechanisms involved in physiological resistance to insecticides. (Lapied, 2009)

Target modification induces a reduction of insecticide sensitivity due to a punctual mutation in the gene coding for a protein target. The best-known mutation described in the *An. gambiae* complex is the sodium channel mutation entitled "kdr" ("knock-down resistance"), inducing a resistance to pyrethroids and organochlorines (Davies et al. 2007). Another mutation is called "Ace-1" and induces resistance to carbamate and to a lesser extent to organochlorines (Weill et al. 2004).

Metabolic resistance is due to an enzyme overexpression able to detoxify or confine insecticides. These enzymes are essentially esterases, P450 oxydases, and glutathione-S-transferase (GST) (Hemingway et al. 2004). The oxidation reactions are involved in resistance to organochlorines, pyrethroids, and organophosphates (Lapied et al. 2009). The esterases are implicated in the resistance to pyrethroids and organophosphates (Hemingway et al. 2004). GST induces resistance to organochlorines and organophosphates (Ranson and Hemingway 2005).

The pyrethroids are the WHO's most highly recommended insecticide family for all vector control strategies due to their safety for human populations. The resistance of pyrethroids, the insecticide family only used for ITNs, could highly decrease the efficacy of ITNs and other vector-control strategies. Consequently, the study of this resistance in Africa was essential. A

recent study has collected publications from 2000 to 2010 to build a distribution map of pyrethroid resistance in Africa due to the "kdr" mutation and metabolic resistance (Figure 15). A large number of resistant vector populations were found in the major *Plasmodium* vector species (*An. gambiae*, *An. arabiensis*, and *An. funestus*) across the continent except in Maghreb and the extreme West of Madagascar (Ranson et al. 2011).

Few studies have analyzed the impact of pyrethroid resistance because of the many confounding factors and the results are contradictory. However, it should be noted that an operational failure of IRS related to the pyrethroid resistance has been highlighted in South Africa (Brooke et al. 2001).

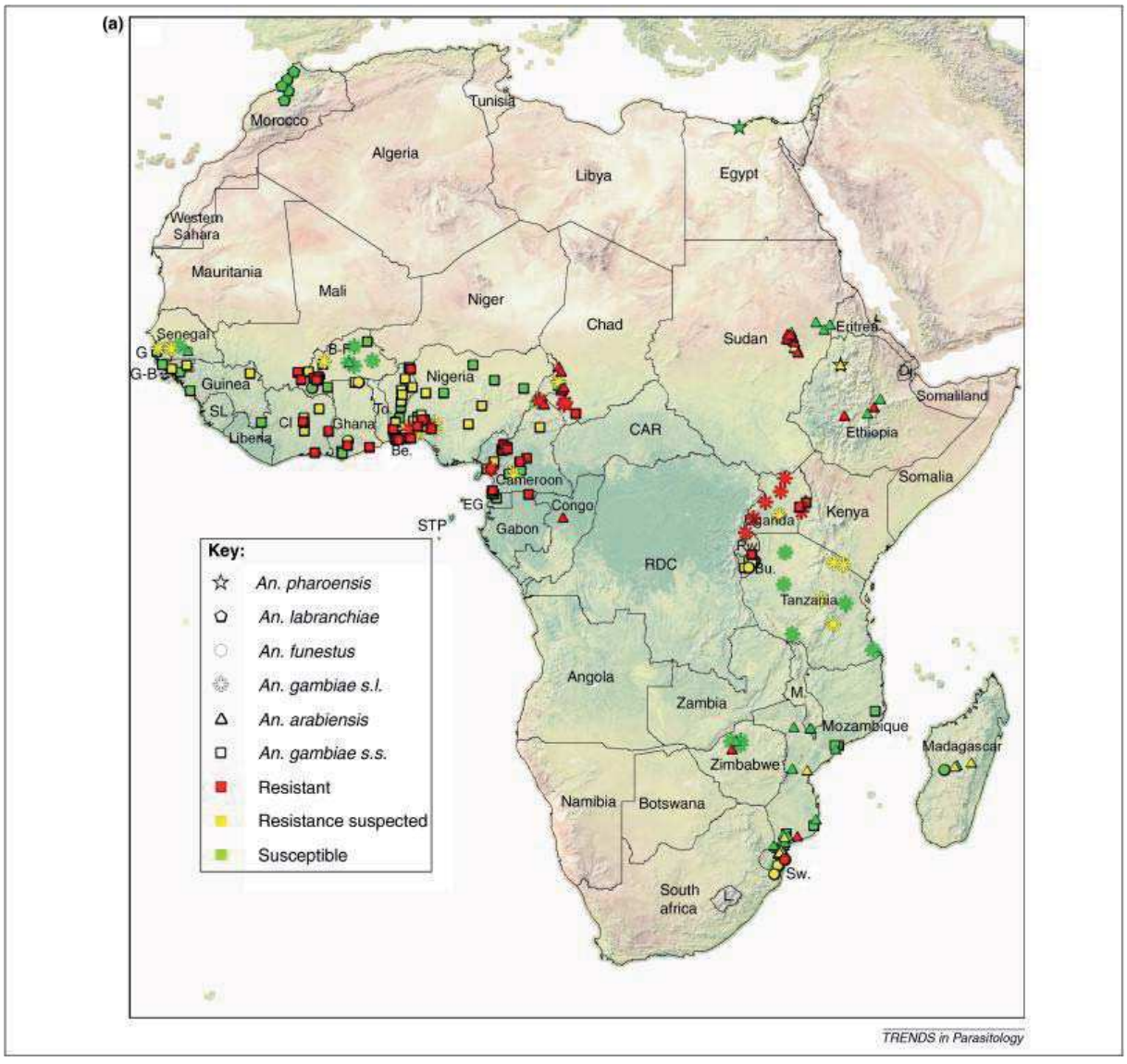

Figure 15. Map representing the distribution of pyrethroid resistance in Africa. (Ranson, 2011)

3.4 Innovative vector control

The resistance of *Anopheles* to the majority of insecticides and especially to pyrethroids (Chandre et al. 1999, N'Guessan et al. 2007) has led to the development of new methods for vector control including biological pathogens and genetic mosquito modification.

Among biological pathogens, the entomopathogenic fungi are being studied. The fungi infect the mosquito by penetrating the cuticle of the larva or adult, multiply, and cause death. The earlier the mosquito is infected by fungi, the earlier it dies, limiting the possibility of transmitting the *Plasmodium* parasite (Scholte et al. 2004). The transinfection of *Anopheles* by *Wolbachia* (bacteria) could make *Anopheles* mosquitoes refractory to *P. falciparum* infection and development (Hughes et al. 2011, Bian et al. 2013).

Genetic control aims to reduce vector competence and the reproductive potential of a vector population by altering or replacing the hereditary material. Genetic vector control is based on two strategies (Alphey et al. 2002). The first concept is to produce mosquitoes with an altered phenotype that could be introduced into the population so that this new phenotype becomes dominant and spreads into the natural population. Generally, this phenotype acts on parasite transmission rather than mosquito biology. The second concept aims to reduce mosquito density. The goal is to release sterile males (Alphey et al. 2010) or insects carrying dominant lethal mutations that will lead to an unsustainable lineage (Thomas et al. 2000). However, beyond the risks involved in introducing genetically modified mosquitoes in the field (Knols et al. 2007), this method is currently only possible in relatively remote areas and with a single vector species (Helinski and Knols 2008, Munhenga et al. 2011, Raghavendra et al. 2011). At this time, this genetic vector control is not approved for the field and continues to be studied to evaluate its feasibility and efficacy.

3.5 Transmission-blocking vaccine

An alternative to reducing malaria transmission is to disrupt the development of the parasite in the mosquito. This is the aim of the transmission-blocking vaccine (TBV). These vaccines induce the production of Ab in humans, targeting either a parasite stage that develops within the mosquito (sexual stage, gametocyte, zygote, oocyst) and/or targeting mosquito antigens. This type of vaccine does not protect the human host against malaria, but prevents the spread of the parasite by limiting the number of infectious vectors (Dinglasan and Jacobs-Lorena 2008, Coutinho-Abreu and Ramalho-Ortigao 2010). To be efficient, these TBVs have to induce high titers of Ab to completely block parasite development and should provide long-

lasting immunity in the vaccinated population (Kubler-Kielb et al. 2007). Several candidate vaccines have been developed targeting antigens at different stages of *P. falciparum* infection and tested in animal models: Pfs25, which is a surface protein of zygotes and ookinetes (Coban et al. 2004, Arakawa et al. 2005, LeBlanc et al. 2008, Kumar et al. 2013); Pfs28, a surface protein of a transitional stage between zygote and ookinete, used in synergy with Pfs25 (Gozar et al. 1998); Pfs48/45, proteins expressed on gametocyte and gamete surfaces (Outchkourov et al. 2007, Outchkourov et al. 2008); and Pfs230, a surface protein of gametocytes (Quakyi et al. 1987). The P25 protein of *P. vivax* (Pvs25) has already been tested as a vaccine target in the mouse (Hisaeda et al. 2000) and the monkey (Arévalo-Herrera et al. 2005). Currently, the TBV candidates Pfs25 and Pvs25 are being tested in a phase 1 trial using different adjuvants (Malkin et al. 2005, Wu et al. 2008). However, the latest study has demonstrated that the formulation of these vaccines causes systematic adverse events in humans. Extensive studies have to be conducted to improve the immunogenicity of TBV Pfs25 and Pvs25 and the effects of adjuvants. The advantage of these vaccines is that the targeted antigens have low polymorphism because sporogonic stage proteins are not subjected to immune pressure in humans. However, the lack of natural boosting presents a drawback for these vaccines as the immune system of humans never meets these parasite antigens.

Another objective of TBV is to target vector antigens acting on vector survival, reducing the size of the vector population or acting on proteins necessary for the parasite's development inside the mosquito (Lal et al. 2001, Lavazec et al. 2007, Lavazec and Bourgouin 2008). Most studies have focused on the midgut antigens blocking the transmission of *P. berghei*, a rodent parasite (Ramasamy and Ramasamy 1990, Lal et al. 1994) as well as *P. falciparum* (Lal et al. 2001) and *P. vivax* (Srikrishnaraj et al. 1995, Lal et al. 2001). All these studies showed that the Ab against midgut antigens prevents the interaction between the ookinete and the midgut membrane and then inhibits the development of *Plasmodium* ookinetes into an oocyst (Lavazec et al. 2007). However, as mentioned for the TBV against parasite antigens, the TBV against midgut antigens lacks natural boosting in humans. To counteract this drawback, salivary proteins as targets of TBV offer a good alternative. The human immune response against salivary antigens is reinforced at each injection of saliva during the blood meal. Mosquito salivary gland invasion by the sporozoites is the last step of parasite development that needs an interaction between sporozoites and membrane receptors from salivary glands (Barreau et al. 1995, Korochkina et al. 2006). Previous studies have demonstrated that sporozoite invasion in salivary glands could be

blocked using Ab against salivary proteins (Barreau et al. 1995). These Ab could be ingested by the mosquito during a blood meal and cross the midgut membrane to reach the target salivary protein (Brennan et al. 2000). Two salivary proteins, SGS proteins and saglin, have been shown to be potential candidates for TBV (Barreau et al. 1999, Okulate et al. 2007). Recently, the genetic variation of the saglin protein in wild *An. gambiae* in Mali has been studied. No evidence of positive selection was found, confirming the potential of the saglin protein as a target for TBV (Crawford et al. 2013).

Context of the thesis

I. Assessing the risk of exposure to the risk of infection

1. Evaluation of malaria transmission risk

Parasite transmission depends on several factors such as the presence of a competent vector (high vectorial capacity), the infection rate of these vectors, and the intensity of the human–vector contact. The risk of malaria transmission can be evaluated by studying the vector and/or directly the human host.

1.1 Entomological indicator of transmission

Different methods of catching anopheline mosquitoes can measure the intensity of human exposure to the vector, thus assessing human–vector contact. Although vector density does not precisely reflect the intensity of malaria transmission, these methods evaluate vector abundance in a given place at a given time and can then characterize the spatiotemporal risk of transmission. Moreover, the assessment of adult vector density is the first parameter to define the entomological inoculation rate (EIR), which is the commonly used measurement of the intensity of malaria transmission. *Anopheles* population sampling is an essential step in a malaria survey program, to identify the vector population to control and evaluate the efficacy of vector-control strategies.

1.1.1 Evaluation of *Anopheles* density

Today, different methods can be used to catch adult *Anopheles* species depending on the aim of the study, the species studied, the cost, convenience, and reproducibility: the human landing catch and the catching trap.

The human landing catch (HLC) consists in a seated individual catching mosquitoes with tubes which land on an exposed body part (generally the legs) to have a blood meal (Coffinet et al. 2009) (Figure 16). Mosquitoes are caught at different sites in a village and rotation between catchers is organized to limit bias related to individual variation in *Anopheles* attraction. This technique is particularly well-adapted to the female of anthropophilic species (Le Goff et al. 1993) and is the best way to evaluate the number of *Anopheles* biting humans, expressed in bites per human per night, and provides information on the seasonal and daily variation and vector aggressiveness. Moreover, this method preserves mosquitoes better than

traps, allowing precise analysis such as morphological identification, dissection as well as biochemical and molecular biological assays. However, this method exposes the catchers to the *Plasmodium* parasite and other pathogens transmitted by mosquito vectors. On enrollment, catchers will be vaccinated against yellow fever (if they have not already done so). Since the study is conducted in malaria-endemic areas, adult collectors who have already acquired immunity against malaria parasites will not receive chemoprophylaxis, but will be medically supervised by local physicians in case of illness and will be treated free of charge for malaria-presumed illness. Indeed, they will receive anti-malaria treatment (ACT) if fever or clinical malaria symptoms are confirmed by microscopy. For ethical concerns, this catching method can only be used by adult males and is not applicable to children under 5 years of age, the major risk population for malaria (Carnevale et al. 1978).

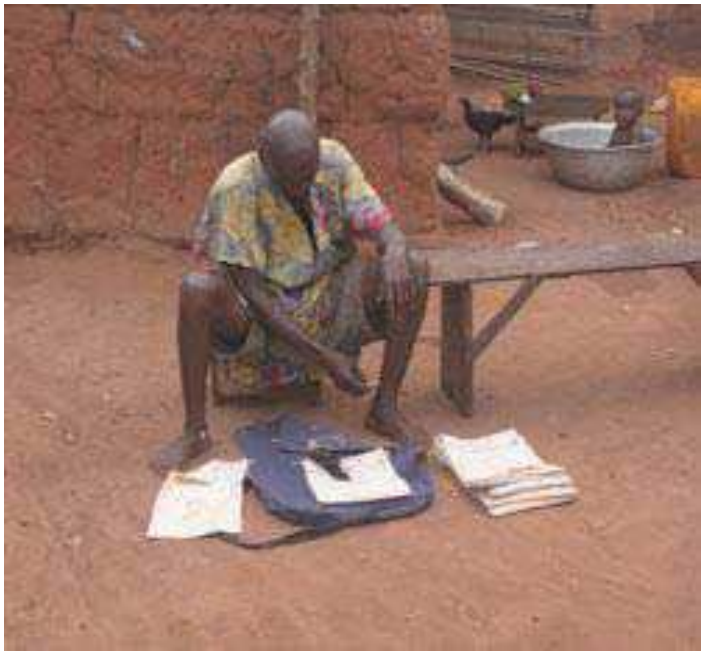

Figure 16. Human landing catch (HLC) in Kindjitokpa, Benin. (Moiroux N, 2001)

To overcome the ethical limitations, catching traps such as the CDC light trap (Center for Disease Control), CDC light trap associated with CO_2 or the Mbitrap have been developed. Different studies have shown variations between traps, leading to bias: these traps have a low specificity catching males and females, nonhematophagous arthropods, zoophilic mosquitoes, and importantly they do not inform on mosquito aggressiveness. For the moment, no adequate alternative methods have been recommended to replace HLCs (Rubio-Palis and Curtis 1992, Le Goff et al. 1993, Mathenge et al. 2005, Coffinet et al. 2009).

It should be noted that none of these methods assess the individual heterogeneity of human exposure to bites and provide only an overall picture of actual human–vector contact.

1.1.2 Entomological inoculation rate

An entomological indicator was developed by Ross (1911) to evaluate the malaria transmission rate from captured *Anopheles* females: the entomological inoculation rate (EIR). The EIR represents the number of infective bites per individual per time period (year, month, day, or night) and is obtained by the mathematical formula:

h = m.a.s where

m.a is the average number of bites delivered to an individual per time period, evaluated by entomological catching (HLCs, traps), and

s is the sporozoite index, which represents the percentage of *Anopheles* possessing sporozoites in the salivary glands. "s" is obtained by salivary gland dissection to visualize sporozoites by microscopy or detection of the circumsporozoite protein (CSP) in the mosquito head and thorax using the ELISA test (CSP-ELISA) (Burkot et al. 1984).

In Africa, different studies have shown high variability of EIR values (Gilles 1993). In holoendemic areas, the EIR can reach a value of 1000, whereas in hypoendemic areas such as Sahel or the Sub-Sahel region, or in urban contexts, the EIR can be below 1. Between these extreme situations, seasonal unstable malaria is defined by an EIR less than 10 and often around 1 or 2, and seasonal stable malaria (the majority of West Africa) is characterized by an EIR between 10 and 100. However, this measurement is strongly influenced by the quality of entomological catching and therefore obtaining precise results is problematic (Githeko et al. 1996, Kelly-Hope and McKenzie 2009). Differences in biting behavior exist, due to factors such as the human's age, larval habitat proximity (Thomas and Lindsay 2000), individual attractiveness, etc. For example, pregnant women and gametocyte carriers are more attractive to *Anopheles* (Lacroix et al. 2005). Carnevale et al showed that adults attract more *Anopheles* than children (Carnevale et al. 1978). The EIR for a given site must be measured over a year and compared to previous years to take into account the distribution of *Anopheles* bites. Indeed, depending on whether unstable or stable malaria is studied, *Anopheles* mosquitoes can be present all the time or gather only during a short period such as the rainy season. EIR measurement lacks precision owing to the heterogeneity of *Anopheles* distribution as well as

to very low sporozoite rates (below 5%), even in highly endemic areas (Mbogo et al. 1995, Drakeley et al. 2003, Corran et al. 2007). Moreover, the EIR is a population/community indicator and cannot appreciate human individual heterogeneity to *Anopheles* exposure. The relation between the EIR and malaria morbidity and mortality is not clearly established (Smith et al. 2001, Mbogo et al. 2003, Smith et al. 2004). This relation is extremely complex and other parameters play a role such as the nature of immunity against malaria, the time of appearance and disappearance of this immunity, whether or not there is access to effective treatment, and human genetic factors.

1.2 Human indicator of transmission

1.2.1 Parasitological criteria

Malaria transmission is also evaluated in the human host using different indexes such as parasite prevalence and density. These two indexes are measured using the diagnostic tests described in section II. 2.2. Diagnosis is based on thick blood film and thin blood smear, which are the referent methods used in the field in most health centers.

Prevalence corresponds to the number of infected (symptomatic and asymptomatic) individuals at a given site and time and is evaluated by the presence of at least one parasite in thick blood. However, only individuals presenting clinical symptoms are tested. Consequently, the prevalence measure presents bias due to undeclared asymptomatic individuals.

Parasite density, which corresponds to the number of parasites per microliter of blood, is also used and is considered by WHO as the referent method to evaluate the efficacy of anti-malaria drug treatment and vector-control strategies. This parameter is the most important in areas of stable malaria, where the clinical manifestations are related to high parasitemia, not only due to the presence of parasites, and where about 90% of carriers are asymptomatic. Parasite density depends on the *Plasmodium* species (average, the highest parasitemia is observed with *P. falciparum* and the lowest with *P. malariae* (Manguin et al. 2008) and are sensitive to different host factors such as the immune status, the use of effective drugs, *Plasmodium* resistance to treatment, and pregnancy or postpartum periods (Diagne et al. 2000). When transmission is low or after drug treatment, parasitemia is low and the detection of parasites in human blood is very laborious and can produce false-negative results.

1.2.2 Serological method against parasite antigens

Malaria transmission can be also evaluated by the assessment of an Ab response to *Plasmodium* antigens. The serological methods used in the field are the RDT described in section II.2.2. Diagnosis is based on the detection of HRP2 or the LDH enzyme. However, RDT presents several limitations and therefore WHO recommends confirming the result by microscopy analysis.

In addition to the detection of *Plasmodium* antigens (by RDT), the human Ab response to different stages of *P. falciparum* antigens are also studied: circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP) of the sporozoite stage, liver-stage antigen 1 (LSA-1), apical merozoite antigen 1 (AMA-1) of the pre-erythrocytic stage, and merozoite surface protein (MSP-1, MSP-2, and MSP-3) of the erythrocytic stage.

These *Plasmodium* antigens produce different Ab subclasses and this varies with age, malaria transmission intensity, and time of antigen exposure (Aribot et al. 1996, Tongren et al. 2006, Wickramarachchi et al. 2006).

For the majority of *P. falciparum* antigens, an age effect is observed and it has been shown that the Ab levels to these antigens increase with age as well as with the number of malaria exposures, inducing a more stable Ab response (Taylor et al. 1996, Akpogheneta et al. 2008). In an unstable malaria area, John CC et al have demonstrated that the Ab against pre-erythrocytic antigens (CSP, TRAP, LSA-1) vary in individuals depending on age and season. The Ab response to CSP and TRAP was lower in children than in adults during the dry season, although no variation was observed between the two age groups during the rainy season, whereas the Ab response to LSA-1 was lower in children than in adults in both seasons (John et al. 2003). In the Kenyan highlands, adults (>15 years) presented a stable Ab response to MSP-1_{19} despite seasonal variation of malaria transmission, whereas the Ab response in children was lower and varied with the season (Badu et al. 2012). In intense malaria transmission areas, the dynamics of the Ab level against different antigens (CSP, AMA-1, MSP-1_{19}, MSP-2) in young children (<5 years old) seems dependent on recent parasite exposure, contrary to older children and adults in whom the association between malaria infection and Ab level is less evident (Proietti et al. 2013). Romi et al showed that the Ab prevalence against CSP in children from 6 to 13 years old was higher in stable malaria areas compared to unstable areas (Romi et al. 1994).

Concerning the longevity of the Ab response, it has been demonstrated in African immigrants in France that Ab against *P. falciparum* could persist for several years (Bouchaud et al. 2005). Studies have proved that the half-life of the Ab against CSP differs from that of blood-stage antigens (Modiano et al. 1996, John et al. 2003). Webster et al showed that the Ab response to CSP has a short life, reflecting recent exposure to *Plasmodium* (Webster et al. 1987, Webster et al. 1992), although the Ab against whole parasite may persist longer in absence of re-infection (Druilhe et al. 1986). The Ab response to CSP seems, however, to reflect the transmission of *P. falciparum* (Esposito et al. 1988, Webster et al. 1992, Metzger et al. 1998), even in asymptomatic individuals (Jelinek et al. 1996). In areas of low but endemic malaria transmission in Thailand, it has been shown that the level of Ab response to blood-stage antigens, AMA-1 and MSP-1, in adults is stably maintained over periods of more than 5 years since the last known malaria infection (Wipasa et al. 2010).

The low Ab level produced for several months or years after antigen exposure without re-infection could reflect the previous and cumulative human exposure to the parasite and becomes an appropriate tool to estimate malaria exposure at the individual level and to chronicle the exposure history rather than malaria transmission. This cumulative Ab response does not distinguish old and recent infections (Bruce-Chwatt et al. 1973, Bruce-Chwatt et al. 1975, Corran et al. 2007). In addition, these antigens, such as AMA-1, which induces a long-lasting specific Ab response, may be good vaccine candidates (Thera et al. 2011).

We have noted that the Ab response to *Plasmodium* antigens is highly complex, is antigen-dependent, and varies according to several parameters such as age (low detection in children), the intensity of malaria transmission (stable or unstable, endemic or seasonal), and also with the genetic background of each individual. Studies have shown that some individuals do not produce Ab against CSP despite malaria exposure (Quakyi et al. 1989), other individuals appear not to develop an Ab response to the most immunogenic antigens such as AMA-1 and yet present an Ab response to MSP-1_{19}, which is less immunogenic (Taylor et al. 1995). Consequently, it has been suggested that the combination of several antigens (pre-erythrocytic and erythrocytic) in addition to CSP would be more sensitive and allow detecting all malaria infections (Orlandi-Pradines et al. 2006, Ambrosino et al. 2010, Campo et al. 2011, Sarr et al. 2011).

1.3 Serological method against vector saliva

For several years, a new serological approach has been highlighted. It has been shown that the human Ab response to arthropod saliva can be used as a biomarker of human exposure to vector bites and therefore to evaluate direct host–vector contact and potentially pathogen transmission. As described above, the Ab response to parasite antigens induces bias due to the effect of the cumulative immune response, making it impossible to distinguish previous and recent infection and the difficulty observing a significant Ab response in children. Consequently, the Ab response to saliva, which does not present an accumulative Ab response and is detectable in young children, could be a good immune-epidemiological indicator to evaluate the exposure to bites and, as a result, to extrapolate to pathogen transmission. This purpose is discussed in the next part in a published chapter of a book titled *Anopheles mosquito – New insights into malaria vector* by Drame et al (Drame et al. 2013b).

2. Human–vector relationship: toward a biomarker of exposure to *Anopheles* malaria vectors

The next part presents how human exposure to the *Anopheles* bites can be assessed from saliva.

In the following review, the functions of mosquito saliva are first detailed (pharmacological and immunological). Then the concept of a biomarker of bite exposure based on Ab response to mosquito salivary proteins is explained. Methods to identify specific *Anopheles* biomarker salivary proteins are also reviewed. Next, the results obtained with the gSG6-P1 peptide are presented (predicted from the specific *Anopheles* gSG6 salivary protein) in order to evaluate the exposure of *Anopheles* bites in different African countries and in different epidemiological malaria settings.

To finish, the drawbacks of this technique are discussed.

Chapter 23

New Salivary Biomarkers of Human Exposure to Malaria Vector Bites

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Additional information is available at the end of the chapter

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1. Introduction

Mosquitoes are the most menacing worldwide arthropod disease vectors. They transmit a broad range of viral, protozoan and metazoan pathogens responsible of the most devastating human and animal diseases [1]. Among the main frequent mosquito-borne diseases, malaria represents the most widespread and serious infection in terms of heavy burden on health and economic development throughout the world. Despite substantial efforts and increasing international funding to eliminate it, malaria is still a major public health problem with nearly a million of deaths per year, especially in children younger than 5 years old (86%) [2]. Approximately two thirds of the world's population live in areas at risk for malaria [3, 4]. Understanding mechanisms that govern its transmission remains therefore a major scientific challenge, but also an essential step in the design and the evaluation of effective control programs [5, 6].

Entomological, parasitological and clinical assessments are routinely used to evaluate the exposure of human populations to *Anopheles* vector bites and the risk of malaria transmission. However, these methods are labor intensive and difficult to sustain on large scales, especially when transmission and exposure levels are low (dry season, high altitude, urban settings or after vector control) [7, 8]. In particular, the entomological inoculation rate (EIR), the gold standard measure for mosquito–human transmission intensity of *Plasmodium*, is highly dependent on the density of human-biting *Anopheles* [9]. This latter is estimated by using trapping methods such as human-landing catches (HLC) of adult mosquitoes, the commonly used for sampling host-seeking mosquitoes and then for assessing the human exposure level.

HLC may be limited because of ethical and logistical constraints to relevantly apply it to children [10]. Transmission estimates based on the prevalence or density of human infection are susceptible to micro-heterogeneity caused by climatic factors and the socioeconomic determinants of the host-seeking behavior [8]. Incidence of disease may be the closest logical correlate of the burden of disease on health systems. However, it can be subject to variability between sites and may not be appropriate for the evaluation of early phase studies of vector control or reliable for epidemic prediction [10]. More recently, serological correlates of transmission intensity have been described, yet they represent long-term rather than short-term exposure data [8]. They are not then suitable in evaluating the short-term impact of vector control programs. Therefore, it is currently emphasized the need to develop new tools assessing reliably human malaria risk and control interventions, and monitoring changes over time at both population and individual levels [5, 6].

Malaria is a parasitic disease caused by protozoan agents of the genus *Plasmodium* (*Aplicomplexa*; *Haemosporida*). Five *Plasmodium* species are pathogen for humans: *P. falciparum*, *P. vivax, P. ovale*, *P. malariae* and *P. knowlesi*. During their complex life cycle in the female *Anopheles* mosquito (*Insecta*; *Diptera*), *Plasmodium* parasites go through several developmental transitions, traverse the midgut and reach the salivary gland (SG) epithelium. They acquire their maturity within SGs of the vector and can be then transmitted by the bite of the female mosquito. This latter needs, during the first days after emergence, to feed on sugar to meet the energy demands of basic metabolism and flight, but also to feed on vertebrate blood for its eggs' development and maturation [11], and therefore to keep perennial its life cycle and indirectly malaria transmission cycle.

Anopheles mouthparts comprise six pieces that form a long stylus allowing to perforate human tissues and to suck the internal liquid. However, it is clear that *Anopheles* mosquito acts not only as syringe injecting parasites during the bite. When taking a blood meal, it also injects into human skin avascular tissue [12] a cocktail of bioactive molecules including enzymes that are injected in human skin by saliva [13, 14]. Some of these salivary compounds are essential to the *Plasmodium* life cycle [15]. They have substantial anti-hemostatic, anti-inflammatory, and immunomodulatory activities that assist the mosquito in the blood-feeding process by inhibiting several defense mechanisms of the human host [16]. Furthermore, many of them are immunogenic and elicit strong immune responses, evidenced by the swelling and itching that accompany a mosquito bite [17]. Specific acquired cellular [18, 19] or/and humoral responses are developed by human individuals when exposed to bites of *Anopheles* mosquitoes [20-23]. These immune responses may play several roles in the pathogen transmission ability and the disease outcomes [24]. In addition, recent studies have demonstrated that the intensity of the antibody response specific to salivary proteins could be a biomarker of the exposure level of human to *Anopheles* bites [22, 25]. Therefore, studying *Anopheles*-human immunological relationships can provide new promising tools for monitoring the real human-*Anopheles* contact and identifying individuals at risk of malaria transmission. It can also allow the development of novel methods for monitoring control and mosquito-release programmes' effectiveness.

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However, whole saliva could be inadequate as a biomarker tool, because it is a cocktail of various molecular components with different nature and biological functions. Some of these elements are ubiquitous and may potentially cause cross-reactivities with common salivary epitopes of other haematophagous arthropods [26]. In addition, a lack of reproducibility between collected whole *Anopheles* saliva batches has been observed and difficulties to obtain sufficient quantities needed for large-scale studies were highlighted [26]. Therefore, specific and antigenic proteins have been identified in the secretome of *Anopheles* mosquitoes and a specific biomarker of *Anopheles* bites was developed by coupling bioinformatic and immuno-epidemiological approaches. This promising candidate, namely, the gSG6-P1 (*An. gambiae* Salivary Gland Protein-6 peptide 1), has been described to be highly antigenic [26]. It has been then validated as a pertinent biomarker assessing specifically and reliably the exposure level to *Anopheles* bites [27-29] and/or the effectiveness of malaria vector control [30] in all age-classes of human populations (newborns, infants, children and adults) from several malaria epidemiological settings (rural, semi-urban and urban areas…) throughout sub-Saharan Africa countries (Senegal, Angola and Benin).

The present chapter contributes therefore to a better understanding of the human-mosquito immunological relationship. It resumes most of the studies highlighting the roles of mosquito saliva on the human physiology and immunology, approaches, techniques, and methods used to develop and validate specific candidate-biomarkers of exposure to *Anopheles* bites and their applications on malaria control in several different epidemiological settings. Effects of various explanatory variables (age, sex, seasonality, differential use of vector control…) on human antibody responses to *Anopheles* salivary antigens are also discussed in the aim to optimize their use in epidemiological and vector-borne disease (VBD) control studies. Finally, different ways of application of such salivary biomarker of exposure of *Anopheles* vector bites in the field of operational research by National Malaria Control Programmes (NMCP) are highlighted.

2. Human host-mosquito relationship: Roles of mosquito saliva

Arthropods represent the vast majority of described metazoan life forms throughout the world, with species' richness estimated between 5 to 10 million [31]. The blood feeding habit has arisen and evolved independently in more than 14,000 species from 400 genera in the arthropod taxonomy [32]. In mosquitoes, only the adult female is hematophagous, whereas both male and female take sugar meals [33]. During the probing and the feeding stages, like all blood-sucking arthropods, female *Anopheles* must circumvent the highly sophisticated barriers represented by human defense systems (Fig. 1): haemostatic and inflammatory reactions, innate and adaptive immune system defenses. Therefore, they express in their saliva potent pharmacological and immunogenic components.

2.1. Pharmacological properties of mosquito saliva

The first-line of the human host non-specific defense to the insect bite is the haemostatic reaction. It provides an immediate response to the vascular injury caused by the intrusion of

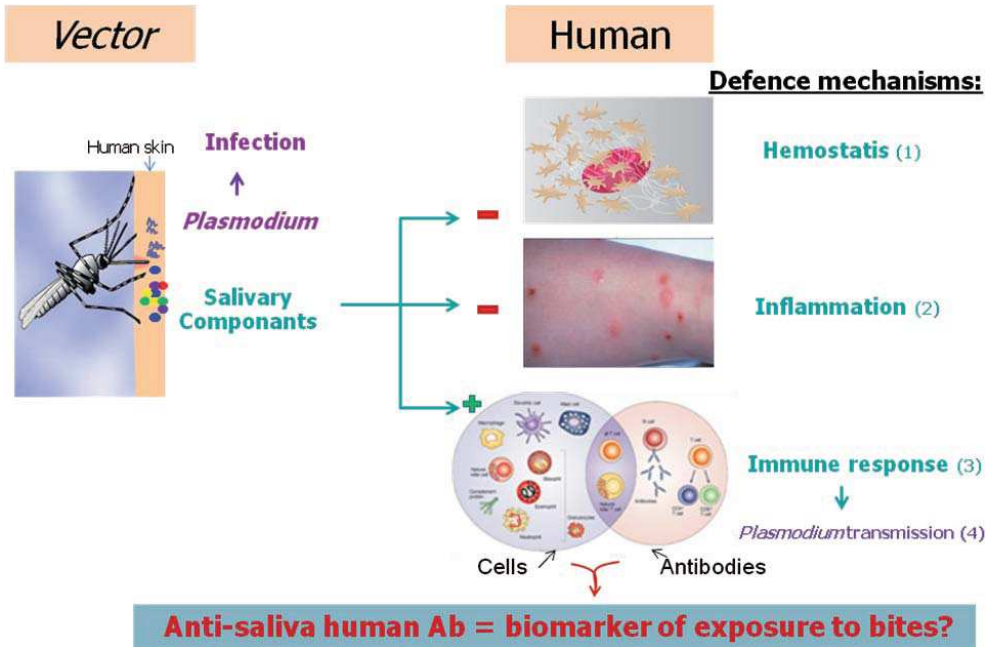

Figure 1. Effects of *Anopheles* saliva on hemostatic, inflammatory and immune reactions of the human to the vector bites.

the mosquito mouthparts in host vessels, thus preventing the extensive loss of host blood [32, 34]. The haemostatic reaction consists of three not physiologically distinct mechanisms: i) the blood coagulation that leads to the production of fibrin clots, ii) the thrombus formation and wound healing mediated by platelet aggregation, and iii) the vasoconstriction that leads to restricted influx of blood to the injured site. Each mechanism is activated by several pathways, in response to different exogenous and endogenous stimuli. Platelet aggregation is the first step in the haemostatic cascade and follows the interaction between blood platelets and the exposed extracellular matrix. This latter contains a large number of adhesive macromolecules such as collagen which is abundant underneath endothelial cells (not found in blood). This interaction results to the activation of platelets by mainly collagen and adenosine diphosphate (ADP, released by damaged cells and by activated platelets), the primary agonists of platelet aggregation. Platelets can be also activated by other agonists such as thrombin (produced by the coagulation cascade) and thromboxane A2 (TXA_2, produced by activated platelets) [35]. Activated platelets release endogeneous secretions such as serotonin and TXA_2, two potent vasoconstrictors. In parallel, the blood coagulation mechanism is getting underway. The main task of the coagulation cascade is to produce fibrin that supports aggregated platelets in a thrombus formation. The coagulation process consists of an enzymatic cascade with two ways of activation, the exogenous and the endogenous, where several amplification points and regulatory mechanisms are known.

However, mosquitoes can successfully engorge on their hosts within a half-minute because antihemostatic components of their saliva facilitate location of blood vessels and the blood sampling [36]. These salivary secretions, named sialogenins (from the Greek *sialo*, saliva; *gen*, origin, source; and *ins* for proteins), are mainly an array of potent anticoagulants, anti-platelets, vasodilators and anti-inflammatory substances [16, 32, 37, 38].

2.1.1. Inhibition of platelet aggregation

Compared to other blood-sucking arthropods like ticks and sand flies, only a limited number of *Anopheles* mosquito sialogenins involved in the inhibition of platelet aggregation have been characterized. *Apyrase* (Adenosine triphosphate (ATP)-diphosphohydrolase EC 3.6.1.5) is ubiquitous for hematophagous arthropods (mosquitoes, bugs, sand flies, fleas, triatomines, and ticks) and hydrolyses ATP and ADP into adenosine monophosphate (AMP) and inorganic phosphate (P_i), thus inhibiting platelet aggregation [16]. Three classes of apyrase have been characterized at the molecular level in different blood-sucking arthropods (reviewed by [39]). One named 5′-nucleotidase family is highly expressed in the salivary gland of *Anopheles gambiae* [40]. The *D7 protein family* is one of the most abundantly expressed sialogenins of mosquitoes. Two classes have been described in the saliva of mosquitoes: long (28–30 kDa) and short (15–20 kDa) forms [41-43]. The D7-related proteins may inhibit activation of host plasma. It has been described in *Anopheles* mosquitoes in a short form and may block the platelet activation by scavenging serotonin (agonist-positive feedback loop to increase platelet aggregation), while it principal function is reported to modulate tonus of vessels (vasoconstriction) [44]. *Anophelin* from *An. stephensi* saliva is a 30-kDa protein that directly binds to immobilized collagen and specifically inhibits collagen-induced platelet aggregation and the intracellular Ca^{2+} increase [45]. It can also act by inhibiting the activity of thrombin which plays a role in concentration of platelet aggregation [46].

2.1.2. Inhibition of blood coagulation cascade

Arthropod anticoagulants mostly target factor X-active (fXa), which plays a central role at the nexus of the intrinsic and extrinsic pathways, as well as an ultimate role of thrombin in driving production of fibrin from fibrinogen. However, *Anopheles* mosquitoes produce an anti-thrombin [38]. In *An. albimanus* for example, *Anophelin* protein has been shown to be a potent anticoagulant that acts as a specific and tight-binding thrombin inhibitor [46], blocking or delaying then the clot formation process until blood meal completion [34]. In addition, a D7-related protein of *An. stephensi* saliva has been characterized as an inhibitor of fXII [47].

2.1.3. Vasodilator effect on host blood vessels

In human, various types of endogenous vasoconstrictors (serotonin, TXA_2, noradrenalin…) are released few seconds after tissue injury in order to stop the blood flow locally at the bite site. Diverse types of vasodilators have been characterized in the saliva of hematophagous arthropods. *Aedes* mosquitoes use sialokinins that mimic the endogenous tachykinin substance P which stimulate the production of nitric oxide (NO), a potent dilator of blood vessels [48, 49]. In contrast, the saliva of the adult female *Anopheles* mosquito has been shown to contain

a myeloperoxidase with a vasodilator activity associated with a catechol oxidase/peroxidase activity [50]. This latter drives the H_2O_2-dependent destruction of noradrenalin and serotonin, two important endogenous vasoconstrictors [50]. In addition, some D7 proteins of *Anopheles* have been described to bind to biogenic amines such as serotonin, histamine, and norepinephrine [44]. These strategies remove the human host's ability to maintain vascular tone at the bite site, resulting to a weak but persistent local vasodilatation [14].

2.2. Immunological effects of mosquito saliva

The tissue injury causes an immediate onset of acute inflammation and innate immunity, which promote tissue repair, prevent colonization of the damaged tissues by opportunistic pathogens and initiates adaptive immunity, which is more specific [51]. These responses mobilize multiple elements such as phagocytes and antigen-presenting cells, cytokine-producing cells, T and B lymphocytes (TL and BL) and complement (classical and alternative pathways). It may result to the development of strong cell and humoral immune reactions, thereby altering physiologically the environment at the bite site and leading to the rejection of the blood-sucker [52]. The saliva of *Anopheles* mosquitoes (like blood-feeding arthropods in general) has selected, during evolution, compounds that can counter these host responses by modulating immune cells and cytokines' production [52, 53]. This certainly allows mosquitoes to complete successfully a blood meal in only few seconds. Immunomodulatory effects of *Anopheles* mosquito saliva can therefore affect the transmission of pathogens and the development of associated pathologies [54]. Understanding the mechanisms which govern this immunomodulation could then allow the development of new prevention tools or strategies against malaria transmission [54-56].

2.2.1. Inhibition of host inflammatory reaction

The host inflammatory reaction following tissue injury consists of the triple response of Lewis: redness, heat and pain, triggering the awareness of the host to the blood sucker action [16]. If redness and heat are ones of the direct consequences of the dilatation of blood vessels, pain is induced by an increased vascular permeability under the effect of ADP, serotonin and histamine released by platelets and mast cells, following activation of the fXII by tissue-exposed collagen [16]. The fXIIa converts prekallikrein to kallikrein, which hydrolyzes blood kininogen to produce the vasodilator peptide, bradykinin. This latter induces TNF-α (Tumor Necrosis Factor alpha) release by neutrophils [57], which in turn stimulates the release of IL (interleukin)-1β and IL-6 from various cell types. These cytokines contribute to the phenomenon of hyperalgesia (increased sensitivity to pain) that accompanies inflammation. Host inflammatory reaction to bites has been described as mast cells-dependent in individuals bitten by *Anopheles* mosquitoes [58]. In contrast to ticks which need to be attached to their host for several hours (tick *Argasidæ*) or weeks (tick *Ixodidæ*), mosquitoes take just few seconds for a successful blood meal. This certainly explains the poverty of anti-inflammatory components in their saliva in contrast to the ticks' one. Nevertheless, some salivary components of *Anopheles* mosquitoes can inhibit the human inflammatory reaction. In particular, a 16kDa D7 family proteins of *An. stephensi* (Hamadarin) inhibits the contact

system by preventing the mutual activation between the fXIIa and the kallikrein in the presence of Zn^{2+} [47].

2.2.2. Modulation of host immune response

A role for arthropod saliva in modifying the outcome of transmission and infection is not a novel idea introduced in the context of mosquitoes and malaria parasites. The increased pathogen infectivity in association with ticks, sand flies, and mosquitoes saliva has been described previously [54]. If ticks that take a long time to engorge must additionally necessitate in their saliva anti-inflammatory and immunosuppressive factors, rapidly feeding dipterans, in particular mosquitoes and sand flies, clearly have evolved salivary factors that directly modulate host immune defenses [52]. One possible explanation is that these molecules have evolved because they have long-term beneficial effects for the populations rather than to the individual at the time of feeding [24]. Although the molecular mechanisms by which mosquito saliva induces alteration of the host immune response are unclear [59, 60], data evidently demonstrate that effects depend on the global regulation of the Th1/Th2 cytokines' balance, as it has been described in sand flies/*Leishmania* model, the most studied striking host-parasite vector system [61]. The Th1 response has been described to lead to a protective immunity and the resistance of the host to intracellular pathogens, while the Th2 response might favor the survivor of pathogens (parasites, virus…) and then the disease transmission and evolution [24]. For mosquitoes, studies have globally shown an enhancement of transmission and disease when pathogens are introduced in the presence of vector saliva. Mosquito saliva is commonly associated with a downregulation of the expression of Th1 and an upregulation of the Th2-type cytokines. In mouse models, mosquito saliva can potentiate the infection of arboviruses [24, 62, 63]. The co-inoculation of Sindbis virus with *Aedes aegypti* salivary gland extract resulted on a reduced interferon- gamma (IFN-γ) expression, when compared to injection of virus alone [64]. It has been also shown that *Ae. aegypti* saliva contains multiple factors that can affect various components of the host immune response [65]. For example, factor Xa inhibitor may inhibit complement activation and leukocyte migration to the bite site [24] and other factors inhibit TNF-α release from activated mast cells [66]. Chickens subcutaneously infected with *P. gallinaceum* sporozoites in the presence of *Aedes fluviatillis* salivary gland homogenates showed a higher level of parasitaemia when compared to those that received only sporozoites [67]. For *Anopheles*, mice exposed to mosquito feeding in tandem with the inoculation of sporozoites had higher parasitemia and an elevated progression to cerebral malaria. This was associated with, in particular, elevated levels of IL-4 and IL-10, suppression of overall transcription in response to infection, and decreased mobility of dendritic cells and monocytes [19]. It was also described that *Anopheles stephensi* saliva downregulates specific antibody (Ab) immune responses by a mechanism that is mast cell and IL-10 -dependent [60]. IL-10, by inhibiting pro-inflammatory and Th1 cytokines, stimulates certain T, mast and B cells and has pleiotropic effects in immunoregulation and inflammation, while IL-4 is the prototypical Th2 cytokine (it differentiates $CD4^+$ T-cells and up-regulates MHC class II production). The enhancement of IL-10 expression could account for reduction in secretion of other cytokines because it inhibits antigen presentation, IFN–γ expression, and macrophage activation [68]. However, some

data have suggested a paradoxical protective role of mosquito saliva against pathogen transmission and disease infection. *Ae. aegypti* saliva can inhibit infection of dendritic cells by dengue virus, and the pre-sensitization of dendritic cells with saliva prior to infection enhanced this inhibition. Moreover, the proportion of dead cells was also reduced in virus-infected dendritic cell cultures exposed to mosquito saliva, and an enhanced production of IL-12 and TNF-α was detected in these cultures [69]. In addition to these effects on cellular immunity, *Anopheles* saliva can also acts on humoral host immune response. Indeed, specific antibodies (immunoglobulins [Ig] G, M and E) to salivary antigens have been described in several studies [20, 22, 23, 25, 56, 70]. However, the implication of these Ab responses in disease pathogenesis or protection is not yet elucidated.

Therefore, future studies are needed for an overall understanding of mosquito saliva effect, especially *Anopheles* mosquito saliva, in pathogen transmission, disease development and pathogenesis.

*2.2.3. Human host-*Anopheles *vector immune relationship and applications*

The study of immunological properties of salivary proteins of *Anopheles* mosquitoes represents a new research thematic which can significantly improve the understanding of *Plasmodium* transmission mechanisms and therefore help for the effective prevention and control of malaria. It can notably lead to major applications in three areas: i) development of vaccines, diagnosis, treatment, ii) prevention of allergies, and iii) development of biomarkers of exposure to bites and malaria disease risk.

The development of parasite transmission-blocking vaccines, by stimulating the immune response against the vector is an attractive alternative way for malaria control. Several studies targeted the effect of Abs specific to the mosquito midgut antigens have shown promising results [71-73]. The study of the immune response induced by vector saliva at the biting site and its potential effect on the transmission and the development of pathogens suggests the possibility to control parasite transmission by vaccinating the host with immunogenic salivary compounds [54, 74]. In a mouse model, it has been shown that two salivary proteins (29 and 100 kDa) of the female *An. gambiae* can induce production of Ab which can block about 75% of the invasion of *An. stephensi* salivary glands by *P. yoelii* sporozoites [75]. In addition, the prior exposition to non infective *An. stephensi* bites induces a Th1 immune response with increased production of IL-12 and IFN-γ. Its effect can subsequently limit future *P. yoelii* infection (reduced rate of liver and blood parasites) and the development of cerebral malaria in mouse [18]. In this context, saliva can be thought as a non-specific "adjuvant" which could be effective at inducing a Th1-biased environment that is known to be protective against malaria infection. However, the development of such vaccines is complex. For example, Ab produced by immunization (with salivary proteins) must be ingested by the mosquito during a bite, cross it midgut and digestive enzymes, migrate to the salivary glands, before they can block the invasion by sporozoites. Nevertheless, the possibility to develop a pan-arthropod vaccine has been recently demonstrated by another mechanism. Indeed, an immune response directed to salivary proteins that adsorb to pathogens can turn the microorganism into an innocent bystander of anti-salivary immunity as it has been recently reported in a salivary

protein (Salp15) from the hard tick *Ixodes scapularis* [76] and vaccine candidate for the control of Lyme disease [77]. Unfortunately, any hematophagous arthropod saliva-based vaccine has not yet been tested on humans.

In the field of allergic reactions to salivary proteins of mosquitoes, the first studies were mainly conducted in Canada and Finland. They concerned *Aedes* and *Culex* mosquitoes which express a panel of allergens in their saliva during the blood feeding time [17, 56, 78]. These proteins can thus be used in recombinant form, as diagnostic tool of the level of human exposure to allergens or in immunotherapy injections for desensitization of human [56, 70, 79]. It exists yet no study highlighting the presence and effect of allergens in the *Anopheles* mosquitoes' saliva.

The study of immunological relationship between human-vector by quantifying specific Ab responses to salivary proteins may also allow the identification and characterization of biological markers for epidemiological assessment of the exposure of individuals and populations to the *Anopheles* bites and thus to the risk of malaria transmission [22]. The development of such biomarkers or indicators (see next chapter) can be a complementary alternative to current referent entomological and parasitological methods which present several limitations especially in low exposure/transmission contexts.

3. Development of biomarkers of human exposure to *Anopheles* bites and indicators of malaria vector control effectiveness

3.1. Validation of concept with whole *Anopheles* saliva

To improve the fight against malaria and regarding numerous limitations described with current entomological and parasitological tools, the World Health Organization (WHO) has emphasized the need of new indicators and methods to evaluate, at individual and population levels, the exposure level to *Anopheles* vectors and the effectiveness of vector control strategies. One promising concept is based on the fact that mosquito saliva injected to the human host during the vector bite is antigenic and can induce an adaptive humoral host response (see Figure 1). Therefore, a logical positive correlation between the human exposure level to *Anopheles* bites and human anti-mosquito saliva Ab level can be expected. In this way, anti-mosquito saliva Ab response can be a pertinent epidemiological biomarker of human exposure to vector bites.

The epidemiological importance of human exposure to the saliva of vectors has been firstly described in Lyme disease [80, 81], leishmaniasis [82] and Chagas disease [83]. During the last decade, studies have provided data on human exposure to anopheline saliva and its interaction with malaria transmission. In particular, Remoue *et al.* [22] have shown that children living in a seasonal malaria transmission region of Senegal developed IgG responses to *An. gambiae* whole saliva (WS). Interestingly, these specific IgG levels were positively associated with an increased rainfall and the *Anopheles* mosquito density, measured by referent entomological methods. Indeed, an increase in the level of IgG was observed according to the *Anopheles* aggressiveness and density in September (Figure 2), the peak of malaria transmission.

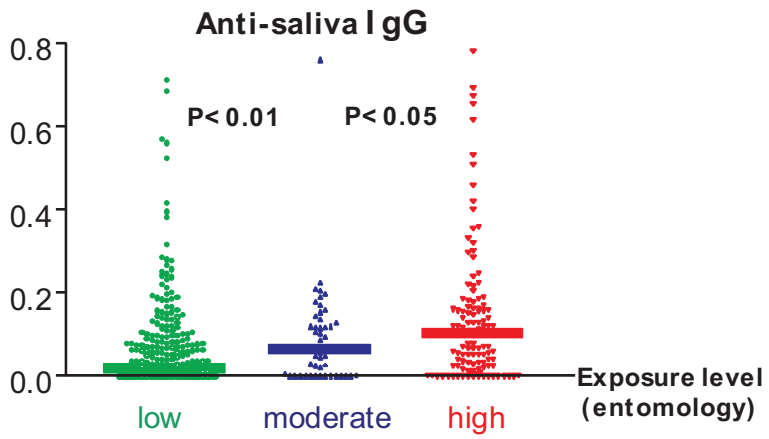

Figure 2. Anti-saliva IgG according to the intensity of exposure [22]. Individual absorbance (OD) values in September are shown for the three groups with different levels of exposure. Bars indicate the median value for each group. Statistical significances between each group by non-parametric Mann–Whitney *U*-test are indicated.

Importantly, IgG response to *An. gambiae* WS can predict clinical malaria cases. Indeed, children who developed a malaria attack in December had higher levels of anti-WS IgG in September of the same year, i.e. three months before they develop the disease (Figure 3) [22].

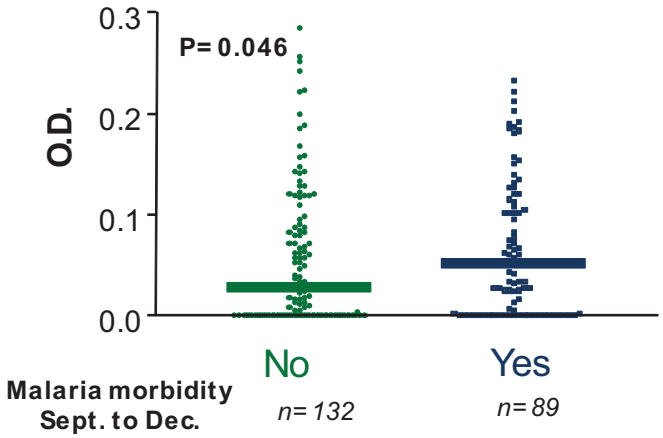

Figure 3. Anti-salivary IgG according to malaria morbidity. The results of individual absorbance (OD) values in September are shown according to subsequent detection of clinical malaria for the age ≥1 year. Bars indicate the median value for each group. Statistical significance between groups is indicated by a non-parametric Mann–Whitney *U*-test).

Anti-mosquito saliva Ab appeared transitional. Soldier travelers transiently exposed to *An. gambiae* bites in endemic areas of Africa (especially Ivory Coast and Gabon) developed specific

IgG responses to anti-*An. gambiae* WS which strongly decreased several weeks after the end of their trip [21]. In addition, anti-*An. gambiae* saliva IgG levels waned rapidly after 6 weeks of Insecticide-Treated Nets (ITNs) well-use in a semi-urban population in Angola, before a new significant increase two months later following the stop of ITN use [84]. Data on human exposure to anopheline saliva and its interaction with malaria were also provided by studies from other none African areas. In South-eastern Asia, it has been described that anti-*An. dirus* salivary protein Ab occur predominantly in patients with acute *P. falciparum* or *P. vivax* malaria; people from non-endemic areas do not carry such Abs [23]. In the Americas, the presence of anti-*Anopheles* saliva Ab has been also described. In adult volunteers from Brazil, anti-*An. darlingi* WS Ab levels increased with *P. vivax* infections [20]. The presence of anti-*An. albimanus* WS Ab with exposure to mosquito bite has been recently described in Haiti [25]. Specific IgG response to *An. gambiae* WS has also been described as an immunological indicator evaluating the efficacy of malaria vector control strategies. Indeed, Drame *et al*. have recently shown in a semi-urban area (Lobito, Provence Benguela) in Angola that specific IgG levels drastically decreased after the introduction of ITNs and this was associated with a drop in parasite load (Figure 4) [84].

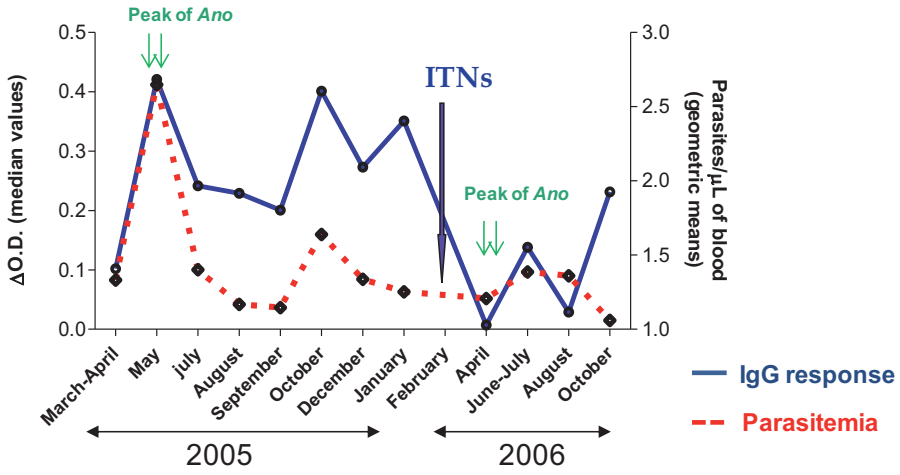

Figure 4. Evolution of anti-*Anopheles* gambiae saliva IgG and *Plasmodium falciparum* infections before and after ITN implementation, (Ano=*Anopheles*).

Anti-*Anopheles* saliva IgG response has also been recently used to evaluate and compare the effectiveness of three malaria vector control strategies in another area (Balombo) of Angola [85]. Indeed, Brosseau *et al.* [85] have investigated over a period of two years (2008-2009) Ab response to *An. gambiae* WS in children between 2 to 9 years old, before and after the introduction of three different malaria vector control methods: deltamethrin treated long lasting impregnated nets (LLIN) and insecticide treated plastic sheeting (ITPS) - Zero Fly®) (ITPS-ZF), deltamethrin impregnated Durable (Wall) Lining (ITPS-DL - Zerovector®) alone, and indoor residual spraying

(IRS) with lambdacyhalothrin alone. They observed considerable decreases in entomological (82.4%), parasitological (54.8%) and immunological criteria analyzed. In particular, the immunological data based on the level of anti-saliva IgG Ab in children of all villages significantly dropped from 2008 to 2009, especially with LLIN+ZF and with IRS (Figure 5).

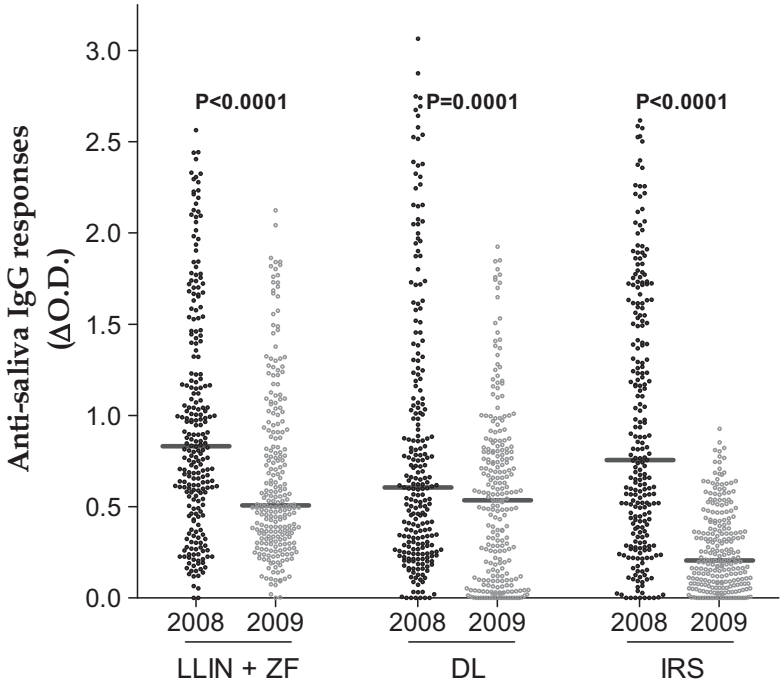

Figure 5. Comparison of median values of the IgG antibody response to *Anopheles* saliva obtained before and after implementation of each vector control method [85].

Taken together, these studies indicated that the estimation of human IgG Ab responses specific to *Anopheles* WS could provide a reliable biomarker for evaluating the *Anopheles* exposure level, the risk of malaria transmission, the disease outcomes and the effectiveness of vector control strategies. However, the pertinence and the practical large-scale application of serological tests for epidemiological purposes have been hampered by several limitations. First, WS is a cocktail of various molecular components with different nature and biological functions. Some components are *Anopheles*-specific and other widely distributed within genus, families, orders or classes of bloodsucking *Diptera* or Arthropods [16]. Therefore, the evaluation of *Anopheles* exposure or vector control effectiveness based on the immunogenicity of WS could be skewed and over or underestimated by possible cross-reactivities between common epitopes between mosquito species or other organisms [26]. Second, the collection of saliva or salivary gland extracts is tedious and time-consuming; therefore it will be difficult or impossible to have an adequate production of mosquito saliva needed for large-scale epidemiological studies [26].

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Third, saliva composition can be affected by several ecological parameters such as age, feeding status or infectivity of *Anopheles* [86],which in turn may influence the anti-saliva immune response measured and may cause a lack of reproducibility between saliva batches. An alternative for optimizing the specificity of this immunological test would thus be to identify *Anopheles* genus-specific proteins [87].

3.2. Methods for the identification of specific *Anopheles* salivary proteins

The isolation of salivary components has been a challenge for many years. Many functional active salivary proteins have been isolated following classical biochemical and molecular biology approaches [88]. Protocols mainly consisted of the isolation of salivary components from hundreds of salivary gland pairs, obtaining amino-terminal or internal peptide sequence of the purified component, screening of a salivary gland library with the information obtained, and isolation of the cDNA or gene of interest (Fig. 6).

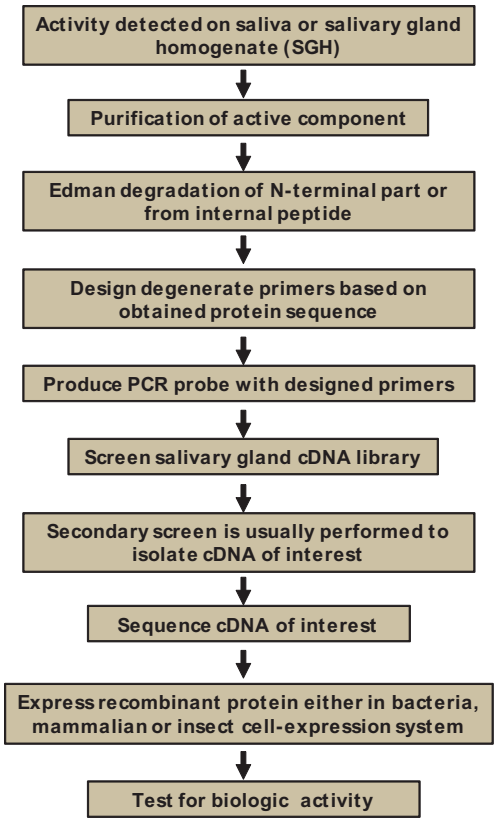

Figure 6. Classical biochemical and molecular biology protocol used for isolation and characterisation of salivary proteins and cDNA from vectors of disease [90].

During the last decade, technical advances in molecular biology have allowed the sequencing of the genome, including transcripts of salivary glands [89], of most disease vectors, comprising *Anopheles* mosquitoes [90]. However, protocols do not allow to obtain entire sequences [89]. Nowadays, researchers have switched from testing one salivary molecule at a time to studying the whole complex of genes and secreted proteins in blood-feeding arthropods using transcriptomic and/or proteomic approaches. The transcriptomic is the complete set of transcripts in an organism for a specific developmental stage or physiological condition. Transcriptomic techniques help to interpret the functional elements of the genome, and to understand the transmission and development of diseases [91]. They aim to catalogue transcript of major *Anopheles* species, including mRNAs, non-coding RNAs and small RNAs; to determine the transcriptional structure of genes and to quantify the changing expression levels of each transcript during development and under different conditions [91]. Proteomic is a large-scale study of the gene expression at the protein level, which ultimately provides direct measurement of protein expression levels [92]. The proteomic revolution is hitting the vector biology field as well as many other fields. The isolation and sequencing of all the proteins from SGs of disease vectors and, more specifically, secreted salivary proteins, is clarifying the complexity of proteins present in the saliva of various blood-feeding arthropods [93]. During the last years, a comprehensive high-throughput approach has been developed (Figure 7) [88]. It combines massive sequencing protocol of high quality full-length salivary gland cDNA libraries, a proteomic approach to isolate a large set of salivary proteins, and high-throughput computational biology and functional assays to analyze and test the biologic activities of these novel molecules. It is a powerful tool which can help easily and rapidly to identify and characterize genes or transcripts encoding for various proteins of SGs (the sialome) of blood-sucking arthropods. This high-throughput approach has then allowed an unprecedented insight into the complexity of salivary gland compounds of mosquito vectors of disease agents, indicating that the diversity of their targets is still larger than previously thought [16].

3.3. Salivary proteins (sialome) of *Anopheles* mosquitoes

The increasing power of large-scale genomic, transcriptomic and proteomic analyses allowed the accumulation of a considerable amount of information on the salivary secretions of blood-sucking arthropods [86]. As far as mosquitoes are concerned, the analysis of salivary transcriptomes of a number of *Anopheles* have allowed the discovery of a variety of genes that matched the sequence of various protein families, providing some clues on the evolution of blood feeding [15, 41-43, 92, 94-100]. Many of the salivary protein sequences are coded by genes related to intrinsic functions of the cell (housekeeping genes). However, the large number of salivary proteins is secreted during plant or blood feeding. Finally, a little number has no similarities to sequences deposited in databases, representing unknown and novel sequences [41, 94, 101]. This emphasizes how much still need to be learned concerning the biological functions of salivary proteins in blood feeding, pathogen transmission and manipulation of host responses.

The analysis of the adult *Anopheles* sialome has shown that secreted proteins and/or peptides (secretome) can be ubiquitous or specific to arthropod classes, orders, families, genus or species

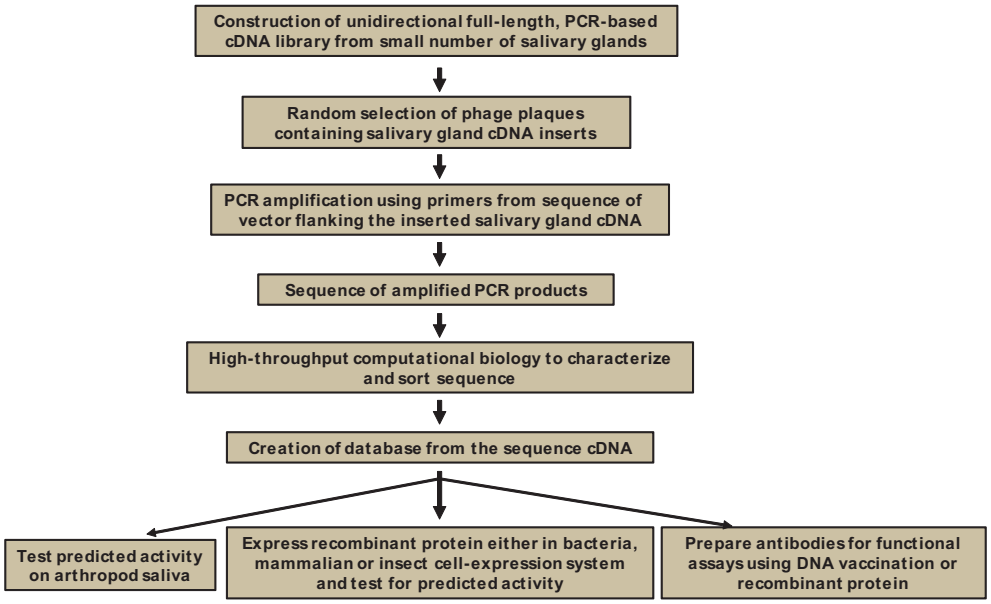

Figure 7. Current high-throughput strategies used for the isolation and characterisation of salivary cDNA and proteins from disease vectors [90].

[44, 101, 102]. In *An. gambiae* salivary gland females over 70 putative secreted salivary proteins have been identified [94].

3.3.1. Ubiquitous salivary proteins

AG5 family proteins are found in the salivary glands of many blood-sucking insects and ticks [102, 103]. In *An. gambiae*, four proteins belonging to this family were identified, but only one (putative gVAG protein precursor) was coding for transcripts enriched in the adult female SGs [94]. A precursor of gVAG protein was also described in *An. funestus* (84% sequence identity) and *An. stephensi* (85% sequence identity) sialome [95, 100]. The function of any AG5 protein in the saliva of any blood-sucking arthropod is still unknown.

Enzymes such as maltase, apyrase, 5′ nucleotidase, and adenosine deaminase, are also secreted during the bite of many blood-sucking arthropods, including *Anopheles* mosquitoes [95]. They generally assist in sugar feeding (maltase) or in degradation of purinergic mediators of platelet aggregation (apyrase, 5′ nucleotidases) and inflammation (adenosine deaminase).

3.3.2. Salivary proteins found exclusively in Diptera

D7 family proteins are specific to SGs of blood-sucking Nematocera, including mosquitoes and sand flies [104, 105]. They are highly represented in the sialome of *Anopheles* mosquitoes in short and long forms [95, 96, 101, 104, 105]. *An. funestus* D7 proteins vary between 64% and

75% identity with their *An. gambiae* closest match [105]. D7 proteins could act as anti-hemostatic factors by trapping agonists of hemostasis [44, 47]. However, further investigations are needed to clearly describe their function.

Other Diptera-specific protein families or peptides have also been described in the sialome of blood-feeding mosquitoes [95]. However their function is still unknown, even if some were known to play a role in antimicrobial property of mosquito saliva.

3.3.3. Protein families found exclusively in mosquitoes

The 30-kDa antigen family found exclusively in the SGs of adult female mosquitoes has been found in both culicine and anopheline mosquitoes [95, 100, 101, 106-108]. Only one gene enriched in SGs of adult females is known in *An. gambiae*. The *An. funestus* homologue is also abundantly expressed and shares 63% identity with the *An. gambiae* orthologue. The function of this protein family is still unknown [95].

The *gSG (An. gambiae Salivary Gland)-5 family* was first discovered in the SGs of *An. gambiae* and shown to be exclusively expressed in the adult female [94, 109]. This protein shows a high similarity to *Aedes* and *Culex* proteins [101]. Transcripts coding for this family were found in the sialotranscriptome of *An. darlingi* with 46% identical to the *An. gambiae* orthologue and only 26% and 23% identical to the culicine proteins [101]. The function of this mosquito-specific protein remains unknown, but its tissue- and sex-specific expression profile suggests it is possibly related to blood feeding.

The *gSG8 family* is highly divergent with members only found in *An. gambiae* and *Ae. aegypti*. In *An. gambiae*, this protein is specifically expressed in female SGs [109], suggesting a likely role in blood feeding.

Various types of *mucins* have been described in the saliva of adult mosquitoes and may function/act as a lubricant of their mouthparts [15, 41, 94, 102]. Three mucins encoding transcripts have been identified in the *An. gambiae* larval SG [110], suggesting the importance of mucins at multiple developmental stages. Mucins may also play a crucial role in *Anopheles* salivary gland invasion by *P. berghei* sporozoites [111]. Several protein families are also represented in this group, including gSG-3, gSG-10, and 13.5-kDa families [101]. These families were also found abundantly expressed in the sialotranscriptome of *An. gambiae* adult male [112], indicating their function is not related specifically to blood feeding.

3.3.4. Protein families found exclusively in Anophelines

Anophelin was described as a short acidic peptide with strong thrombin inhibitory activity in *An. albimanus* [46]. *An. funestus* anophelin is 59% identical to the *An. gambiae* orthologue [95], and *An. darlingi* anophelin is 86% identical to *An. albimanus* [101].

The 8.2-kDa family is represented in several *Anopheles* species. In *An. funestus* the peptide have 42% identity with the 8.2-kDa salivary peptide of *An. stephensi* and similar proteins from *An. gambiae* and *An. darlingi* [95]. In *An. gambiae*, this peptide was found enriched in adult female SGs, suggesting a role in blood feeding.

The 6.2-kDa family was first described in a sialotranscriptome of *An. gambiae* [94], where it was found enriched in adult female SGs compared to other tissues. The *An. funestus* member of this family is 61% identical to the *An. gambiae* [95], and 53% to an *An. darlingi* [101] homologues.

The SG-1 family proteins appear to be exclusively expressed in the female SGs of *Anopheles* mosquitoes and not observed in other tissues [94, 101]. However, their function remains to be determined.

The SG-2 family proteins were identified from *An. gambiae* saliva and shown to be expressed in female SGs and adult males but not in other tissues [113]. Related, but very divergent, sequences were obtained from salivary transcriptomes of other anopheline species [95, 101]. Because this protein family is expressed in both male and female *An. gambiae*, and due to its relatively small size, it may display antimicrobial function [101].

The *hyp 8.2* and *hyp 6.2 proteins* are similarly enriched in *An. gambiae* adult female SGs [94]. *An. stephensi* and *An. funestus* also have members of these protein families.

The *SG-7/Anophensin family* is also unique to anophelines. In *An. gambiae*, it is highly enriched in female SGs [94]. More recently, the *An. stephensi* homologue was determined to inhibit kallikrein and production of bradykinin, a pain-producing substance [114]. Four putative alleles representing the homologue(s) of gSG7 in *An. darlingi* were identified. These *An. darlingi* transcripts have no more than 45% identity to the *An. gambiae* gSG7 and *An. stephensi* anophensin [101].

The *SG6 protein* is a small protein first described in *An. gambiae* [109] and a unique sequence codes for a mature peptide/protein of ~10 kDa (116 amino-acids) with ten cysteine residues making probably five disulphide bonds. A homologue was later found in the sialotranscriptome of *An. stephensi* [100] and *An. funestus* [95]. *An. funestus* SG6/fSG6 (f for funestus) has 81% and 76% identities with *An. stephensi* and *An. gambiae* polypeptides, respectively. It is not found in the transcriptomes of the Culicinae subfamily members analyzed so far, i.e. *C. pipiens quinquefasciatus*, *Ae. aegypti* and *Ae. albopictus* [108, 115, 116]. In *An. gambiae*, the transcript coding for gSG6 (g for *gambiae*) was found to be 16 times more expressed in SGs of adult females than in males [94]. The gSG6 protein plays some essential blood feeding role and was recruited in the anopheline subfamily most probably after the separation of the lineage which gave origin to *Cellia* and *Anopheles* subgenera [99]. The gSG6 protein, because immunogenic, can be therefore a reliable indicator of human exposure specific to *Anopheles* mosquito bites [99], vectors of malaria.

3.4. Specific salivary biomarker of exposure to *Anopheles* bites: The gSG6-P1 peptide candidate

The SG6 salivary protein has been reported to be immunogenic in travelers exposed for short periods to *Anopheles* bites [21], and in Senegalese children living in a malaria endemic area by an immuno-proteomic, coupling 2D immunoblot and mass spectrometry [117], and by an ELISA [26] approaches. Recently, its immunogenicity has been confirmed in individuals from a malaria hyperendemic area of Burkina Faso [118, 119], by using a recombinant form expressed as purified N-terminal His-tagged recombinant protein in the *E. coli* vector pET28b(+) (Novagen) [99, 119].

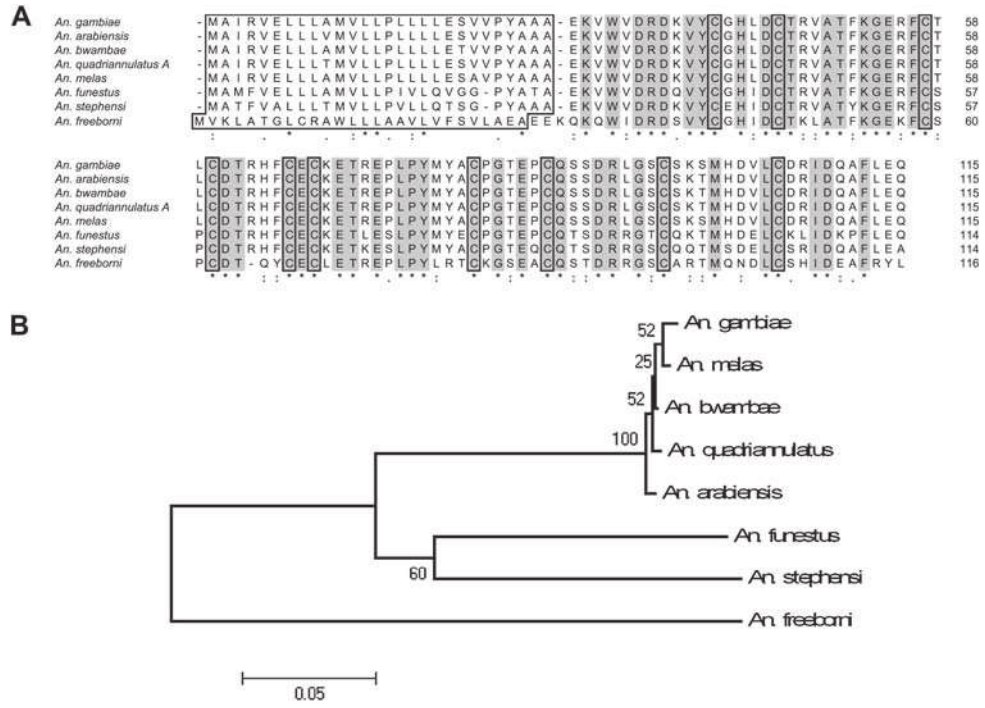

Figure 8. Sequences of the anopheline gSG6 proteins [99]. (A) Clustal alignment of anopheline gSG6 proteins. Signal peptides and conserved Cysteines are boxed. Conserved sites are shaded. (B) Phylogenetic tree (NJ algorithm, bootstrapped 10,000 times) constructed from the alignment of the nucleotide sequence encoding the mature gSG6 polypeptides.

In particular, increased anti-gSG6 IgG levels were observed in exposed individuals during the malaria transmission/rainy season [119]. In addition, anti-gSG6 IgG response appeared to be a reliable serological indicator of exposure to bites of the main African malaria vectors (*An. gambiae*, *An. arabiensis* and *An. funestus*) in the same area [119]. However, gSG6 recombinant protein has been described to relatively generate a high background in control sera from individuals not exposed to *Anopheles* bites, and considerable variations in specific Ab response between children supposed to be similarly exposed to *Anopheles* bites [26]. Therefore, with the objective of optimizing *Anopheles* specificity and reproducibility of the immunological assay, a peptide design approach was undertaken using bioinformatic tools [26].

3.4.1. Identification and sequence of gSG6-P1 peptide

Several algorithms were employed for prediction of potential immunogenic sites of *the* gSG6 protein by using bioinformatics. The prediction of immunogenicity was based on the determination of physico-chemical properties of the amino-acid (AA) sequences with BcePred and FIMM databases and on the identification of MHC class 2 binding regions using the ProPred-2 online service. This led to define five gSG6 peptides (gSG6-P1 to gSG6-P5) of 20 to 27 AA

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residues in length (Fig. 9), overlapping by at least 3 residues and spanning the entire sequence of the mature gSG6 protein. Both predictive methods for putative linear B-cell epitopes (FIMM and BcePred) assigned the highest immunogenicity to gSG6-P1, gSG6-P2, gSG6-P3, and then gSG6-P4.

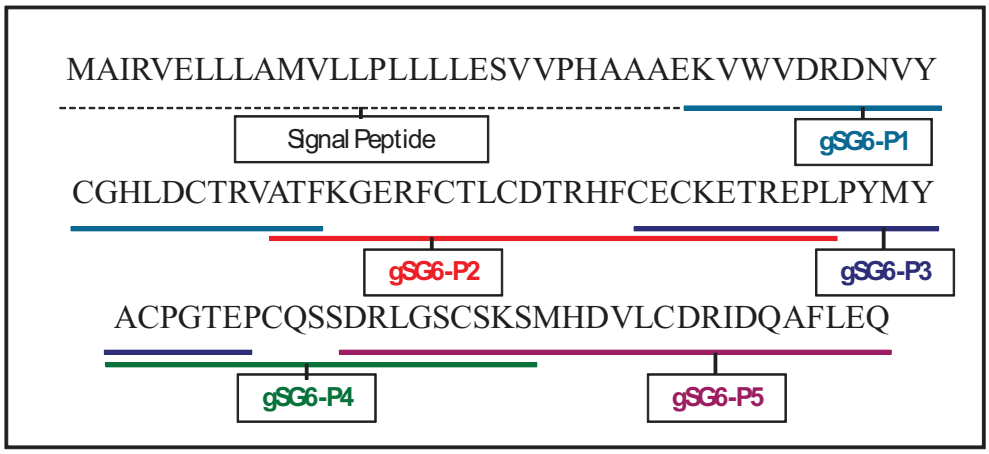

Figure 9. Amino-acid sequence of gSG6 Peptides. Amino-acid sequence of the SG6 protein of *Anopheles gambiae* (gi: 13537666) is presented and sequences of the selected peptides, gSG6-P1 to gSG6-P5, are underlined. Signal peptide (SP) sequence is indicating by dotted underline [26].

Similarities were also searched using the Blast family programs, including both the genome/EST libraries of other vector arthropods available in Vectorbase and of pathogens/ organisms in non-redundant GenBank CDS databases. No relevant identity was found with proteins of other blood-sucking arthropods. Indeed, the longest perfect match was 6 AAs between a putative protein from *Pediculus humanus* and gSG6-P2 and gSG6-P3 peptides. In the case of gSG6-P1, the best match was 4 AAs in length with *Culex pipiens quinquefasciatus* salivary adenosine deaminase. Moreover, no relevant similarity was found with sequences from pathogens or other organisms. The highest hits of gSG6-P1 were with the cyanobacterium *Microcystis aeruginosa* (3 AAs) and with *Ostreococcus* OsV5 virus (4 AAs). Altogether, this analysis confirmed the *bona fide* high specificity of the five selected gSG6 peptides for the *Anopheles* species. Peptides were then synthesized.

3.4.2. Antigenicity of gSG6 peptides

IgG Ab responses to the five gSG6 peptides were evaluated by ELISA in a randomly selected subsample of children (n<30) living in a rural area of Senegal. All peptides were immunogenic, but the intensity of the IgG level was clearly peptide-dependent; weak immunogenicity was observed for gSG6-P3, gSG6-P4 and gSG6-P5, whereas gSG6-P1 and gSG6-P2 appeared highly immunogenic (Fig. 10).

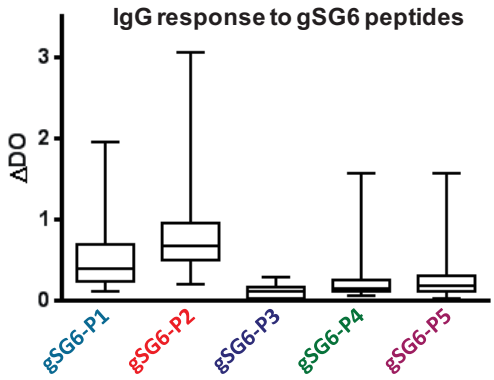

Figure 10. IgG antibody response according to gSG6 peptides [26]. For each peptide, the IgG Ab level was evaluated in a subsample of exposed children. Results at the peak of the season of *Anopheles* exposure are reported according to gSG6 peptides. Results are presented by box plot graph where lines of the boxes represent the 75th percentile, median and 25th percentile of individual average ΔOD values; whiskers represent the lower and upper adjacent values.

3.4.3. Validation as a biomarker of exposure in several epidemiological settings

The specific IgG level to the two most antigenic gSG6 peptides (gSG6-P1 et gSG6-P2) was then evaluated according to the level of exposure (estimated by entomological data) in a larger sample (n=241) of children living in a malaria seasonal area [26]. A positive trend was found for both peptides, but only significant for gSG6-P1 (Figure 11). Altogether, these results indicated that only the IgG response to gSG6-P1 is suitable to be a pertinent biomarker of exposure to *Anopheles* bites and thus to risk of malaria.

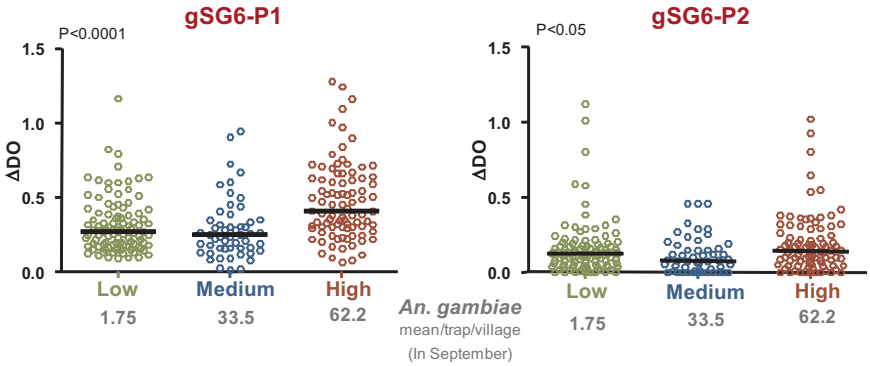

Figure 11. IgG response to gSG6-P1 and gSG6-P2 according to intensity of exposure to *Anopheles* gambiae bites [26]. Individual ΔOD (Optical Density) values in September (peak of the season of *Anopheles* exposure) are shown for the three different exposure groups. Results are presented for the same children (n=241) for gSG6-P1 (A) and gSG6-P2 (B). Exposure groups were defined by entomological data. Bars indicate median value for each exposure group. Statistical significance between the 3 groups is indicated (non-parametric Mann-Whitney U-test).

Therefore, the gSG6-P1 was selected as the most pertinent candidate as marker of exposure. Indeed, this peptide appeared to satisfy several requirements that an exposure biomarker should fulfill. First, it thus far appears to be specific to *Anopheles* genus and therefore, no relevant cross-reactivity phenomena with epitopes from other proteins of arthropods or pathogens would be expected. Second, because it is of a synthetic nature, it guarantees high reproducibility of the immunological assay. Third, it elicits a specific Ab response which correlates well with the level of exposure to *An. gambiae* bites.

3.4.3.1. Biomarker of Anopheles *vector bites*

As previously suggested, anti-gSG6-P1 IgG response was described as a biomarker of *An. gambiae* bites in children living in Senegalese villages where malaria transmission seasonally and moderately occurred [26]. In the same area, a specific IgG response to the peptide has been detected in 36% of children living in villages where very few *An. gambiae,* or none, were collected by classical entomological methods [28]. This deals with a high sensitivity and specificity of the gSG6-P1 epitope(s) after a low immunological boost induced by weak bites exposure. This result points to the potential use of such serological tool as an epidemiological biomarker of *An. gambiae* bites in very low exposure areas, where the sensitivity of current entomological methods of malaria risk assessment is weak.

One study aimed to evaluate the risk of malaria transmission in children and adults living in urban area of Senegal (Dakar region) by using the gSG6-P1 peptide biomarker. Results showed considerable individual variations in anti-gSG6-P1 IgG levels between and within districts, in spite of a context of a global low *Anopheles* exposure level and malaria transmission [27]. Despite this individual heterogeneity, the median level of specific IgG and the percentage of immune responders differed significantly between districts. In addition, a positive association was observed between the exposure levels to *An. gambiae* bites, estimated by classical entomological methods, and the median IgG levels or the percentage of immune responders reflecting the real contact between human populations and *Anopheles* mosquitoes [27]. Differences in exposure levels to *An. gambiae* bites could then partly explain district and/or group-variations in anti-gSG6-P1 IgG Ab response as previously described in a low-exposure rural area of Senegal [28]. Interestingly, in urban Dakar area, immunological parameters seemed to better discriminate the *Anopheles* exposure level between different groups compared to referent entomological data. Moreover, in this study, some discrepancies were observed in the correlation between immunological parameters and the exposure level to *An. gambiae* bites assessed by entomological data in districts. This suggests the main role of the human behavior influencing the contact with vectors. A differential use of Vector Control Measures (ITNs, sprays, curtains) can for example drastically reduce human-vector contact. Many household characteristics (height, type, use of air conditioning, well-closed windows), which can differ between districts, could also be crucial factors. Importantly, the effect of these factors may be not taken into account by assessing the mosquito exposure level and malaria risk with classical entomological tools. This strengthens the usefulness of such biomarker as an alternative tool in the evaluation of exposure levels to *Anopheles* bites, especially in low/very low exposure, where current entomological methods can give inaccurate estimations of the human-mosquito contact [27].

In a population from a malaria hyperendemic area of Burkina Faso, the use of gSG6 recombinant protein as reliable indicator of exposure to the 3 main African malaria vectors (*An. gambiae s.s.*, *An. arabiensis* and *An. funestus*) has been suggested [119]. This probably could be relied to a wide cross-reactivity between SG6 sequences of principal *Anopheles* vectors, which highly share identical epitopes between species. Moreover, the gSG6-P1 peptide has been used to accurately evaluate the exposure level to *An. funestus* bites in a rural area in Senegal [29]. Indeed, two-thirds of 2-9 years old children from this area developed an IgG response to gSG6-P1, in an area where *An. funestus* only was reported. In addition, IgG response increased during the *An. funestus* exposure season, and a positive association was observed with the level of exposure to *An. funestus* bites [29]. This result deals with the cross-reactivity between *An. gambiae* gSG6-P1 and *An. funestus* fSG6-P1 sequences which share a high level of identity. Indeed, these sequences differ only by the substitution of two AAs: asparagine by glutamine (position 9) and leucine by isoleucine (position 15) (Fig. 12).

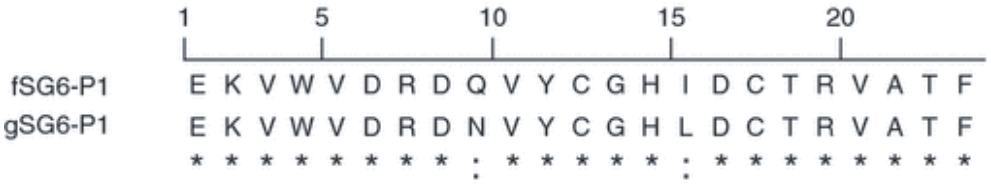

Figure 12. Sequences of the SG6-P1 salivary peptide [29]. Sequences are shown for *An. funestus* (fSG6-P1), for *An. gambiae* (gSG6-P1). Identities are marked with '*' and strong AA conservations with ':'.

AAs from fSG6-P1 are close in terms of polarity and charge to those from *An. gambiae* gSG6-P1. The main consequence is that individuals exposed to *An. funestus* bites can sufficiently develop a specific Ab response against gSG6-P1 *An. gambiae* antigen. This observation, in conjunction with present results, suggests that these substitutions do not alter the synthesis and the recognition of specific Ab because epitope appears to be conserved.

All mentioned studies were conducted on subjects older than 1 year. However, to be more relevant in epidemiological surveys and studies on malaria, such biomarker tool must pertinently be applicable to all human age-classes, including newborns and young infants (<1 year old) who can be also bitten by *Anopheles* and at high risk of malaria transmission [120]. In this way, a recent study has indicated that human Ab responses to gSG6-P1 biomarker help to assess *Anopheles* exposure level and the risk of malaria in younger than 1 year old infants living in moderate to high transmission area of Benin (Drame *et al.*, submitted).

Indeed, the presence of anti-gSG6-P1 IgG and IgM in the blood of respectively 93.28 and 41.79% of 3-months old infants (the majority of infants) and their gradual increasing levels until 12 months (Fig. 13), whatever the *Anopheles* exposure level or the season. These observations are consistent with the development and maturation patterns of the newborn immune system during the first months of life. Indeed, the immature human immune system completes its maturation during infancy following exposition to antigens. Therefore, newborns are naive and increasingly susceptible to infectious agents; their immune system is not or insufficiently

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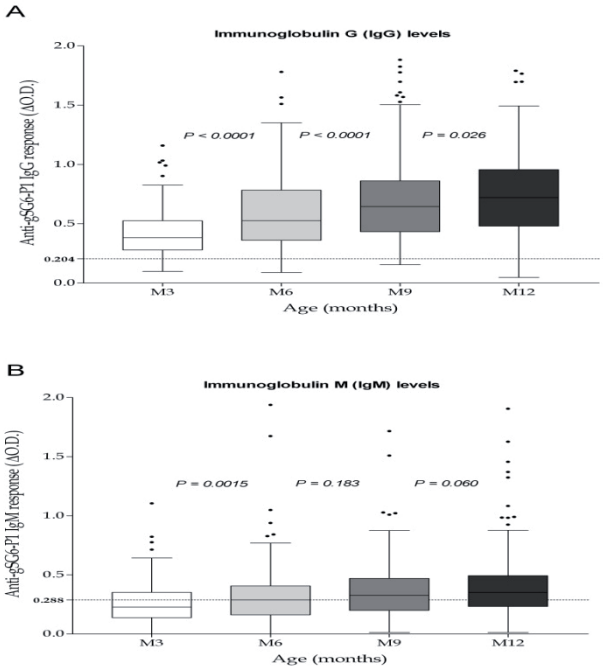

Figure 13. IgG and IgM responses to *Anopheles* gSG6-P1 salivary peptide in the first year-life. Individual IgG (A) and IgM (B) responses to the *Anopheles* gSG6-P1 are represented for infants in months 3 (white), 6 (light-gray), 9 (dark-gray) and 12 (black box) after their birth. Horizontal lines in the boxes indicate medians of the individual data. Horizontal black dotted lines represent the cut-off of IgG (0.204) and IgM (0.288) responder. Statistical significant differences between all age groups (multivariate linear mixed model analysis) are indicated.

stimulated by antigens. In endemic malaria transmission area, they are progressively exposed to salivary antigens of *Anopheles* [121], probably explaining the progressive increase of anti-gSG6-P1 IgG and IgM from 3 to 12 months-old. Individual or population factors and behaviors enhancing the level of the human-*Anopheles* contact with age can play a crucial role on accelerating this gradual acquisition [122, 123].

3.4.3.2. Factors of variation of antibody response to gSG6-P1 and their consequences

Specific gSG6-P1 Ab responses can be influenced by several determinant factors in their variations between individuals, districts, villages, regions... Therefore, identifying effects of human intrinsic (gender, age…) and extrinsic (period of sampling, use of vector control measure…) factors will be useful to the application of the gSG6-P1 biomarker in epidemiological studies or monitoring, evaluation and surveillance of risk of malaria programmes.

Effect of age

Studies have globally reported an increasing anti-gSG6-P1 Ab level according to individual age. In a moderate transmission semi-urban area in Angola, the lowest and highest specific

IgG levels have been described in young children (0-7 years old) and in teenagers/ adults (>14 years old) respectively [30]. In a low malaria transmission urban area (Dakar region) in Senegal, specific IgG levels were significantly higher in adults (>18 years old) compared to 6-10 years old children and in this latter group compared to those aged from 2 to 5 years [27] [124]. In Tori Bossito, moderate-high rural transmission area of Benin, both anti-gSG6-P1 IgG and IgM levels were low at 3 months of age and gradually increased until 12 months after birth (Drame *et al.*, *submitted*). The increase of specific IgG response with age is consistent with the gradual acquired immunity against *Anopheles* mosquito saliva [30] following the development of individual factors and behaviors enhancing the probability of human-vector contact [122, 123]. However, few data have reported a decrease of IgG levels to gSG6-P1 peptide [28] or to SG6 protein [118] with age. In particular, in Senegalese children (0 to 60 months old), the highest specific IgG levels were reported in the youngest children in spite of a probable very weak exposure to *An. gambiae* [30]. It can be explained by a passive IgG transfer from mother to child during pregnancy or breastfeeding as recently reported in young infants from Benin (Drame *et al.*, *submitted*). This represents a way of overestimation of the assessment of human-*Anopheles* contact level and the risk of malaria in young infants by using anti-gSG6-P1 IgG Ab. Therefore, the evaluation of specific IgM Ab levels could be a relevant solution to bias in IgG measurements. Indeed, IgM Ab, in a form of polymers (usually pentamers) in the human organism, could not cross the maternal-foetal barrier [125] and are the first Ab to appear in response to initial or primary exposure to antigen [126]. Interestingly, in Tori Bossito, specific IgM levels seemed to be a serological marker only during the first 6-months of exposure. In infants older to 6 months, the assessment of gSG6-P1-specific IgG showed a more pertinent evaluation of exposure level.

Effect of sex

Some studies have reported higher levels of anti-gSG6-P1 in female individuals (children and women) compared to males (children and men) [27, 30] ([124]; Drame *et al.*, *submitted*). However, this difference was not significant, suggesting that it might be only physiological.

The season of *Anopheles* exposure

The season of individual sampling may be also a factor of confusion in the use gSG6-P1 biomarker in epidemiological studies on malaria risk assessment or control. Indeed, significant seasonally variations in anti-gSG6-P1 IgG or/and IgM levels have been reported in studies conducted in newborns, children or/and adults from endemic malaria areas in Senegal [27-29, 124], Angola [30] and Benin (Drame *et al.*, submitted). In Senegal, in particular, specific gSG6-P1 in urban children and adults steadily waned from the beginning (October) to the end (December) of the study, due to an important drop in human exposure level to *An. gambiae s. l.* bites from the end of rainfalls (October) to the beginning of the dry season (December) [127, 128].

One direct application of a salivary biomarker of exposure could serve in the elaboration of maps representing the risk of exposure to *Anopheles* bites. Such immuno-epidemiological marker might represent a quantitative tool applied to field conditions and a complementary tool to those currently available, such as entomological, ecological and environmental data [59, 129]. It could represent a geographic indicator of the risks of malaria transmission and thus a

useful tool for predicting malaria morbidity risk as previously described [22]. Furthermore, it may represent a powerful tool for evaluation of vector control strategies (impregnated bednet, intradomiciliary aspersion, etc.) and could here constitute a direct criterion for effectiveness and appropriate use (malaria control program) [84].

3.4.3.3. Indicator of malaria vector control effectiveness

Long and short-term evaluation of ITN efficacy

A longitudinal study associating parasitological, entomological and immunological assessments of the efficacy of ITN-based strategies using the gSG6-P1 biomarker has been conducted in a malaria-endemic area in Angola. Human IgG responses to gSG6-P1 peptide were evaluated in 105 individuals (adults and children) before and after the introduction of ITNs and compared to entomo-parasitological data. A significant decrease of anti-gSG6-P1 IgG response was observed just after the effective use of ITNs (Fig. 14). The drop in gSG6-P1 IgG levels was associated with a considerable decrease of *P. falciparum* parasitaemia, the current WHO criterion for vector control efficacy [130]. It was particularly marked in April-August 2006, corresponding to the season peak of *An. gambiae* exposure. Interestingly, the entomological data indicated that this season-dependent peak was of similar intensity before (2005) and after (2006) ITN use, suggesting ITN installation had no impact on *An. gambiae* density, probably because of the low percentage of the overall human population covered in the studied area [131]. This study indicated also that the drop of anti-gSG6-P1 IgG response was associated with correct ITN use and not due to low *Anopheles* density. In addition, this was observed in all age groups studied (<7 years, 7–14 years, and >14 years), suggesting that this biomarker is relevant for ITN evaluation in all age groups. This rapid decrease after correct ITN usage appears to be a special property of anti-gSG6-P1 IgG which is short-lived (4-6 weeks) in the absence of ongoing antigenic stimulation, at/for all age classes.

The response does not seem to build up but wanes rapidly, when exposure failed. This property represents a major strength when using such salivary biomarker of exposure for evaluating the efficacy of vector control. In addition, using a response threshold (ΔOD=0.204) combined with ΔOD_{ITNs} - the difference between April (after ITNs) and January 2006 (before) - makes possible the use of this operational biomarker at individual level (Fig. 15). The threshold response (TR) represents the non-specific background IgG response (the cut-off of immune response) and was calculated in non-*Anopheles* exposed individuals (n= 14- neg; North of France) by using this formula: TR= mean (ΔDO_{neg}) + 3SD = 0.204. An exposed individual was then classified as an immune responder if its ΔOD> 0.204. If the ΔOD_{ITNs} value is comprised between -0.204 and +0.204, no clear difference in exposure level to *Anopheles* bites can be defined.
In contrast, if the individual ΔOD_{ITNs} value <–0.204, it could be concluded with a high level of confidence that this individual is benefiting from ITN installation. The ΔOD_{ITNs} parameter could therefore provide a measure of ITN efficacy at the individual level. An individual biomarker would also be relevant at the large-scale operational studies or surveillance in the field, e.g. in National Malaria Control Programs (NMCP). In addition, the high sensitivity and specificity of the gSG6-P1 Ab response make it ideal for the evaluation of low-level ex-

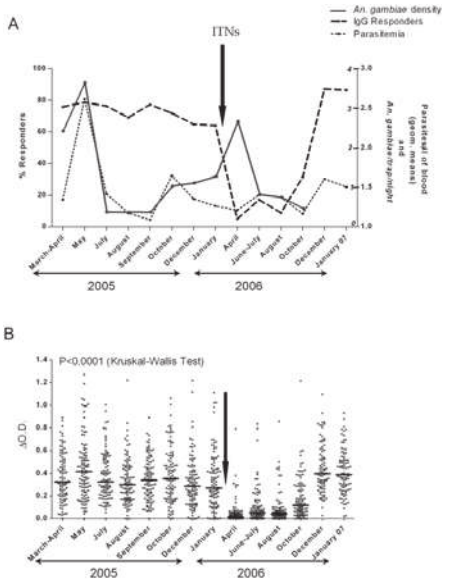

Figure 14. IgG Ab responses to gSG6-P1 before and after ITN use [30]. The percentage (%) of anti-gSG6-P1 IgG immune responders (thick-dotted line) in the "immunological" sub-population (n=105), before (2005) and after (2006 and January 2007) the installation of ITNs (A). These results are presented together with the intensity of *P. falciparum* infection (mean parasitaemia – fine-dotted line) measured in the same population and the mean of number of *An. gambiae* (solid line) in the studied area (A). Entomological data were not available in December 2006 and January 2007 (the last two months of the study). Arrows indicate the installation of Insecticide Treated Nets (ITNs) in February 2006. Individual anti-gSG6-P1 IgG levels (ΔOD) are presented before (2005) and after (2006) the installation of ITNs (B). Bars indicate the median value for each studied month. Statistically significant differences between months are indicated.

posure to *Anopheles bites* [27, 28], even when exposure or transmission is curtailed by NMCP efforts. Taken together, the estimation of human IgG responses to *Anopheles* gSG6-P1 could provide a reliable indicator for evaluating the efficacy of ITN-based strategies against malaria vectors, at individual and population levels, even after vector control generating particular low exposure/transmission contexts. This salivary biomarker is a relevant tool for the evaluation of short-term efficacy as well as longer-term monitoring of malaria VCMs.

Evaluation of effectiveness of diverse vector control measures

A recent cross-sectional study conducted from October to December 2008 on 2,774 residents (children and adults) of 45 districts of urban Dakar (Senegal) has validated IgG responses to gSG6-P1 as an epidemiological indicator evaluating the effectiveness of a range of VCMs. Indeed, in this area, IgG levels to gSG6-P1 as well as the use of diverse malaria VCMs (ITNs, mosquito coils, spray bombs, ventilation and/or incense) highly varied between districts [124]. This difference of use suggests some socio-economical and cultural discrepancies between householders as described in large cities of Ivory Coast [132] and Tanzania [123]. At the district level, specific IgG levels significantly decreased with VCM use in children as well as in adults.

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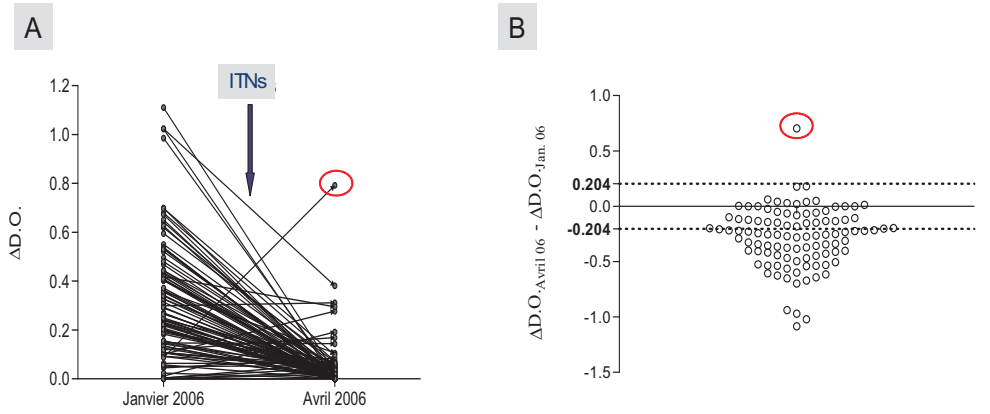

Figure 15. IgG response to gSG6-P1 as biomarker for short-term ITN efficacy. Changes in individual IgG levels (ΔOD) are presented between "just before" (January 2006) and "just after" (April 2006) ITN introduction (n=105; children and adults) (A). The arrow indicates the installation of Insecticide Treated Nets (ITNs) in February 2006. Individual IgG level changes from January (before) to April are presented (B) by individual $\Delta OD_{ITNs,}$ value ($\Delta OD_{ITNs=}\Delta OD_{April06,}$ - $\Delta OD_{January06}$). The threshold of specific IgG responders (TR=0.204) is indicated (dotted line). Significant positive (ΔOD>0.204) or negative (ΔOD<–0.204) changes are therefore individually presented.

Among used VCM, ITNs, the 1st chosen preventive method (43.35% rate of use), by reducing drastically the human-*Anopheles* contact level and specific IgG levels in children as well as in adults, were by far the most efficient whatever age, period of sampling or the exposure level to mosquito bites. Spray bombs were secondarily associated to a decrease of specific IgG level, due certainly to their power and fast knock-down action. But, their effects can be limited by the non-persistence of used products and some socio-economic considerations [133]. In addition, they only have been recently adopted and are more expensive in the majority of sub-Saharan Africa cities [133], explaining their less frequent use (9.57% rate of use) in the Dakar area. The non-effect of mosquito coil use is surprising, regardless to their well-adoption by residents (36.68% of rate of use), but it can be explained by their power deterrent effect which tends to push *Anopheles* vectors outside where they can remain active [133]. However, the protection ensured by ITN use seemed to be insufficient because anti-gSG6-P1 IgG levels in ITN users were specifically high in some periods of fairly high exposure to *Anopheles* bites. Changes in *An. arabiensis* behaviour, the major malaria vector in the area, can also explain this lack of protection. It can bite outside the rooms/ habitations with a maximal activity around 10.00 pm, when people are not in bed and ITNs not hanged [123]. Therefore, ITNs must be associated to a complementary VCM for an effective protection against *Anopheles* bites.

Taken together, these results suggest that the assessment of human IgG responses to *Anopheles* gSG6-P1 salivary peptide can provide a reliable evaluation of the effectiveness of malaria vector control in urban settings of Dakar whatever the age, sex, level of exposure to bites or period of malaria transmission. Therefore, this salivary biomarker can be used to compare the effectiveness of different anti-malaria vector strategies in order to identify the most suitable for a given area.

Comparing effectiveness of combined or not vector control measures

In parallel to an entomological and parasitological evaluation, IgG responses to gSG6-P1 were also used to assess, in a randomized controlled trial in 28 villages in southern Benin, four malaria vector control interventions: Long-Lasting Insecticide-treated Net (LLIN) targeted coverage to pregnant women and children younger than 6 years (TLLIN, reference group), LLIN universal coverage of all sleeping units (ULLIN), TLLIN plus full coverage of carbamate-indoor residual spraying (IRS) applied every 8 months (TLLIN+IRS), and ULLIN plus full coverage of carbamate-treated plastic sheeting (CTPS) lined up to the upper part of the household walls (ULLIN+CTPS). Results from this study have shown that specific IgG levels were similar in the 4 groups before intervention and only significantly lower in the ULLIN group compared to the others after intervention. In contrast to immunological data, clinical incidence density of malaria, the prevalence and parasite density of asymptomatic infections, and the density and aggressiveness of *Anopheles* mosquitoes, were not significantly different between the four groups before as well as after interventions [134]. These findings mean that LLIN used along by all the population of a given area may be more suitable in reducing the contact between human populations and the *Anopheles* vectors, even if any effect on malaria morbidity, infection, and transmission was not observed. Therefore, combining anti-vector tools do not undeniably reduce individual exposure to malaria vectors, even if significant effect on reducing more rapidly malaria transmission and burden has been reported [135]. These findings confirm that anti-vector saliva Ab response as a biomarker of exposure is also important for NMCPs and should help the design of more cost-effective strategies for malaria control and elimination.

3.4.4. Importance to develop a specific biomarker of infecting Anopheles *bites*

Recent data have shown that the use of the gSG6-P1 biomarker for the assessment of the differential risk of the disease transmission may have some limitations in high exposure areas (Drame *et al.*, submitted). Indeed, the gSG6-P1 assesses the exposure level to both infective and not infective *Anopheles* bites. In malaria hyperendemic areas, resident people are highly exposed to mainly not infective bites and present almost all Ab specific to gSG6-P1 levels relatively high. Therefore it should be relevant to develop a biomarker of exposure specific to infective bites in order to assess the human risk of malaria transmission in such contexts. Such epidemiological parameter would be important to define in the context of malaria control. The transmission depends on the density of competent *Anopheles*, of their *Plasmodium* infective rate and of the intensity of human-vector contact. In addition, current methods to measure the intensity of malaria transmission show several limitations, especially in low transmission areas. The EIR (entomological inoculation rate) is a commonly used metric rate that estimates the number of bites by infectious mosquitoes per person per unit time. It is the product of the "human biting rate" – the number of bites per person per day by vector mosquitoes – and the fraction of vector mosquitoes that are infectious (the "sporozoite rate"). The classical method to estimate the density of sporozoites in mosquitoes is the dissection of salivary glands and the sporozoites counting under microscope. But in area of low exposure and because few mosquitoes are infected, many mosquitoes must be caught and dissected. The salivary glands

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dissection is a tedious technique which required well trained and studious personnel. Moreover this technique cannot differentiate *Plasmodium* species. Another technique named CSP-ELISA detects the CSP (Circumsporozoite protein) parasite surface protein and is generally done on head/thorax of mosquitoes. However the CSP protein is expressed at the oocyst stage, consequently the CSP can be detected in the mosquito before the sporozoites have reached the salivary glands (until 2-3 days) [136, 137]. Therefore, this method induced a bias with an overestimation of sporozoites index [138, 139]. Other traditional epidemiological estimates mainly based on parasitological tests are very sensitive and specific allowing the determination of parasite species, but the examination of finger prick and thick blood smear is also labour intensive and time-consuming requiring well trained staff for a reliable examination [140]. To improve the measure of transmission, antibody responses against parasite proteins (CSP, AMA1, MSP1, MSP3, etc…) could be used but several studies have highlighted limits of this approach. Actually, people exposed to malaria can be seropositive during several months [141, 142], even after transmission has stopped [141] or in the context of low transmission [143]. So by using this method we are not able to distinguish old and new infection which is particularly important in the context of evaluation of the effectiveness of vector control program. Considering these limits, these serological parameters seem inappropriate to assess the malaria exposure at the individual level. Some proteomic and transcriptomic studies highlighted that the composition of *Anopheles* salivary glands could be modified with the presence of *Plasmodium* parasite [15, 144, 145]. Therefore, the development of a biomarker specific of infective bites based on the analysis of antibody response against salivary proteins should represent an alternative method to assess the parasite transmission to the human.

The principle of biomarker of infective bites is based on the use of immunogenic salivary protein like marker of transmission. The expression of some salivary proteins could be induced or regulated when the salivary glands are infected. Therefore, if one of such protein presents also immunogenic properties, we can probably use the specific immune response to this protein like a marker of transmission in human. Such a biomarker will be also particularly relevant in the context of re-emergence after malaria transmission reduction or in area of low exposure. This tool will allow focusing the intervention (vector control strategies and drugs distribution) on the most exposed and the most susceptible population.

4. Conclusions

In the present chapter, we have described the development of a biomarker (the *An. gambiae* gSG6-P1 peptide) of *Anopheles* mosquito bites by using an original approach coupling bioinformatic tools and immuno-epidemiological assays. Then, measurements of IgG level specific to gSG6-P1 at individual as well as population level, represent a tool/biomarker for accurately evaluate the level of human exposure to *Anopheles* bites and the risk of malaria in all age-classes of populations (newborns, infants, children, adults) living in various settings (very-low, low, moderate, and high malaria transmission areas) of rural, semi-urban and urban regions of Senegal, Angola and Benin. In the majority of these areas, this biomarker appeared to be promising and complementary to classical entomological methods, because it can give a reliable

evaluation of the individual contact with anthropophilic *Anopheles* even if exposure to bites is low/very low (urban area). Therefore, such biomarker would be particularly relevant in places where malaria transmission is low, e.g. in foci of urban, high-altitude or seasonal malaria, and in travelers in endemic areas. This chapter has also shown that the availability of such a biomarker could allow the evaluation of the exposure to the main *P. falciparum* vectors (*An. gambiae s.s.*, *An. arabiensis*, *An. funestus*, *An. melas*) in Africa where different species of malaria vector co-inhabit. One direct application of such a gSG6 peptide marker of exposure could be in the elaboration of maps representing the risk of exposure to *Anopheles* bites. It could represent a geographic indicator of the risks of malaria transmission and thus a useful tool for predicting malaria morbidity risk as previously described. Furthermore, it represents a powerful and reliable tool for the evaluation of the effectiveness of vector control strategies. Such an indicator could also represent an alternative to classical entomological-parasitological monitoring methods for measuring and following the effectiveness of vector control strategies used by the National Malaria Control Programmes in various settings across Africa. Finally, this biomarker approach could be similarly applied to vector-control strategies for other mosquito-borne diseases such as emergent or re-emergent arbovirus diseases and trypanosomiasis.

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Moreover, since this chapter was published in 2013, other studies have demonstrated that in addition to assessing the exposure to *Anopheles* bites, the gSG6-P1 peptide (derived from the gSG6 protein) appears to be an indicator of a malaria reservoir or risk of infection during the dry season in northern Senegal (Sagna et al. 2013a). It has also been shown that the Ab response to the gSG6 protein is positively associated with malaria incidence (Stone et al. 2012).

II. Objectives of the thesis

1. Validation of new biomarker of exposure to *Anopheles* bites

As stated above, the gSG6 protein and gSG6-P1 peptide derived from this protein have already been validated for estimating human exposure to *Anopheles* bites in different transmission contexts. The gSG6-P1 peptide has also been validated as an indicator to assess the efficacy of insecticide-treated nets. However, as has been mentioned for the Ab response to *Plasmodium* antigens and as has been shown with the gSG6-P1 peptide, high heterogeneity in the Ab response has been observed between individuals from the same area. A study reported by Drame et al observed a very low or no Ab response to the gSG6-P1 peptide in children, whereas *Anopheles* are still present, before the interruption of human–vector contact using ITNs (Drame et al. 2010a). Given inter-individual variation and the lack of sensitivity of the gSG6-P1 peptide in identifying all exposed individuals, and within the objective of improving this salivary protein-based tool, it seems valuable to evaluate the Ab response to other salivary antigens to combine them in order to increase sensitivity and detect the entire range of exposure to *Anopheles* bites. The final objective is then to obtain an optimal biomarker that can detect all individuals exposed to vector bites and measure the actual level of human exposure in different exposure contexts (very low, urban malaria, malaria pre-elimination areas, after ITN implementation, etc.).

The first part of this thesis is therefore the evaluation of the Ab response against another biomarker candidate, the cE5 salivary protein, which could be used as a biomarker of human exposure to *Anopheles* bites and an indicator of ITN efficacy.

2. Identification of biomarkers of exposure to *Anopheles* infective bites

Given the updated scientific and financial focus on malaria elimination (Roberts and Enserink 2007, Grabowsky 2008), the most appropriate and sensitive tools are required to measure and monitor transmission (Greenwood 2008, Hay et al. 2008), to evaluate the efficacy of different control strategies (drug treatment, vector control, vaccines), and to survey post-elimination areas to prevent reintroduction of malaria transmission.

Tools already available were optimized years ago for areas where malaria is quite stable or at least easily detectable during the peak of the rainy season. These tools presented poorer efficacy (with a lack of sensitivity) in the new deployment contexts of integrated malaria control strategies at the country scale, thus reaching the pre-elimination stage. Consequently, new complementary techniques must now be associated. The serology method used against parasite antigens seems to be a good approach. Nevertheless, the Ab response against *Plasmodium* antigens induces bias due to the effect of the cumulative immune response making it impossible to distinguish previous and recent infection and the problems observing a response in children.

As described above, one promising approach is the study of human–vector contact, representing the transmission stage, using the human Ab response to salivary proteins as a biomarker of exposure to *Anopheles* bites. However, malaria is only transmitted by infective bites, which account for less than 5% of the total bites (Beier et al. 1999, Drakeley et al. 2003). Consequently, human exposure to bites does not fully represent malaria transmission. Therefore, the development of a tool that will differentiate noninfective and infective bites of *Anopheles* is necessary.

The second part of the thesis is the identification of salivary proteins that can serve as candidate exposure biomarkers specifically for *Anopheles* infective bites.

RESULTS

Chapter 1: Validation of a new biomarker of exposure to *Anopheles* bites.

In different studies, it has been noticed that the Ab response to salivary gSG6 protein and gSG6-P1 peptide was heterogeneous between individuals from a same area, and which probably presented thus the same level of exposure to *Anopheles* bites evaluated by entomological methods. Even if, it is clearly possible that individuals are differently exposed to *Anopheles* bites within the same area, the biomarker tool has to be optimized. In order to increase the sensitivity and to assess the different levels of individual exposure, new salivary proteins as potential complementary biomarker candidates have to be therefore identified and tested.

The criteria of an optimal biomarker of human exposure based on the Ab response to salivary proteins are i) the *Anopheles* specificity, ii) the antigenic properties of salivary protein, iii) the facility to produce the antigen to insure the reproducibility of the immunological assay (ELISA), iv) the low/moderate antigenicity (i.e., level of specific Ab response) of the antigen to avoid a cumulative Ab response with time and to observe a rapid decrease after the stop of exposure and v) to observe a positive association of the level of Ab response to candidate with the level of exposure.

The *Anopheles gambiae* cE5 protein, an *Anopheles*-specific thrombin-inhibitor protein only found in female salivary glands (Ronca et al. 2012), has been recently identified and has been shown as being antigenic by inducing a specific Ab response in human populations bitten by *An. gambiae* in Burkina Faso (Arcà B., unpublished data).
The present study aims to assess if the specific IgG Ab response against cE5 protein, produced in recombinant form, could be associated with the exposure level of *An. gambiae* bites, before ITNs implementation and if this protein candidate could be used as a biomarker of ITNs efficacy after their implementation. To this end, this work is based on the longitudinal survey in Angola, where the well-used of ITNs has been shown to decrease the malaria transmission despite the unchanged density of mosquitoes (Drame et al. 2010b).

This work is the subject of a publication in preparation for submission in *Microbes and Infection*.

The *Anopheles gambiae* cE5 salivary protein: an additional biomarker to evaluate the efficacy of insecticide-treated nets in malaria vector control.

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Abstract

In order to improve malaria vector control, new complementary indicators to evaluate the effectiveness of vector control strategies are needed, as recommended by the World Health Organization. In this study, the immunogenic cE5 salivary protein, which is specific to the *Anopheles* genus, was tested as biomarker of exposure to *Anopheles* bites and as a tool to evaluate the efficacy of insecticide treated nets. A longitudinal study carried out in Angola, and including entomological and parasitological data, was used to assess the IgG response to cE5 in both children and adults, before and after the application of insecticide treated nets. A positive association between IgG and exposure levels was observed only in children (< 14 years) before insecticide treated nets implementation. Moreover, a drop of the specific IgG response was also observed only in children after bed nets installation. Overall, the cE5 protein was sensitive enough to detect even a weak exposure of children to *Anopheles* bites; moreover, it appeared to be a reliable biomarker to evaluate the efficacy of insecticide treated nets already shortly after their application.

Keywords: cE5 salivary protein; *Anopheles gambiae*; biomarker; children exposure; insecticide treated nets

1. Introduction

Despite substantial efforts and increasing international funding to eliminate malaria, it is still the most important parasitic disease causing 660 000 deaths and 219 million cases per year. *Plasmodium falciparum*, the most dangerous *Plasmodium* species is responsible of ~90% of cases, with more than 85% of deaths occuring in children from sub-Saharan Africa, where the major parasite vectors are members of the *Anopheles gambiae* complex [1].

Nowadays, no vaccine is available, and malaria containment in endemic areas is largely based on control measures against the parasites (mainly artemisin-based combination therapy) and the vectors (insecticide-based control). Different vector control strategies are employed, such as for example Indoor Residual Spraying (IRS) or insecticide treated plastic sheeting, but Insecticide Treated Nets (ITNs) are the most efficient and used strategies to decrease the human-vector contact for reducing malaria transmission and morbidity. However, parasite resistance to antimalarial treatment [2] and mosquito vector resistances to insecticides [3, 4] impair the control effectiveness.

Currently, the WHO recommendation for phase 3 evaluation of ITNs efficacy is based on the assessment of the number of *P. falciparum* parasites in the human blood. Entomological methods are also used to evaluate the vector control strategies by assessing the *Anopheles* mosquito density, its aggressivity and the Entomological Inoculation rate (EIR). However, these techniques present several limitations when it comes to large-scale field studies, particularly in areas of low exposure and transmission (dry season, high altitude, urban setting) or also in highly endemic areas after vector control, such as after implementation of ITNs. Moreover, some entomological methods (CDC traps, IRS) cannot be applied for evaluating the human-vector contact at the individual level and human landing catches on adult volunteers, which is the reference method to evaluate exposure at the individual level, may raise ethical concerns and it is not applicable to children. Considering these limits, new complementary indicators are needed for a better evaluation of the efficacy of vector control strategies. One recent approach is based on the concept that human exposure to arthropod vector bites can be assessed by monitoring the anti-saliva antibody response [5, 6]. This approach has been investigated for several vectors that carry human pathogens such as *Ixodides* ticks (Borrelia) [7], *Triatoma* (Chagas disease) [8-10], *Lutzomia* sand sand flies (Leishmania) [11, 12], *Aedes* (Dengue and Chikungunya) [13-15], *Glossina* (African Trypanosomiasis) [16, 17], and *Culex* (Filariasis) [18, 19]. When blood sucking arthropods bite their hosts, they inject saliva while searching for blood and during their meal. Saliva is known to carry several bioactive molecules counteracting the human haemostatic [20] and

inflammatory reactions to favor the successful blood meal [21, 22]. In addition salivary proteins, at least some of them, are immunogenic and can induce an antibody (Ab) response in human. The IgG Ab response to whole *Anopheles* saliva has been shown to represent an effective biomarker of human exposure to bites of malaria vectors [23-26]. Such epidemiological biomarker could be also used as tool to evaluate the efficacy of bednets or other vector control strategies decreasing the human-vector contact [27-30]. However, the use of whole saliva has several limitations. First, some saliva compounds are ubiquitous in arthropod species and can induce immune cross-reactivity. Moreover, saliva collection is fastidious and the composition of different batches may depend from physiological factors such as age [31] inducing a lack of reproducibility. For these reasons, a pertinent biomarker can't be based on whole saliva and should better rely on a single protein/peptide. In this respect, the gSG6 salivary protein has been identified as specific to mosquitoes of the *Anopheles* genus [32, 33] and demonstrated as immunogenic [34]. It has been shown that the human IgG response to this protein can be used as a serological indicator of exposure to *An. gambiae* [35, 36]. The gSG6 protein has been first identified in *An. gambiae* [37, 38], and found to be highly conserved among *Anopheles* species [39, 40]; as a consequence it can be also pertinent as biomarker of exposure to bites of *An. arabiensis* and *An. funestus* [41].]. In addition, the gSG6-based peptide gSG6-P1 has been validated as a suitable biomarker to evaluate the level of human exposure to *An. gambiae* [34, 42-45] and *An. funestus* [46]. More recently, this peptide has also been employed to evaluate different vector control strategies in Senegal during an operational effectiveness evaluation [47] and in Angola during Phase 3 evaluation [48]. In this last study, a very low IgG response to the gSG6-P1 peptide was observed in several children already before the installation of ITNs. Consequently, no change of the anti-gSG6 IgG response was found after the installation of ITNs in a rather large proportion of individuals (39%). These results suggested that this peptide may not be sensitive enough to detect very low levels of exposure to *Anopheles* bites. To verify this hypothesis and to optimize the biomarker toolbox it looked interesting to test other *An. gambiae* salivary proteins as potential biomarkers of human exposure to *Anopheles* bites and for the evaluation of efficacy of vector control strategies. In this respect the *Anopheles*-specific salivary protein cE5, the *An. gambiae* member of the anophelin family of thrombin-inhibitors [49, 50], was recently identified as highly immunogenic to humans (B. Arcà, Sapienza University of Rome, manuscript in preparation) and, therefore, appeared especially suitable to this purpose.

The aim of the present study was to assess if the specific IgG Ab response against the recombinant cE5 protein could be associated with the level of exposure to *An. gambiae* bites,

before and after ITNs implementation, and if it could be used as a biomarker of ITNs efficacy. To this end sera from a longitudinal survey in Angola, where application of ITNs had already been shown to decrease malaria transmission despite the unchanged density of mosquitoes [27], were used to measure the IgG antibody response against the cE5 protein.

2. Materials and methods

2.1. *Ethics Statement*

This study was conducted in accordance with the Edinburgh revision of the Helsinki Declaration, and was approved by the National Malaria Control Program of the Minister of Health of Angola (October 17th 2008), the only one Ethical authority in 2008 for approving studies on malaria research in Angola. Written informed consent (signed by the head of each household) was obtained for all individuals enrolled in the study, by the SONAMET Malaria Control Program (MCP) which control malaria infection of all workers for SONAMET and their family. This consent procedure was regularly approved by SONAMET workers, who benefited to several malaria studies/survey by MCP, and was approved by the involved Ethical authority in Angola.

2.2. *Study population*

A two-year follow-up longitudinal survey in malaria-endemic area was performed in Angola with epidemiological, parasitological and entomological data previously available.

This study was conducted in Lobito, a coastal city of Western Angola, from March 2005 to December 2006. In February 2006, Long Lasting Insecticide Nets (LLINs) were given to the families. The site is in the tropical Savannah with a rainy season from October to May, with approximately 600-700 millimeters of rain per year. The duration of malaria transmission season varies between 7 and 12 months with a peak between March and May. The major malaria vector is *Anopheles gambiae s.l.* complex [27].

The studied population has been previously described [27, 48]. Briefly, all workers of the Société Nationale de Métallurgie (SONAMET) Company lived in 250 households in the Bella Vista district. Residents were followed in the SONAMET in-patient clinic. In 2004, the presence of malaria parasite was diagnosed in 60 households (positive, at least, in one member) by the SONAMET MPC. Twenty-one of these 60 households were then randomly selected for the present study. In total, 230 individuals (children and adults) were included for

longitudinal follow-up, with evaluation every 6 weeks on two 2 periods: from March 2005 to January 2006 and from April 2006 to December 2006. In February 2006, Long-Lasting Insecticide Nets (LLIN) treated with deltamethrin (Permanet®) were distributed to the families (according to the number of rooms and beds per households). At each visit, thick blood smear and dried blood spot (filter paper) samples were collected from each individual for parasitological tests and immunological analysis, respectively. Parasite density (parasitemia) was calculated as the number of *P. falciparum* per microliter of blood and presented as the geometric mean of parasitemia values (x+1), as previously indicated [27]. Immunological tests were performed on a sub-sample of the whole study population (n=73; 35 children aged from 0 to 6 years old (mean: 3.48; 95% CI: 2.92-4.04), 22 children from 7 to 14 years old (mean: 9.68; 95% CI: 8.67-10.69) and 16 individuals aged more than 14 years old (mean: 26; 95% CI: 20.56-31.44)) for whom blood spots were available for at least 10 of 12 visits. Filter papers were kept at 4°C in Silicagel before testing.

2.3. *Entomological analysis and survey of LLIN use*

Mosquitoes were collected every six weeks during the study at 6 reference households, representative of the studied area. *An. gambiae* density was evaluated using capture by CDC light trap from 7h PM to 7h AM for two consecutive nights. PCR was used to confirm species to estimate the number of *An. gambiae*/trap/night.

After the introduction of LLIN, their use by individuals and their quality were inspected the night before each blood sampling by the MCP team. Information on ITNs use was then collected for all studied individuals by questionnaires, covering: i) the number of installed LLINs, ii) the number of exchanged LLINs, and iii) the number of damaged LLINs (hole, torn, etc.), as previously described [27].

2.4. *Evaluation of human IgG antibody level (ELISA)*

Standardized dried blood spots (0.6cm diameter) were eluted by incubation in 200µL of phosphate buffer saline (PBS-Tween 0.1%) at 4°C for 24 hours. Enzyme-Linked ImmunoSorbent Assays (ELISA) were carried out on eluates to measure the level of IgG Ab reacting to the cE5 antigen. The cE5 recombinant protein was expressed and purified as previously described [49]. Maxisorp plates (Nunc, Roskilde, Denmark) were coated with cE5 protein (5µg/mL) in carbonate/bicarbonate buffer (0.1 M $NaHCO_3$, 0.1M Na_2CO_3, pH9.6) at 37°C for 2h30. After five washings (distilled water-Tween 1%), wells were blocked (1h at 37°C) in Blocking Buffer (Pierce® Protein-Free, Thermo Scientific, Rockford, USA), washed

again and each eluate was incubated at 4°C overnight at a 1/20 dilution (PBS-Tween 1%). Eluate samples were analyzed in duplicate with the antigen and once without antigen. After washing, plates were incubated (1h30 at 37°C) with a mouse biotinylated Ab against human IgG (BD Pharmingen, San Diego, CA, USA) at a 1/2000 dilution. After washing, peroxydase conjugated streptavidin (Amersham, Les Ulis, France) was added at a 1/2000 dilution for 1h at 37°C. After washing, colorimetric development was carried out using ABTS (2,2'-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium; Sigma, St Louis, MO, USA) in 50 mM citrate buffer (pH 4) containing 0.003% H_2O_2. Optical density (OD) was measured at 405nm. IgG levels were expressed as final OD (ΔOD) calculated for each eluate as the mean OD value with antigen minus the OD value without antigen. Intra- and inter- assay variation of samples was below 20%. Eluates presenting duplicates with coefficient variation (CV) ≥20% were excluded from the analysis and have been re-assessed by ELISA. In parallel, specific IgG Ab levels were also evaluated in individuals (n= 32) living in France and with no known exposure to *An. gambiae* mosquitoes and were used to calculate the specific immune response threshold (TR). A subject was considered as an "immune responder" if his ΔOD was higher than the TR = mean ($\Delta DO_{unexposed}$) + 3SD = 0.257.

2.5. *Statistical analysis*

All data were analyzed withGraphPad Prism5 Software® (San Diego, CA, USA). After checking the non-Gaussian distribution, the non-parametric Mann-Whitney U-test was used to compare Ab levels between two independent groups, the Wilcoxon matched pairs test was used for comparison between two paired groups, the non-parametric Kruskall-Wallis test for comparison between more than two groups. The Fisher's exact test was used for the comparison of two proportions. All differences were considered significant at $p<0.05$.

3. Results

3.1. *IgG Ab response to cE5 before and after ITNs use according to age groups*

The anti-cE5 IgG level was followed during the longitudinal study according to the three age groups: 0-6 years, 7-14 years and > 14 years old (Figure 17). Seasonal variation of IgG level was observed in the two youngest groups (0-6 years: $p<0.001$; 7-14 years: $p<0.001$) before the ITNs implementation (March 2005 to January 2006), in contrast to adult group

(>14 years: p>0.05). In April 2006, only two months after the installation and well-use of ITNs (February 2006), a high drop of anti-cE5 IgG level was only observed for children groups (0-6 years: p<0.0001; 7-14 years: p<0.001). In contrast, no significant decrease was observed after the introduction of ITNs for > 14 years group (p>0.05).

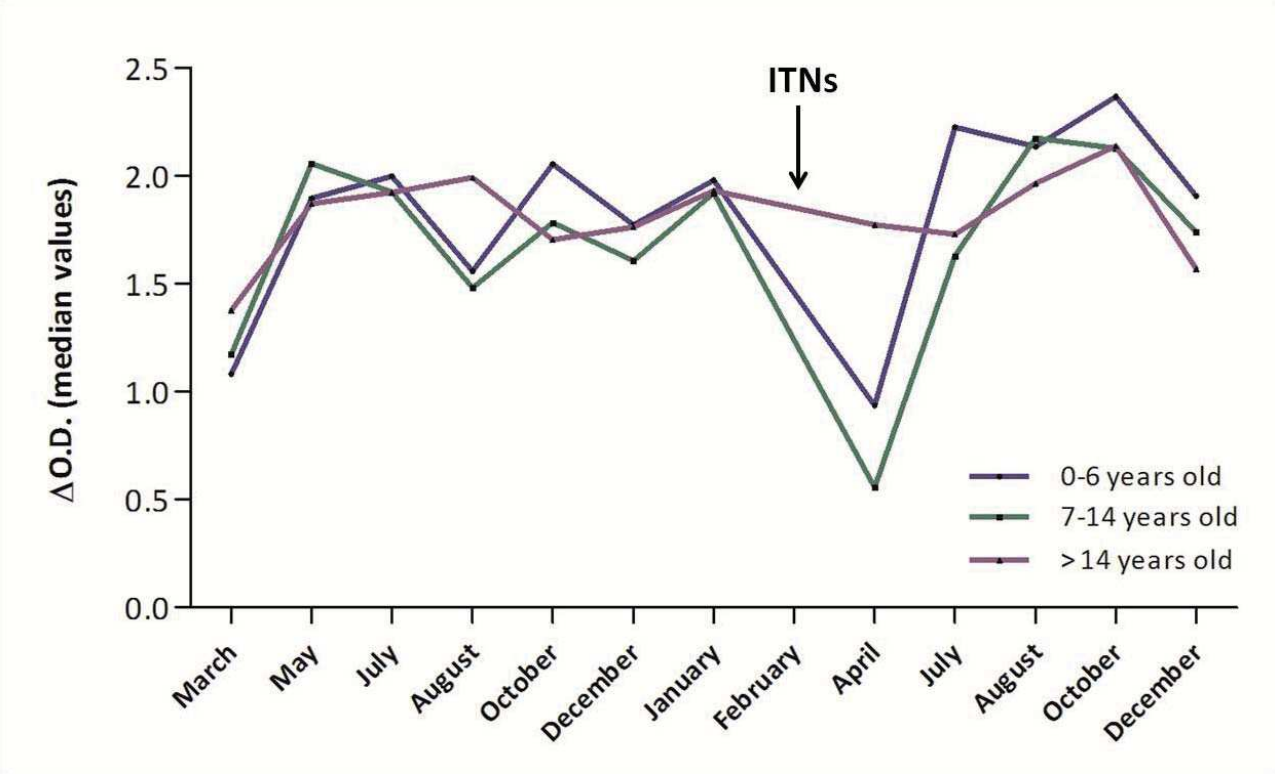

Figure 17. IgG Ab response to cE5 before and after ITNs use according to age group.
Median values of anti-cE5 IgG level are presented according to the three age groups: 0-6 years old (blue line; n=35), 7-14 years old (green line; n=22) and >14 years old (purple line, n=16). The arrow indicates the installation of ITNs in February 2006. Seasonal variation for each age group was tested using Kruskal-Wallis test: 0-6 years old, P<0.0001; 7-14 years old, P<0.0001; >14 years old, P>0.05.

3.2. *IgG Ab response to cE5 before and after ITNs use in children <14 years old*

According to the age-dependent results mentioned above the individual IgG response against cE5 protein is only presented in children <14 years old before (March 2005 to January 2006) and after (April 2006 to December 2006) installation of ITNs (February 2006) (Figure 18). Considerable individual variation of the anti-cE5 IgG level was observed during every visit, suggesting that the IgG response was specific to the individual exposure. At population level (median values), a seasonal variation of the IgG response to the cE5 protein was also observed (p<0.001). The first peak of specific IgG response was associated with the peak of

Anopheles density in May 2005. We observed then a decrease from July to August 2005 and a second peak of IgG response was associated with a rise of *An. gambiae* density in October 2005 ($p<0.05$), stable to January 2005. Interestingly, after the well-use of ITNs, a significant decrease of the anti-cE5 IgG level was observed in April 2006 ($p<0.0001$), despite the fact that the number of *An. gambiae* mosquitoes peaked this month. Moreover, according to visit, the specific IgG Ab response in April 2006 was not significantly different compared to March 2005 but significantly lower compared to May 2005 ($p<0.0001$). This IgG decrease in April 2006 was also associated to the decline of *P. falciparum* parasitemia, as previously described [27, 48]. The decrease of the parasitemia during a period of increase of the mosquito population highlights the clear efficacy of ITNs in decreasing the human-vector contact. Afterwards, from July to December 2006, the anti-cE5 IgG level increased despite the wane of *Anopheles* density. This phenomenon was also observed in previous studies and explained by the incorrect use and/or the damage of the nets.

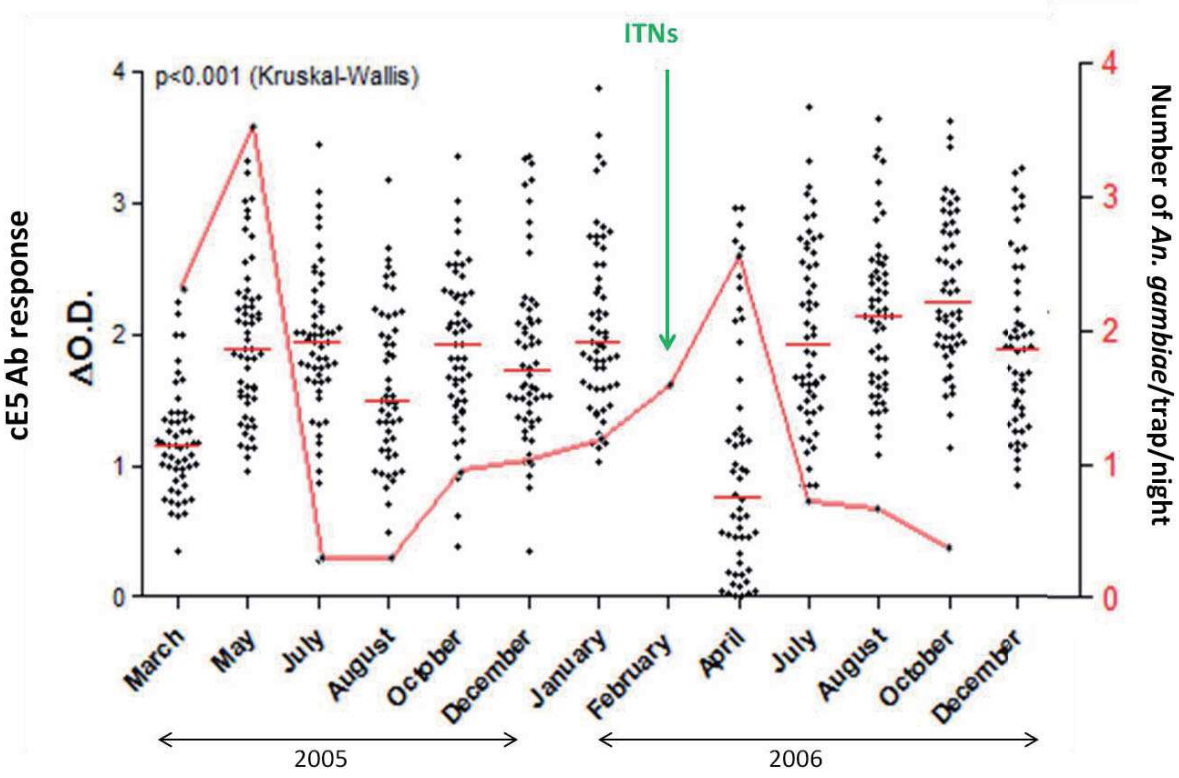

Figure 18. Individual anti-cE5 IgG levels (ΔO.D.) before (2005) and after (2006) the installation of ITNs in children <14 years old and entomological data (mean of number of *An. gambiae*) in red curve.

The red bars represent the median value for each studied month. The arrow indicates the installation of ITNs in February 2006.

3.3. *Individual evolution of anti-cE5 IgG level in children and short-term ITNs efficacy*

The relationship between ITN implementation and anti-cE5 IgG levels was also analyzed at individual level by comparing immunological results in April 2006 (just after ITN) to January 2006 (just before). As previously observed at population level (Figure 17), no significant difference was observed in the adult group when the anti-cE5 IgG levels were compared before and after introduction of ITNs (data not show). On the contrary in children up to 14 years of age the IgG response to the cE5 protein decreased in most individuals, even if some of them presented increased or unchanged IgG responses (Figure 19A). A similar pronounced drop of the specific IgG response was individually observed between January 2006 and April 2006 in both young (0-6 years) (fiure.193B) and older children (7-14 years) (figure 19C).

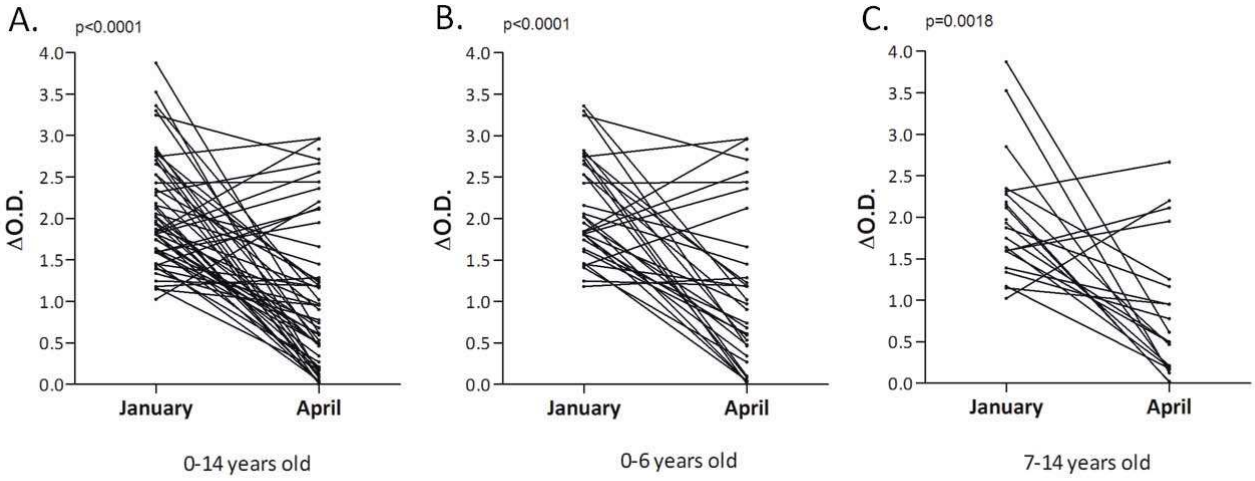

Figure 19. Changes in anti-cE5 IgG levels before and after the introduction of ITNs in children <14 years old.

Individual changes in Ab response from January 2006 (just before ITNs) to April 2006 (just after ITNs) are presented according to age group (A): 0-14 years old, (B): 0-6 years old, (C): 7-14 years old. Statistical differences are indicated (Wilkoxon matched-pairs).

To evaluate the trend in the anti-cE5 IgG response before and after ITN implementation (positive, negative or unchanged), a new indicator was determined as represented by the difference between the response in April and in January ($\Delta OD_{ITNs} = \Delta OD_{April} - \Delta OD_{January}$) (Figure 20). A threshold value of 0.257 was obtained measuring the anti-cE5 response in 32 individuals living in France and with no history of exposure to Afrotropical anophelines. By applying this threshold and analyzing the ΔOD_{ITNs} it was found

that 75.5 % (40/53) of children <14 years old presented a decrease (ΔOD_{ITNs}<-0.257) of their anti-cE5 IgG response, 15.1% (8/53) showed an increase (ΔOD_{ITNs}>0.257) and 9.4% (5/53) revealed no significant change (-0.257< ΔOD_{ITNs}<0.257) (Figure 20A). Similar proportions were observed when the two children age groups were analyzed independently (compared using the Fisher's exact test). Indeed, a decrease in the specific IgG response was found in 75.8% (25/33) of 0-6 years old children and in 75% (15/20) of 7-14 years old children (no significant difference between the two proportion, p>0.05). Instead, only 12.1% (4/33) of 0-6 years and 20% (4/20) of 7-14 years presented an increased response (no significant difference between the two proportion, p>0.05)whereas 12.1% (4/33) of 0-6 years and 0.05% (1/20) of 7-14 years showed unchanged IgG level (no significant difference between the two proportion, p>0.05) (Figure 20B and 20C).

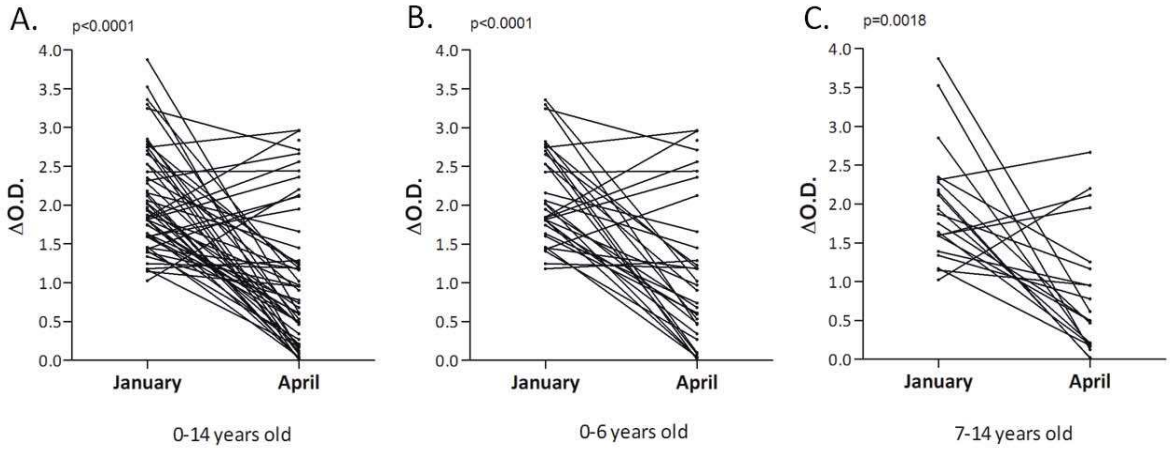

Figure 20. IgG response to cE5 protein as biomarker for short-term ITNs efficacy in children <14 years old.

Individual IgG level changes from January 2006 to April 2006 are presented by individual ΔOD_{ITNs} value ($\Delta OD_{ITNs}=\Delta OD_{April} - \Delta OD_{January}$). The threshold of specific responders (TR=0.257) is indicated (dotted line). Significant positive (ΔOD_{ITNs}>0.257) or negative (ΔOD_{ITNs}<0.257) changes are therefore individually presented according to age (A): 0-14 years old, (B): 0-6 years old, (C): 7-14 years old.

4. Discussion

In the present study, the IgG Ab response to the cE5 recombinant salivary protein was investigated in a two-year longitudinal study in children and adults before and after the installation of ITNs. As previously reported parasitological data showed that the ITNs were effective in decreasing the parasite density, the current WHO criterion for evaluating vector control efficacy, whereas the *Anopheles gambiae* mosquitoes were still present [27, 48]. This cohort appeared therefore an adequate support to evaluate whether the Ab response to specific salivary protein might be a good biomarker of human exposure and, by consequences, a reliable indicator to evaluate the real efficacy of ITNs on human-vector contact.

The IgG Ab response against cE5 was found to be different in the three age groups (0-6, 7-14 and >14 years old) in which the studied population was divided. For individuals older than 14 years old, no significant seasonal variation of specific IgG level and no decrease after ITNs installation were noticed. This observation could be at least in part explained by the different sleeping schedule of adults, which may result in a lower protection by ITNs from *Anopheles* bites. However, it is likely that the kinetic of appearance and disappearance of the anti-cE5 response also contributes to the absence of seasonal variation. Since adults are generally more intensively exposed to *Anopheles* bites they may establish a stronger and more persistant specific Ab response to the cE5 antigen, which may then need more time to vanish. Moreover, this suggestion is strengthened by the fact that in the same adults the IgG response to the gSG6-P1 peptide, which is known to be short-lived, decreased after ITNs implementation indicating that the adult-vector contact was indeed disrupted [48]. For the two youngest group (<14 years old), seasonal variation of IgG response was detected before the ITNs implementation and a significant decrease was observed just two months after the introduction of ITNs. A positive association between IgG response against cE5, exposure to *Anopheles* bites and parasite density was observed, indicating the potential validity of this antigen as candidate biomarker of children exposure to *An. gambiae*. Two months after the introduction of ITNs (April 2006), a rapid decrease of the Ab response was observed, and this accompanied a decline of *P. falciparum* parasitemia. Therefore, the proper use of ITNs appeared to effectively reduce the human-vector contact, even during the period corresponding to the peak of the *An. gambiae* population, resulting in a decreased IgG response against the cE5 protein in these younger groups. These observations are fully in agreement with previous results obtained analyzing the IgG response to whole saliva and to the gSG6-P1 peptide [27, 48], which also showed a drop after ITN installation without effect

on the mosquito density. Overall our observations indicate that the drop of the anti-cE5 IgG response was associated with the correct use of ITNs, disrupting the human-vector contact, and not due to a wane of *Anopheles* density. Noteworthy, in July 2006 the IgG response against the cE5 protein, as well as the *P. falciparum* parasitemia [27], increased and stayed high in the following months, despite the seasonal decline of mosquito density. One possible explanation could be the incorrect use and/or the damage of the nets, an hypothesis that is supported by a survey on ITNs use indicating that only 53% of ITNs were well-installed and undamaged in June 2006 in the total population [27].

The data reported here also show the high immunogenicity of the cE5 protein, as indicated by the high intensity of the Ab response found during this two years longitudinal study. This also confirms previous unpublished observations indicating cE5 as more immunogenic than gSG6 (B. Arcà, Sapienza University of Rome, manuscript in preparation). In a previous study, including the same group of individuals analyzed here, Drame *et al,* showed that the anti-gSG6-P1 IgG response was very low in some young and older children in January 2006, before the introduction of ITNs [48]. Consequently approximately 39% of the subjects showed no significant change of the IgG response to gSG6-P1 between January 2006 and April 2006. Here, in the same individuals, the Ab response against cE5 was higher during the same period, suggesting that the absence of IgG response against the gGS6-P1 peptide was most likely due to the lower sensitivity of the peptide rather than to the absence of exposure to *Anopheles* bites. In this context the use of the cE5 protein could allow detecting individuals bitten very weakly and presenting a low Ab response to gSG6-P1 peptide, maybe not detectable by ELISA.

The kinetics of accumulation and the persistance of the anti-cE5 IgG response in adults suggest that the IgG response to cE5 may not be pertinent as biomarker in individuals continuously exposed for several years to *Anopheles* bites. Instead it may be more useful to evaluate exposure to malaria vectors in individuals transiently exposed to *Anopheles* bites, such as travelers or soldiers living in malaria transmission areas just for a few weeks. In addition, the results reported here in children up to 14 years of age reveal the potential application of this protein as biomarker of exposure to *Anopheles* bites and for evaluating the efficacy of ITNs in children population. Moreover, children less than 5 years old are the first vulnerable/susceptible population to malaria and so the first target for preventive measures as ITNs. In conclusion, further studies are needed to validate the use of the cE5 salivary protein in other context of malaria transmission and also in individuals occasionally exposed to *Anopheles* bites such as travelers and soldiers. It is known that *An. funestus* and *An.*

arabiensis are important vector of malaria in Tropical Africa and can play a major role in malaria transmission when *An. gambiae* is not present. It is highly predictable that the IgG response to the *An. gambiae* cE5 may function as indicator of exposure to the closely related *An. arabiensis*. Since the *An. gambiae* and the *An. funestus* proteins share only 57% identity and 74% similarity, other studies will be needed to evaluate the cross-reactivity and verify the possibility to use the cE5 protein to evaluate exposure to all three main Afrotropical malaria vectors.

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Summary of results

The objective of this study was to test whether the Ab response to the salivary cE5 protein could be used as biomarker of human exposure to *Anopheles* bites and as indicator of ITNs efficacy. A longitudinal study in Angola, including entomological and parasitological data was used to assess the IgG response to cE5, in children and adults, before and after the insecticide treated nets implementation. A positive association between the IgG level and the exposure level was observed only in children (<14 years) before insecticide treated nets implementation. Moreover, after the bed nets installation, a drop of the specific IgG response was also only observed in children allowing an evaluation of the short-time efficacy of bed nets and at individual level. Moreover, the high antigenicity of this protein appeared to be pertinent to detect very low exposure in children. In adults, no seasonal variation and no decrease of the specific IgG response was observed suggesting a possible accumulation of the Ab response with age due to a long time and a constant history of exposure to the *Anopheles* bites. In conclusion, the IgG response to cE5 could be a reliable biomarker of exposure to *Anopheles* bites and could also evaluate the short-term efficacy of insecticide treated nets in children. However in the field, one optimal biomarker tool must be able to evaluate the bite exposure in all age groups of population. This point is discussed in the general discussion session.

Chapter 2: Identification of an immuno-epidemiological biomarker of exposure to infective *Anopheles* bites

Because infective bites represent a very low proportion of all *Anopheles* bites received by individual, it could be interesting to develop a biomarker tool specific and sensitive which could distinguishes non-infective and infective bites. Such a new and complementary indicator may identify individuals who have been specifically exposed to infective bites but also to evaluate the efficacy of vector control strategies only against the infective bites and by consequences, on the malaria transmission.

To achieve this objective, salivary proteins specific to infective bites have to be identified. The modification of saliva composition by *Plasmodium* infection could be a basis to identify such proteins. This effect of parasite on saliva has already been shown in salivary glands of *An. gambiae* infected by *P. berghei* (murine *Plasmodium*) (Choumet et al. 2007, Rosinski-Chupin et al. 2007, Zocevic et al. 2013). If the expression of an immunogenic salivary protein is modified by *Plasmodium*, the human Ab response against this protein could be also modified. Ideally, one or more immunogenic proteins which are only expressed in presence of the parasite could represent the optimal biomarker of infective bites. However, if one or more upregulated immunogenic proteins could be identified when salivary glands are infected, we can suppose that the specific Ab responses to these proteins could be also increased allowing the discrimination between infective and non-infective bites. This specific biomarker could be also combined with a biomarker of human exposure to all *Anopheles* bites and/or with a *P. falciparum* antigen to increase the sensitivity of the tool.

Currently, no study has investigated changes in salivary proteins expression in *An. gambiae* infected by wild *P. falciparum*, the natural complex of human malaria.
To resolve this question, experimental infections in semi-field condition of *An. gambiae* with wild *P. falciparum* were performed in Cameroon (October 2011):

i) Blood samples and selection of gametocyte carriers

All procedures involving human subjects were approved by the Cameroonian national ethic committee (statement 099/CNE/SE/09).

The detection and selection of asymptomatic gametocyte carriers have enrolled children from 5 to 11 years old in schools from Mfou area in Yaoundé (Figure 21). A thick blood film has been realized in children having a parental signed statement and colored with Giemsa to detect trophozoites and gametocytes of *P. falciparum*. Children with a parasite density more than 50 parasites/µL were treated with ACT. Children presenting a gametocyte rate more than 20 parasites/µL were selected for experimental infections. The day after, the gametocyte carriers were brought to the laboratory and a thick blood film was removed again to verify the gametocytemia. For each carrier, 5mL of venous blood were collected, spin down at 2000g for 2 min at 37°C and the carrier serum was replaced by non-immune serum to avoid the effects of factors which could block the parasite development (Boudin et al. 2005). The reconstituted blood was used for the blood feeding of mosquitoes.

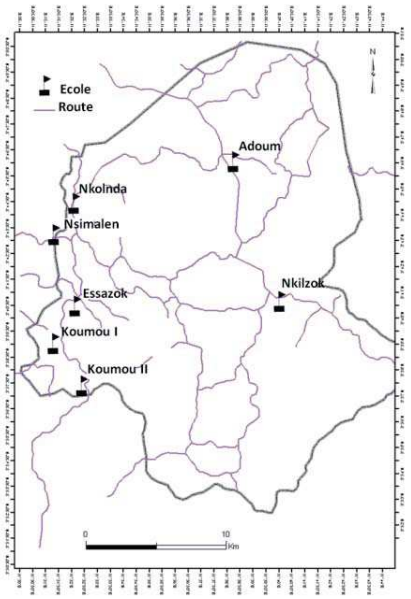

Figure 21. Localisation of selected school in Youndé.

ii) Experimental infections

Females *An. gambiae* (laboratory mosquitoes, KIS strain), 3 to 5 days old, were starved for 24 hours and then were gorged on blood, either infected or not, using an adapted system (Figure 22).

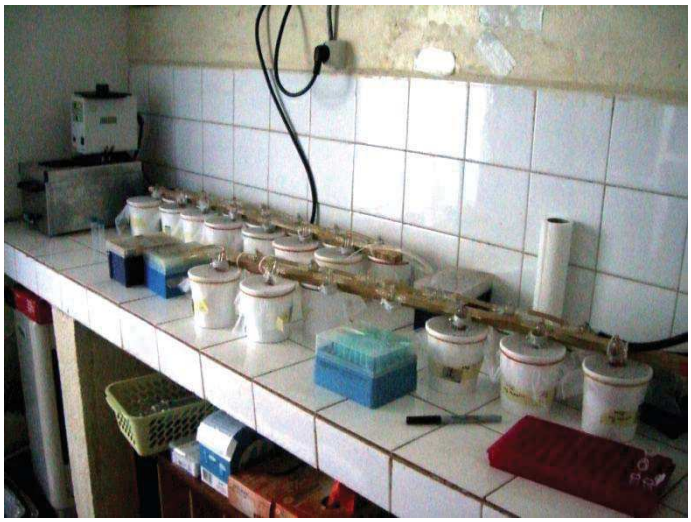

Figure 22. System used for the blood feeding of *An. gambiae*.
Mosquitoes in the cups gorged on the infected or non-infected blood across the parafilm membrane. Blood are maintained at 37°C via a water bath.

This system is composed of a water bath heated at 37°C and blood feeding cells. Each cell is composed of an internal circuit for the thermal regulation with circulating water at 37°C and an external circuit for the blood introduction. The parafilm® membrane is disposed on the basal part of the cells and a volume of 400µL of reconstituted blood (red blood cells + non-immune serum) is introduced in the cells. For the present proteomic experiments, non-infected salivary glands were also necessary. To obtain them, the same batches of blood were inactivated by heating (43°C) during 12 min to kill the parasites (gametocytes inactivation) before use (Mendes et al. 2011). Mosquitoes were fed on the infected or non-infected blood for 30-45 min. Unfed and partially fed females were removed and discarded. Fed female mosquitoes were maintained in the cups in the insectary. Eight days post-infection, the midguts of some of them were dissected to verify the presence of oocysts and confirmed the effectiveness of the infection and to verify if the parasites were actually killed in the non-infected blood feeding. Salivary glands of the mosquitoes were dissected 14 post-infection.

To verify the infection by wild *P. falciparum* and the non-infection of salivary glands, several methods could be employed: the CSP-ELISA assay, the multiplex PCR and a quantitative PCR. The last method was developed by a PhD student in the lab for the detection of *P. falciparum* in the midguts of *An. gambiae*. Here, I tested if we could use this qPCR to detect *P. falciparum* in the small DNA amount extracted from salivary glands. The following article published in *Malaria Journal* presents and compared these techniques.

Marie A, Boissière A, Tchioffo TM, Poinsignon A, Awono-Ambéné PH, Morlais I, Remoue F and Cornelie S. Evaluation of a real-time quantitative PCR to measure the wild *Plasmodium falciparum* infectivity rate in salivary glands of *Anopheles gambiae*. *Malaria J* 2013, **12**:224

MALARIA JOURNAL

METHODOLOGY Open Access

Evaluation of a real-time quantitative PCR to measure the wild *Plasmodium falciparum* infectivity rate in salivary glands of *Anopheles gambiae*

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Abstract

Background: Evaluation of malaria sporozoite rates in the salivary glands of *Anopheles gambiae* is essential for estimating the number of infective mosquitoes, and consequently, the entomological inoculation rate (EIR). EIR is a key indicator for evaluating the risk of malaria transmission. Although the enzyme-linked immunosorbent assay specific for detecting the circumsporozoite protein (CSP-ELISA) is routinely used in the field, it presents several limitations. A multiplex PCR can also be used to detect the four species of *Plasmodium* in salivary glands. The aim of this study was to evaluate the efficacy of a real-time quantitative PCR in detecting and quantifying wild *Plasmodium falciparum* in the salivary glands of *An. gambiae*.

Methods: *Anopheles gambiae* (n=364) were experimentally infected with blood from *P. falciparum* gametocyte carriers, and *P. falciparum* in the sporozoite stage were detected in salivary glands by using a real-time quantitative PCR (qPCR) assay. The sensitivity and specificity of this qPCR were compared with the multiplex PCR applied from the Padley method. CSP-ELISA was also performed on carcasses of the same mosquitoes.

Results: The prevalence of *P. falciparum* and the intensity of infection were evaluated using qPCR. This method had a limit of detection of six sporozoites per µL based on standard curves. The number of *P. falciparum* genomes in the salivary gland samples reached 9,262 parasites/µL (mean: 254.5; 95% CI: 163.5-345.6). The qPCR showed a similar sensitivity (100%) and a high specificity (60%) compared to the multiplex PCR. The agreement between the two methods was "substantial" ($\kappa = 0.63$, $P < 0.05$). The number of *P. falciparum*-positive mosquitoes evaluated with the qPCR (76%), multiplex PCR (59%), and CSP-ELISA (83%) was significantly different ($P < 0.005$).

Conclusions: The qPCR assay can be used to detect *P. falciparum* in salivary glands of *An. gambiae*. The qPCR is highly sensitive and is more specific than multiplex PCR, allowing an accurate measure of infective *An. gambiae*. The results also showed that the CSP-ELISA overestimates the sporozoite rate, detecting sporozoites in the haemolymph in addition to the salivary glands.

Keywords: *Plasmodium falciparum*, *Anopheles gambiae*, Salivary Glands, Quantitative PCR, Multiplex PCR, CSP-ELISA

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Background

In malaria endemic countries, *Plasmodium falciparum* is transmitted to the human host by the bite from a female *Anopheles* mosquito. *Anopheles gambiae sensu stricto* (*s.s.*) is the most widespread malaria vector throughout the afrotropical belt. In the context of malaria eradication, it is essential for malaria-surveillance programmes to estimate accurately the risk of malaria transmission. Currently, the main indicator of *Plasmodium* transmission is the measure of the entomological inoculation rate (EIR) [1], which is the number of infective mosquito bites per human per night. In field settings, the EIR is commonly estimated by using captured adult mosquitoes. Evaluation of infection prevalence in salivary glands can be measured by counting sporozoites by microscopy [2] or by using the enzyme-linked immunosorbent assay on the head-thorax of the mosquito to detect the surface circumsporozoite protein (CSP-ELISA) [3]. Both methods are known to be labour intensive and it has been shown that CSP-ELISA overestimates the real infection rate by detecting the CSP from the oocysts bursting, two to three days before the sporozoites actually reach the salivary glands [2,4].

Research efforts in recent decades have led to the development of molecular biology tools for detecting *Plasmodium falciparum* in human blood [5] and in mosquito samples [6]. Among these, a multiplex PCR was developed by Padley *et al* to detect the four major species of *Plasmodium* (*P. falciparum*, *Plasmodium malariae*, *Plasmodium ovale,* and *Plasmodium vivax*) in human blood samples [7] and was applied to detect them in *Anopheles* mosquitoes. Multiplex PCR is based on the detection of a Small SubUnit of ribosomal RNA (SSU rRNA) of each *Plasmodium* species but it requires a significant amount of parasite DNA, which is not easily achieved with small tissues like a single pair of salivary glands. Specific and sensitive methods such as quantitative PCR (qPCR) have also been developed to measure the prevalence and intensity of infection in human blood samples [8,9]. In mosquito samples, quantification of *P. falciparum* oocysts in *Anopheles stephensi* [10] and in wild *An. gambiae s.s.* [11] has also been achieved through real-time PCR. The latter study evaluated the difference in susceptibility of malaria infection (oocyst stage) between the M and S molecular form of *An. gambiae s.s.* in Cameroon. In addition, Vernick *et al* [12] estimated the infection prevalence of *P. falciparum* (parasite culture) in *An. gambiae* (insectary-reared mosquitoes) by reverse transcriptase PCR using specific sequences of the Small SubUnit of ribosomal RNA (SSU rRNA) of the sporogonic stages. Recently, a duplex real-time PCR was developed for the detection of the four *Plasmodium* species in field mosquitoes from Benin based on species-specific primers and probes for the gene encoding the small subunit (18S) of *Plasmodium* rRNA [13]. However, in this study, the use of the head-thorax of mosquitoes leads to an inaccurate estimation of the EIR, which should be based only on the sporozoites present in salivary glands.

Therefore, it is important to develop sensitive and rapid diagnostic tools for detecting *Plasmodium* in salivary glands of the *Anopheles* vectors, as this will reveal the true proportion of infective mosquitoes and, consequently, only those that can transmit malaria parasites. The aim of the present study was to evaluate the sensitivity and the specificity of a quantitative PCR method in the detection of wild *P. falciparum* sporozoites in *An. gambiae* salivary glands. First, the qPCR assay based on the mitochondrial cytochrome c oxydase subunit 1 (COX-1) gene described by Boissiere *et al* [11] was tested on infected salivary glands to detect and quantify *P. falciparum*. A comparison of the qPCR method with a multiplex PCR based on the Padley method was also made to identify the most sensitive method. Finally, a comparison of the infectivity rates obtained with these two techniques with those obtained with the CSP-ELISA was performed on the carcasses of mosquitoes without salivary glands. CSP-ELISA was considered the current reference method used in the field. In this paper, experiments were conducted in semi-field conditions. *Anopheles gambiae* mosquitoes were fed on blood from asymptomatic children containing high similar gametocyte densities (from 52.7 to 60.6 gametocytes/μL). In natural settings, mosquito infectivity rate depends on several factors such as gametocyte density, sex ratio and multiclonality of parasites [14-17]. In consequence, this original approach allowed to mimic field conditions, and thereby to evaluate the potential application of this qPCR in field settings. Data showed that qPCR is highly sensitive but more specific than the multiplex PCR. Moreover, this study confirmed that the CPS-ELISA overestimates the infectivity rate by detecting the circulating sporozoites in addition to those present in salivary glands.

Methods

Ethics statements

All procedures involving human subjects used in this study were approved by the Cameroonian National Ethical Committee (statement 099/CNE/SE/09). Children identified as gametocyte carriers were enrolled as volunteers after their parents or legal guardians have signed an informed consent form.

Mosquito collection

The Kisumu strain of *An. gambiae* was provided by the Laboratoire de Lutte contre les Insectes Nuisibles, Institut de Recherche pour le Développement, France. The colony was established and maintained at the insectary in OCEAC (Yaoundé, Cameroon) for the experimental infections. Adult mosquitoes were maintained in

standard insectary conditions (27±2°C, 85±5% RH, and 12 h light/dark) and provided with 6% sterile sucrose solution.

Experimental infections and salivary gland dissection

Female mosquitoes were fed on *P. falciparum* gametocyte carriers. Infectious feeding was performed as previously described [18,19]. Females, three to five days old, were starved for 24 h and allowed to feed on human blood containing *P. falciparum* gametocytes for 35 min. Unfed and partially fed mosquitoes were removed by aspiration and discarded. Fully engorged females were kept in the insectary until dissections 14 days after the infectious blood meal. Mosquitoes were cold-anaesthetized and salivary glands were dissected in 10 μL of buffer containing 7 M urea, 2 M thiourea, and 4% CHAPS (GE, Healthcare). Samples were kept frozen individually at −20°C until processing.

CSP-ELISA assay

After the dissection of salivary glands, the carcass-thorax-head were tested by ELISA for the presence of *P. falciparum* CSP as described by Burkot and modified by Wirtz *et al* [20]. The monoclonal antibody and positive controls were provided by the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). Mosquitoes were considered positive when the optical density (OD) was higher than the mean plus three standard deviations of the negative controls (OD=0.059).

DNA extraction

DNA extraction from the salivary glands was performed using DNAzol® (Molecular Research Center, Inc, Cincinnati, OH, USA) according to the manufacturer's instructions. Extracted DNAs were eluted in a final volume of 20 μL water and were stored at −20°C. DNA extraction was checked for the presence of mosquito DNA by specific PCR for *An. gambiae* species [21].

Identification of *Plasmodium falciparum* by multiplex PCR

The infection status of *P. falciparum* was determined by multiplex PCR as previously described [7] based on the detection of a Small SubUnit of ribosomal RNA of each *Plasmodium* species with five primers: universal reverse *Plasmodium* primer 5′-GTATCTGATCGTCTTCACTCC C-3′, *P. malariae forward* 5′-CGTTAAGAATAAACGC CAAGCG-3′, *P. falciparum forward* 5′- ACAGACGGGT AGTCATGATTGAG-3′, *P. ovale forward* 5′-CTGTTC TTTGCATTCCTTATGC-3′, and *P. vivax forward* 5′-CG GCTTGGAAGTCCTTGT-3′. PCR was performed on 5 μL of eluted DNA with the Taq Hot Start Master mix (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. PCR amplification was carried out under the following conditions: an initial incubation cycle to activate the enzyme for 45 sec at 95°C followed by 43 cycles of amplification involving 45 sec at 95°C, 90 sec at 60°C and a final extension of 5 min at 72°C.

Quantitative real-time PCR

qPCR was performed on 1 μL of eluted DNA with the EvaGreen® dye (5X HOT Pol EvaGreen® qPCR Mix Plus (ROX), Euromedex, Souffelweyersheim, France) in the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Specific primers used for the qPCR were 5′-TTACATCAGGAATGTTATTGC-3′ and 5′-ATATTG GATCTCCTGCAAAT-3′ [9,22]. They amplified a 120-bp sequence of the *P. falciparum* cytochrome c oxidase subunit 1 (Cox1) mitochondrial gene. The reaction mixture was prepared following the procedure of Boissière *et al* [11]. Absolute qPCR was performed following the amplification program of an initial melting cycle for 15 min at 95°C followed by 40 amplification cycles at 95°C for 15 sec and 58°C for 30 sec. The melting temperature was determined using a dissociation curve. Curves were generated after amplification: at 95°C for 15 sec (DNA denaturation), at 60°C for 30 sec (double stranded DNA), and at 95°C for 15 sec (single stranded DNA). Fluorescence was monitored allowing the identification of the specific melting point. As described by Boissière *et al* [11], standard curves using 3D7 strain DNA were generated from serial dilution methods and resulting in a quantification range of 6 to 60,000 genomes/μL. These standards were used to determine the concentration of sporozoites in the salivary glands of *An. gambiae* [11].

Statistical analysis

Statistical analyses were performed using the statistical software R [23], and all differences were considered significant at P values of <0.05. The means of the amplification efficiencies between the standard samples and the salivary gland samples were compared using the Mann-Whitney-Wilcoxon test. Cohen's kappa co-efficient (κ) was calculated to measure the agreement between the qPCR and multiplex PCR. Methods were compared using the McNemar test.

Results

The detection threshold of the method was determined by using a five-fold serial dilution of genomic DNA isolated from a 3D7 culture of *P. falciparum,* allowing a quantification range from 6 to 60,000 genomes/μL, as previously described [11]. The reproducibility of the test was confirmed by using a composite of 53 standard curves showing a standard deviation <0.75 and a regression value equal to 0.998 for the five data points (Figure 1). The means of the amplification efficiencies per amplicon for the cultured parasites and the salivary gland samples were 94.3% (± 0.6) and 94.1% (± 0.3), respectively, and the difference was not significant (P=0.065). This result showed that the

prevalence and intensity of infection can be evaluated using this method. Absolute quantification of *P. falciparum* genomes in *Anopheles* salivary gland samples was based on the calibration curve (composite of the 53 standard curves) with a detection limit of six genomes/μL (120 sporozoites by pair of salivary glands in this study). *Plasmodium falciparum* parasitaemia in salivary gland samples reached 9,262 parasites/μL, with an infection mean of 254.5 parasites/μL (95% CI: 163.5-345.6). This result showed an heterogeneity in the *Plasmodium* infection intensity among mosquitoes, as observed in the salivary glands. For the first time, the optimized qPCR enabled specific detection and quantification of total *Plasmodium* parasitaemia (genome/μL) in *An. gambiae* salivary glands.

The prevalence of *P. falciparum* was assessed on DNA extracted from the salivary glands of 364 *An. gambiae* using qPCR and multiplex PCR. The qPCR revealed 276 positive (76%) and 88 negative (24%) salivary glands for *P. falciparum,* whereas the presence of *Plasmodium* DNA was found in 217 (60%) salivary glands by multiplex PCR (Table 1). The statistical analysis showed that differences obtained by both methods were significant (McNemar test: multiplex PCR *vs* qPCR, P <0.001). The 217 positive salivary glands with multiplex PCR were confirmed as positive with qPCR. Among the 147 negative salivary glands detected with multiplex PCR, 88 were also negative with qPCR and 59 were identified as positive. The qPCR method presented high values of sensitivity of 100% (Se = (217/217)*100) and specificity of 60% (Sp = (88/147)*100) when compared to the multiplex PCR, considered here as the reference test. The agreement between qPCR and multiplex PCR was "substantial" (κ = 0.63 and P <0.001). In the field and especially in the context of malaria eradication or low transmission, the number of sporozoites in salivary glands could be very low and multiplex PCR may not be sensitive enough to detect low infection rates. Furthermore, this method requires a high amount of DNA template and therefore it seems unsuitable for investigating infection in mosquito salivary glands. This may explain the low positivity rate detected in the present study (59%). Moreover in *Plasmodium spp*, Cox1 mitochondrial gene is present in higher quantity than the SSU rRNA gene. Indeed, mitochondrial DNA is composed to approximately 20 copies per cell [24], whereas the SSU rRNA genes are presented in 4-8 copies [25]. Consequently, the use of the Cox1 gene for the qPCR increases its specificity. qPCR, known for its sensitivity and for the small amount of DNA required, thus seemed a feasible way to detect *Plasmodium* in *Anopheles* salivary glands. Using this technique, a 1.25-fold higher prevalence rate of *P. falciparum* infection compared to the multiplex PCR and a detection limit of six sporozoites/μL were observed. Interestingly, false-negative samples determined by multiplex PCR were detected as positive using qPCR. In addition, this method estimates the *Plasmodium* intensity level in contrast to multiplex PCR, even in very small biological samples like the single pair of salivary glands used here. This method could open the way for determining the relationship between the sporozoite load in salivary glands and the infectiousness of the *Anopheles* mosquito. However, this qPCR approach identified only *P. falciparum* species, in contrast to multiplex PCR. Although *P. falciparum* is the species causing the majority of clinical cases of malaria in Africa, a recent study in rural Benin has shown that *P. faciparum* accounted for 91% of the malaria infections,

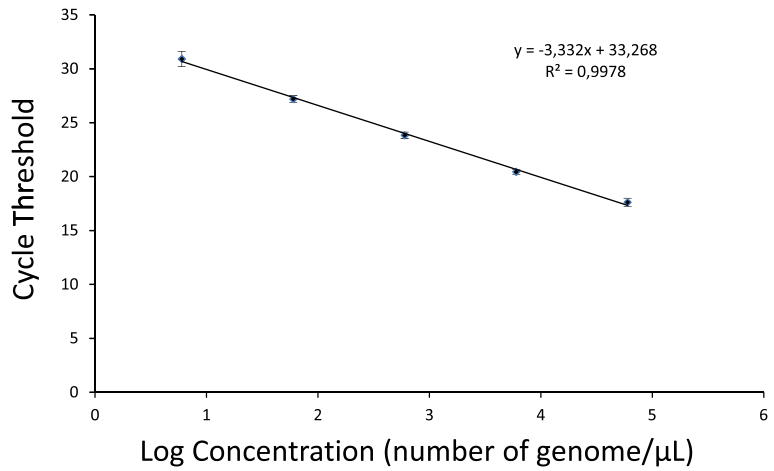

Figure 1 Standard curve of qPCR using serial dilutions of DNA from cultured parasites. Calibration curve was generated using 53 calibrations curves. The curve is based on the known DNA concentration (genomes/μL) and shows the reproducibility. Error bars show the standard deviation for each DNA standard from 6 to 60, 000 genomes/μL [11]. © Boissiere *et al* [11].

Table 1 Comparison of qPCR and multiplex PCR techniques for detection of *Plasmodium falciparum* sporozoites in salivary glands of *Anopheles gambiae*

Salivary glands samples	qPCR positive	qPCR negative	Total	Sensitivity	Specificity	Kappa (κ)
PCR positive	217	0	217 (60%)	100%	60%	0.63
PCR negative	59	88	147 (40%)			
Total	276 (76%)	88 (24%)	364 (100%)			

McNemar test: qPCR *vs* multiplex PCR, P <0.001.

evaluated by thick blood smears. Mixed infections with *P. malariae* or *P. ovale* were also detected at 3% and 2% of the tested slides, respectively [26]. A multiplex qPCR was developed to discriminate the four species of *Plasmodium* in human blood samples [27,28], and one was developed very recently by Sandeu *et al* in mosquitoes [13]. However, the latter method was performed and optimized using a duplex qPCR on the head-thorax of mosquitoes, consequently using DNA from both the circulating sporozoites and those in the salivary glands [13].

Some multiplex qPCR assays have also used the EvaGreen® dye, as it was done in the present study [29,30]. Therefore, it seems possible to adapt the present qPCR method so as to carry out multiplex qPCR detection of the four species of *Plasmodium*. Evagreen® dye is a DNA-binding dye with many features that make it superior to the SYBR® Green I for qPCR [29,31]. Furthermore, this dye is compatible with all common real-time PCR cyclers [32] and is currently about half the price (€0.16 per reaction) of the SYBR® Green (€0.53 per reaction) commonly used. The duplex qPCR performed by Sandeu *et al* used the Taqman technique (€1.12 per reaction). In conclusion, the qPCR developed here is cost-effective and therefore suitable for large field studies. It is also cheaper than the multiplex PCR (€1.60 per reaction).

Detection of the presence of the *Plasmodium* parasite by CSP-ELISA was also tested on the head-thorax carcasses of the same mosquitoes without salivary glands, thus detecting only circulating sporozoites. A total of 302 mosquitoes were found to be *P. falciparum* positive (range OD: from 0.164 to 2.420) (Table 2). The results of the CSP-ELISA showed that a higher number of positive mosquitoes (83%) were detected compared to multiplex PCR (60%) and qPCR (76%). The statistical analysis revealed statistically significant differences between the three methods (McNemar test: CSP-ELISA *vs* qPCR, P <0.001; CSP-ELISA *vs* PCR, P <0.001) (Table 2). Of the 302 *Plasmodium*-infected head-thorax-carcasse samples, 261 were found to be positive while 41 samples were negative using the qPCR method. According to these results, 11.2% of the mosquitoes were found to be *Plasmodium* positive in the head-thorax-carcasses but not in the salivary glands, meaning that circulating sporozoites can be detected using CSP-ELISA even in non-infective mosquito. This finding is in accordance with other studies [2,33,34] showing that the CSP-ELISA assay (performed on head-thorax including salivary gland) overestimates the sporozoite rate in mosquitoes by detecting circulating sporozoites. Indeed, parasites covered by CSP, spread into the haemolymph for two to three days before they reach the salivary glands [35]. Moreover, it has been shown that only 10-20% of sporozoites reach the salivary glands [36-38] and that some mosquitoes could be refractory to the entrance of sporozoites in salivary glands [39]. Consequently, CSP-ELISA, which is routinely used in the field, detects infected mosquitoes but not necessarily the infective ones.

Conclusion

Estimation of malaria transmission requires sensitive and specific tools for the evaluation of infective mosquitoes, i e, detection of sporozoites in *Anopheles* salivary glands. This study showed that real-time quantitative PCR can be used to detect and quantify sporozoites of wild *P. falciparum* in the salivary glands of *An. gambiae.* This qPCR can be performed on small samples such as the DNA of *P. falciparum* sporozoites extracted from a

Table 2 Comparison of CSP-ELISA with qPCR and multiplex PCR for detection of *Plasmodium falciparum* sporozoites

	CSP-ELISA positive	CSP-ELISA negative	Total
qPCR positive	261	15	276 (76%)
qPCR negative	41	47	88 (24%)
Total	302 (83%)	62 (17%)	364 (100%)
PCR positive	211	6	217 (60%)
PCR negative	91	56	147 (40%)
Total	302 (83%)	62 (17%)	364 (100%)

McNemar test: CSP-ELISA *vs* qPCR, *P* <0.001; CSP-ELISA *vs* PCR, P <0.001.
CSP-ELISA was performed on head-thorax-carcasses, and qPCR and multiplex PCR were performed on salivary glands DNA.

single pair of salivary glands of *An. gambiae* with a sensitivity of six genomes/μL. In the present study, the real-time quantitative PCR was compared for the first time with multiplex PCR and CSP-ELISA methods.

qPCR is highly sensitive but more specific than multiplex PCR. Moreover, qPCR with EvaGreen® dye is reliable, reproducible, and cost-effective. This method is feasible for evaluating the *P. falciparum* infection rate in the salivary glands and it can lead to an accurate estimation of the risk of transmission in field settings, which were overestimated by CSP-ELISA. Improving the estimation of the EIR with this method could have significant implications on vector control strategies and on the evaluation of their effectiveness.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AM, AB, IM and SC conceived and designed the experiments. AM, AB, MTT and IM carried out the experiments. AM and AB analysed the data. FR, PHAA and IM contributed reagents/materials/analysis tools. AM, AB, AP and SC wrote the paper. All authors read and approved the final manuscript.

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In this study, we showed that the real time quantitative PCR assay to detect *P. falciparum* in *An. gambiae* salivary glands was highly sensitive and more specific than the multiplex PCR allowing an accurate detection of sporozoites in a small DNA sample as well as their quantification. Moreover, we demonstrated that the CSP-ELISA assay, the referent method used in the field to evaluate the malaria risk of transmission, overestimate the number of infectious mosquitoes.

iii) Proteomic approach

To compare the expression of salivary proteins in *P. falciparum*-infected and non-infected salivary glands of *An. gambiae*, a proteomic approach combining the 2D-Differential gel electrophoresis (2D-DIGE) and the mass spectrometry (Figure 23) was undertaken.

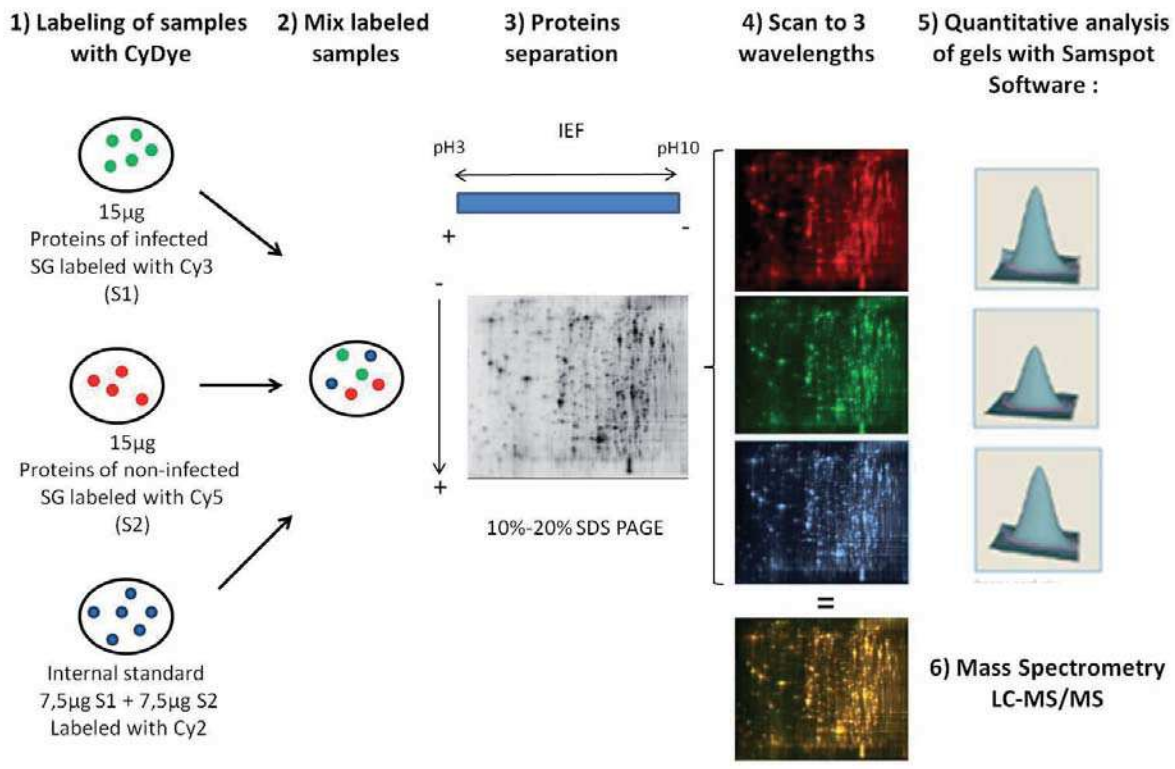

Figure 23. Proteomic approach combining 2D-Differential Gel Electrophoresis (2D-DIGE) and mass spectrometry.
Each sample of salivary proteins (infected and non-infected) are labeled with different Dyes (Cy3 and Cy5) and an internal standard composed of the two samples is labeled with a third Dye (Cy2). All samples are pooled and proteins are separated on the same gel according their isoelectric point and molecular weight. Gels are scanned at different wavelengths and analyzed with the Samespot software to quantify and compare the protein expression in each condition. Spots of interest are excised manually and analyzed by mass spectrometry to identify the proteins.

The following article, in preparation for submission in *Parasites and Vectors*, presents the study of the differential expression of salivary proteins between non-infected and wild *P. falciparum*-infected salivary glands of *An. gambiae* in the aim to identify one or more candidate proteins as biomarker of infective bites.

Modulation of salivary proteins expression of *Anopheles gambiae* by wild *Plasmodium falciparum* infection: Toward the identification of biomarkers of infective bites.

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Abstract

Background

Malaria is the major parasitic disease worldwide and was due to *Plasmodium* infection. The objective of malaria integrated control programs is the decrease of malaria transmission which needs specific tools to be accurately assessed. In areas where the transmission is low or has been profoundly decreased, new complementary tools have to be developed to improve the surveillance. A recent approach, based on the human antibody response to *Anopheles* salivary proteins, has been shown efficient for evaluating the human exposure to *Anopheles* bites. The aim of the present study is to identify new *An. gambiae* salivary proteins as potential candidate biomarker only specific to human exposure to *P. falciparum*-infective bites.

Methods

Experimental infections of *An. gambiae* by wild *P. falciparum* have been performed in semi-field conditions. First, the proteomic method**,** combining 2D-DIGE and mass spectrometry, was carried out to identify the over-expressed salivary proteins in infected salivary glands, compared to uninfected *An. gambiae* controls. Subsequently, a peptide design of each candidate was performed *in silico* and their antigenicity was tested by epitope-mapping technique using blood from individuals exposed to *Anopheles* bites.

Results

Five salivary proteins (gSG6, gSG1b, TRIO, SG5 and the long form D7) which were presented in overexpressed spots in the infected salivary glands, are found to be antigenic in children exposed to the *Anopheles* bites and could be thus selected as candidate biomarker. Moreover, results showed that the presence of wild *P. falciparum* in salivary glands induce the modulation of expression of several salivary proteins and appeared also to induce post-translationnal modifications.

Conclusions

This study is, to our knowledge, the first which compares the sialome of *An. gambiae* infected or not by wild *P. falciparum* allowing to mimic the natural conditions of infection. It is a first step to understand the close interactions between the parasite and the mosquito salivary glands. In addition, our results indicate the potential to define a new biomarker specific to the infective bites of *Anopheles* which could, in the future, improve the evaluation of malaria transmission and of the efficacy of malaria control strategies.

Keyword

Anopheles gambiae, wild *Plasmodium falciparum*, salivary proteins, biomarker, infective bites, proteomic

Background

In Sub-Saharan Africa, *Anopheles gambiae* is the main vector of *Plasmodium falciparum*, the most murderer of the five human *Plasmodium* species, responsible of malaria. Over Half million of deaths (660 000) occurred per year, especially in children under 5 years old [1]. The lack of vaccines, the resistance to anti-malaria drugs [2] and the availability of drug treatment (the artemisinin-based combination therapies (ACT)) involve that the major method to decrease malaria is the vector control of *Anopheles* populations. However, the spread of *Anopheles* resistances to insecticide impaired the efficacy of vector control strategies [3, 4]. In a context of malaria elimination in some areas, integrated malaria controls have been implemented to reduce the malaria burden. Consequently in these areas where the transmission has profoundly decreased, but also in urban settings or high altitude where the *Anopheles* exposure and malaria transmission can be very low, the current methods (clinical, parasitological and entomological assessments) are not enough sensitive to evaluate the human exposure to *Anopheles* bites and the risk of malaria transmission. Indeed it appeared difficult to have reliable/pertinent information in terms of parasite detection and catching mosquitoes in these contexts. It is therefore necessary to develop complementary tools easy to sustain a large scale. To this objective, one promising approach is to evaluate the real human-vector contact for assessing the risk of transmission and the measure of the human Antibody (Ab) response against *Anopheles* salivary proteins was pertinent [5]. Indeed, during its blood meal, mosquitoes inject saliva in the human skin inducing a humoral response. This concept has been validated using the whole saliva extracts (WSE) of *An. gambiae* [6] and in other hematophagous arthropods, such as *Aedes* [7-9], *Culex* [10, 11] and *Glossina* [12, 13]. However, some salivary proteins are ubiquitous in arthropods and then the response observed against WSE could reflect the exposure to numerous arthropods. For this reason, a biomarker of human exposure to bites must be directed to genus- or species-specific epitopes. Based on previous studies, the gSG6 protein has been shown specific to *Anopheles* genus and immunogenic [14, 15] and therefore was selected. Its validation as biomarker of exposure to *Anopheles* bites has been studied in Burkina Faso [16, 17] and in Tanzania [18]. To optimize the specificity and the useful of biomarker, a peptide design of this protein was performed. The gSG6-P1 peptide has then been identified as antigenic and the Ab response to this peptide was positively associated with the level of exposure to *Anopheles* bites [19]. This peptide has also been validated as biomarker in different malaria transmission areas such as rural low exposure in Senegal [20, 21], in Kenya [22], in urban settings in Senegal [23] and for

exposure to *An. funestus* in Senegal [24]. However, we hypothesize that Ab response to the gSG6 protein and the gSG6-P1 peptide could evaluate at the same time the non-infective and infective bites of *Anopheles*. Settings of malaria transmission could be very different according field conditions from unstable to stable malaria with sporozoite rates (number of infectious *Anopheles*) ranging from 0.1% to 8% [25, 26]. In area of low transmission, the exposure to all *Anopheles* bites does not then closely represent the malaria transmission risk [27]. Moreover, hotspot of malaria transmission exist in all epidemiological settings, in maintaining transmission in low transmission seasons and fuelling transmission in high transmission seasons, and the detection of these hotspot will allow to concentrate the integrated malaria controls [28]. Consequently, a new biomarker specific to infective bites has to be developed to precisely assess malaria risk in these particular settings.

Salivary glands are the crucial organ for the development and the transmission of parasite to a host. Indeed the *Plasmodium* interacts with salivary proteins to enter in salivary glands [29-32]. To survive and multiply in this organ, parasites have to counteract the immune system, use the vector metabolism by modifying the salivary proteins expression [33]. Many studies have already demonstrated the modification of salivary proteins expression when the arthropods are infected by a pathogen. Transcriptomic studies have shown that genes of salivary proteins were up- or down-regulated in *Ixodes scapularis* nymphs infected by flavivirus [34], in *Rhipicephalus microplus* infected by *Anaplasma marginale* [35], in *Culex quinquefasciatus* infected by West Nile virus [36] and in *Aedes aegypti* infected by different serotypes of dengue virus (DENV) [37]. Proteomic analyses indicated that salivary proteins were modulated in *Glossina pallipides* infected by salivary gland hypertrophy virus [38], in *Ae. aegypti* infected by DENV serotype 2 (DENV-2) [39], in *Ae. albopictus* infected by DENV-2 [40] and in *Ae. aegypti* infected by chikungunya virus (CHIKV) [41]. Concerning the *Plasmodium* parasites, several studies have investigated the change of salivary proteins expression in *Anopheles* infected by murine *Plasmodium*. These reports revealed that infection by *P. berghei* modified the expression of several salivary proteins in *An. gambiae* [33, 42, 43] and in *An. stephensi* [44]. In these studies, the presence of pathogen in salivary glands strongly affects the composition saliva promoting pathogen's survival and transmission. Modification of salivary content could also allow the identification of a biomarker of parasite transmission.

The present study aimed to identify salivary proteins as potential biomarker of *An. gambiae* infective bites. In natural settings, mosquito infectivity rate depends on several factors such as

gametocyte density, sex ratio and multiclonality of parasites [45-48]. Consequently, the modification of salivary proteins expression could be linked to the infection rate in salivary glands. To mimic field conditions, and thereby to evaluate at best the salivary protein modulation, experiments were thus conducted in semi-field conditions. *Anopheles gambiae* mosquitoes were fed on blood from asymptomatic children containing high similar gametocyte densities. Comparison of sialome in wild *P. falciparum*-infected *versus* non-infected salivary glands of *An. gambiae* was achieved by 2D-Differential Gel Electrophoresis (2D-DIGE) and mass spectrometry (LC-MS/MS). A peptide design was carried out on protein candidates and their immunogenicity has been tested in human living in malaria area. These methods allowed us to identify several *Anopheles* salivary proteins as candidate biomarker of *An. gambiae* infective bites.

Methods

Ethics statement

Experimental infection involving human subjects were approved by the Cameroonian national ethical committee (statement 099/CNE/SE/09). Children identified as gametocytes carriers were enrolled as volunteers after their parents or legal guardians had signed an informed consent form.

Mosquitoes collection

Kisumu strain of *An. gambiae* mosquitoes was obtained from M.N Lacroix, laboratoire de Lutte contre les Insectes Nuisibles, Institut de Recherche pour le Developpement, France. Eggs were reared at insectarium in OCEAC (Yaounde, Cameroon). Adult mosquitoes were maintained in controlled conditions (27 ±2°C, 85 ± 5% RH, and 12h light/dark) and provided with 6% sterile sucrose solution.

Experimental infections

Female mosquitoes were fed on *P. falciparum* gametocytes carriers. Infectious feeding was performed as previously described [49, 50]. Females, 3 to 5 days old, were starved for 24 h and allowed to feed on the blood containing *P. falciparum* gametocytes (from 52.7 to 60.6 gametocytes/µL) for 35 min. Non-infected salivary glands were obtained by feeding female mosquitoes on the blood from the same donors but heated at 43°C during 12 min for gametocyte inactivation. Unfed and partially fed mosquitoes were removed by aspiration and discarded. Fully engorged females were kept in insectarium until dissections 14 days after the

infectious blood meal. Salivary glands were dissected in buffer containing Urea, 7M; Thiourea, 2M; CHAPS, 4%. Samples were frozen individually until processing.

Protein samples preparation

Infected and non-infected salivary glands were lysed in liquid nitrogen and homogenates were then centrifuged for 20 min at 30 000 g at 17°C. The supernatants, named salivary gland extracts (SGE), were collected, purified by 2D Cleanup Kit (GE Healthcare) and protein concentrations were measured using a Coomassie Plus Protein kit (Pierce).

Bidimensionnel Differential Gel Electrophoresis (2D-DIGE)

For 2D-DIGE, Pf-infected and non infected proteins samples were compared using the CyDye DIGE Fluors for Ettan DIGE (GE Healthcare).

15 µg of SGE from Pf-infected and non-infected samples were labeled with 150 pmol/µL of either Cy3 or Cy5 following manufacturer recommendations. An internal standard constituted by 7.5 µg of each SGE was labeled with Cy2. A dye swap was performed to ensure that the modifications observed between the two conditions were not due to different efficiencies in dye labeling. Labeled SGE were pooled together in a final volume of 180 µL with rehydration buffer (7M Urea, 2M Thiourea, 4% CHAPS, 0.5% Triton X-100, 40mM Tris-HCl) prior to the separation by isoelectric focusing (IEF). IEF was performed with 11 cm Immobiline DryStrip, pH 3-11 non linear (NL) (GE Healthcare, Germany). Strips were rehydrated during the night at 20°C. Running conditions were: temperature 20°C; current 50µA per strip; 60 V (step) for 1h, 1000 V (gradient) for 2h, 6000 V (gradient) for 2h and then 6000 V steps up to 30000 Vh. The second dimension was carried out on 10-20% SDS-PAGE gels (Biorad, Marnes la Coquette, France) at 30 V for 20 min and then 200 V until the bromophenol blue front had reached the bottom of the gel. Gels were scanned using a Typhoon 9400 imager (GE healthcare). Images were acquired at 100µm pixel resolution under nonsaturating conditions and were analyzed with Progenesis Samespots 3.3 software. First, PCA analysis was carried out to verify that the gels from two conditions (Pf-infected and non-infected) were distributed in two distinct groups. Secondly, statistical analysis was done by an ANOVA test ($p<0.005$) for all spots in both groups and a second statistical analysis taking into account possible false positives was then performed with a cut-off of 1.4 fold either direction (up- and down-expression) and with $q<0.005$ and power >0.9. The q-value represents therefore the p value adjusted by the False Discovery Rate (FDR). Details are indicated in: http://www.nonlinear.com/support/progenesis/samspots/faq/pq-values.aspx.

Protein identification by LC-MS/MS

For the spot excision, gels were stained with the PageBlue Protein Staining Solution (Fermentas).

Trypsin digestion

Enzymatic in-gel digestion was performed according to the Shevchenko modified protocol [51]. Briefly, gel slices were destained by three washes in 50% acetonitrile, 50 mM Triethylammonium bicarbonate buffer and incubated overnight at 25 °C (with shaking) with 300 ng trypsin (Gold, Promega, Charbonnières, France) in 100 mM Triethylammonium bicarbonate buffer. Tryptic fragments were extracted with 50% acetonitrile and 5% formic acid, and dehydrated in a vacuum centrifuge.

Nano LC-MS/MS analysis

Peptide samples were dehydrated in a vacuum centrifuge, solubilized in 2 µl of 0.1% formic acid-2% acetonitrile and analyzed online by nano-flow HPLC-nanoelectrospray ionization using a LTQ Orbitrap XL mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific, San Jose, CA) coupled with an Ultimate 3000 HPLC (Dionex). Desalting and pre-concentration of samples were performed on-line on a Pepmap® precolumn (0.3 mm x 10 mm). A gradient consisting of 0-40 % A in 30 min, 80 % B in 15 min (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1 % formic acid in acetonitrile) at 300 nl/min was used to elute peptides from the capillary (0.075 mm x 150 mm) reverse-phase column (Pepmap®, Dionex). LC-MS/MS experiments comprised cycles of 5 events; an MS^1 scan with orbitrap mass analysis at 60000 resolution followed by CID of the five most abundant precursors. Fragment ions generated by CID were detected at the linear trap. Normalized collision energy of 35 eV and activation time of 30 ms were used for CID. All Spectra were recorded under positive ion mode using the Xcalibur 2.0.7 software (Thermo Fisher Scientific). Spectra were acquired with the instrument operating in the information-dependent acquisition mode throughout the HPLC gradient. The mass scanning range was m/z 400-2000 and standard mass spectrometric conditions for all experiments were: spray voltage, 2.2 kV; no sheath and auxiliary gas flow; heated capillary temperature, 200 °C; capillary voltage, 40 V and tube lens, 120 V. For all full scan measurements with the Orbitrap detector a lock-mass ion from ambient air (m/z 445.120024) was used as an internal calibrant as described [1].

All MS/MS spectra were searched against the Insecta entries of either SwissProt or TrEMBL databases (http://www.uniprot.org/ ; v 2012_07) by using the Proteome Discover software v 1.3 (Thermo Fisher Scientific) and Mascot v 2.3 algorithm (http://www.matrixscience.com/)

with trypsin enzyme specificity and one trypsin missed cleavage. Carbamidomethylation was set as fixed cystein modification and oxidation was set as variable methionine modification for searches. A peptide mass tolerance or 5 ppm and a fragment mass tolerance of 0.5 Da were allowed for identification.

Management and validation of mass spectrometry data will be carried out using Proteome Discoverer software v 1.3 ($p<0.01$ for 2 peptides or more/protein).

Peptide design

The design of potential immunogenic peptides was investigated *in silico* approach. The identification of putative B-cell epitopes was performed with BcePred [52], ABCpred [53], BepiPred [54] and SVMTrip [55] databases. Sequence alignments were performed with the Blastp program in Vectorbase database [56] and in UniProtKB database to compare the peptide sequences with known genomes or EST libraries. The peptides were selected when at least 3 algorithms to 4 predicted the same epitopes.

Peptide array

Experiments were performed with EpiFlag® methodology (Innobiochips, Lille, France). Eighteen peptides of 18 to 27 amino acids were synthesized by solid phase peptide synthesis with an automated peptide synthesizer (Intavis AG, Köln, Germany) using the Fmoc/tert-butyl strategy on a 20 µmol scale on a Rink-ChemMatrix® (PCAS BioMatrix Inc, Saint-Jean-sur-Richelieu, Quebec) resin. Following their elongation, peptides were deprotected and cleaved for 3h at RT using TFA/water/triisopropylsilane/EDT (1850 µL/50µL/50µL/50µL), precipitated in diethyl ether/n-heptane, 1/1 v/v, purified by RP-HPLC on a 120 Å 5 µm C18 Nucleosil column using a linear water/acetonitrile gradient containing 0.05 % TFA by vol (6 mL/min, detection at 215 nm) and lyophilized.

Each peptide characterized by RP-HPLC and MALDI-TOF MS was dissolved to a final concentration of 0.1 mM in 0.01 M PBS, pH 7.4, and printed on amine-modified glass slides (Arrayit, Sunnyvale, US) in duplicate.

Peptide arrays were blocked for 1 h at RT with PBS-M (0.01 M PBS, pH7.4, 0.05% Tween 20 and 2.5% non-fat milk). Saturated microarrays were washed with PBS containing 0.05% Tween 20. Human sera were diluted 1:10 in PBS-M and incubated overnight at 4°C. Microarrays were then washed 3 times with PBS containing 0.05% Tween 20. After washing, microarrays were revealed using a AlexaFluor 555-labeled goat polyclonal anti-human IgG antibody (Life Technology, Saint Aubin, France) at 1 µg/mL in PBS-M, for 1 h at RT.

Microarrays were washed, rinsed with distilled water, and dried. The glass slides were scanned with a TECAN LS-reloaded scanner (Tecan, Männedorf, Switzerland): PMT = 150. Data were extracted using Array-Pro® Analyzer Software.

Human serum were eluated dried blood spots from 42 children aged from 1 to 8 years old from Senegal in order to evaluate the Ab response to different salivary peptide candidates.

This procedure involving human subjects was approved by the National Ethics Committee of the Ministry of Health of Senegal (October 2008; 0084/MSP/DS/CNRS, ClinicalTrials.gov ID: NCT01545115). Oral and written informed consents were obtained from the parents or the legal guardians of the children.

Results and Discussion

Difference in sialome composition between *P. falciparum*-infected and non-infected salivary glands of *An. gambiae*

The salivary glands were dissected 14 days post-infection, time period for the parasite to reach the salivary glands. The infectivity status of each pair of salivary glands was verified by quantitative PCR [57]. The differential expression of sialome between *P. falciparum*-infected and non-infected salivary glands of *An. gambiae* was assessed by 2D-DIGE. In overall, 4 biological replicates were performed (figure 24a). After the ANOVA analysis and adjustment using the FDR approach, 207 spots showed a significant differential profile ($q<0.01$ and power >0.9) with a modulation from 1.3 fold to 8.8 fold. Among them, 73 spots were over-expressed and 128 under-expressed. After the colloidal coomassie blue staining, 43 visible spots could be excised for LC-MS/MS identification (figure 24a). Among them, 23 spots presented a 1.4 fold to 2.3 overexpression in *P. falciparum*-infected salivary glands whereas the 19 spots presented a 1.4 fold to 2.6 underexpression (figure 24b).

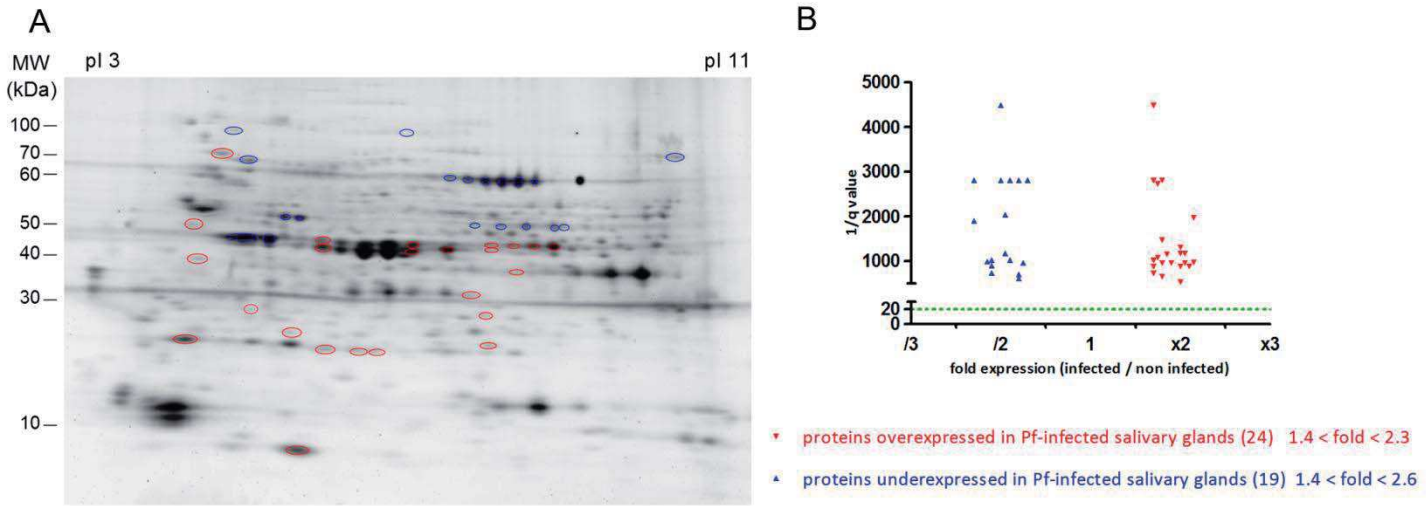

Figure 24. Differential salivary protein expression of *An. gambiae* infected or not by wild *P. falciparum*.
(A) 2D-DIGE gel. 43 spot differentially expressed are indicated by circle. Red circles represent the 24 overexpressed spots and the 19 blue circles represent the underexpressed spots. The pI and weight scales are indicated in the figure. (B) Differences in protein expression is represented in function of the expression ratio (infected / non infected) and significance ratio (q value). Horizontal dotted line indicates the significance threshold of $q<0.05$ (or $1/q>20$) according to the Samspot analysis.

These fold changes were in accordance with another study comparing the modification of *An. gambiae* sialome infected by *P. berghei* using the iTRAQ technology in which the modulations are in the same range from 1.5 to 1.95. Nevertheless, in this previous study, the expression of only five proteins was found altered [42]. Another study using the *An. gambiae*-*P. berghei* experimental model has shown that the expression of 7 salivary proteins was changed from 3.5 to 12 fold with 2D-PAGE technique [43]. One major difference from the present study was the high amount of spots whose the expression has been modulated by *P. falciparum* compared to the two previous studies. This could be due to the use of different *Plasmodium* species.

Identification of overexpressed proteins in *P. falciparum*-infected salivary glands of *An. gambiae*

Among the 43 overexpressed spots, the ubiquinol cytochrome c reductase iron-sulfur subunit and the gSG6 were identified as unique protein inside one spot (Figure 25 and table 1).

The ubiquinol-cytochrome c reductase iron-sulfur subunit (Rieske subunit) is an essential component of the complex III implicated in the oxydative phosphorylation in the aim to produce energy. The increase of the expression of enzyme involved to the oxidative phosphorylation has been already observed in the head of *An. gambiae* infected by *P. berghei*. Other proteins playing a role in the metabolic processes have also their expression modulated [58]. The presence of *P. falciparum* in *An. gambiae* could thus increase the energy metabolism necessary to the life of mosquito cells.

The gSG6 protein was first identified in *An. gambiae* [59] but also found in *An. stephensi* [14] and *An. funestus* [60] and is specifically expressed in salivary glands in female mosquitoes [61]. This protein has not been found in *Culex* [62] and *Aedes* [63, 64] mosquitoes, suggesting that it is specific to *Anophelinae* mosquitoes.

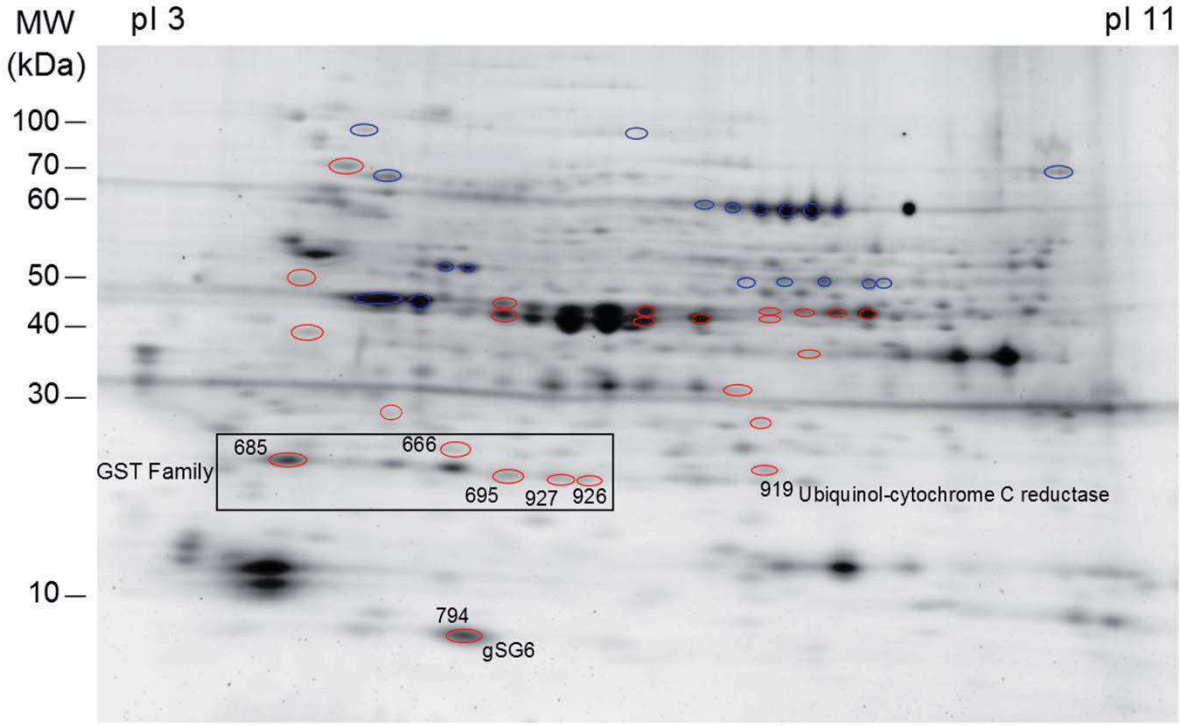

Figure 25. Overexpressed proteins in *P. falciparum*-infected SG of *An. gambiae* (2D-DIGE gel).
Unique proteins presented in overexpressed spot are indicated by number. The pI and weight scales are indicated in the figure.

Table 1. Up-regulated proteins in *P. falciparum*-infected salivary glands of *An. gambiae*.

Spot	Accession number (UniProtKB/TrEMBL)	Protein identification	Fold	Molecular mass (kDA)	pI	Mascot score	Cover sequence (%)
666	P46428_ANOGA	GST S1	2.3	23.2	5.29	7	29.06
	Q9GPL9_ANOGA	GST E1		25.3	5.66	4	17.86
685	P46428_ANOGA	GST S1	2.2	23.2	5.29	9	33.99
695	Q93113_ANOGA	GST D1 iso D	2.0	23.4	6.34	5	25.36
	Q93112_ANOGA	GST D1 iso C		23.8	6.55	3	20.57
927	Q93112_ANOGA	GST D1 iso C	2.3	23.8	6.55	8	36.36
926	Q93112_ANOGA	GST D1 iso C	2.0	23.8	6.55	5	17.70
919	Q7PWI1_ANOGA	Ubiquinol cyt c reductase iron sulfur subunit	2.0	28.1	8.53	3	9.43
794	Q9BIH5_ANOGA	gSG6	1.8	13.1	5.49	14	45.22

A recent study has demonstrated that this protein plays a role in the blood-feeding [65]. In the present study, the infection of salivary glands of *An. gambiae* by wild *P. falciparum* induced an overexpression of the gSG6 protein. However, other proteomic studies in murine models of infection have shown that gSG6 protein was down-regulated in *An. gambiae* infected by *P. berghei* [42, 43]. These divergent results could be explained by different aspects. The first is the *Plasmodium* and *Anopheles* species used for the experiments. Indeed, in the two previous proteomic studies, *An. gambiae* was infected by *P. berghei*. However, in the field, *P. berghei* infects naturally *An. dureni* [66]. In our study, the natural complex of human malaria, *An. gambiae* and *P. falciparum* was used. It is known that a strong relationship between pathogens and their hosts exist and which generate mutual co-evolution and a co-adaptation [67]. Therefore, we could suggest that *An. gambiae* reacts differentially to the two species of *Plasmodium* and the contradictory results could be due to the use of unnatural complex. The second aspect is the use of laboratory parasite strain versus wild *parasite*. The use of laboratory strain induces a bias to the multiclonality, important factor playing a role in the infectivity rate of *Anopheles* [48]. Moreover, the intensity of the mosquito infection depends on the gametocyte density [46] and the sex ratio [47]. We could hypothesize that the opposite results could be also due to the laboratory *P. berghei* rate injected to the mice which could influenced the number of gametocytes sucking by the mosquito and the number of sporozoites found in salivary glands. In the present study, the gametocyte rates measured in infected individuals were relatively low and ranged from 52.7 to 60.6 gametocytes/µL of blood. The parasite density may impact the physiology of the mosquito vector and especially salivary proteins. Our results indicated that to "mimic natural system" of *Anopheles* infections could reveal strong modulations in vector's physiology which cannot be observed with experimental models. Concerning the gSG6 protein, Lombardo *et al*, have demonstrated that the decrease of gSG6 expression induced an increase of the probing time of *An. gambiae* [65]. Our study indicated that the *P. falciparum* infection induced an increase (1.8 fold overexpression) of the gSG6 protein which could thus potentially lead to a shorter probing time. However, murine models of infection showed divergent results with no effect of *P.berghei* or *P. yoelli* infection on the probing time of *An. gambiae* [68] and *An. stephensi* [69] respectively. In regard to all these studies, other experiments have to be done to confirm the exact function of the gSG6 protein on the probing stage.

In our study, several proteins belonging to the Gluthation S-Transferase (GST) family were also up-regulated: the GST S1, the GST E1, the GST D1 isoform D and the GST D1 isoform C. The GST S1 and GST D1 isoform C were identified in several closed spots (Figure 2 and table 1). This could be due to post-translational modifications such as glycosylation, phosphorylation, acetylation. The GST proteins are enzymes which are strongly involved not only in diverse biological processes in almost organisms such as detoxification of endogenous and xenobiotic compounds but also in protein transport and protection against oxidative stress [70, 71]. This family possesses conserved domains and is found in the majority of arthropods such as *Ae. aegypti* [72], *Cx. quiquefasciatus* [73]. The delta and epsilon classes of GST are insect-specific [74]. The GST D1 isoform C and D belongs to the delta class. These genes are rapidly diverging suggesting a role in the adaptation of insects in different ecological niches and may be involved in the detoxification of environmental xenobiotics. This hypothesis is supported by the implication of this delta class in the insecticide resistance [75]. The GST E1 belongs to the epsilon class implicated in the detoxification of insecticides and in the resistance to DDT [76-78]. They also have peroxidase activity which could be involved in the protection against secondary effects of oxidative stress [79]. The GST S1 protein belongs to the sigma class. This class of GST is found in indirect flight muscles suggesting a structural role. However it could also protect against the deleterious effects produced by oxidative stress [71]. It has been shown that the malaria infection in mosquitoes induced an oxidative stress producing reactive oxygen species [80]. So the increase of GST family protein probably counteract the negative effects produced by insect cells in response to the infection.

For the other spots, the identification of up- or down-regulated protein was more complex. Numerous proteins were identified in each spot which not allowed to know which proteins have their expression modified (supplemental data, Annexe). Moreover, several proteins were found at a time in up- and down-regulated spots, certainly due to post-translational modifications, which allowed not to conclude on the modulation of their expression. However, some of identified proteins in overexpressed spots involving in a glycolyse pathway (triophosphate isomerase, fructose biphosphate aldolase, phosphoglycerate mutase and glyceraldehydes-3-phosphate dehydrogenase) were also found up-regulated during DENV-3 infection in cell line of *Ae. albopictus* [81], DENV-2 or CHIKV-infected midgut of *Ae. aegypti* [41] and also in *P. berghei*-infected head of *An. gambiae* [58]. In addition, proteins implied to the lipid metabolism were found in overexpressed spots in agreement with a previous transcriptomic study [33]. All these studies strengthen our hypothesis that these

identified proteins are certainly upregulated. Moreover, these metabolic pathways are involved in the energy production which is in accordance with the overexpression of the ubiquinol-cytochrome c reductase iron-sulfur subunit. *P. falciparum* infection seems to interfere with the metabolic process of *An. gambiae* salivary glands. This has been already observed during the influenza virus [82] or *Leishmania* [83] infection.

Surprisingly, very few *P. falciparum* proteins were found in over- and down-expressed spots. This result could be due to the protein extraction protocol that is not enough hard to disrupt sporozoites. We could also suppose that the salivary gland proteins were in majority amount and the ratio or proportion salivary gland proteins/ parasite proteins was high, not allowing the detection of the *P. falciparum* proteins.

Identification/selection of candidate biomarker of exposure to *Anopheles* infective bites

The selection of candidates as biomarker, only specific to infective bites was based on several criterion: i) overexpressed proteins are considered or proteins found in overexpressed spots with a high percentage of cover sequence and a high number of identified peptides, suggesting thus that they are mostly in the spot, ii) the presence of a peptide signal in the protein sequence, meaning that they are secreted in the saliva and consequently injected into the human skin during the blood feeding and, by consequences which potentially induce an Ab response in human if antigenic, iii) the specificity of proteins to *Anopheles* genus.

Among the overexpressed proteins previously mentioned, the ubiquinol-cytochrome c reductase Rieske subunit and the GST proteins are common in many organisms and are not secreted proteins. Consequently they are not suitable to be select as protein candidate for biomarker specific to *Anopheles*.

The gSG6 protein has been previoulsy shown as biomarker of exposure to *Anopheles* bites [15-18] and some peptides have also been designed from this protein. Among them, gSG6-P1 and gSG6-P2 peptides were antigenic but only the gSG6-P1 peptide seemed positively associated with the level of human exposure to *Anopheles* [19], in different context of exposure [20-23]. In our study, the expression of gSG6 protein was clearly increased in presence of wild *P. falciparum*. This result suggests that this protein could be also a potential candidate as biomarker of infective bites. Recent studies strengthen this point by

demonstrating that Ab response to the gSG6 recombinant protein was associated with the malaria incidence in Tanzania [18] and that the gSG6-P1 peptide could be an indicator of infection risk during dry season (very low exposure/transmission) in Northern Senegal [84].

Other candidate proteins as biomarker were selected in the some overexpressed spots: the gSG1b, the TRIO protein, the long form D7 and the SG5 (Figure 26 and Table 2).

The gSG1b and the TRIO protein have already been found overexpressed in *P. berghei*-infected salivary glands of *An. gambiae* [43]. This result supports the fact that, in the present study, these proteins, identified among others in overexpressed spots, seems clearly upregulated. The long form D7 and the SG5 proteins were selected although their sequences matched with other arthropods but with a low identity. The long form D7 protein presented 35.1% of identity with *Ae. Aegypti* ($e=8e^{-51}$), 34.1% with *Cx. quiquefasciatus* ($e=5e^{-53}$), 31.3% with *G. morsitans* ($e=4e^{-04}$), 27.3% with *Phlebotomus papatasi* ($e=2e^{-10}$) *and Lutzomia longipalpis* ($e=3e^{-11}$). The SG5 presented 27.1% of identity with *Ae. Aegypti* (e=1e-30) and 24.3% with *Cx. quiquefasciatus* (e=1e-34). The specificity of these proteins as biomarker of exposure to *Anopheles* genus will have to be verified and, for example, an animal model of exposure could be used. The Ab response against these proteins or peptides derived from them can be assessed in rabbits exclusively bitted by *Ae. albopictus*, *Ae. aegypti* or *Cx. quiquefasciatus*. However, the use of peptides of these proteins should one approach and opportunity to decrease the possible immune cross-reactivity.

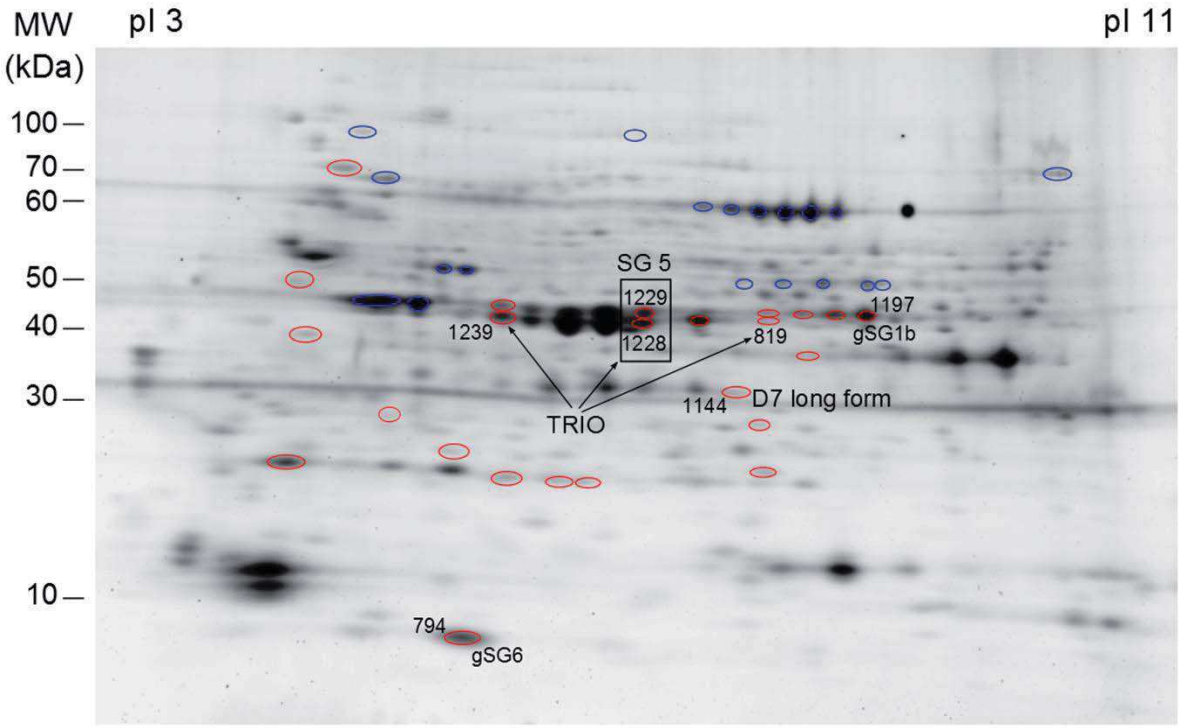

Figure 26. Candidate proteins as biomarker of *Anopheles* infective bites (2D-DIGE gel).
Candidate proteins are indicated by number. The pI and weight scales are indicated in the figure.

Table 2. Identification/selection of candidate proteins as biomarker of *Anopheles* infective bites.

Protein identification	Accession number (UniProtKB/TrEMBL)	Spot	Fold	Mascot score	Cover sequence (%)	Peptide number	Molecular mass (kDA)	pI
gSG6 SP	Q9BIH5_ANOGA	794	1.8	14	45.22	14	13.1	5.49
gSG1b SP	Q9BIH6_ANOGA	1197	1.6	59	46.23	59	43.6	7.58
long form D7 SP	Q7PJ76_ANOGA	1144	1.4	15	40.19	15	35.6	5.90
SG5 SP	Q9BIH7_ANOGA	1228	2.0	15	29.22	15	38.2	6.47
		1229	1.6	14	29.52	14		
TRIO protein SP	Q8WR22_ANOGA	819	1.4	12	31.46	12	43.7	6.46
		1228	2.0	21	36.83	21		
		1229	1.6	15	29.41	15		
		1239	1.4	25	40.15	25		

SP means signal peptide

Peptide design and antigenicity of selected peptides as potential candidate biomarker

In the aim to optimize the *Anopheles* specificity and to avoid the limits in the production of recombinant protein and batch-to-batch variations, a peptide design of the four proteins, previously selected (gSG1b, long form D7, SG5, TRIO protein), was performed for assessing their antigenic properties.

The bioinformatic tools allowed prediction of potential epitopes for each protein. The crossing of data generated by the four algorithms allowed to define 2 peptides for gSG1b (gSG1b-P1 and gSG1b-P2), 7 peptides for the long form D7 (D7-P1 to D7-P7), 5 peptides for SG5 (SG5-P1 to SG5-P5) and 2 peptides for TRIO protein (TRIO-P1 and TRIO-P2) of 18 to 27 amino acid residues in length (Table 3).

Table 3. Peptide design of candidate proteins.

Protein identification	Accession number (UniProtKB/TrEMBL)	candidat peptide biomarker	peptide sequence
gSG6 [SP]	Q9BIH5_ANOGA	gSG6-P1	EKVWVDRDNVYCGHLDCTRVATFK
		gSG6-P2	ATFKGERFCTLCDTRHFCECKETREPL
long form D7 [SP]	Q7PJ76_ANOGA	D7-P1	FKALDPEEAWYVYERCHEDHLPS
		D7-P2	DHLPSGPNRETYLKTWKFWK
		D7-P3	GLQMYDEKTNTFKPETVPVQHEAYK
		D7-P4	SRKIYHGTVDSVAKIYEAKPEIKKQ
		D7-P5	NKSDLEPEVRSVLASCTGTQAYDYY
		D7-P6	CTGTQAYDYYSCLLNSPVKEDFRN
		D7-P7	GKVYEGPEKVKEELKKLNY
SG5 [SP]	Q9BIH7_ANOGA	SG5-P1	GSLDPLDEEDIRTEQPTSCV
		SG5-P2	VLVSIKSRMMAYTNDAVAKFEHL
		SG5-P3	EECHDKLADHLAEQRREIDAAQ
		SG5-P4	AEQRREIDAAQQLMGEPYRKMDG
		SG5-P5	RRQLMKQNEREVVEKSKS
TRIO protein [SP]	Q8WR22_ANOGA	TRIO-P1	PLTCIRWRSQNPASPAGSLGGKDVV
		TRIO-P2	LGGKDVVSKIDAAMANFKTLF
gSG1b [SP]	Q9BIH6_ANOGA	gSG1b-P1	FEVCLPEIRKDPATAGLVTEV
		gSG1b-P2	KKHMVASKDYESYLGALFAADA

SP means signal peptide

All these peptides have been aligned using the Blastp program in Vectorbase to search similarities with other hematophagous arthropods and using Blastp program in UniProtKB to research similarities with human infectious organisms to avoid immune cross-reactivity. No relevant identity was found as indicated by the low scores observed (few amino acids (aa) consecutively matched and high rate of e-value, i.e. e>0.03). The D7-P1 peptide showed low identity with *Cx. quiquefasciatus* (4aa consecutive, e=0.036) and *Ae. Aegypti* (4aa consecutive, e=0.3); D7-P2 peptide matched with a low identity with *Ae. aegypti* (4aa consecutive, e=0.62); D7-P3 peptide with *Cx. quiquefasciatus* (3aa consecutive, e=0.13) and *Trichomonas vaginalis* (6aa consecutive, e=3.1). D7-P5 peptide showed low identity with *G. morsitans* (3aa consecutive, e=0.87); D7-P6 peptide also matched with a low identity with *Ae. Aegypti* (2aa consecutive, e=0.046) and *Cx. quiquefasciatus* (3aa consecutive, e=0.23) and D7-P7 peptide with *Leptospira weilii* (6aa consecutive, e=5). SG5-P2 peptide presented low identity with *Cronobacter sakazakii* (8aa consecutive, e=6.7); SG5-P4 peptide matched weakly with *Rhodnius prolixus* (3aa, e=0.25) and SG5-P5 peptide with *Trypanosoma cruzi* (3aa consecutive, e=9) presented a low identity. This analysis showed that all selected peptides possessed a high specificity for *Anopheles* species.

The antigenicity (level of specific IgG Abs) of gSG6-P1 and gSG6-P2 peptides has already shown in a previous study [19]. The antigenicity of all other peptides has been assessed by epitope-mapping approach, by using serum from children known to be exposed to *Anopheles* bites (n=42) (Figure 27). All peptides appeared to be antigenic but different level of antigenicity between them were observed. The D7-P1, SG5-P2, SG5-P3, SG5-P4, SG5-P5 TRIO-P1, TRIO-P2, SG1b-P1 and SG1b-P2 seemed to have a lower antigenicity than the D7-P2, D7-P3, D7-P4, D7-P5, D7-P6, D7-P7 and SG5-P1 peptides. A high antigenicity could be one of pertinent criterion for the identification of specific biomarker of infective bites, but the most important criterion is clearly that this biomarker can differentiate uninfective and infective bites of *Anopheles*. The next step of this work will be to evaluate at large scale the Ab response to all these peptides for comparing their antigenicity between individuals infected by *P. falciparum* (individuals previously bitten by infected bites) and individuals exposed to *Anopheles* bites and identified as not infected. The immuno-epidemiological evaluation of various cohorts from different epidemiological malaria (confirmed by several entomological, parasitological and immunological tools) will allow to validate one or several combined peptide candidates (gSG6-P1, gSG6-P2, gSG1b-P1 and gSG1b-P2, D7-P1 to D7-

P7, SG5-P1 to SG5-P5, TRIO-P1 and TRIO-P2) as specific biomarker of human exposure to only *Anopheles* infective bites.

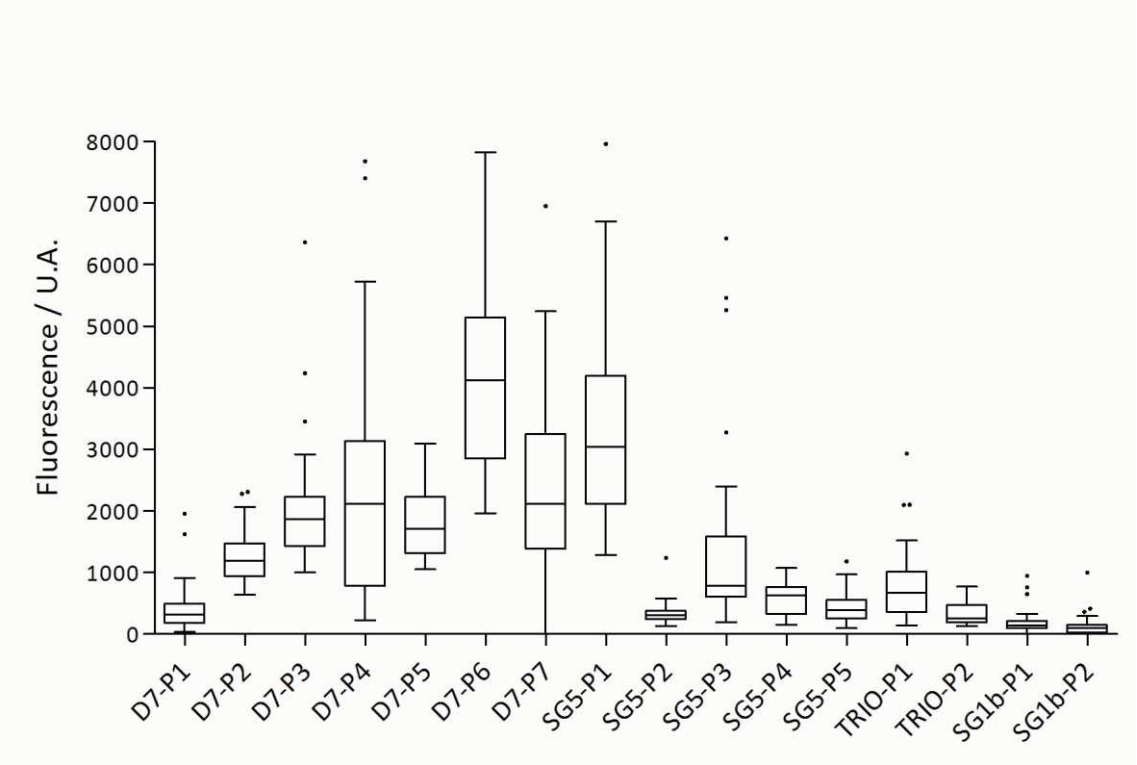

Figure 27. IgG Ab response according to the different peptides.
The IgG antibody level was evaluated in a sample of children exposed to *Anopheles* bites living in Senegal. Box plots display the median value, 25th and 75th percentile. Whiskers represent 5–95 percentiles and dots the outliers.

Conclusion

In this study, five salivary proteins, the gSG6, gSG1b, long form D7, SG5 and TRIO were selected as candidate biomarker of exposure to *Anopheles* infective bites in order to evaluate the risk of malaria transmission. *Anopheles*-specific and immunogenic peptides were designed from these proteins *in silico*: gSG1b-P1 and gSG1b-P2, D7-P1 to D7-P7, SG5-P1 to SG5-P5, TRIO-P1 and TRIO-P2. Their immunogenicity was tested and validated using blood from children exposed to *Anopheles* bites. These results are the first step toward the development of a biomarker of exposure to *Anopheles* infective bites. This tool is essential to evaluate the malaria transmission in area of low transmission such as urban settings, highlands and area where the *P. falciparum* transmission decreased highly thanks to the malaria integrated control strategies. The next step is to verify whether all these peptides in addition to the gSG6-P1 and gSG6-P2 peptides can differentiate uninfective from infective bites of *An. gambiae*.

Moreover, this present study gave some results about the effect of the presence of wild *P. falciparum* parasite on the expression of proteins in *An. gambiae* salivary glands. The parasite up- and down-regulates the sialome expression, but seems also to induce post-translational modifications. This study and further studies provide key elements to understand how the insect cells act to protect themselves against the infection and how *P. falciparum* manipulate the cellular machinery of salivary glands and the behavior of *Anopheles* mosquitoes. Such studies will give us information on potential target for transmission blocking vaccine.

List of abbreviations

ACT: artemisin-based combination therapies; Ab: antibody; WSE: whole saliva extract; ITNs: insecticide treated nets; CHIKV: chikungunya virus; DENV: dengue virus; 2D-DIGE: bidimensional differential gel electrophoresis; SGE: salivary gland extract; FDR: false discovery rate; GST: gluthatione S transferase; aa: amino acid.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AM, MR, FC, VC, FR and SC designed the study and experiments. AM, MTT and IM performed the experimental infection. AM and PH carried out the proteomic experiments. MS performed the mass spectrometry analysis. PH and ED assisted in the gel analysis and extraction. AM, PH, MS, ED and SC analyzed the data. AM, SC and FR wrote the manuscript. All authors read and approved the final manuscript.

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DISCUSSION

PERSPECTIVES

To orient appropriate strategies for malaria elimination and to evaluate better the efficacy of control methods, the indicators measuring the risk of transmission should be more sensitive.

The referent methods usually used to assess the level and the risk of human exposure to *Anopheles* bites are entomological methods based on adult mosquito sampling. However, the majority of the sampling methods present bias (insect populations favored depending on their ecology and blood feeding behavior (indoor or outdoor), catching of nonhematophagous arthropods), and numerous limitations (sensitivity, problems sustaining a large-scale field, high cost). In addition, all methods, except for the HLC (human landing catching), provide information on the density of vectors but not the anthropophilic rate and thus actual human–vector contact. Moreover, these methods can estimate human exposure at the population level but provide no information on the heterogeneity of exposure between individuals. These limitations are exacerbated in areas of low exposure and/or after vector control strategies, thus in area with a weak number of collected *Anopheles* population. Nevertheless, these entomological methods are used to estimate the risk of transmission via the EIR. The HLC method is the best way to evaluate the aggressiveness of mosquitoes, but it raises ethical concerns and is not applicable in children under 5 years of age, the major at-risk population.

To overcome these limitations, an immuno-epidemiological approach has been developed based on the human Ab response to salivary *Anopheles* proteins to evaluate human–vector contact and thus the risk of human exposure and transmission (Remoue et al. 2005). Over the past several years, it has been shown that the Ab response to saliva of different vectors (*Triatoma*, *Ixodides*, *Phlebotomus*, *Aedes*, *Anopheles*, etc.) reflects the level of human exposure to the bites of these vectors (Schwartz et al. 1991, Barral et al. 2000, Nascimento et al. 2001, Doucoure et al. 2012a, Doucoure et al. 2012b). In the perspective of using this specific biomarker at a large scale in the field, the study of the Ab response to whole saliva extract is not adequat. These whole salivary extracts were obtained by dissection or salivation, and are therefore labor-intensive. The salivary protein compositions may also differ according to different parameters such as age, sex, infection status, and insecticide resistance, inducing reproducibility problems. Moreover, some salivary proteins are ubiquitous and could cause immunogenic cross-reactivity.

To bypass these constraints, colleagues have selected an immunogenic salivary protein first described in *An. gambiae* (Lanfrancotti et al. 2002) but also found in *An. stephensi*

(Valenzuela et al. 2003) and *An. funestus* (Calvo et al. 2007): the gSG6 protein. This protein is specific to *Anopheles* mosquitoes, is more expressed in the salivary gland of adult females (Arca et al. 2005), and seems to play a role in blood feeding (Lombardo et al. 2009). Because it possesses all these criteria, this protein has been evaluated as a biomarker of human exposure to *Anopheles* bites (Poinsignon et al. 2008, Rizzo et al. 2011a, Rizzo et al. 2011b, Ali et al. 2012). However, like whole saliva, the production of recombinant protein could present limitations and batch-to-batch variations have led to favoring the production of chemically synthesized peptides to optimize *Anopheles* specificity and the reproducibility of the immunological assay (ELISA). One peptide, gSG6-P1, was selected because a positive and significant association with the different levels of *Anopheles* exposure was observed. This peptide was then validated in different contexts of exposure to *Anopheles* bites (low exposure, highlands, urban malaria) in children and adults (Poinsignon et al. 2009, Badu et al. 2012, Drame et al. 2012, Sagna et al. 2013b). Moreover, this peptide has also been validated to evaluate the efficacy of ITNs (Drame et al. 2010a, Drame et al. 2013a).

However, we have noted that the Ab responses to whole saliva and gSG6-P1 are heterogeneous between individuals from areas with similar levels of exposure. Moreover, in some very young children, it has been observed that the Ab response to gGS6-P1 is very low or nonexistent, whereas no vector control strategies such as ITNS have been implemented without disrupting human–vector contact (Drame et al. 2010a). As demonstrated for the Ab response to different *Plasmodium* antigens, human individuals react differently to an epitope and very young children produce a very low Ab response to CSP. This suggests that this single antigen appeared not to be sufficient to evaluate the risk of transmission in populations. It could be assumed that the same principle can be applied to the salivary antigens for the evaluation of human exposure to *Anopheles* bites. Concerning the estimation of the risk of transmission, it has been shown that less than 5% of the bites are infective; consequently, the Ab response to salivary antigens could overestimate the risk of transmission if infective and noninfective bites are not distinguished. Moreover, as is known in other infectious diseases (Woolhouse et al. 1997) and demonstrated for malaria (Smith et al. 2005), 20% of individuals receive 80% of all infections. These 20% of individuals represent small groups of households or transmission hotspots that play an essential role in malaria transmission in all epidemiological contexts. Indeed, these hotspots can fuel transmission in high-transmission seasons and maintain transmission in low-transmission seasons, such as dry season (Bousema et al. 2012b). It has been shown that the use of hotspot-targeted interventions was more

efficient in reducing malaria transmission than using interventions throughout the community (Bousema et al. 2013). Consequently, the detection of these transmission hotspots is an important strategy and can be evaluate by the human exposure to infected mosquitoes.

Therefore, to improve this biomarker tool based on the specific human Ab response to salivary proteins of *An. gambiae*, we focused our studies on two aspects. The first was to validate another salivary protein, probably more sensitive than the gSG6-P1 peptide, as a biomarker of human exposure to *Anopheles* bites. The second was to identify new salivary proteins to evaluate the exposure to *Anopheles* infective bites specifically (Figure 28).

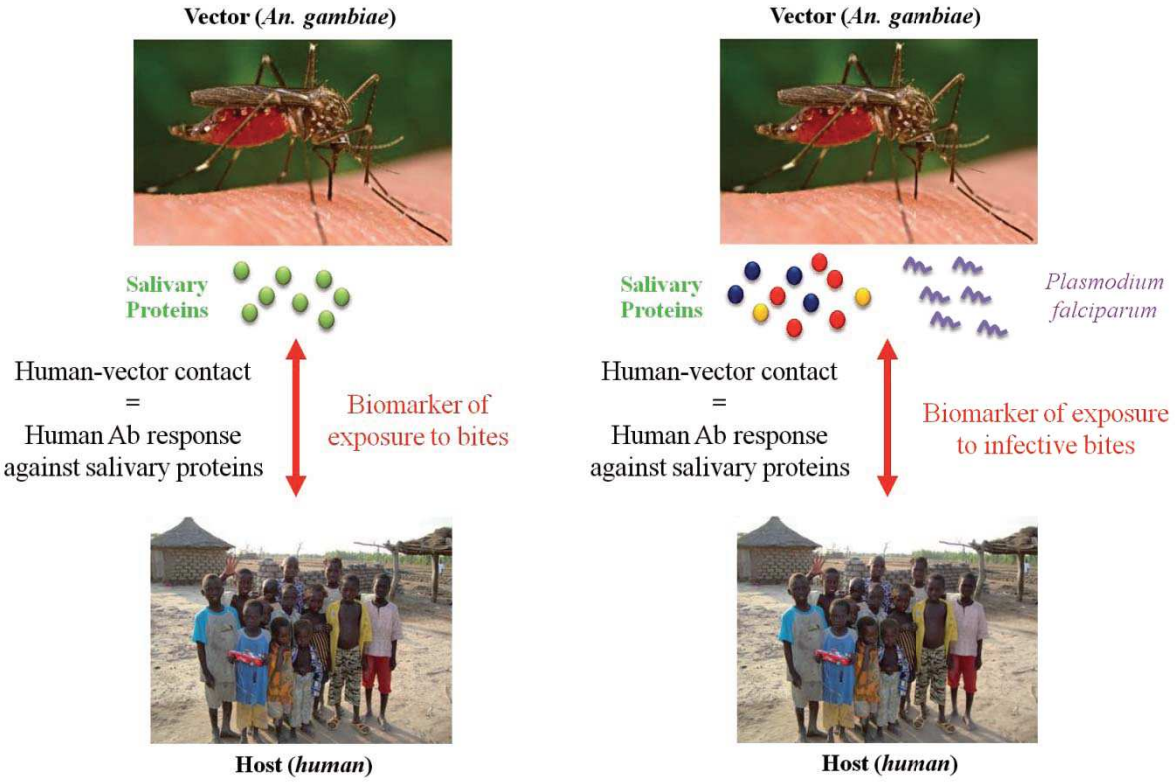

Figure 28. Goals of the thesis

1. Validation of a new salivary protein as a biomarker

The first part of my PhD was to validate another salivary protein as a biomarker to improve the existing biomarker tool.

The criteria for an optimal exposure biomarker based on the human Ab response to salivary proteins are:

- *Anopheles* specificity,

- antigenicity of the salivary proteins,

- capacity to produce the antigen easily to ensure the reproducibility of the immunological assay (ELISA),

- low/moderate antigenicity of the candidate biomarker to avoid a cumulative Ab response over time and to observe a rapid decrease after ceasing exposure, thus after the stop of an immune stimulation by saliva

- positive association of the Ab response to the candidate biomarker with the level of exposure to bites.

Based on the previous study from B. Arcà's team, the cE5 protein was selected. This protein is specific to the *Anopheles* genus and is only found in female salivary glands. It plays an anti-thrombin inhibitor role to facilitate the blood meal by preventing the blood coagulation (Ronca et al. 2012). This protein has been produced by B. Arcà's team and has been observed to be antigenic in human populations from Burkina Faso who develop a specific Ab response (B. Arcà, Sapienza University of Rome, manuscript in preparation).

In this thesis, the goal was to validate the cE5 protein, produced in recombinant form, as a biomarker of human exposure to *Anopheles* bites, and this validation was assessed using two complementary approaches: we examined i) whether the Ab response to cE5 is positively associated with the exposure level (evaluated by the classical entomological methods, referred to as the positive approach), and ii) whether it could be used as a biomarker to assess the efficacy of ITNs by observing a decrease in the specific Ab level after interrupting human exposure to bites by the well-used ITNs (the negative approach). To this end, we used a longitudinal study conducted in Lobito, a coastal city of Angola. This survey is a 2-year

follow-up with epidemiological, parasitological, and entomological data. Previous studies, using parasitological tests, the referent method recommended by the WHO, have demonstrated that the ITNs protect human individuals by decreasing malaria transmission despite the unchanged density of mosquito vectors. This has been confirmed using whole saliva and the gSG6-P1 peptide as well. This cohort was therefore an adequate support to validate another salivary protein as a biomarker of exposure (before ITNs use) and a reliable indicator to evaluate the efficacy of ITNs on human–vector contact (comparison between before and after ITNs implementation).

For our immunological tests with the cE5 protein, a subsample of the whole studied population (n=73; 35 children, 0–6 years old (mean: 3.5 years), 22 children, 7–14 years old (mean: 9.7 years) and 16 individuals over 14 years of age (mean: 26 years)) was used. The specific IgG level against cE5 was different in the three age groups. For people older than 14 years of age, no significant seasonal variation of IgG response to cE5 before ITNs installation and no decrease in specific Ab level after ITNs installation were noted. This means that the Ab response to cE5 is not positively associated with the bite exposure level in this age group. One explanation could stem from the sleeping schedule of older people, which may result in lower protection by ITNs, enhancing the probability of contact with mosquitoes. It may also be possible that the Ab response to this protein in adults could persist longer, thus presenting a long time period of cumulative Ab response, because they have been exposed to bites longer than children during their lifetime. For children under 14 years of age, before the installation of ITNs, a positive association between the Ab response to cE5 and the exposure level was observed and the ITNs introduction induced a rapid drop of the IgG level observed 2 months later. These results suggested that the cE5 could be an indicator of exposure to *Anopheles* bites and could evaluate the short-term efficacy of ITNs only in children. Moreover, some tested children with a very weak IgG response to the gSG6-P1 peptide presented a high IgG response to cE5. This suggests that this salivary protein could identify children bitten by *Anopheles* who are not positive for the gSG6-P1 peptide. The cE5 seems more sensitive or more pertinent to evaluating the exposure to *Anopheles* bites in children.

However, all the children in the whole studied population are not studied in our study due to a limitation of the recombinant protein amount. Furthermore, in the field, human–vector contact and the efficacy of vector control strategies have to be evaluated for all age groups. Consequently, within the objective of improving a biomarker tool for application in the field, the use of this recombinant protein seems compromise given that i) it cannot be used in

teenagers and adults and ii) due to the limits in the production of recombinant protein. However, this protein has a good potential for the estimation of exposure to *Anopheles* bites in children, a group that appears to be less well assessed by the gSG6-P1 peptide, and this in the particular context of low exposure to *Anopheles* as observed in our studied area in Angola. We also observed that the Ab response to salivary proteins is heterogeneous in the exposed populations, confirming that the immunogenicity of an antigen is different in individuals due to genetic factors. So the use of an antigen combination could be a good technique to assess all individuals bitten by *Anopheles* mosquitoes. For all these reasons, other studies need to be conducted to determine how to use this protein for all the age groups within the population. It may be possible to test different antigenic cE5 peptides. Despite the loss of the conformational epitope, the use of chemically produced peptides makes it possible to circumvent the limitations in the production of recombinant protein and the possible batch-to-batch variations. Moreover, a protein possesses several epitopes that could be in immunological competition and individuals cannot develop an Ab response against all the epitopes and we can observe a decrease of specificity/antigenicity of the biomarker (because not focalize to one or two epitopes). Some epitopes could induce a higher or lower Ab response. In addition, some epitopes, such as the gSG6-P1 peptide, could not induce a persistent Ab response over time. Consequently, it would be valuable to identify one or more epitopes, not necessarily with the highest antigenic response but epitopes that do not induce a persistence of the Ab response and detect children bitten by *Anopheles* not evaluated with the gSG6-P1 peptide. The study of several salivary antigens therefore appeared to be highly pertinent to improve the biomarker tool in order to detect bite exposure in each individual. Moreover, this tool would be optimal in the field if it can be used in all contexts of human exposure to mosquito bites. Consequently, other studies have to be carried out to evaluate the potential use in areas with low, medium, and high exposure and with or without seasonal variations to *Anopheles* bites. In addition, orthologues of the cE5 protein are also found in other *Anopheles* species such as *An. stephensi* and *An. albimanus*. As for the gSG6-P1 peptide, the immune cross-reaction has to be studied to estimate the species or genus specificity of this protein and the immunogenic peptides derived from it.

Other research topic will be to find salivary proteins specific to *Anopheles* species. This could make it possible to evaluate the real implication of the different species of *Anopheles* in malaria transmission. Indeed, in Africa, *An. gambiae* is considered as the major vector of malaria, but *An. arabiensis* and *An. funestus* are also important vectors of *Plasmodium*. Each

species has specific ecologic niches but could be also found in sympatry and their presences vary depending on the season: for example, *An. funestus* is more resistant to drought than *An. gambiae* and takes over malaria transmission. It has also been observed that *An. funestus* can change its feeding behavior due to the ITNs implementation and bite later in the morning when humans become active (Moiroux et al. 2012). Moreover, the *Anopheles* species, which transmit *Plasmodium* in Asia, are different from those in Africa. A recent proteomic study has shown that species-specific salivary proteins exist and are antigenic, thus having a potential as a biomarker of bite exposure specific to *Anopheles* species. However, differentiating *Anopheles* species belonging to the same complex seems very complex (Ali et al. 2012, Fontaine et al. 2012). Consequently, further studies have to be conducted to improve the biomarker tool allowing both the assessment of the human exposure to different species of *Anopheles* and the development of the same tool for Asia.

2. Identification of salivary proteins as a biomarker of infective bites

The second part of my PhD project was to identify salivary proteins that could be used as biomarkers of exposure specifically to *Anopheles* infective bites. In the field, the majority of bites are not infective (Beier et al. 1999, Drakeley et al. 2003). A low number of *Anopheles* mosquitoes are infected by *Plasmodium* parasites and consequently the measurement of human–vector contact does not accurately represent the risk of malaria transmission. This also stems from the length of the sporogonic cycle in *Anopheles* mosquitoes – approximately 14 days for *P. falciparum* in *An. gambiae* – consequently, only old females are infectious (Figure 7).

The new idea was then to identify one or more salivary proteins exclusively expressed in presence of *P. falciparum*, and consequently detecting the human Ab response against these proteins would be used as a precise indicator of parasite transmission. To determine these salivary proteins, a proteomic approach combining 2D-DIGE and mass spectrometry LC/MS-MS was carried out to compare the differential expression of proteins between *P. falciparum*-infected and uninfected salivary glands of *An. gambiae*. To remain closest to natural settings, *An. gambiae* were experimentally infected with *P. falciparum* in semi-field conditions. Mosquito infection depends on different factors such as *P. falciparum* multiclonality, the sex ratio, and the density of gametocytes (Mitri et al. 2009, Mendes et al. 2011, Bousema et al. 2012a, Nsango et al. 2012), and these parameters cannot be obtained with a parasite culture.

Consequently, *An. gambiae* mosquitoes were fed on blood from asymptomatic children carrying gametocytes.

2.1 Success of experimental infections

To verify the success of the salivary gland infections 14 days post-infection, the time required for the parasite to reach the salivary glands, several methods were employed: 1) the CSP-ELISA assay, the current method used in the field to evaluate the *P. falciparum* infection rate of mosquitoes; 2) multiplex PCR allowing the detection of the four human *Plasmodium* spp.; and 3) real-time quantitative PCR to detect *P. falciparum*, developed by Boissiere *et al.* (Boissière et al. 2013). The first method is based on the detection of a surface protein (CSP) of *P. falciparum* in the head-thorax of the mosquitoes. Before performing this assay, salivary glands were dissected to extract proteins and DNA. The two other methods were carried out on the salivary gland DNA. These three methods were compared: the results showed that the qPCR is highly sensitive and more specific than multiplex PCR. However, multiplex PCR has the advantage of detecting several species of human *Plasmodium*. It could be highly valuable to improve this qPCR to detect the five *Plasmodium* species affecting humans in order to evaluate the distribution of these species, the infection rate for each species in *Anopheles*, and mixed infections. Even if *P. falciparum* is the most dangerous species in terms of malaria morbidity and mortality, the other species must also be taken into account. In addition, this study also demonstrated that the CSP-ELISA overestimates the mosquito infection rate, a result that is in accordance with other studies (Fontenille et al. 2001, Durnez et al. 2011). Yet, this method is recommended by the WHO and used to determine the EIR. In a context of malaria elimination, it is essential to have accurate measures of malaria risk of transmission to better adapt control strategies. The qPCR seems to be a good approach to assess the *Plasmodium* infection rate of *Anopheles* mosquitoes (Marie et al. 2013). However, the risk of malaria transmission cannot be assessed only by the EIR. The assessment of the human Ab response to salivary proteins specifically expressed in *P. falciparum*-infected salivary glands could be a relevant complementary indicator to evaluate the human–vector–pathogen contact and thus more precisely represent parasite transmission.

2.2 Identification of a biomarker of exposure to infective bites

To develop a biomarker of infective bites, the first step was to compare the expression of salivary proteins in *P. falciparum*-infected versus uninfected salivary glands of *An. gambiae*

using the 2D-DIGE approach to identify a potential candidate biomarker. The first result was to observe any protein that was found exclusively expressed in presence of *P. falciparum*. Consequently, another hypothesis was that the human Ab response to an overexpressed salivary protein would be increased after an infective bite and could therefore be used as a biomarker of transmission. The selection of biomarker candidates was based on several criteria:

i) the selected proteins were identified as overexpressed proteins or proteins found in overexpressed spots with a high percentage of cover sequence and a high number of identified peptides, thus suggesting that they are potentially overexpressed;

ii) the presence of a peptide signal in the protein sequence, meaning that they are secreted in saliva and consequently injected into the human skin during blood feeding, which could induce the development of a specific Ab response in humans;

iii) the *Anopheles* genus specificity of proteins.

In the present study, five salivary proteins presented in overexpressed spots were selected as candidate biomarkers according to the criteria listed above: the gSG6, the gSG1b, the TRIO protein, the long form D7, and the SG5.

The gSG6 protein has already been validated as a biomarker of human exposure to *Anopheles* bites. Here, its overexpression in presence of *P. falciparum* suggests that it could be used as a specific biomarker of exposure to infective bites. This hypothesis has already been strengthened by recent studies showing that the IgG response to the gSG6 recombinant protein was associated with malaria incidence in Tanzania (Stone et al. 2012) and that the gSG6-P1 peptide could be an indicator of malaria infection risk, in the particular context of the dry season in Senegal (Sagna et al. 2013a).

Aiming to use this biomarker in the field, to have a straightforward production, and to improve *Anopheles* specificity, an epitope design of the other four proteins was applied using a bioinformatics tool and two peptides were defined for gSG1b (gSG1b-P1 and gSG1b-P2), seven peptides for the long form D7 (D7-P1 to D7-P7), five peptides for SG5 (SG5-P1 to SG5-P5), and two peptides for TRIO protein (TRIO-P1 and TRIO-P2), 18–27 amino acid residues long. The antigenicity of each epitope was tested by epitope mapping technology using sera from Senegalese children exposed to *Anopheles* bites. All peptides are antigenic

but present different intensities of the specific Ab response. As mentioned previously, the high antigenicity seems to be the first idea of a good factor to evaluate the exposure to infective bites. However, the most important criterion is that this Ab response can differentiate an *Anopheles* infective from an uninfective bite.

2.3 Perspectives

The next step will be to test these epitopes as specific biomarkers of exposure to infective bites and also to verify *Anopheles* specificity. Indeed, the protein sequence of two selected proteins, the long form D7 and the SG5, present identity with sequences of other arthropods but with a low identity. Nevertheless, the selected epitopes of these proteins present no relevant identity with other proteins of arthropods and human infectious organisms, suggesting a high *Anopheles* specificity. However, it could be useful to test this specificity, which could be done using animal models. The Ab response to all peptides can be assessed in rabbits exclusively bitten by *An. gambiae*, *Ae. albopictus*, *Ae. aegypti*, and *Cx. quinquefasciatus*.

Then, to evaluate the use of these peptides as biomarkers of infective bites, several methods could be employed. The first is experimentation with rabbits or mice. The protocol would compare the Ab response to each peptide in animals bitten by uninfected *An. gambiae*, in animals bitten by *P. falciparum*-infected *An. gambiae*, and those not bitten. Each animal would be bitten by the same number of mosquitoes and different numbers of mosquito bites will be tested. The same protocol could be applied to human experiments in collaboration with R. Sauerwein from the University Nijmegen Medical Center in The Netherlands, where human malaria challenge models by varied infective bites are used for vaccine development (Sauerwein et al. 2011, Bijker et al. 2013). These experiments would make it possible to determine whether infective and noninfective bites can be differentiated and to define a detection threshold of the number of infective bites. Nevertheless, the candidate salivary proteins used are both expressed in *P. falciparum*-infected and -uninfected salivary glands. The only difference is that their expression is increased, so we assume that the Ab response will also increase. Consequently, the use of a salivary protein whose expression is unchanged in the two conditions, and which would be an indicator of exposure to all types of bites, could be used to define a threshold of differentiation. The gSG6 protein cannot be used because the present study has shown that it is overexpressed in presence of *P. falciparum* and consequently potentially a biomarker of infective bites. Therefore, the study of the cE5 protein and particularly peptides derived from it could be useful. In this context, the future

experiments presented above must compare the Ab response to a biomarker of exposure to bites (for example, against the cE5 protein) to the Ab response to a biomarker of exposure to infective bites (for example, against the gSG6 protein or the gSG6-P1 peptide). To determine a threshold from the Ab response against cE5, it is necessary that for a same number of uninfective bites, a same level of Ab response to both antigens has to be observed. This idea is illustrated in Figure 29.

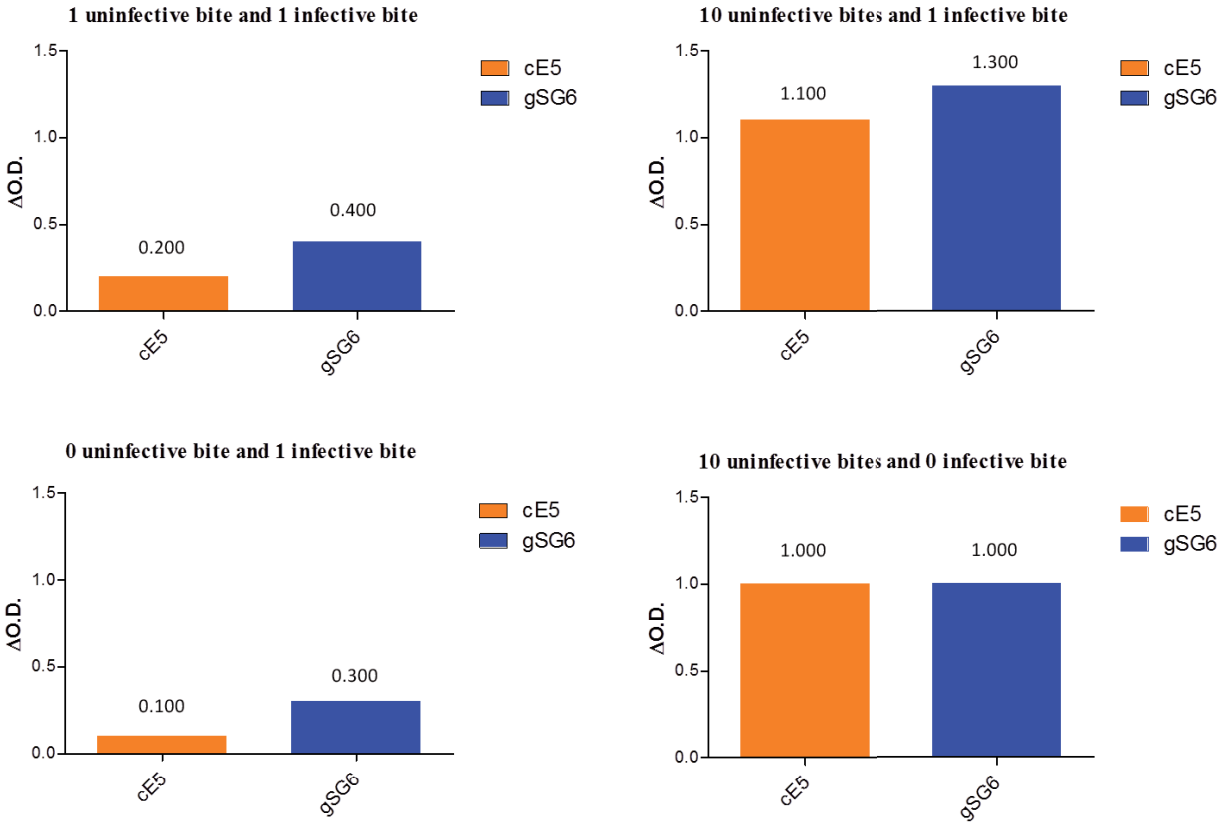

Figure 29. Illustration of the Ab response to noninfective and infective bites in different exposure situations for an individual.
cE5 represents the biomarker of all the bites. gSG6 represents the biomarker of infective bites. The two proteins cause an Ab response against an infective or noninfective bite. However, the Ab response against gSG6 should be higher for an infective bite than a noninfective bite. For this example, the value of the Ab response to cE5 or gSG6 for a noninfective bite is 0.100 and the value of the Ab response for an infective bite for cE5 is 0 and for gSG6 is 0.300.

However, this concept can only be relevant if the Ab response against these two antigens is similar after exposure to a same number of uninfective bites. Yet, in the same individual, the Ab response to different antigens is generally not the same, and the Ab response to the same antigen between individuals could be different. Consequently, the development of a biomarker of infective bites from an overexpressed salivary protein seems to be complex and

particularly with proteins with a low fold overexpression. Indeed, the fold overexpression of the five candidate proteins varies from 1.4- to 2.0-fold, which is low. The question can be raised of whether this difference of expression is sufficient to differentiate the Ab response against noninfective and infective bites. The initial hypothesis was to identify one or more proteins exclusively expressed in *P. falciparum*-infected salivary glands to observe an Ab response specific to infective bites. However, according to the co-evolution that exists between the two protagonists, it is possible that the low cost of the parasite in the mosquito does not induce the synthesis of specific proteins but only a regulation of existing proteins. To find these potential salivary proteins, additional studies must be conducted. Further proteomic methods could be used to compare the modulation of expression of salivary proteins such as the iTRAQ^{TM}, SELDI-TOF, or HPLC. We could also carry out 2D-DIGE experiments reducing the pH condition to obtain gels with more dispatched proteins facilitating spot excision and consequently allowing better protein identification. The combination of several proteomic approaches could ensure good coverage of salivary proteins of varied isoelectric points and molecular weights. A transcriptomic approach could also be used, but it must be kept in mind that what is observed at the transcriptomic level does not necessarily reflect what exists at the proteomic level. Consequently, this technique will give us clues but has to be checked at the protein level.

Moreover, in this study, we focused on the *An. gambiae–P. falciparum* complex, which represents the most important complex responsible for malaria in Africa. However, other *Anopheles* species and other *Plasmodium* species are also responsible for malaria cases in Africa and in the other parts of the world. Other studies could be conducted to find salivary proteins specific to infective bites in the different *Anopheles–Plasmodium* species complexes. This will provide more information on the implication of the different complexes on malaria transmission worldwide.

3. *P. falciparum-An. gambiae* interaction

This proteomic study is also the first to investigate the *P. falciparum–An. gambiae* interaction at the salivary gland level. This analysis has highlighted the impact of the parasite on the expression of salivary proteins. Indeed, 207 spots were found over- or down-expressed, but the parasite also seems to induce post-translational modifications such as phosphorylation, glycosylation, acetylation, and protein degradation. The majority of the spots (164) could not

be excised manually because blue coloration, preventing protein identification. This was disappointing because among these spots, some had up to eight-fold overexpression. This suggests that the proteins presented in these spots play an important role in parasite survival or in mosquito defense. Moreover, such an overexpressed protein could potentially be a good candidate as a biomarker of infective bites.

Among 7 excised spots, two proteins (gSG6 and the ubiquinol cytochrome c reductase iron subunit) and the GST family proteins have been identified as overexpressed when the salivary glands are infected by *P. falciparum*.

The consequences of the increase of gSG6 protein expression are not known. One study showed that the decrease of this protein expression induced an increase in probing time (Lombardo et al. 2009); however, other studies showed that the infection of *An. gambiae* or *An. stephensi* by murine *Plasmodium* had no effect on probing time. Consequently, further studies will be conducted to confirm the potential role of the gSG6 protein and of the infection of *An. gambiae* by *P. falciparum* on the probing time process. This could have an impact on vectorial competence, which may be increased or decreased.

The ubiquinol-cytochrome c reductase iron-sulfur subunit (Rieske subunit) is a component of the complex III involved in oxidative phosphorylation in order to produce energy. The increase of this protein during the *P. falciparum* infection in salivary glands of *An. gambiae* seems to increase energy metabolism.

The GST proteins are enzymes that are strongly involved not only in diverse biological processes in most organisms such as detoxification of endogenous and xenobiotic compounds, but also in protein transport and protection against oxidative stress (Hayes and Pulford 1995, Singh et al. 2001). The increase of the GST family proteins probably counteracts the negative effects produced by insect cells in response to malaria infection, which produces reactive oxygen species (Dimopoulos et al. 2002).

In the 36 other excised spots, many proteins have been identified in each spot, making it impossible to know which proteins are over- or down-expressed. Nevertheless, some proteins involved in the glycolysis pathway and lipid metabolism are found in overexpressed spots and in accordance with other studies about the modulation of mosquito protein expression by pathogens (Lefevre et al. 2007, Rosinski-Chupin et al. 2007, Patramool et al. 2011, Tchankouo-Nguetcheu et al. 2012), we suggest that *P. falciparum* could also increase the expression of these proteins.

Further proteomic and transcriptomic studies, such as the RNA-seq, micro-array, and SAGE techniques, could make it possible to compare the mRNA in the two conditions and provide key elements to understand how the insect cells act to protect themselves against infection and how *P. falciparum* manipulates the cellular machinery of the salivary glands. Several studies have demonstrated that the *Plasmodium spp*. manipulate the behavior of *Anopheles spp*. vectors (Rossignol et al. 1984, Koella and Packer 1996), particularly at the blood feeding step, generally when the infective stage of the parasite, the sporozoites, has reached the salivary gland (Wekesa et al. 1992, Koella et al. 1998). A differential proteomic approach using the *An. gambiae-P. berghei* experimental model has shown that *Plasmodium* manipulates the vector's behavior through altered neuronal functions, by increasing the glucose oxidation in the head of the mosquito (Lefevre et al. 2007). This study and the next could also help identify targets for parasite control in the *Anopheles* salivary glands using the TBV method. Indeed, studies have shown that Abs fixed specifically to salivary proteins effectively inhibit salivary gland invasion by sporozoites (Barreau et al. 1995, Brennan et al. 2000). This could prevent the transmission and the spread of malaria.

It has also been shown that the expression of salivary proteins of *Cx. quinquefasciatus* differed between insecticide-susceptible or -resistant mosquitoes (Djegbe et al. 2011). The same results have been demonstrated in the study of susceptible and resistant *An. gambiae* (Cornelie, submitted). In these two studies, the expression of the D7 long form protein, involved in the blood feeding process, was underexpressed. It could be useful to study the impact of the *P. falciparum* presence in salivary glands of insecticide-resistant *An. gambiae* on parasite transmission.

4. Conclusion

All these results will allow us to improve the biomarker tool to detect human exposure to *Anopheles* infective and noninfective bites. The combination of several salivary proteins and peptides will make it possible to evaluate the human–vector contact in all populations at the individual level and to evaluate the real risk of malaria transmission by distinguishing noninfective and infective bites. In all transmission contexts (low, moderate, or high), malaria hotspot detection is essential. Therefore, the use of a combination of biomarker candidates: biomarker of bites, biomarker of infective bites, and parasite antigens (AMA-1 or CSP) can

be envisaged to optimize indicators of malaria transmission and/or of vector control strategies. However, parasite antigens could only be used in children due to the Ab accumulation observed in adults such that recent infections cannot be detected. In the post-elimination context, the biomarker of exposure to bites is sufficient and can detect individuals exposed to *Anopheles* bites, who could become a malaria risk population if the parasite is reintroduced in the area. This immuno-epidemiological tool also allows us to map the *Anopheles* distribution and malaria transmission to orient appropriate strategies for malaria elimination and also to better evaluate the efficacy of control methods. These results also highlighted for the first time the impact of wild *P. falciparum* on the expression of *An. gambiae* salivary proteins and could help identify targets for the control of *Plasmodium* development in the mosquito and consequently stop transmission.

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ANNEXE

Supplemental data

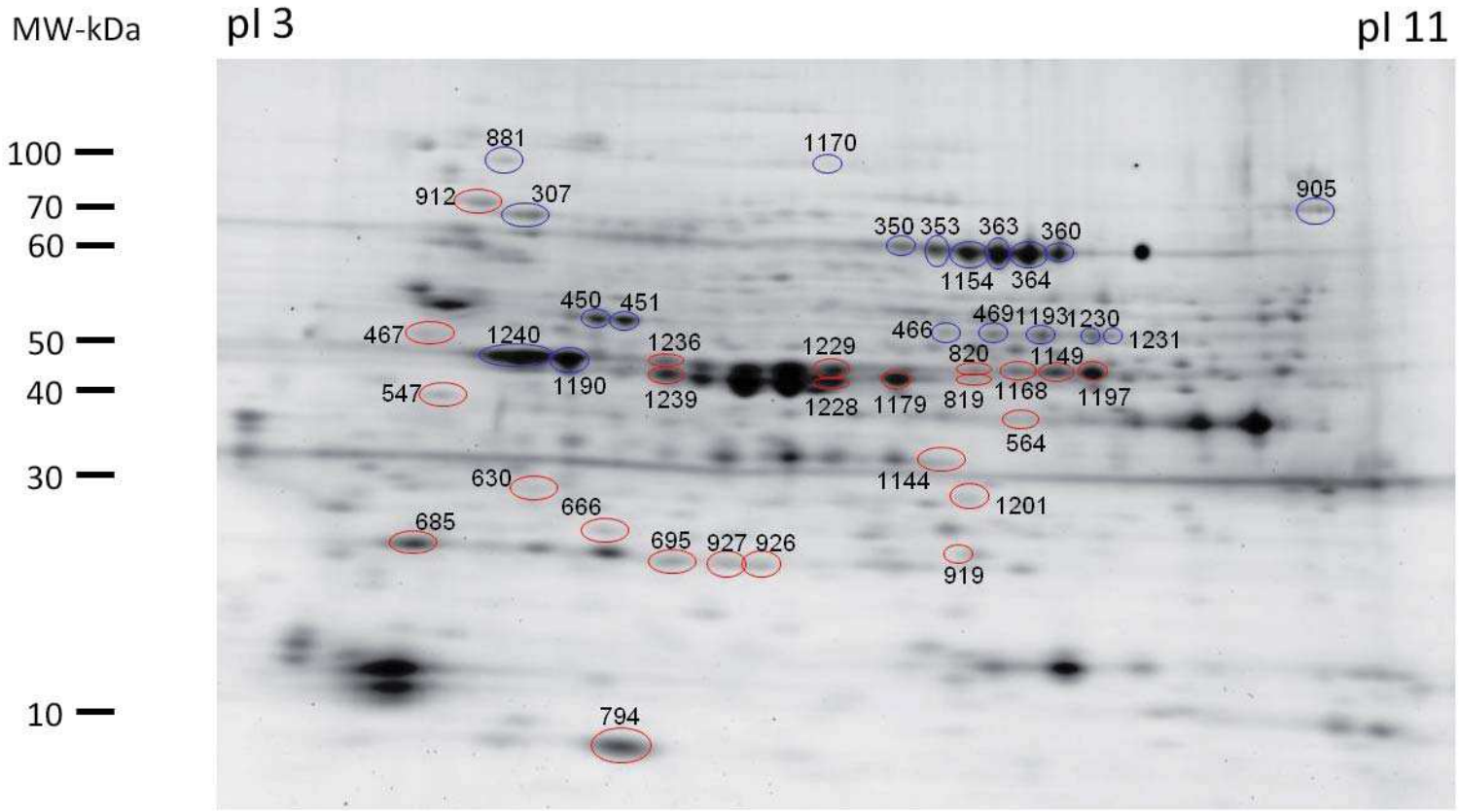

Figure 30. 2D-DIGE gel. 43 spot differentially expressed are indicated by circle and number. Red circles represent the 24 overexpressed spots and the 19 blue circles represent the underexpressed spots. The pI and weight scales are indicated in the figure

Protein identification in overexpressed spots (red circles in figure)

Spot 794

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q9BIH5	GSG6 protein (Precursor) OS=Anopheles gambiae GN=gSG6 PE=4 SV=1 - [Q9BIH5_ANOGA]	45,22	1	0	6	14	13,1	5,49

Spot 926

Accession	Description	Coverage	Proteins	Unique Peptides	Peptides	PSMs	MW [kDa]	calc. pI
Q93112	Glutathione S-transferase 1, isoform C OS=Anopheles gambiae GN=GstD1 PE=1 SV=2 - [GST1C_ANOGA]	17,70	1	4	4	5	23,8	6,55
Q93113	Glutathione S-transferase 1, isoform D OS=Anopheles gambiae GN=GstD1 PE=1 SV=1 - [GST1D_ANOGA]	12,44	1	1	2	2	23,4	6,34
A0NEA9	AGAP005393-PA OS=Anopheles gambiae GN=AGAP005393 PE=3 SV=2 - [A0NEA9_ANOGA]	14,08	1	0	2	2	23,6	6,39

Spot 927

Accession	Description	Coverage	Proteins	Unique Peptides	Peptides	PSMs	MW [kDa]	calc. pI
Q93112	Glutathione S-transferase 1, isoform C OS=Anopheles gambiae GN=GstD1 PE=1 SV=2 - [GST1C_ANOGA]	36,36	1	6	6	8	23,8	6,55
A0NEA9	AGAP005393-PA OS=Anopheles gambiae GN=AGAP005393 PE=3 SV=2 - [A0NEA9_ANOGA]	25,82	1	0	4	4	23,6	6,39

Spot 695

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q93113	Glutathione S-transferase 1, isoform D OS=Anopheles gambiae GN=GstD1 PE=1 SV=1 - [GST1D_ANOGA]	25,36	1	2	4	5	23,4	6,34
Q93112	Glutathione S-transferase 1, isoform C OS=Anopheles gambiae GN=GstD1 PE=1 SV=2 - [GST1C_ANOGA]	20,57	1	1	3	3	23,8	6,55
Q7QHC8	AGAP011131-PA OS=Anopheles gambiae GN=AGAP011131 PE=4 SV=3 - [Q7QHC8_ANOGA]	23,26	1	2	3	3	19,5	5,95
Q7PTJ0	AGAP011054-PA OS=Anopheles gambiae GN=TPX2 PE=4 SV=2 - [Q7PTJ0_ANOGA]	12,76	1	0	2	2	22,0	5,90
Q5TPP7	AGAP004759-PA OS=Anopheles gambiae GN=AGAP004759 PE=3 SV=1 - [Q5TPP7_ANOGA]	12,04	1	0	2	2	22,1	6,23
Q7PD30	AGAP012804-PA OS=Anopheles gambiae str. PEST GN=AgaP_AGAP012804 PE=3 SV=4 - [Q7PD30_ANOGA]	10,09	1	0	2	2	26,2	8,19

Protéines Q5TPP7 et Q7PD30 identifiées avec les 2 mêmes peptides

Spot 919

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7PWI1	Ubiquinol-cytochrome c reductase iron-sulfur subunit OS=Anopheles gambiae GN=AGAP008955 PE=3 SV=4 - [Q7PWI1_ANOGA]	9,43	1	2	2	3	28,1	8,53

Spot 666

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q8T0Y8	Proteasome subunit alpha type OS=Anopheles gambiae GN=AGAP001995 PE=2 SV=1 - [Q8T0Y8_ANOGA]	43,16	1	0	8	11	25,8	5,90
A7UT37	AGAP004559-PA OS=Anopheles gambiae GN=AgaP_AGAP004559 PE=3 SV=2 - [A7UT37_ANOGA]	27,91	1	0	6	9	24,3	6,01
Q7PXW5	Triosephosphate isomerase OS=Anopheles gambiae GN=AgaP_AGAP001630 PE=3 SV=4 - [Q7PXW5_ANOGA]	38,06	1	0	7	9	26,3	6,34
P46428	Glutathione S-transferase OS=Anopheles gambiae GN=GstS1 PE=2 SV=4 - [GST_ANOGA]	29,06	1	5	5	7	23,2	5,29
Q9GPL9	AGAP009195-PA OS=Anopheles gambiae GN=GSTE1 PE=2 SV=1 - [Q9GPL9_ANOGA]	17,86	1	3	3	4	25,3	5,66
Q7QGK4	Eukaryotic translation initiation factor 3 subunit K OS=Anopheles gambiae GN=AGAP011580 PE=3 SV=2 - [EIF3K_ANOGA]	9,05	1	2	2	2	25,8	5,96
Q8WQJ8	AGAP009193-PA OS=Anopheles gambiae GN=GSTe4 PE=2 SV=1 - [Q8WQJ8_ANOGA]	12,89	1	2	2	2	25,3	6,40

Spot 630

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7PSE5	AGAP009323-PA OS=Anopheles gambiae GN=AGAP009323 PE=4 SV=2 - [Q7PSE5_ANOGA]	50,74	1	11	12	19	30,1	5,73
Q7Q9Y6	AGAP004528-PA OS=Anopheles gambiae GN=AGAP004528 PE=4 SV=2 - [Q7Q9Y6_ANOGA]	43,70	1	7	7	12	27,5	5,41
Q7PJ67	AGAP008225-PA OS=Anopheles gambiae GN=AgTpp PE=2 SV=4 - [Q7PJ67_ANOGA]	30,65	1	9	9	12	29,4	5,96
Q7QK64	AGAP002170-PA OS=Anopheles gambiae GN=AgaP_AGAP002170 PE=3 SV=4 - [Q7QK64_ANOGA]	39,75	1	7	7	10	26,4	6,54
Q7Q3B2	AGAP007793-PA (Fragment) OS=Anopheles gambiae GN=AGAP007793 PE=4 SV=4 - [Q7Q3B2_ANOGA]	30,45	1	8	8	8	37,4	6,58
Q7PS09	Probable methylthioribulose-1-phosphate dehydratase OS=Anopheles gambiae GN=AGAP000470 PE=3 SV=5 - [MTNB_ANOGA]	24,68	1	5	5	7	26,4	5,81
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	17,19	1	6	6	7	63,4	7,02
Q7QHT9	AGAP011353-PA (Fragment) OS=Anopheles gambiae GN=AGAP011353 PE=4 SV=2 - [Q7QHT9_ANOGA]	21,26	1	4	4	6	29,1	6,04
Q7Q5G0	AGAP006456-PA OS=Anopheles gambiae GN=AGAP006456 PE=3 SV=2 - [Q7Q5G0_ANOGA]	29,39	1	6	6	6	29,9	6,27
Q7PQV7	ADP,ATP carrier protein 2 OS=Anopheles gambiae GN=AGAP002358 PE=3 SV=2 - [ADT2_ANOGA]	15,33	1	0	4	4	32,9	9,72
A7URN4	AGAP007172-PA OS=Anopheles gambiae GN=AGAP007172 PE=3 SV=1 - [A7URN4_ANOGA]	11,02	1	0	3	3	29,5	6,95
Q7QJ05	AGAP007172-PB OS=Anopheles gambiae GN=AGAP007172 PE=3 SV=4 - [Q7QJ05_ANOGA]	12,73	1	0	3	3	25,6	5,48

F1C3T7	Actin (Fragment) OS=Timema genevievae PE=3 SV=1 - [F1C3T7_9NEOP]	9,45	1	0	2	2	30,9	5,66
F1C3V4	Actin (Fragment) OS=Timema podura PE=3 SV=1 - [F1C3V4_TIMPD]	9,45	1	0	2	2	30,9	5,49
E3XAW7	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_18280 PE=4 SV=1 - [E3XAW7_ANODA]	3,48	1	2	2	2	34,8	5,95
A0NBC2	AGAP007643-PA OS=Anopheles gambiae GN=AGAP007643 PE=3 SV=1 - [A0NBC2_ANOGA]	10,08	1	0	2	2	28,2	4,93
A7UR78	AGAP007643-PC OS=Anopheles gambiae GN=AGAP007643 PE=3 SV=1 - [A7UR78_ANOGA]	10,08	1	0	2	2	28,3	5,11

Protéines A7URN4 et Q7QJ05 identifiées avec les 3 mêmes peptides

Protéines F1C3T7 et F1C3V4 (non ANOGA) identifiées avec les 2 mêmes peptides

Spot 1201

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7PQZ4	AGAP002401-PA OS=Anopheles gambiae GN=AGAP002401 PE=3 SV=1 - [Q7PQZ4_ANOGA]	38,50	1	9	9	16	25,7	7,28
Q5TNW4	AGAP010130-PA OS=Anopheles gambiae GN=AGAP010130 PE=4 SV=3 - [Q5TNW4_ANOGA]	25,80	1	6	6	7	31,6	8,60
A7UVK8	AGAP002076-PA OS=Anopheles gambiae GN=AgaP_AGAP002076 PE=4 SV=2 - [A7UVK8_ANOGA]	8,84	1	0	4	6	71,3	5,47
Q7Q9Y3	AGAP004533-PA OS=Anopheles gambiae GN=AgaP_AGAP004533 PE=3 SV=5 - [Q7Q9Y3_ANOGA]	17,21	1	4	4	6	36,9	6,81
Q7PXI5	Phosphoglycerate mutase OS=Anopheles gambiae GN=AGAP001420 PE=3 SV=2 - [Q7PXI5_ANOGA]	16,86	1	5	5	5	28,7	6,81
A7URV6	AGAP006936-PB OS=Anopheles gambiae GN=AGAP006936 PE=4 SV=1 - [A7URV6_ANOGA]	14,81	1	0	3	4	32,7	8,46
Q7QIL1	AGAP006936-PA OS=Anopheles gambiae GN=AGAP006936 PE=4 SV=4 - [Q7QIL1_ANOGA]	14,38	1	0	3	4	33,2	8,35
Q7PZC0	AGAP011833-PA OS=Anopheles gambiae GN=AGAP011833 PE=3 SV=2 - [Q7PZC0_ANOGA]	18,73	1	4	4	4	32,4	8,57
Q7Q6V7	AGAP005645-PA OS=Anopheles gambiae GN=AGAP005645 PE=3 SV=4 - [Q7Q6V7_ANOGA]	18,78	1	3	3	3	26,5	6,96
A0NAF9	AGAP012883-PA (Fragment) OS=Anopheles gambiae str. PEST GN=AgaP_AGAP012883 PE=4 SV=2 - [A0NAF9_ANOGA]	12,76	1	0	2	3	21,8	5,92
Q7QAZ5	AGAP004271-PA OS=Anopheles gambiae GN=AGAP004271 PE=4 SV=3 - [Q7QAZ5_ANOGA]	10,00	1	0	2	3	27,8	6,44
Q7Q415	AGAP008150-PA OS=Anopheles gambiae GN=AGAP008150 PE=4 SV=4 - [Q7Q415_ANOGA]	11,70	1	3	3	3	30,6	6,87
Q7QKJ2	AGAP010488-PA (Fragment) OS=Anopheles gambiae GN=AGAP010488 PE=3 SV=4 - [Q7QKJ2_ANOGA]	12,08	1	2	2	2	34,4	8,48

Spot 1144

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7PJ76	AGAP008278-PA OS=Anopheles gambiae GN=AGAP008278 PE=4 SV=1 - [Q7PJ76_ANOGA]	40,19	1	10	10	15	35,6	5,90
Q7Q6C1	AGAP005929-PA OS=Anopheles gambiae GN=AGAP005929 PE=4 SV=4 - [Q7Q6C1_ANOGA]	41,75	1	9	9	15	34,1	6,96
Q7Q254	AGAP004031-PA OS=Anopheles gambiae GN=AgaP_AGAP004031 PE=4 SV=4 - [Q7Q254_ANOGA]	34,94	1	8	8	11	34,6	8,57
O97413	Putative gVAG protein (Precursor) OS=Anopheles gambiae GN=gVAG PE=2 SV=2 - [O97413_ANOGA]	40,00	1	7	7	8	28,9	8,91
Q7QBJ8	AGAP003141-PA OS=Anopheles gambiae GN=AgaP_AGAP003141 PE=4 SV=5 - [Q7QBJ8_ANOGA]	21,40	1	5	5	7	31,6	7,01
F5HMB4	AGAP001957-PB OS=Anopheles gambiae GN=AgaP_AGAP001957 PE=4 SV=1 - [F5HMB4_ANOGA]	19,85	1	0	5	5	31,6	6,06

Q7PUN0	AGAP001957-PA OS=Anopheles gambiae GN=AGAP001957 PE=4 SV=4 - [Q7PUN0_ANOGA]	19,85	1	0	5	5	31,4	6,54
F5HL87	AGAP013231-PA OS=Anopheles gambiae GN=AgaP_AGAP013231 PE=4 SV=1 - [F5HL87_ANOGA]	15,88	1	0	3	3	30,6	6,96
Q7QKM8	AGAP012662-PA (Fragment) OS=Anopheles gambiae str. PEST GN=AgaP_AGAP012662 PE=4 SV=3 - [Q7QKM8_ANOGA]	14,29	1	0	3	3	33,8	7,88
F5HKI4	AGAP002879-PB OS=Anopheles gambiae GN=AgaP_AGAP002879 PE=3 SV=1 - [F5HKI4_ANOGA]	1,47	1	0	2	2	202,1	5,92
F5HKI5	AGAP002879-PC OS=Anopheles gambiae GN=AgaP_AGAP002879 PE=3 SV=1 - [F5HKI5_ANOGA]	2,83	1	0	2	2	106,0	8,06
Q7QCZ7	AGAP002879-PA OS=Anopheles gambiae GN=AgaP_AGAP002879 PE=3 SV=5 - [Q7QCZ7_ANOGA]	1,49	1	0	2	2	199,4	5,83
Q7Q122	AGAP010011-PA OS=Anopheles gambiae GN=AGAP010011 PE=4 SV=2 - [Q7Q122_ANOGA]	9,83	1	2	2	2	33,9	6,84
Q7PYE7	AGAP001903-PA OS=Anopheles gambiae GN=AGAP001903 PE=3 SV=4 - [Q7PYE7_ANOGA]	9,20	1	2	2	2	35,4	9,06

Protéines F5HMB4 etQ7PUN0 identifiées avec les 5 mêmes peptides

Protéines F5HL87 et Q7QKM8 identifiées avec les 3 mêmes peptides

Protéines F5HKI4, F5HKI5 et Q7QKM8 identifiées avec les 2 mêmes peptides

Spot 685

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q8MUR9	Glutathione S-transferase S1-2 (Fragment) OS=Anopheles gambiae GN=GSTs1 PE=2 SV=1 - [Q8MUR9_ANOGA]	46,67	1	6	9	17	22,3	5,00
Q7QDJ9	AGAP003415-PA OS=Anopheles gambiae GN=AGAP003415 PE=4 SV=2 - [Q7QDJ9_ANOGA]	38,64	1	7	7	12	24,2	5,02
P46428	Glutathione S-transferase OS=Anopheles gambiae GN=GstS1 PE=2 SV=4 - [GST_ANOGA]	33,99	1	2	5	9	23,2	5,29
Q7PTC6	AGAP007666-PA OS=Anopheles gambiae GN=AGAP007666 PE=4 SV=3 - [Q7PTC6_ANOGA]	34,88	1	6	6	6	24,0	5,25
Q7QC60	AGAP002465-PA OS=Anopheles gambiae GN=HCH PE=2 SV=4 - [Q7QC60_ANOGA]	20,83	1	4	4	5	24,6	5,20
Q5TWA3	AGAP000941-PA OS=Anopheles gambiae GN=AGAP000941 PE=3 SV=3 - [Q5TWA3_ANOGA]	26,74	1	3	3	5	20,9	5,21
Q7PY23	AGAP001711-PA OS=Anopheles gambiae GN=AgaP_AGAP001711 PE=3 SV=5 - [Q7PY23_ANOGA]	16,06	1	3	3	3	24,7	5,72

Peptides communs entre Q8MUR9 et P45428 mais peptides spécifiques également pour chaque protéine

Spot 564

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7PZJ8	AGAP011948-PA OS=Anopheles gambiae GN=AGAP011948 PE=4 SV=3 - [Q7PZJ8_ANOGA]	20,78	1	5	5	5	41,0	8,46
Q7PLZ4	AGAP009610-PA OS=Anopheles gambiae GN=AGAP009610 PE=3 SV=4 - [Q7PLZ4_ANOGA]	14,72	1	3	3	5	35,1	7,06
Q7Q1U8	Glyceraldehyde-3-phosphate dehydrogenase OS=Anopheles gambiae GN=AGAP009623 PE=3 SV=2 - [Q7Q1U8_ANOGA]	12,95	1	3	3	4	35,4	8,32
Q8MUR9	Glutathione S-transferase S1-2 (Fragment) OS=Anopheles gambiae GN=GSTs1 PE=2 SV=1 - [Q8MUR9_ANOGA]	15,38	1	0	3	3	22,3	5,00

O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	5,99	1	0	2	3	46,2	8,38
Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	5,99	1	0	2	3	46,1	8,21
Q7PVV6	AGAP009173-PA OS=Anopheles gambiae GN=AGAP009173 PE=3 SV=4 - [Q7PVV6_ANOGA]	13,69	1	3	3	3	36,7	7,71
Q7Q484	AGAP008279-PA (Fragment) OS=Anopheles gambiae GN=AGAP008279 PE=4 SV=4 - [Q7Q484_ANOGA]	7,17	1	2	2	2	36,8	8,02

ProtéinesO97407 et Q7QFQ0 identifiées avec les 2 mêmes peptides

Spot 547

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7PZ81	40S ribosomal protein SA OS=Anopheles gambiae GN=AGAP011777 PE=3 SV=3 - [RSSA_ANOGA]	41,40	1	8	10	18	31,4	5,10
Q7QJG1	AGAP007500-PA OS=Anopheles gambiae GN=AGAP007500 PE=4 SV=4 - [Q7QJG1_ANOGA]	13,32	1	4	4	4	41,7	5,94
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	10,23	1	0	3	3	39,0	5,44
Q7Q7K6	AGAP011516-PA (Fragment) OS=Anopheles gambiae GN=AGAP011516 PE=3 SV=4 - [Q7Q7K6_ANOGA]	9,42	1	0	3	3	42,3	5,72
Q8MUR9	Glutathione S-transferase S1-2 (Fragment) OS=Anopheles gambiae GN=GSTs1 PE=2 SV=1 - [Q8MUR9_ANOGA]	14,87	1	0	2	3	22,3	5,00
O97413	Putative gVAG protein (Precursor) OS=Anopheles gambiae GN=gVAG PE=2 SV=2 - [O97413_ANOGA]	13,08	1	0	2	2	28,9	8,91
Q7PNX0	AGAP006421-PA OS=Anopheles gambiae GN=AGAP006421 PE=4 SV=3 - [Q7PNX0_ANOGA]	13,08	1	0	2	2	28,9	8,82
Q7Q484	AGAP008279-PA (Fragment) OS=Anopheles gambiae GN=AGAP008279 PE=4 SV=4 - [Q7Q484_ANOGA]	7,17	1	2	2	2	36,8	8,02
Q7QCF9	AGAP002608-PA OS=Anopheles gambiae GN=AgaP_AGAP002608 PE=3 SV=5 - [Q7QCF9_ANOGA]	8,23	1	1	2	2	34,4	5,27
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	4,41	1	0	2	2	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	4,61	1	0	2	2	47,1	7,80
F5HLR5	AGAP003352-PB OS=Anopheles gambiae GN=AgaP_AGAP003352 PE=4 SV=1 - [F5HLR5_ANOGA]	7,95	1	0	2	2	42,7	5,82
F5HLR6	AGAP003352-PC OS=Anopheles gambiae GN=AgaP_AGAP003352 PE=4 SV=1 - [F5HLR6_ANOGA]	9,20	1	0	2	2	37,0	6,15
Q7PCP9	AGAP003352-PA OS=Anopheles gambiae GN=AgaP_AGAP003352 PE=4 SV=4 - [Q7PCP9_ANOGA]	9,37	1	0	2	2	35,8	5,30
F5HM48	AGAP013423-PA OS=Anopheles gambiae GN=AgaP_AGAP013423 PE=4 SV=1 - [F5HM48_ANOGA]	7,63	1	2	2	2	42,7	7,59

Protéines Q7Q7K5 et Q7Q7K6 identifiées avec les 3 mêmes peptides
Protéines O97413 et Q7PNX0 identifiées avec les 2 mêmes peptides
Protéines F7IW82 et Q2TLV8 identifiées avec les 2 mêmes peptides
Protéines F5HLR5, F5HLR6 et Q7PCP9 identifiées avec les 2 mêmes peptides

Spot 467

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
E3XEC7	ATP synthase subunit beta OS=Anopheles darlingi GN=AND_21925 PE=3 SV=1 - [E3XEC7_ANODA]	26,04	1	0	9	12	53,7	5,12

Q17FL3	ATP synthase subunit beta OS=Aedes aegypti GN=AAEL003393 PE=3 SV=1 - [Q17FL3_AEDAE]	25,99	1	0	9	12	53,9	5,12
Q17H12	ATP synthase subunit beta OS=Aedes aegypti GN=AAEL002827 PE=3 SV=1 - [Q17H12_AEDAE]	25,99	1	0	9	12	53,9	5,12
Q7PSI4	AGAP010929-PA OS=Anopheles gambiae GN=AGAP010929 PE=3 SV=4 - [Q7PSI4_ANOGA]	17,90	1	0	6	10	50,1	4,86
Q7PPI8	AGAP004940-PA OS=Anopheles gambiae GN=AGAP004940 PE=4 SV=3 - [Q7PPI8_ANOGA]	27,03	1	7	7	9	43,5	5,35
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	18,47	1	0	5	6	39,0	5,44
Q7Q7K6	AGAP011516-PA (Fragment) OS=Anopheles gambiae GN=AGAP011516 PE=3 SV=4 - [Q7Q7K6_ANOGA]	17,02	1	0	5	6	42,3	5,72
F5HJ17	AGAP000969-PE OS=Anopheles gambiae GN=AgaP_AGAP000969 PE=4 SV=1 - [F5HJ17_ANOGA]	15,57	1	0	4	4	40,8	5,10
F5HJ18	AGAP000969-PB OS=Anopheles gambiae GN=AgaP_AGAP000969 PE=4 SV=1 - [F5HJ18_ANOGA]	13,19	1	0	4	4	48,4	4,65
F5HJ20	AGAP000969-PC OS=Anopheles gambiae GN=AgaP_AGAP000969 PE=4 SV=1 - [F5HJ20_ANOGA]	14,62	1	0	4	4	43,7	5,05
Q7PFC9	AGAP000969-PA OS=Anopheles gambiae GN=AgaP_AGAP000969 PE=4 SV=5 - [Q7PFC9_ANOGA]	13,73	1	0	4	4	46,7	5,01
Q7QE00	AGAP010723-PA (Fragment) OS=Anopheles gambiae GN=AGAP010723 PE=4 SV=3 - [Q7QE00_ANOGA]	8,98	1	3	3	4	48,2	5,16
Q2WG66	Heat shock protein 90 OS=Plutella xylostella GN=hsp90 PE=2 SV=1 - [Q2WG66_PLUXY]	3,91	1	1	2	2	82,3	5,07
H2KMF4	AGAP009863-PA OS=Anopheles gambiae GN=AgaP_AGAP009863 PE=3 SV=1 - [H2KMF4_ANOGA]	5,94	1	2	2	2	45,6	5,67
Q8MUR9	Glutathione S-transferase S1-2 (Fragment) OS=Anopheles gambiae GN=GSTs1 PE=2 SV=1 - [Q8MUR9_ANOGA]	14,87	1	0	2	2	22,3	5,00
F5HML9	AGAP012996-PA OS=Anopheles gambiae GN=AgaP_AGAP012996 PE=4 SV=1 - [F5HML9_ANOGA]	4,93	1	2	2	2	51,5	5,07
Q5TMX9	AGAP012407-PA (Fragment) OS=Anopheles gambiae GN=AGAP012407 PE=3 SV=3 - [Q5TMX9_ANOGA]	4,87	1	0	2	2	53,1	5,10
Q7PZ25	Probable dynactin subunit 2 OS=Anopheles gambiae GN=Dmn PE=3 SV=3 - [DCTN2_ANOGA]	9,04	1	2	2	2	42,8	5,11
O97415	Putative uncharacterized protein (Precursor) OS=Anopheles gambiae PE=2 SV=2 - [O97415_ANOGA]	6,48	1	0	2	2	46,5	9,41
Q7PRT6	AGAP000609-PA OS=Anopheles gambiae GN=AGAP000609 PE=4 SV=1 - [Q7PRT6_ANOGA]	6,48	1	0	2	2	46,5	9,41

Protéines E3XEC7, Q17FL3 et Q17H12 identifiées avec les 9 mêmes peptides
Protéines Q7Q7K5 et Q7Q7K6 identifiées avec les 5 mêmes peptides
Protéines F5HJ17, F5HJ18, F5HJ20 et Q7PFC9 identifiées avec les 4 mêmes peptides
Protéines O97415 et Q7PRT6 identifiées avec les 2 mêmes peptides

Spot 1168

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	44,16	1	18	18	42	43,6	7,34
Q7Q6L2	AGAP005781-PA OS=Anopheles gambiae GN=AGAP005781 PE=3 SV=4 - [Q7Q6L2_ANOGA]	41,08	1	12	12	25	46,6	7,49
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	38,07	1	0	10	15	39,0	5,44
Q7Q609	AGAP006099-PA OS=Anopheles gambiae GN=AGAP006099 PE=3 SV=3 - [Q7Q609_ANOGA]	38,50	1	13	13	15	45,1	8,59
O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	23,19	1	0	10	13	46,2	8,38
Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	23,19	1	0	10	13	46,1	8,21

Q7PNP8	AGAP005662-PA OS=Anopheles gambiae GN=AGAP005662 PE=3 SV=2 - [Q7PNP8_ANOGA]	27,92	1	8	8	9	45,4	8,06
F5HKV6	Fructose-bisphosphate aldolase OS=Anopheles gambiae GN=AgaP_AGAP002564 PE=3 SV=1 - [F5HKV6_ANOGA]	35,54	1	8	8	9	39,2	7,64
Q7QIC5	AGAP006780-PA OS=Anopheles gambiae GN=AGAP006780 PE=3 SV=3 - [Q7QIC5_ANOGA]	20,28	1	7	7	9	47,0	8,03
Q7PXH7	AGAP001407-PA OS=Anopheles gambiae GN=AgaP_AGAP001407 PE=3 SV=5 - [Q7PXH7_ANOGA]	18,37	1	0	6	7	44,3	7,03
Q7Q4N7	AGAP008501-PA (Fragment) OS=Anopheles gambiae GN=AGAP008501 PE=3 SV=3 - [Q7Q4N7_ANOGA]	18,31	1	6	6	7	45,5	7,20
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	19,13	1	6	6	6	44,5	6,25
Q7QEV4	AGAP000167-PA OS=Anopheles gambiae GN=AgaP_AGAP000167 PE=4 SV=5 - [Q7QEV4_ANOGA]	20,42	1	5	5	6	36,4	7,37
Q7PYV6	AGAP002127-PA OS=Anopheles gambiae GN=AGAP002127 PE=3 SV=2 - [Q7PYV6_ANOGA]	11,17	1	0	3	5	42,6	7,06
Q7QHS3	AGAP011329-PA (Fragment) OS=Anopheles gambiae GN=AGAP011329 PE=3 SV=4 - [Q7QHS3_ANOGA]	14,29	1	5	5	5	43,5	8,09
Q7PQQ3	Isocitrate dehydrogenase [NADP] OS=Anopheles gambiae GN=AgaP_AGAP003168 PE=3 SV=5 - [Q7PQQ3_ANOGA]	9,21	1	3	3	4	50,5	8,63
Q7QC97	AGAP002518-PA OS=Anopheles gambiae GN=AgaP_AGAP002518 PE=3 SV=4 - [Q7QC97_ANOGA]	6,45	1	4	4	4	86,2	7,36
Q9BIH7	AGAP004334-PA (Precursor) OS=Anopheles gambiae GN=gSG5 PE=2 SV=1 - [Q9BIH7_ANOGA]	10,84	1	3	3	3	38,2	6,47
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	3,94	1	0	2	2	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	4,13	1	0	2	2	47,1	7,80
Q8WR22	TRIO protein (Fragment) OS=Anopheles gambiae PE=2 SV=1 - [Q8WR22_ANOGA]	5,88	1	0	2	2	43,7	6,46
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	5,88	1	0	2	2	43,8	6,46
Q7PWZ1	Aminomethyltransferase OS=Anopheles gambiae GN=AGAP001124 PE=3 SV=3 - [Q7PWZ1_ANOGA]	6,99	1	2	2	2	45,7	8,51
Q7QIF7	AGAP006821-PA OS=Anopheles gambiae GN=AGAP006821 PE=3 SV=2 - [Q7QIF7_ANOGA]	5,53	1	0	2	2	41,6	8,32
Q7QER0	AGAP000106-PA OS=Anopheles gambiae GN=AgaP_AGAP000106 PE=4 SV=5 - [Q7QER0_ANOGA]	6,16	1	2	2	2	51,1	8,41

Protéines O97407 et Q7QFQ0 identifiées avec les 10 mêmes peptides

Protéines F7IW82 et Q2TLV8 identifiées avec les 2 mêmes peptides

Protéines Q8WR22 et Q7QPUJ5 identifiées avec les 2 mêmes peptides

Spot 1149

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	42,08	1	16	17	53	43,6	7,34
Q7Q6L2	AGAP005781-PA OS=Anopheles gambiae GN=AGAP005781 PE=3 SV=4 - [Q7Q6L2_ANOGA]	51,64	1	15	16	23	46,6	7,49
Q7Q609	AGAP006099-PA OS=Anopheles gambiae GN=AGAP006099 PE=3 SV=3 - [Q7Q609_ANOGA]	48,06	1	15	15	19	45,1	8,59
Q7QHS3	AGAP011329-PA (Fragment) OS=Anopheles gambiae GN=AGAP011329 PE=3 SV=4 - [Q7QHS3_ANOGA]	49,15	1	14	14	18	43,5	8,09
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	52,56	1	11	12	17	39,0	5,44
O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	30,92	1	0	11	14	46,2	8,38

Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	30,92	1	0	11	14	46,1	8,21
Q7PNP8	AGAP005662-PA OS=Anopheles gambiae GN=AGAP005662 PE=3 SV=2 - [Q7PNP8_ANOGA]	34,61	1	10	10	13	45,4	8,06
Q7PQQ3	Isocitrate dehydrogenase [NADP] OS=Anopheles gambiae GN=AgaP_AGAP003168 PE=3 SV=5 - [Q7PQQ3_ANOGA]	24,72	1	8	8	12	50,5	8,63
Q7Q4N7	AGAP008501-PA (Fragment) OS=Anopheles gambiae GN=AGAP008501 PE=3 SV=3 - [Q7Q4N7_ANOGA]	29,40	1	8	8	11	45,5	7,20
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	27,81	1	7	7	9	44,5	6,25
F5HKV6	Fructose-bisphosphate aldolase OS=Anopheles gambiae GN=AgaP_AGAP002564 PE=3 SV=1 - [F5HKV6_ANOGA]	31,68	1	8	8	8	39,2	7,64
Q7QER0	AGAP000106-PA OS=Anopheles gambiae GN=AgaP_AGAP000106 PE=4 SV=5 - [Q7QER0_ANOGA]	17,83	1	7	7	7	51,1	8,41
Q7QIF7	AGAP006821-PA OS=Anopheles gambiae GN=AGAP006821 PE=3 SV=2 - [Q7QIF7_ANOGA]	8,79	1	0	3	4	41,6	8,32
Q7PXH7	AGAP001407-PA OS=Anopheles gambiae GN=AgaP_AGAP001407 PE=3 SV=5 - [Q7PXH7_ANOGA]	9,44	1	0	3	3	44,3	7,03
O97413	Putative gVAG protein (Precursor) OS=Anopheles gambiae GN=gVAG PE=2 SV=2 - [O97413_ANOGA]	15,38	1	0	2	2	28,9	8,91
Q7PNX0	AGAP006421-PA OS=Anopheles gambiae GN=AGAP006421 PE=4 SV=3 - [Q7PNX0_ANOGA]	15,38	1	0	2	2	28,9	8,82
Q8WR39	Antigen 5-related 1 protein OS=Anopheles gambiae PE=2 SV=1 - [Q8WR39_ANOGA]	22,47	1	0	2	2	19,7	9,03
Q8WR22	TRIO protein (Fragment) OS=Anopheles gambiae PE=2 SV=1 - [Q8WR22_ANOGA]	6,39	1	0	2	2	43,7	6,46
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	6,39	1	0	2	2	43,8	6,46

Protéines O97407 et Q7QFQ0 identifiées avec les 11 mêmes peptides

Protéines O97413, Q7PNX0 et Q8WR39 identifiées avec les 2 mêmes peptides

Protéines Q8WR22 et Q7QPUJ5 identifiées avec les 2 mêmes peptides

Spot 1149_Plasmodium

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
PFI1105w	organism=Plasmodium_falciparum_3D7 | product=phosphoglycerate kinase | location=Pf3D7_09:913220-914470(+) | length=416	40,63	1	12	12	13	45,4	7,83

Spot 1179

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	34,81	1	10	11	14	43,6	7,34
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	33,76	1	8	8	12	43,8	6,46
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	14,29	1	3	4	4	44,5	6,25

Spot 1197

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q9BIH6	GSG1b protein (Precursor) OS=Anopheles gambiae GN=gSG1b PE=2 SV=1 - [Q9BIH6_ANOGA]	46,23	1	17	18	59	43,6	7,58
Q7Q609	AGAP006099-PA OS=Anopheles gambiae GN=AGAP006099 PE=3 SV=3 - [Q7Q609_ANOGA]	55,35	1	15	15	20	45,1	8,59
Q7PQQ3	Isocitrate dehydrogenase [NADP] OS=Anopheles gambiae GN=AgaP_AGAP003168 PE=3 SV=5 - [Q7PQQ3_ANOGA]	33,48	1	11	11	18	50,5	8,63
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	50,57	1	3	10	14	39,0	5,44
O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	28,43	1	0	10	14	46,2	8,38
Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	28,43	1	0	10	14	46,1	8,21
F5HKV6	Fructose-bisphosphate aldolase OS=Anopheles gambiae GN=AgaP_AGAP002564 PE=3 SV=1 - [F5HKV6_ANOGA]	35,54	1	8	8	9	39,2	7,64
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	23,72	1	7	7	8	44,5	6,25
Q7Q6L2	AGAP005781-PA OS=Anopheles gambiae GN=AGAP005781 PE=3 SV=4 - [Q7Q6L2_ANOGA]	21,13	1	6	7	8	46,6	7,49
Q7PWZ1	Aminomethyltransferase OS=Anopheles gambiae GN=AGAP001124 PE=3 SV=3 - [Q7PWZ1_ANOGA]	24,82	1	6	6	7	45,7	8,51
Q7PNP8	AGAP005662-PA OS=Anopheles gambiae GN=AGAP005662 PE=3 SV=2 - [Q7PNP8_ANOGA]	17,18	1	5	5	5	45,4	8,06
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	6,26	1	0	3	3	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	6,55	1	0	3	3	47,1	7,80
Q7QHS3	AGAP011329-PA (Fragment) OS=Anopheles gambiae GN=AGAP011329 PE=3 SV=4 - [Q7QHS3_ANOGA]	10,90	1	3	3	3	43,5	8,09

Protéines O97407 et Q7QFQ0 identifiées avec les 10 mêmes peptides

Protéines F7IW82 et Q2TLV8 identifiées avec les 3 mêmes peptides

Spot 1228

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	40,31	1	12	13	21	44,5	6,25
Q8WR22	TRIO protein (Fragment) OS=Anopheles gambiae PE=2 SV=1 - [Q8WR22_ANOGA]	36,83	1	3	11	21	43,7	6,46
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	36,83	1	2	11	21	43,8	6,46
A7UTS9	AGAP005627-PC OS=Anopheles gambiae GN=AGAP005627 PE=3 SV=1 - [A7UTS9_ANOGA]	34,93	1	0	11	18	39,7	6,47
Q7PIQ5	AGAP005627-PA OS=Anopheles gambiae GN=AGAP005627 PE=3 SV=3 - [Q7PIQ5_ANOGA]	31,23	1	0	11	18	43,7	6,04
Q9BIH7	AGAP004334-PA (Precursor) OS=Anopheles gambiae GN=gSG5 PE=2 SV=1 - [Q9BIH7_ANOGA]	29,22	1	9	9	15	38,2	6,47
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	31,69	1	9	9	13	43,6	7,34

F5HL20	AGAP003238-PC OS=Anopheles gambiae GN=AgaP_AGAP003238 PE=4 SV=1 - [F5HL20_ANOGA]	15,53	1	4	4	4	40,1	8,19
F5HKV6	Fructose-bisphosphate aldolase OS=Anopheles gambiae GN=AgaP_AGAP002564 PE=3 SV=1 - [F5HKV6_ANOGA]	14,33	1	3	3	3	39,2	7,64
Q7QIC5	AGAP006780-PA OS=Anopheles gambiae GN=AGAP006780 PE=3 SV=3 - [Q7QIC5_ANOGA]	7,93	1	3	3	3	47,0	8,03
Q7QC97	AGAP002518-PA OS=Anopheles gambiae GN=AgaP_AGAP002518 PE=3 SV=4 - [Q7QC97_ANOGA]	5,18	1	3	3	3	86,2	7,36
Q7Q8X4	AGAP010499-PA (Fragment) OS=Anopheles gambiae GN=AGAP010499 PE=3 SV=4 - [Q7Q8X4_ANOGA]	13,11	1	3	3	3	37,4	6,23
Q7PH36	AGAP004097-PA OS=Anopheles gambiae GN=AgaP_AGAP004097 PE=4 SV=5 - [Q7PH36_ANOGA]	8,01	1	3	3	3	42,4	7,46
Q7Q164	AGAP009945-PA OS=Anopheles gambiae GN=AGAP009945 PE=4 SV=3 - [Q7Q164_ANOGA]	15,04	1	3	3	3	37,3	6,68
Q7PXB3	AGAP001318-PA OS=Anopheles gambiae GN=AGAP001318 PE=3 SV=3 - [Q7PXB3_ANOGA]	7,36	1	2	2	3	40,6	6,70
Q7QB79	Aspartate aminotransferase OS=Anopheles gambiae GN=AgaP_AGAP004142 PE=3 SV=4 - [Q7QB79_ANOGA]	4,46	1	0	2	2	44,8	6,79
O97413	Putative gVAG protein (Precursor) OS=Anopheles gambiae GN=gVAG PE=2 SV=2 - [O97413_ANOGA]	13,08	1	0	2	2	28,9	8,91
Q7PNX0	AGAP006421-PA OS=Anopheles gambiae GN=AGAP006421 PE=4 SV=3 - [Q7PNX0_ANOGA]	13,08	1	0	2	2	28,9	8,82
Q7PWF1	Glutamine synthetase OS=Anopheles gambiae GN=AGAP008988 PE=3 SV=2 - [Q7PWF1_ANOGA]	10,45	1	2	2	2	44,5	7,62
Q7QKG7	AGAP009439-PA (Fragment) OS=Anopheles gambiae GN=AGAP009439 PE=4 SV=4 - [Q7QKG7_ANOGA]	6,30	1	2	2	2	38,0	9,04
A7UUE0	AGAP006576-PA OS=Anopheles gambiae GN=AGAP006576 PE=4 SV=1 - [A7UUE0_ANOGA]	4,99	1	0	2	2	53,2	8,40
Q7PI68	AGAP006576-PC OS=Anopheles gambiae GN=AGAP006576 PE=4 SV=4 - [Q7PI68_ANOGA]	6,87	1	0	2	2	38,4	6,73
Q7Q1H8	AGAP009783-PA (Fragment) OS=Anopheles gambiae GN=AGAP009783 PE=3 SV=3 - [Q7Q1H8_ANOGA]	4,80	1	0	2	2	45,6	6,70
A7UUV1	AGAP004773-PA OS=Anopheles gambiae GN=AGAP004773 PE=4 SV=1 - [A7UUV1_ANOGA]	6,63	1	0	2	2	41,2	7,62
Q7Q2S3	AGAP004786-PA OS=Anopheles gambiae GN=AGAP004786 PE=4 SV=3 - [Q7Q2S3_ANOGA]	6,30	1	0	2	2	43,3	7,88

Peptides communs entre Q8WR22 et Q7PUJ5 mais peptides spécifiques également pour chaque protéine

Protéines A7UTS9 et Q7PIQ5 identifiées avec les 11 mêmes peptides

Protéines O97413 et Q7PNX0 identifiées avec les 2 mêmes peptides

Protéines A7UUE0 et Q7PI68 identifiées avec les 2 mêmes peptides

Spot 1229

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	40,31	1	12	13	16	44,5	6,25
Q8WR22	TRIO protein (Fragment) OS=Anopheles gambiae PE=2 SV=1 - [Q8WR22_ANOGA]	29,41	1	2	9	15	43,7	6,46
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	31,71	1	2	9	15	43,8	6,46
Q9BIH7	AGAP004334-PA (Precursor) OS=Anopheles gambiae GN=gSG5 PE=2 SV=1 - [Q9BIH7_ANOGA]	29,52	1	10	10	14	38,2	6,47
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	28,41	1	0	8	10	39,0	5,44
Q7PWF1	Glutamine synthetase OS=Anopheles gambiae GN=AGAP008988 PE=3 SV=2 - [Q7PWF1_ANOGA]	23,13	1	5	6	7	44,5	7,62
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	21,04	1	5	6	7	43,6	7,34

O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	13,22	1	0	5	6	46,2	8,38
Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	13,22	1	0	5	6	46,1	8,21
Q7PUT4	AGAP001153-PA OS=Anopheles gambiae GN=AGAP001153 PE=4 SV=4 - [Q7PUT4_ANOGA]	12,69	1	4	4	5	45,1	6,89
Q7QB79	Aspartate aminotransferase OS=Anopheles gambiae GN=AgaP_AGAP004142 PE=3 SV=4 - [Q7QB79_ANOGA]	10,64	1	4	4	4	44,8	6,79
Q7Q609	AGAP006099-PA OS=Anopheles gambiae GN=AGAP006099 PE=3 SV=3 - [Q7Q609_ANOGA]	11,62	1	4	4	4	45,1	8,59
Q7Q180	AGAP009926-PA OS=Anopheles gambiae GN=AGAP009926 PE=4 SV=4 - [Q7Q180_ANOGA]	8,31	1	2	2	3	41,7	6,48
Q7Q6L2	AGAP005781-PA OS=Anopheles gambiae GN=AGAP005781 PE=3 SV=4 - [Q7Q6L2_ANOGA]	7,51	1	0	2	2	46,6	7,49

Peptides communs entre Q8WR22 et Q7PUJ5 mais peptides spécifiques également pour chaque protéine

Protéines O97407 et Q7QFQ0 identifiées avec les 5 mêmes peptides

Spot 1236

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	45,92	1	13	13	27	44,5	6,25
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	53,69	1	4	12	21	39,0	5,44
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	48,34	1	11	12	17	43,8	6,46
Q7Q068	Eukaryotic translation initiation factor 3 subunit M OS=Anopheles gambiae GN=AGAP012281 PE=3 SV=2 - [EIF3M_ANOGA]	30,65	1	9	9	9	44,1	5,85
O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	10,97	1	0	4	4	46,2	8,38
Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	10,97	1	0	4	4	46,1	8,21
O97415	Putative uncharacterized protein (Precursor) OS=Anopheles gambiae PE=2 SV=2 - [O97415_ANOGA]	11,72	1	0	4	4	46,5	9,41
Q7PRT6	AGAP000609-PA OS=Anopheles gambiae GN=AGAP000609 PE=4 SV=1 - [Q7PRT6_ANOGA]	11,72	1	0	4	4	46,5	9,41
Q7Q609	AGAP006099-PA OS=Anopheles gambiae GN=AGAP006099 PE=3 SV=3 - [Q7Q609_ANOGA]	10,93	1	4	4	4	45,1	8,59
Q5TVB9	AGAP003762-PA OS=Anopheles gambiae GN=AgaP_AGAP003762 PE=4 SV=2 - [Q5TVB9_ANOGA]	8,40	1	3	3	4	47,2	6,14
Q7Q6F9	AGAP005865-PA OS=Anopheles gambiae GN=AGAP005865 PE=4 SV=3 - [Q7Q6F9_ANOGA]	12,50	1	3	3	4	45,5	6,10
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	10,91	1	3	3	3	43,6	7,34
Q9BIH7	AGAP004334-PA (Precursor) OS=Anopheles gambiae GN=gSG5 PE=2 SV=1 - [Q9BIH7_ANOGA]	7,83	1	2	2	3	38,2	6,47
Q7PSK1	AGAP010821-PA (Fragment) OS=Anopheles gambiae GN=AGAP010821 PE=4 SV=4 - [Q7PSK1_ANOGA]	5,51	1	2	2	3	40,4	6,25
A7USV9	AGAP002350-PC OS=Anopheles gambiae GN=AgaP_AGAP002350 PE=4 SV=2 - [A7USV9_ANOGA]	10,32	1	0	3	3	45,5	5,20
A7USW0	AGAP002350-PE OS=Anopheles gambiae GN=AGAP002350 PE=4 SV=1 - [A7USW0_ANOGA]	10,80	1	0	3	3	43,5	4,89
A7USW1	AGAP002350-PD OS=Anopheles gambiae GN=AgaP_AGAP002350 PE=4 SV=2 - [A7USW1_ANOGA]	10,48	1	0	3	3	44,9	5,19
Q7PWF1	Glutamine synthetase OS=Anopheles gambiae GN=AGAP008988 PE=3 SV=2 - [Q7PWF1_ANOGA]	8,21	1	2	2	2	44,5	7,62

Protéines O97407 et Q7QFQ0 identifiées avec les 4 mêmes peptides

Protéines O97415 et Q7PTR6 identifiées avec les 4 mêmes peptides

Protéines A7USV9, A7USW0 et A7USW1 identifiées avec les 3 mêmes peptides

Spot 1239

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	47,31	1	4	14	29	43,8	6,46
Q8WR22	TRIO protein (Fragment) OS=Anopheles gambiae PE=2 SV=1 - [Q8WR22_ANOGA]	40,15	1	2	12	25	43,7	6,46
Q7Q1H8	AGAP009783-PA (Fragment) OS=Anopheles gambiae GN=AGAP009783 PE=3 SV=3 - [Q7Q1H8_ANOGA]	41,73	1	13	13	23	45,6	6,70
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	35,46	1	11	11	19	44,5	6,25
A7UTS9	AGAP005627-PC OS=Anopheles gambiae GN=AGAP005627 PE=3 SV=1 - [A7UTS9_ANOGA]	32,11	1	0	8	10	39,7	6,47
Q7PIQ5	AGAP005627-PA OS=Anopheles gambiae GN=AGAP005627 PE=3 SV=3 - [Q7PIQ5_ANOGA]	28,72	1	0	8	10	43,7	6,04
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	15,63	1	0	4	6	39,0	5,44
F5HKV6	Fructose-bisphosphate aldolase OS=Anopheles gambiae GN=AgaP_AGAP002564 PE=3 SV=1 - [F5HKV6_ANOGA]	10,74	1	0	3	4	39,2	7,64
Q7PGI9	Fructose-bisphosphate aldolase OS=Anopheles gambiae GN=AGAP002564 PE=3 SV=4 - [Q7PGI9_ANOGA]	10,71	1	0	3	4	39,5	8,24
Q9BIH7	AGAP004334-PA (Precursor) OS=Anopheles gambiae GN=gSG5 PE=2 SV=1 - [Q9BIH7_ANOGA]	7,83	1	2	2	3	38,2	6,47
Q7QHF8	AGAP011172-PA OS=Anopheles gambiae GN=AGAP011172 PE=3 SV=3 - [Q7QHF8_ANOGA]	6,08	1	2	2	3	43,4	6,48
Q7Q806	AGAP004918-PA OS=Anopheles gambiae GN=AGAP004918 PE=4 SV=3 - [Q7Q806_ANOGA]	13,44	1	3	3	3	34,9	5,88
A7UVP8	AGAP001299-PA OS=Anopheles gambiae GN=AGAP001299 PE=4 SV=1 - [A7UVP8_ANOGA]	11,67	1	3	3	3	39,9	6,25
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	8,83	1	0	2	2	43,6	7,34
Q9BIH6	GSG1b protein (Precursor) OS=Anopheles gambiae GN=gSG1b PE=2 SV=1 - [Q9BIH6_ANOGA]	8,83	1	0	2	2	43,6	7,58
Q7QC97	AGAP002518-PA OS=Anopheles gambiae GN=AgaP_AGAP002518 PE=3 SV=4 - [Q7QC97_ANOGA]	3,79	1	0	2	2	86,2	7,36
Q7PH36	AGAP004097-PA OS=Anopheles gambiae GN=AgaP_AGAP004097 PE=4 SV=5 - [Q7PH36_ANOGA]	5,43	1	2	2	2	42,4	7,46
F5HL19	AGAP003238-PD OS=Anopheles gambiae GN=AgaP_AGAP003238 PE=4 SV=1 - [F5HL19_ANOGA]	7,26	1	0	2	2	40,6	8,00
F5HL20	AGAP003238-PC OS=Anopheles gambiae GN=AgaP_AGAP003238 PE=4 SV=1 - [F5HL20_ANOGA]	7,36	1	0	2	2	40,1	8,19
Q7QBC4	AGAP003238-PA OS=Anopheles gambiae GN=AgaP_AGAP003238 PE=4 SV=5 - [Q7QBC4_ANOGA]	7,32	1	0	2	2	40,6	8,21
Q7QC19	AGAP002408-PA OS=Anopheles gambiae GN=AGAP002408 PE=4 SV=3 - [Q7QC19_ANOGA]	5,87	1	2	2	2	40,4	6,01

Peptides communs entre Q8WR22 et Q7PUJ5 mais peptides spécifiques également pour chaque protéine

Protéines A7UTS9 et Q7PIQ5 identifiées avec les 8 mêmes peptides

Protéines F5HKV6 et Q7PGI9 identifiées avec les 3 mêmes peptides

Protéines Q7QFL2 et Q9BIH6 identifiées avec les 2 mêmes peptides

Protéines F5HL19, F5HL20 et Q7QBC4 identifiées avec les 2 mêmes peptides

Spot 819

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	37,40	1	13	14	26	43,6	7,34
Q7QIC5	AGAP006780-PA OS=Anopheles gambiae GN=AGAP006780 PE=3 SV=3 - [Q7QIC5_ANOGA]	31,93	1	12	12	18	47,0	8,03
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	36,06	1	2	10	15	43,8	6,46
Q8WR22	TRIO protein (Fragment) OS=Anopheles gambiae PE=2 SV=1 - [Q8WR22_ANOGA]	31,46	1	2	9	12	43,7	6,46
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	28,83	1	8	8	11	44,5	6,25
Q7PNP8	AGAP005662-PA OS=Anopheles gambiae GN=AGAP005662 PE=3 SV=2 - [Q7PNP8_ANOGA]	26,49	1	8	8	10	45,4	8,06
F5HKV6	Fructose-bisphosphate aldolase OS=Anopheles gambiae GN=AgaP_AGAP002564 PE=3 SV=1 - [F5HKV6_ANOGA]	42,98	1	9	9	10	39,2	7,64
Q7Q609	AGAP006099-PA OS=Anopheles gambiae GN=AGAP006099 PE=3 SV=3 - [Q7Q609_ANOGA]	22,32	1	8	8	9	45,1	8,59
Q7Q6L2	AGAP005781-PA OS=Anopheles gambiae GN=AGAP005781 PE=3 SV=4 - [Q7Q6L2_ANOGA]	19,72	1	5	6	8	46,6	7,49
Q7QB79	Aspartate aminotransferase OS=Anopheles gambiae GN=AgaP_AGAP004142 PE=3 SV=4 - [Q7QB79_ANOGA]	20,05	1	7	7	7	44,8	6,79
Q7QC97	AGAP002518-PA OS=Anopheles gambiae GN=AgaP_AGAP002518 PE=3 SV=4 - [Q7QC97_ANOGA]	8,47	1	6	6	6	86,2	7,36
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	14,49	1	0	4	5	39,0	5,44
Q7PXH7	AGAP001407-PA OS=Anopheles gambiae GN=AgaP_AGAP001407 PE=3 SV=5 - [Q7PXH7_ANOGA]	15,31	1	0	5	5	44,3	7,03
Q7PYI1	AGAP001951-PA OS=Anopheles gambiae GN=AgaP_AGAP001951 PE=4 SV=5 - [Q7PYI1_ANOGA]	7,92	1	0	3	4	43,7	8,07
F5HIU6	AGAP002192-PB OS=Anopheles gambiae GN=AgaP_AGAP002192 PE=3 SV=1 - [F5HIU6_ANOGA]	9,41	1	0	3	4	43,5	7,27
Q7QK78	AGAP002192-PA (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP002192 PE=3 SV=5 - [Q7QK78_ANOGA]	9,64	1	0	3	4	42,4	7,24
Q7QIF7	AGAP006821-PA OS=Anopheles gambiae GN=AGAP006821 PE=3 SV=2 - [Q7QIF7_ANOGA]	8,54	1	3	3	4	41,6	8,32
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	4,04	1	0	2	2	63,4	7,02
Q9UB34	Putative 5'-nucleotidase (Precursor) OS=Anopheles gambiae GN=5Ntd PE=2 SV=1 - [Q9UB34_ANOGA]	4,04	1	0	2	2	63,4	6,89
O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	5,99	1	0	2	2	46,2	8,38
Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	5,99	1	0	2	2	46,1	8,21
Q7Q6H4	AGAP005845-PA OS=Anopheles gambiae GN=AGAP005845 PE=4 SV=4 - [Q7Q6H4_ANOGA]	5,45	1	0	2	2	44,0	7,75
Q7PWZ1	Aminomethyltransferase OS=Anopheles gambiae GN=AGAP001124 PE=3 SV=3 - [Q7PWZ1_ANOGA]	5,54	1	2	2	2	45,7	8,51
A7UUE0	AGAP006576-PA OS=Anopheles gambiae GN=AGAP006576 PE=4 SV=1 - [A7UUE0_ANOGA]	4,99	1	0	2	2	53,2	8,40
Q7PI68	AGAP006576-PC OS=Anopheles gambiae GN=AGAP006576 PE=4 SV=4 - [Q7PI68_ANOGA]	6,87	1	0	2	2	38,4	6,73
F5HL19	AGAP003238-PD OS=Anopheles gambiae GN=AgaP_AGAP003238 PE=4 SV=1 - [F5HL19_ANOGA]	7,53	1	0	2	2	40,6	8,00
F5HL20	AGAP003238-PC OS=Anopheles gambiae GN=AgaP_AGAP003238 PE=4 SV=1 - [F5HL20_ANOGA]	7,63	1	0	2	2	40,1	8,19
F5HL21	AGAP003238-PB OS=Anopheles gambiae GN=AgaP_AGAP003238 PE=4 SV=1 - [F5HL21_ANOGA]	14,74	1	0	2	2	21,1	9,60
Q7QBC4	AGAP003238-PA OS=Anopheles gambiae GN=AgaP_AGAP003238 PE=4 SV=5 - [Q7QBC4_ANOGA]	7,59	1	0	2	2	40,6	8,21

Q7QG51	AGAP009506-PA OS=Anopheles gambiae GN=AGAP009506 PE=4 SV=3 - [Q7QG51_ANOGA]	5,43	1	2	2	2	38,9	6,84

Protéines F5HIU6 et Q7QK78 identifiées avec les 4 mêmes peptides
Protéines Q5TVM9 et Q9UB34 identifiées avec les 2 mêmes peptides
Protéines O97407 et Q7QFQ0 identifiées avec les 2 mêmes peptides
Protéines A7UUE0 et Q7PI68 identifiées avec les 2 mêmes peptides
Protéines F5HL19, F5HL20, F5HL21 et Q7QBC4 identifiées avec les 2 mêmes peptides

Spot 820

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	42,08	1	17	17	37	43,6	7,34
Q7Q6L2	AGAP005781-PA OS=Anopheles gambiae GN=AGAP005781 PE=3 SV=4 - [Q7Q6L2_ANOGA]	34,98	1	12	13	21	46,6	7,49
D0ES27	Beta actin OS=Polyrhachis vicina PE=2 SV=1 - [D0ES27_9HYME]	32,45	1	5	10	13	41,8	5,48
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	31,53	1	0	9	13	39,0	5,44
Q7PNP8	AGAP005662-PA OS=Anopheles gambiae GN=AGAP005662 PE=3 SV=2 - [Q7PNP8_ANOGA]	32,46	1	9	9	12	45,4	8,06
Q7Q609	AGAP006099-PA OS=Anopheles gambiae GN=AGAP006099 PE=3 SV=3 - [Q7Q609_ANOGA]	32,12	1	11	11	12	45,1	8,59
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	16,88	1	5	5	10	43,8	6,46
Q7PXH7	AGAP001407-PA OS=Anopheles gambiae GN=AgaP_AGAP001407 PE=3 SV=5 - [Q7PXH7_ANOGA]	18,37	1	0	6	8	44,3	7,03
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	28,83	1	8	8	8	44,5	6,25
O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	13,47	1	0	6	7	46,2	8,38
Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	13,47	1	0	6	7	46,1	8,21
F5HKV6	Fructose-bisphosphate aldolase OS=Anopheles gambiae GN=AgaP_AGAP002564 PE=3 SV=1 - [F5HKV6_ANOGA]	23,97	1	6	6	7	39,2	7,64
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	19,49	1	0	7	7	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	20,39	1	0	7	7	47,1	7,80
F5HIU6	AGAP002192-PB OS=Anopheles gambiae GN=AgaP_AGAP002192 PE=3 SV=1 - [F5HIU6_ANOGA]	19,85	1	0	6	7	43,5	7,27
Q7QK78	AGAP002192-PA (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP002192 PE=3 SV=5 - [Q7QK78_ANOGA]	20,31	1	0	6	7	42,4	7,24
Q7Q4N7	AGAP008501-PA (Fragment) OS=Anopheles gambiae GN=AGAP008501 PE=3 SV=3 - [Q7Q4N7_ANOGA]	18,31	1	6	6	7	45,5	7,20
Q7QB79	Aspartate aminotransferase OS=Anopheles gambiae GN=AgaP_AGAP004142 PE=3 SV=4 - [Q7QB79_ANOGA]	13,86	1	5	5	6	44,8	6,79
Q7QHS3	AGAP011329-PA (Fragment) OS=Anopheles gambiae GN=AGAP011329 PE=3 SV=4 - [Q7QHS3_ANOGA]	10,90	1	5	5	6	43,5	8,09
Q7QEG0	AGAP000672-PA OS=Anopheles gambiae GN=AGAP000672 PE=4 SV=2 - [Q7QEG0_ANOGA]	13,07	1	4	4	5	44,5	6,64
Q9BIH7	AGAP004334-PA (Precursor) OS=Anopheles gambiae GN=gSG5 PE=2 SV=1 - [Q9BIH7_ANOGA]	14,16	1	4	4	5	38,2	6,47
Q7PQQ3	Isocitrate dehydrogenase [NADP] OS=Anopheles gambiae GN=AgaP_AGAP003168 PE=3 SV=5 - [Q7PQQ3_ANOGA]	13,03	1	4	4	4	50,5	8,63
Q7QIC5	AGAP006780-PA OS=Anopheles gambiae GN=AGAP006780 PE=3 SV=3 - [Q7QIC5_ANOGA]	7,93	1	3	3	3	47,0	8,03

Q7QAH2	Methionine aminopeptidase OS=Anopheles gambiae GN=AgaP_AGAP003700 PE=3 SV=5 - [Q7QAH2_ANOGA]	6,42	1	0	2	2	42,3	6,84
Q7PYV6	AGAP002127-PA OS=Anopheles gambiae GN=AGAP002127 PE=3 SV=2 - [Q7PYV6_ANOGA]	6,38	1	0	2	2	42,6	7,06
Q7PZL5	AGAP011971-PA OS=Anopheles gambiae GN=AGAP011971 PE=3 SV=2 - [Q7PZL5_ANOGA]	4,48	1	0	2	2	61,8	8,46
Q9TW03	Apyrase (Precursor) OS=Anopheles gambiae GN=apy PE=2 SV=1 - [Q9TW03_ANOGA]	4,49	1	0	2	2	61,7	8,69

Peptides communs entre D0ES27 et Q7Q7K5 mais peptides spécifiques également pour chaque protéine
Protéines O97407 et Q7QFQ0 identifiées avec les 6 mêmes peptides
Protéines F7IW82 et Q2TLV8 identifiées avec les 7 mêmes peptides
Protéines F5HIU6 et Q7QK78 identifiées avec les 6 mêmes peptides
Protéines Q7PZL5 et Q9TW03 identifiées avec les 2 mêmes peptides

Spot 820_Plasmodium

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
PFI1105w	organism=Plasmodium_falciparum_3D7 | product=phosphoglycerate kinase | location=Pf3D7_09:913220-914470(+) | length=416	5,77	1	2	2	3	45,4	7,83

Spot 912

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7PQK5	AGAP004192-PA OS=Anopheles gambiae GN=AGAP004192 PE=3 SV=3 - [Q7PQK5_ANOGA]	39,15	1	20	24	37	72,7	5,30
A7UVK8	AGAP002076-PA OS=Anopheles gambiae GN=AgaP_AGAP002076 PE=4 SV=2 - [A7UVK8_ANOGA]	24,70	1	0	14	20	71,3	5,47
Q7PJP4	AGAP003995-PA OS=Anopheles gambiae GN=AGAP003995 PE=4 SV=4 - [Q7PJP4_ANOGA]	9,89	1	5	5	5	73,0	5,31
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	15,63	1	0	4	4	39,0	5,44
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	11,48	1	3	3	3	44,5	6,25
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	10,91	1	3	3	3	43,6	7,34
A7UT63	AGAP005076-PB OS=Anopheles gambiae GN=AGAP005076 PE=4 SV=1 - [A7UT63_ANOGA]	6,72	1	0	2	3	40,5	6,35
Q7Q9R7	AGAP005076-PD OS=Anopheles gambiae GN=AGAP005076 PE=4 SV=3 - [Q7Q9R7_ANOGA]	8,57	1	0	2	3	31,8	5,11
Q7Q9R8	AGAP005076-PA OS=Anopheles gambiae GN=AGAP005076 PE=4 SV=4 - [Q7Q9R8_ANOGA]	3,49	1	0	2	3	73,7	6,09
Q7PXY3	AGAP001653-PA OS=Anopheles gambiae GN=AgaP_AGAP001653 PE=4 SV=4 - [Q7PXY3_ANOGA]	3,72	1	0	2	2	79,1	6,87
A7URE8	AGAP007396-PA OS=Anopheles gambiae GN=AGAP007396 PE=4 SV=1 - [A7URE8_ANOGA]	3,17	1	0	2	2	90,3	5,07
Q7QJB6	AGAP007396-PB OS=Anopheles gambiae GN=AGAP007396 PE=4 SV=4 - [Q7QJB6_ANOGA]	3,96	1	0	2	2	71,7	5,44

Peptides communs entre Q7PQK5 et A7UVK8 mais peptides spécifiques également pour chaque protéine
Protéines A7UT63, Q7Q9R7 et Q7Q9R8 identifiées avec les 2 mêmes peptides
Protéines A7URE8 et Q7QJB6 identifiées avec les 2 mêmes peptides

Spot 912_Plasmodium

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
PFI0875w	organism=Plasmodium_falciparum_3D7 | product=heat shock protein 70 | location=Pf3D7_09:737975-740266(+) | length=652	9,82	1	3	5	7	72,3	5,31
MAL7P1.228	organism=Plasmodium_falciparum_3D7 | product=heat shock protein 70, putative | location=Pf3D7_07:106224-108526(+) | length=661	4,39	1	1	2	4	73,3	5,71
PF13_0201	organism=Plasmodium_falciparum_3D7 | product=sporozoite surface protein 2 | location=Pf3D7_13:1465093-1466817(-) | length=574	5,23	1	2	2	2	64,7	5,07

Peptides communs entre PFI0875w et mal7p1.228 mais peptides spécifiques également pour chaque protéine

Protein identification in underexpressed spots (blue circles in figure)

Spot 1154

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	47,54	1	22	22	45	63,4	7,02
Q7QHE9	AGAP011161-PA OS=Anopheles gambiae GN=AGAP011161 PE=4 SV=4 - [Q7QHE9_ANOGA]	30,62	1	14	14	22	68,0	6,83
E3WRA7	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_05111 PE=3 SV=1 - [E3WRA7_ANODA]	6,29	1	10	11	14	207,9	6,48
Q7Q8E6	AGAP008667-PA OS=Anopheles gambiae GN=AGAP008667 PE=3 SV=4 - [Q7Q8E6_ANOGA]	14,23	1	8	8	11	77,7	7,74
Q7Q3L6	Phosphorylase OS=Anopheles gambiae GN=AgGp PE=2 SV=3 - [Q7Q3L6_ANOGA]	9,74	1	8	8	10	96,8	6,48
Q7QDA2	AGAP003023-PA OS=Anopheles gambiae GN=AGAP003023 PE=4 SV=4 - [Q7QDA2_ANOGA]	17,55	1	8	8	10	66,8	6,98
Q7PRL0	AGAP010735-PA (Fragment) OS=Anopheles gambiae GN=AGAP010735 PE=4 SV=4 - [Q7PRL0_ANOGA]	8,08	1	4	4	5	139,2	8,63
Q7PZJ0	AGAP011938-PA OS=Anopheles gambiae GN=AGAP011938 PE=4 SV=4 - [Q7PZJ0_ANOGA]	7,23	1	4	4	4	68,1	7,18
Q7Q161	Putative oxidoreductase GLYR1 homolog OS=Anopheles gambiae GN=AGAP009949 PE=3 SV=5 - [GLYR1_ANOGA]	9,54	1	4	4	4	61,5	7,42
Q7Q1U7	AGAP009624-PA (Fragment) OS=Anopheles gambiae GN=AGAP009624 PE=3 SV=4 - [Q7Q1U7_ANOGA]	5,88	1	3	3	3	69,9	6,77
F5HJ84	AGAP003742-PB OS=Anopheles gambiae GN=AgaP_AGAP003742 PE=4 SV=1 - [F5HJ84_ANOGA]	5,18	1	0	2	2	48,7	6,54
Q7PFU0	AGAP003742-PA OS=Anopheles gambiae GN=AgaP_AGAP003742 PE=4 SV=5 - [Q7PFU0_ANOGA]	4,70	1	0	2	2	53,7	7,21

Protéines F5HJ84 et Q7PFU0 identifiées avec les 2 mêmes peptides

Spot 1231

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	40,05	1,00	0	14	20	47,105	7,7964
Q7QA76	AGAP004396-PA OS=Anopheles gambiae GN=AGAP004396 PE=4 SV=3 - [Q7QA76_ANOGA]	33,26	1,00	11	11	16	51,941	8,2358
Q7PQM3	6-phosphogluconate dehydrogenase, decarboxylating OS=Anopheles gambiae GN=AGAP004197 PE=3 SV=4 - [Q7PQM3_ANOGA]	24,27	1,00	9	9	15	53,108	7,4888
Q7PYD5	AGAP001884-PA OS=Anopheles gambiae GN=AgaP_AGAP001884 PE=3 SV=4 - [Q7PYD5_ANOGA]	28,03	1,00	9	9	12	54,013	8,5874
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	11,65	1,00	0	3	4	39,021	5,4399
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	10,91	1,00	3	3	3	43,573	7,3423
Q7PT29	Elongation factor 1-alpha OS=Anopheles gambiae GN=AGAP007406 PE=3 SV=3 - [Q7PT29_ANOGA]	4,97	1,00	0	2	2	50,351	9,0854
Q7PUN2	AGAP001969-PA OS=Anopheles gambiae GN=AgaP_AGAP001969 PE=4 SV=3 - [Q7PUN2_ANOGA]	29,45	1,00	2	2	2	34,895	6,9761

Spot 1240

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
E3XBU6	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_19827 PE=3 SV=1 - [E3XBU6_ANODA]	63,30	1	2	16	42	41,5	5,58
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	36,73	1	11	11	15	44,5	6,25
Q7QA95	AGAP004352-PA OS=Anopheles gambiae GN=AGAP004352 PE=3 SV=4 - [Q7QA95_ANOGA]	33,57	1	11	11	12	46,1	6,29
Q7PHG4	AGAP005246-PD OS=Anopheles gambiae GN=SRPN10 PE=3 SV=2 - [Q7PHG4_ANOGA]	26,05	1	9	9	10	42,6	5,48
O97415	Putative uncharacterized protein (Precursor) OS=Anopheles gambiae PE=2 SV=2 - [O97415_ANOGA]	20,70	1	0	8	10	46,5	9,41
Q7PRT6	AGAP000609-PA OS=Anopheles gambiae GN=AGAP000609 PE=4 SV=1 - [Q7PRT6_ANOGA]	20,70	1	0	8	10	46,5	9,41
O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	15,96	1	0	6	6	46,2	8,38
Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	15,96	1	0	6	6	46,1	8,21
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	11,43	1	3	3	4	43,6	7,34
Q7QHC7	AGAP011130-PA OS=Anopheles gambiae GN=AGAP011130 PE=4 SV=4 - [Q7QHC7_ANOGA]	11,78	1	4	4	4	45,9	6,24
C0L2G8	GNBP OS=Anopheles gambiae PE=4 SV=1 - [C0L2G8_ANOGA]	12,15	1	0	4	4	44,0	5,44
E9JZN8	Fasciclin (Fragment) OS=Anopheles gambiae M PE=4 SV=1 - [E9JZN8_ANOGA]	16,67	1	0	2	3	20,3	6,55
E9JZQ7	Fasciclin (Fragment) OS=Anopheles gambiae M PE=4 SV=1 - [E9JZQ7_ANOGA]	16,67	1	0	2	3	20,3	6,55
Q7PSV0	AGAP000935-PA OS=Anopheles gambiae GN=AgaP_AGAP000935 PE=3 SV=4 - [Q7PSV0_ANOGA]	3,80	1	0	2	2	52,2	6,29
Q8WR22	TRIO protein (Fragment) OS=Anopheles gambiae PE=2 SV=1 - [Q8WR22_ANOGA]	5,88	1	0	2	2	43,7	6,46
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	5,88	1	0	2	2	43,8	6,46
Q9BIH7	AGAP004334-PA (Precursor) OS=Anopheles gambiae GN=gSG5 PE=2 SV=1 - [Q9BIH7_ANOGA]	6,63	1	2	2	2	38,2	6,47

Peptides communs entre E3XBU6 et Q7Q7K5 mais peptides spécifiques pour la protéine d'ANODA (E3XUB6)
Protéines O97415 et Q7PRT6 identifiées avec les 8 mêmes peptides
Protéines O97407 et Q7QFQ0 identifiées avec les 6 mêmes peptides
Protéines E9JZN8 et E9JZQ7 identifiées avec les 2 mêmes peptides
Protéines Q8WR22 et Q7PUJ5 identifiées avec les 2 mêmes peptides

Spot 469

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	39,32	1	0	12	21	47,1	7,80
Q7Q3D8	AGAP007827-PA OS=Anopheles gambiae GN=AGAP007827 PE=3 SV=2 - [Q7Q3D8_ANOGA]	26,10	1	8	9	14	46,6	6,92
Q7QIP5	Elongation factor Tu OS=Anopheles gambiae GN=AGAP006996 PE=3 SV=4 - [Q7QIP5_ANOGA]	30,04	1	10	10	12	51,4	8,13
Q7PQM3	6-phosphogluconate dehydrogenase, decarboxylating OS=Anopheles gambiae GN=AGAP004197 PE=3 SV=4 - [Q7PQM3_ANOGA]	19,29	1	7	7	10	53,1	7,49
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	28,41	1	0	8	9	39,0	5,44
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	8,95	1	0	4	5	63,4	7,02
Q9UB34	Putative 5'-nucleotidase (Precursor) OS=Anopheles gambiae GN=5Ntd PE=2 SV=1 - [Q9UB34_ANOGA]	8,95	1	0	4	5	63,4	6,89
Q7Q3V3	AGAP008061-PA OS=Anopheles gambiae GN=AGAP008061 PE=3 SV=3 - [Q7Q3V3_ANOGA]	12,75	1	5	5	5	49,6	7,20
Q7Q850	Phosphoglycerate kinase OS=Anopheles gambiae GN=AGAP008802 PE=3 SV=2 - [Q7Q850_ANOGA]	15,18	1	4	4	4	43,8	7,44
Q7PYD5	AGAP001884-PA OS=Anopheles gambiae GN=AgaP_AGAP001884 PE=3 SV=4 - [Q7PYD5_ANOGA]	10,54	1	4	4	4	54,0	8,59
Q7PNJ7	AGAP000883-PA OS=Anopheles gambiae GN=AgaP_AGAP000883 PE=4 SV=4 - [Q7PNJ7_ANOGA]	8,20	1	3	3	3	48,5	7,68
Q7PUN2	AGAP001969-PA OS=Anopheles gambiae GN=AgaP_AGAP001969 PE=4 SV=3 - [Q7PUN2_ANOGA]	32,36	1	3	3	3	34,9	6,98
Q7Q685	AGAP005981-PA OS=Anopheles gambiae GN=AGAP005981 PE=3 SV=4 - [Q7Q685_ANOGA]	7,25	1	2	2	3	44,4	6,68
Q7PPE7	Pyruvate kinase OS=Anopheles gambiae GN=AgaP_AGAP004596 PE=3 SV=5 - [Q7PPE7_ANOGA]	4,05	1	0	2	2	56,2	7,65
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	6,75	1	0	2	2	43,6	7,34
Q9BIH6	GSG1b protein (Precursor) OS=Anopheles gambiae GN=gSG1b PE=2 SV=1 - [Q9BIH6_ANOGA]	6,75	1	0	2	2	43,6	7,58
Q7QA76	AGAP004396-PA OS=Anopheles gambiae GN=AGAP004396 PE=4 SV=3 - [Q7QA76_ANOGA]	5,44	1	0	2	2	51,9	8,24
Q7PJT7	Adenylyl cyclase-associated protein OS=Anopheles gambiae GN=AGAP010175 PE=3 SV=4 - [Q7PJT7_ANOGA]	2,66	1	2	2	2	85,3	5,57

Protéines Q5TVM9 et Q9UB34 identifiées avec les 4 mêmes peptides
Protéines Q7QFL2 et Q9BIH6 identifiées avec les 2 mêmes peptides

Spot 1170

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7Q3L6	Phosphorylase OS=Anopheles gambiae GN=AgGp PE=2 SV=3 - [Q7Q3L6_ANOGA]	12,11	1	9	9	11	96,8	6,48

Q7QIX8	AGAP007123-PA OS=Anopheles gambiae GN=AGAP007123 PE=3 SV=4 - [Q7QIX8_ANOGA]	14,60	1	9	9	11	99,7	6,81
Q7PTN2	AGAP009441-PA OS=Anopheles gambiae GN=AGAP009441 PE=4 SV=2 - [Q7PTN2_ANOGA]	9,72	1	7	7	10	94,5	6,48
Q7Q978	AGAP004877-PA OS=Anopheles gambiae GN=AGAP004877 PE=4 SV=4 - [Q7Q978_ANOGA]	12,24	1	8	9	9	102,8	5,53
Q7Q8H4	AGAP008632-PA (Fragment) OS=Anopheles gambiae GN=AGAP008632 PE=4 SV=3 - [Q7Q8H4_ANOGA]	10,57	1	8	8	9	100,2	6,51
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	15,05	1	4	4	4	44,5	6,25
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	7,54	1	0	3	4	63,4	7,02
Q9UB34	Putative 5'-nucleotidase (Precursor) OS=Anopheles gambiae GN=5Ntd PE=2 SV=1 - [Q9UB34_ANOGA]	7,54	1	0	3	4	63,4	6,89
F5HJN1	AGAP000255-PE (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP000255 PE=4 SV=1 - [F5HJN1_ANOGA]	5,32	1	0	4	4	104,1	5,94
F5HJN2	AGAP000255-PA (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP000255 PE=4 SV=1 - [F5HJN2_ANOGA]	5,49	1	0	4	4	100,9	6,24
F5HJN4	AGAP000255-PD (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP000255 PE=4 SV=1 - [F5HJN4_ANOGA]	6,17	1	0	4	4	88,5	6,55
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	13,35	1	0	3	3	39,0	5,44
Q7PNV2	AGAP006371-PA OS=Anopheles gambiae GN=AGAP006371 PE=3 SV=4 - [Q7PNV2_ANOGA]	4,70	1	2	2	3	51,6	5,14
A7UU84	AGAP006366-PD OS=Anopheles gambiae GN=AGAP006366 PE=4 SV=1 - [A7UU84_ANOGA]	1,78	1	0	2	3	113,9	7,08
A7UU86	AGAP006366-PA OS=Anopheles gambiae GN=AGAP006366 PE=4 SV=1 - [A7UU86_ANOGA]	1,70	1	0	2	3	118,7	7,43
A7UU87	AGAP006366-PB OS=Anopheles gambiae GN=AGAP006366 PE=4 SV=1 - [A7UU87_ANOGA]	1,74	1	0	2	3	116,3	7,15
Q7PIB4	AGAP006366-PC OS=Anopheles gambiae GN=AGAP006366 PE=4 SV=4 - [Q7PIB4_ANOGA]	1,77	1	0	2	3	114,5	7,15
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	7,66	1	0	2	2	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	8,01	1	0	2	2	47,1	7,80
Q7QC97	AGAP002518-PA OS=Anopheles gambiae GN=AgaP_AGAP002518 PE=3 SV=4 - [Q7QC97_ANOGA]	3,54	1	2	2	2	86,2	7,36
Q7Q7V6	AGAP005003-PA OS=Anopheles gambiae GN=AGAP005003 PE=4 SV=4 - [Q7Q7V6_ANOGA]	3,08	1	2	2	2	91,4	6,77

Protéines Q5TVM9 et Q9UB34 identifiées avec les 3 mêmes peptides

Protéines F5HJN1, F5HJN2 et F5HJN4 identifiées avec les 4 mêmes peptides

Protéines A7UU84, A7UU86, A7UU87 et Q7PIB4 identifiées avec les 2 mêmes peptides

Protéines F7IW82 et Q2TLV8 identifiées avec les 2 mêmes peptides

Spot 1190

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	59,94	1	0	14	45	39,0	5,44
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	36,22	1	11	11	15	44,5	6,25
Q7PUJ5	AGAP001374-PA OS=Anopheles gambiae GN=AGAP001374 PE=4 SV=2 - [Q7PUJ5_ANOGA]	34,78	1	9	9	13	43,8	6,46
Q7QHC7	AGAP011130-PA OS=Anopheles gambiae GN=AGAP011130 PE=4 SV=4 - [Q7QHC7_ANOGA]	33,83	1	10	10	13	45,9	6,24
Q7QFL2	AGAP000548-PA OS=Anopheles gambiae GN=AGAP000548 PE=4 SV=2 - [Q7QFL2_ANOGA]	30,65	1	8	9	12	43,6	7,34

E3XEE3	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_21949 PE=3 SV=1 - [E3XEE3_ANODA]	35,37	1	2	9	11	41,8	5,68
O97407	SG1 protein (Precursor) OS=Anopheles gambiae GN=SG1 PE=2 SV=1 - [O97407_ANOGA]	22,69	1	0	9	10	46,2	8,38
Q7QFQ0	AGAP000612-PA OS=Anopheles gambiae GN=AGAP000612 PE=4 SV=1 - [Q7QFQ0_ANOGA]	22,69	1	0	9	10	46,1	8,21
Q7PSV0	AGAP000935-PA OS=Anopheles gambiae GN=AgaP_AGAP000935 PE=3 SV=4 - [Q7PSV0_ANOGA]	19,83	1	6	7	7	52,2	6,29
O97415	Putative uncharacterized protein (Precursor) OS=Anopheles gambiae PE=2 SV=2 - [O97415_ANOGA]	11,97	1	0	6	7	46,5	9,41
Q7PRT6	AGAP000609-PA OS=Anopheles gambiae GN=AGAP000609 PE=4 SV=1 - [Q7PRT6_ANOGA]	11,97	1	0	6	7	46,5	9,41
Q7PHG4	AGAP005246-PD OS=Anopheles gambiae GN=SRPN10 PE=3 SV=2 - [Q7PHG4_ANOGA]	20,26	1	0	6	6	42,6	5,48
Q8WSX7	AGAP005246-PA OS=Anopheles gambiae GN=spi21F PE=3 SV=1 - [Q8WSX7_ANOGA]	19,49	1	0	6	6	44,0	5,77
Q8WSX9	AGAP005246-PC OS=Anopheles gambiae GN=spi21F PE=3 SV=1 - [Q8WSX9_ANOGA]	20,16	1	0	6	6	42,4	5,24
Q8WSY0	AGAP005246-PE OS=Anopheles gambiae GN=spi21F PE=3 SV=1 - [Q8WSY0_ANOGA]	20,32	1	0	6	6	42,1	5,22
Q7Q609	AGAP006099-PA OS=Anopheles gambiae GN=AGAP006099 PE=3 SV=3 - [Q7Q609_ANOGA]	12,76	1	4	4	5	45,1	8,59
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	7,66	1	0	3	3	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	8,01	1	0	3	3	47,1	7,80
Q9BIH7	AGAP004334-PA (Precursor) OS=Anopheles gambiae GN=gSG5 PE=2 SV=1 - [Q9BIH7_ANOGA]	6,63	1	2	2	3	38,2	6,47
Q7Q229	AGAP010792-PA (Fragment) OS=Anopheles gambiae GN=AGAP010792 PE=4 SV=3 - [Q7Q229_ANOGA]	6,75	1	0	2	2	44,4	8,56
Q7Q068	Eukaryotic translation initiation factor 3 subunit M OS=Anopheles gambiae GN=AGAP012281 PE=3 SV=2 - [EIF3M_ANOGA]	6,49	1	2	2	2	44,1	5,85
Q5TVB9	AGAP003762-PA OS=Anopheles gambiae GN=AgaP_AGAP003762 PE=4 SV=2 - [Q5TVB9_ANOGA]	6,17	1	2	2	2	47,2	6,14
Q7PQM2	AGAP004203-PA OS=Anopheles gambiae GN=AGAP004203 PE=4 SV=2 - [Q7PQM2_ANOGA]	1,32	1	0	2	2	239,3	5,92
Q9NAW9	Vitellogenin 1 OS=Anopheles gambiae GN=VgT1 PE=4 SV=1 - [Q9NAW9_ANOGA]	1,32	1	0	2	2	239,2	5,95
Q7Q4R7	AGAP000909-PA OS=Anopheles gambiae GN=AgaP_AGAP000909 PE=4 SV=5 - [Q7Q4R7_ANOGA]	5,43	1	2	2	2	46,5	5,87
Q7QF41	AGAP000291-PA OS=Anopheles gambiae GN=AgaP_AGAP000291 PE=4 SV=4 - [Q7QF41_ANOGA]	5,13	1	2	2	2	48,5	6,61

Peptides communs entre QQ7K5 et E3XEE3 (ANODA) mais peptides spécifiques également pour chaque protéine. Moins de peptides identfiés chez E3XEE3

Protéines O97407 et Q7QFQ0 identifiées avec les 9 mêmes peptides

Protéines O97415 et Q7PRT6 identifiées avec les 6 mêmes peptides

Protéines Q7PHG4, Q8WSX7, Q8WSX9 et Q8WSY0 identifiées avec les 6 mêmes peptides

Protéines F7IW82 et Q2TLV8 identifiées avec les 3 mêmes peptides

Protéines Q7PQM2 et Q9NAW9 identifiées avec les 2 mêmes peptides

Spot 350

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	49,82	1	21	21	38	63,4	7,02
Q7PZJ0	AGAP011938-PA OS=Anopheles gambiae GN=AGAP011938 PE=4 SV=4 - [Q7PZJ0_ANOGA]	36,97	1	15	16	20	68,1	7,18
Q7Q343	AGAP011476-PA OS=Anopheles gambiae GN=AGAP011476 PE=4 SV=4 - [Q7Q343_ANOGA]	16,56	1	13	13	17	101,6	5,68

E3WRA7	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_05111 PE=3 SV=1 - [E3WRA7_ANODA]	5,97	1	9	9	13	207,9	6,48
Q7QAV9	AGAP003517-PA OS=Anopheles gambiae GN=AGAP003517 PE=4 SV=2 - [Q7QAV9_ANOGA]	10,34	1	5	5	6	66,0	6,61
F5HMV2	AGAP001256-PC OS=Anopheles gambiae GN=AgaP_AGAP001256 PE=3 SV=1 - [F5HMV2_ANOGA]	11,26	1	0	6	6	74,6	7,01
F5HMV3	AGAP001256-PB OS=Anopheles gambiae GN=AgaP_AGAP001256 PE=3 SV=1 - [F5HMV3_ANOGA]	11,38	1	0	6	6	74,1	6,76
Q7PX77	AGAP001256-PA OS=Anopheles gambiae GN=AGAP001256 PE=3 SV=4 - [Q7PX77_ANOGA]	11,94	1	0	6	6	70,6	6,61
Q7PJP4	AGAP003995-PA OS=Anopheles gambiae GN=AGAP003995 PE=4 SV=4 - [Q7PJP4_ANOGA]	9,89	1	5	5	5	73,0	5,31
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	12,78	1	0	3	4	39,0	5,44
Q7Q161	Putative oxidoreductase GLYR1 homolog OS=Anopheles gambiae GN=AGAP009949 PE=3 SV=5 - [GLYR1_ANOGA]	9,72	1	4	4	4	61,5	7,42
Q7PGI2	AGAP002503-PA OS=Anopheles gambiae GN=AgaP_AGAP002503 PE=4 SV=5 - [Q7PGI2_ANOGA]	5,77	1	3	3	3	65,1	7,36
Q5TTG1	V-type proton ATPase catalytic subunit A OS=Anopheles gambiae GN=Vha68-2 PE=3 SV=1 - [VATA_ANOGA]	4,40	1	0	2	2	68,2	5,39
O97413	Putative gVAG protein (Precursor) OS=Anopheles gambiae GN=gVAG PE=2 SV=2 - [O97413_ANOGA]	12,31	1	0	2	2	28,9	8,91
Q7PNX0	AGAP006421-PA OS=Anopheles gambiae GN=AGAP006421 PE=4 SV=3 - [Q7PNX0_ANOGA]	12,31	1	0	2	2	28,9	8,82
Q7QHE9	AGAP011161-PA OS=Anopheles gambiae GN=AGAP011161 PE=4 SV=4 - [Q7QHE9_ANOGA]	4,15	1	2	2	2	68,0	6,83
Q7QBK9	AGAP003124-PA OS=Anopheles gambiae GN=AGAP003124 PE=4 SV=2 - [Q7QBK9_ANOGA]	4,36	1	0	2	2	65,4	6,77

Protéines F5HMV2, F5HMV3 et Q7PX77 identifiées avec les 6 mêmes peptides

Protéines O97413 et Q7PNX0 identifiées avec les 2 mêmes peptides

Spot 353

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	52,81	1	22	22	51	63,4	7,02
Q7PZJ0	AGAP011938-PA OS=Anopheles gambiae GN=AGAP011938 PE=4 SV=4 - [Q7PZJ0_ANOGA]	27,39	1	13	14	18	68,1	7,18
E3WRA7	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_05111 PE=3 SV=1 - [E3WRA7_ANODA]	6,67	1	7	12	15	207,9	6,48
Q7QHE9	AGAP011161-PA OS=Anopheles gambiae GN=AGAP011161 PE=4 SV=4 - [Q7QHE9_ANOGA]	17,07	1	8	8	10	68,0	6,83
Q7Q343	AGAP011476-PA OS=Anopheles gambiae GN=AGAP011476 PE=4 SV=4 - [Q7Q343_ANOGA]	9,67	1	7	7	8	101,6	5,68
Q7Q870	AGAP008769-PA (Fragment) OS=Anopheles gambiae GN=AGAP008769 PE=3 SV=4 - [Q7Q870_ANOGA]	10,74	1	2	6	7	67,7	7,02
Q7QAV9	AGAP003517-PA OS=Anopheles gambiae GN=AGAP003517 PE=4 SV=2 - [Q7QAV9_ANOGA]	10,85	1	6	6	6	66,0	6,61
Q7Q3L6	Phosphorylase OS=Anopheles gambiae GN=AgGp PE=2 SV=3 - [Q7Q3L6_ANOGA]	4,28	1	0	3	3	96,8	6,48
Q7Q161	Putative oxidoreductase GLYR1 homolog OS=Anopheles gambiae GN=AGAP009949 PE=3 SV=5 - [GLYR1_ANOGA]	6,71	1	3	3	3	61,5	7,42
F1C3T7	Actin (Fragment) OS=Timema genevievae PE=3 SV=1 - [F1C3T7_9NEOP]	9,82	1	0	2	2	30,9	5,66
F1C3V4	Actin (Fragment) OS=Timema podura PE=3 SV=1 - [F1C3V4_TIMPD]	9,82	1	0	2	2	30,9	5,49

Peptides communs entre E3WRA7 (ANODA) et Q7Q870 mais peptides spécifiques également pour chaque protéine. Moins de peptides identfiés chez Q7Q7870

Protéines F1C3T7 (NEOP) et F1C3V4 (TIMPD) identifiées avec les 2 mêmes peptides

Spot 360

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	43,86	1	18	18	30	63,4	7,02
F5HLN3	AGAP004437-PC OS=Anopheles gambiae GN=AgaP_AGAP004437 PE=3 SV=1 - [F5HLN3_ANOGA]	13,01	1	0	9	9	81,8	8,05
F5HLN4	AGAP004437-PA OS=Anopheles gambiae GN=AGAP004437 PE=3 SV=1 - [F5HLN4_ANOGA]	13,09	1	0	9	9	81,2	7,80
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	6,12	1	2	2	2	44,5	6,25
Q7PPE7	Pyruvate kinase OS=Anopheles gambiae GN=AgaP_AGAP004596 PE=3 SV=5 - [Q7PPE7_ANOGA]	4,05	1	0	2	2	56,2	7,65

Protéines F5HLN3 et F5HLN4 identifiées avec les 9 mêmes peptides

Spot 363

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	54,39	1	27	27	71	63,4	7,02
Q7QHE9	AGAP011161-PA OS=Anopheles gambiae GN=AGAP011161 PE=4 SV=4 - [Q7QHE9_ANOGA]	31,42	1	15	15	20	68,0	6,83
Q7Q8E6	AGAP008667-PA OS=Anopheles gambiae GN=AGAP008667 PE=3 SV=4 - [Q7Q8E6_ANOGA]	9,67	1	5	5	6	77,7	7,74
Q7PRL0	AGAP010735-PA (Fragment) OS=Anopheles gambiae GN=AGAP010735 PE=4 SV=4 - [Q7PRL0_ANOGA]	10,69	1	5	5	5	139,2	8,63
E3WRA7	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_05111 PE=3 SV=1 - [E3WRA7_ANODA]	1,23	1	2	2	2	207,9	6,48

Spot 364

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	57,37	1	30	30	77	63,4	7,02
E3WRA7	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_05111 PE=3 SV=1 - [E3WRA7_ANODA]	6,13	1	10	11	13	207,9	6,48
Q7PRL0	AGAP010735-PA (Fragment) OS=Anopheles gambiae GN=AGAP010735 PE=4 SV=4 - [Q7PRL0_ANOGA]	13,95	1	7	7	9	139,2	8,63
Q7QHE9	AGAP011161-PA OS=Anopheles gambiae GN=AGAP011161 PE=4 SV=4 - [Q7QHE9_ANOGA]	11,16	1	5	5	7	68,0	6,83
O97413	Putative gVAG protein (Precursor) OS=Anopheles gambiae GN=gVAG PE=2 SV=2 - [O97413_ANOGA]	25,00	1	0	4	4	28,9	8,91
Q7PNX0	AGAP006421-PA OS=Anopheles gambiae GN=AGAP006421 PE=4 SV=3 - [Q7PNX0_ANOGA]	25,00	1	0	4	4	28,9	8,82
Q7Q3L6	Phosphorylase OS=Anopheles gambiae GN=AgGp PE=2 SV=3 - [Q7Q3L6_ANOGA]	2,97	1	0	2	2	96,8	6,48
Q7PNQ9	AGAP005576-PA OS=Anopheles gambiae GN=AGAP005576 PE=3 SV=3 - [Q7PNQ9_ANOGA]	5,16	1	2	2	2	60,2	6,99

Protéines O97413 et Q7PNX0 identifiées avec les 4 mêmes peptides

Spot 881

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7PIQ3	AGAP005630-PA OS=Anopheles gambiae GN=AGAP005630 PE=3 SV=3 - [Q7PIQ3_ANOGA]	51,37	1	31	31	51	89,2	5,29
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	23,86	1	8	8	9	63,4	7,02
Q7PKQ5	Alpha-actinin, sarcomeric OS=Anopheles gambiae GN=Actn PE=3 SV=2 - [ACTN_ANOGA]	7,70	1	0	6	6	106,5	5,85
A7XXV5	Actin OS=Monochamus alternatus PE=2 SV=1 - [A7XXV5_MONAT]	20,48	1	0	5	5	41,7	5,49
E3WRS7	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_05571 PE=3 SV=1 - [E3WRS7_ANODA]	20,48	1	0	5	5	41,6	5,48
Q7PTN2	AGAP009441-PA OS=Anopheles gambiae GN=AGAP009441 PE=4 SV=2 - [Q7PTN2_ANOGA]	3,91	1	0	3	4	94,5	6,48
Q7Q343	AGAP011476-PA OS=Anopheles gambiae GN=AGAP011476 PE=4 SV=4 - [Q7Q343_ANOGA]	4,22	1	3	3	3	101,6	5,68
Q5TRG5	AGAP005728-PA OS=Anopheles gambiae GN=AGAP005728 PE=4 SV=3 - [Q5TRG5_ANOGA]	3,46	1	3	3	3	117,0	5,94
Q7PQK5	AGAP004192-PA OS=Anopheles gambiae GN=AGAP004192 PE=3 SV=3 - [Q7PQK5_ANOGA]	4,10	1	0	2	2	72,7	5,30
A7UVK8	AGAP002076-PA OS=Anopheles gambiae GN=AgaP_AGAP002076 PE=4 SV=2 - [A7UVK8_ANOGA]	4,12	1	0	2	2	71,3	5,47
Q7Q3L6	Phosphorylase OS=Anopheles gambiae GN=AgGp PE=2 SV=3 - [Q7Q3L6_ANOGA]	2,85	1	0	2	2	96,8	6,48
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	4,41	1	0	2	2	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	4,61	1	0	2	2	47,1	7,80
Q17NG8	Phosphorylase OS=Aedes aegypti GN=AAEL000703 PE=3 SV=1 - [Q17NG8_AEDAE]	2,84	1	0	2	2	96,9	6,33
Q7QJQ1	AGAP007612-PA OS=Anopheles gambiae GN=AGAP007612 PE=4 SV=4 - [Q7QJQ1_ANOGA]	3,31	1	2	2	2	92,1	5,08

Protéines F7IW82 et Q2TLV8 identifiées avec les 2 mêmes peptides

Protéines Q7PQK5 et A7UVK8 identifiées avec les 2 mêmes peptides

Spot 450

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7Q716	AGAP005558-PA OS=Anopheles gambiae GN=AGAP005558 PE=3 SV=1 - [Q7Q716_ANOGA]	49,58	1	15	16	32	51,8	6,14
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	45,45	1	9	9	22	39,0	5,44
E3XEC7	ATP synthase subunit beta OS=Anopheles darlingi GN=AND_21925 PE=3 SV=1 - [E3XEC7_ANODA]	33,00	1	0	12	15	53,7	5,12
Q7PSV0	AGAP000935-PA OS=Anopheles gambiae GN=AgaP_AGAP000935 PE=3 SV=4 - [Q7PSV0_ANOGA]	31,65	1	10	11	14	52,2	6,29
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	29,70	1	0	10	12	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	31,07	1	0	10	12	47,1	7,80
Q7QJF7	AGAP007494-PA OS=Anopheles gambiae GN=AGAP007494 PE=4 SV=4 - [Q7QJF7_ANOGA]	28,74	1	10	10	12	48,4	6,13
Q9UB34	Putative 5'-nucleotidase (Precursor) OS=Anopheles gambiae GN=5Ntd PE=2 SV=1 - [Q9UB34_ANOGA]	16,32	1	6	6	6	63,4	6,89
Q7Q6H2	AGAP005847-PA OS=Anopheles gambiae GN=AGAP005847 PE=4 SV=3 - [Q7Q6H2_ANOGA]	9,15	1	4	4	5	52,0	5,91

Q7PUU0	AGAP001919-PA OS=Anopheles gambiae GN=AGAP001919 PE=3 SV=3 - [Q7PUU0_ANOGA]	12,13	1	4	4	5	48,5	5,58
Q7PUN2	AGAP001969-PA OS=Anopheles gambiae GN=AgaP_AGAP001969 PE=4 SV=3 - [Q7PUN2_ANOGA]	33,66	1	4	4	4	34,9	6,98
Q7QHG0	AGAP011174-PA OS=Anopheles gambiae GN=AGAP011174 PE=3 SV=2 - [Q7QHG0_ANOGA]	11,06	1	0	4	4	48,6	5,64
Q7PW65	AGAP009075-PA (Fragment) OS=Anopheles gambiae GN=AGAP009075 PE=4 SV=4 - [Q7PW65_ANOGA]	12,54	1	2	3	4	36,8	7,24
Q7PMS7	AGAP004775-PA OS=Anopheles gambiae GN=AGAP004775 PE=3 SV=3 - [Q7PMS7_ANOGA]	10,44	1	4	4	4	52,9	5,99
Q7PVP3	AGAP009224-PA OS=Anopheles gambiae GN=AGAP009224 PE=4 SV=2 - [Q7PVP3_ANOGA]	9,31	1	3	3	3	41,0	6,99
E3WQU9	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_04792 PE=4 SV=1 - [E3WQU9_ANODA]	7,48	1	2	3	3	54,7	5,45
E3X8V8	Putative uncharacterized protein OS=Anopheles darlingi GN=AND_16684 PE=3 SV=1 - [E3X8V8_ANODA]	8,46	1	0	3	3	49,9	6,06
Q7PT29	Elongation factor 1-alpha OS=Anopheles gambiae GN=AGAP007406 PE=3 SV=3 - [Q7PT29_ANOGA]	4,97	1	0	2	2	50,4	9,09
H2KMF4	AGAP009863-PA OS=Anopheles gambiae GN=AgaP_AGAP009863 PE=3 SV=1 - [H2KMF4_ANOGA]	5,94	1	2	2	2	45,6	5,67
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	6,12	1	2	2	2	44,5	6,25
Q7Q410	AGAP008141-PA (Fragment) OS=Anopheles gambiae GN=AGAP008141 PE=3 SV=4 - [Q7Q410_ANOGA]	4,12	1	2	2	2	52,2	6,23
Q7Q1D3	AGAP009841-PA OS=Anopheles gambiae GN=AGAP009841 PE=4 SV=3 - [Q7Q1D3_ANOGA]	7,32	1	0	2	2	36,2	5,74

Peptides communs entre Q7Q716 et Q7PSV0 mais peptides spécifiques également pour chaque protéine. Moins de peptides identfiés chez Q7PSV0.
Protéines F7IW82 et Q2TLV8 identifiées avec les 10 mêmes peptides
Peptides communs entre Q7PW65 et E3WQU9 (ANODA) mais peptides spécifiques également pour chaque protéine.

Spot 450_Plasmodium

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
MAL8P1.17	organism=Plasmodium_falciparum_3D7 | product=protein disulfide isomerase | location=Pf3D7_08:1207860-1209466(-) | length=483	20,70	1	6	8	11	55,5	5,78
PFL1725w	organism=Plasmodium_falciparum_3D7 | product=ATP synthase beta chain, mitochondrial precursor, putative | location=Pf3D7_12:1487072-1488679(+) | length=535	13,64	1	6	6	9	58,4	6,42

Peptides communs entre E3XEC7 (ANODA, identifié dans 450) et PFL1725w mais 1 peptide spécifique également pour PFL1725w. Moins de peptides identfiés chez PFL1725w

Spot 451

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q9UB34	Putative 5'-nucleotidase (Precursor) OS=Anopheles gambiae GN=5Ntd PE=2 SV=1 - [Q9UB34_ANOGA]	17,89	1	6	6	9	63,4	6,89
Q5TV62	AGAP000607-PA OS=Anopheles gambiae GN=AgaP_AGAP000607 PE=4 SV=2 - [Q5TV62_ANOGA]	11,22	1	2	2	2	44,5	6,25
Q7Q716	AGAP005558-PA OS=Anopheles gambiae GN=AGAP005558 PE=3 SV=1 - [Q7Q716_ANOGA]	6,14	1	2	2	2	51,8	6,14

Spot 466

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	38,05	1	0	13	19	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	39,81	1	0	13	19	47,1	7,80
Q7QBI1	AGAP003165-PA OS=Anopheles gambiae GN=AgaP_AGAP003165 PE=3 SV=4 - [Q7QBI1_ANOGA]	22,75	1	9	9	10	52,9	6,95
E3XEC7	ATP synthase subunit beta OS=Anopheles darlingi GN=AND_21925 PE=3 SV=1 - [E3XEC7_ANODA]	13,12	1	0	5	6	53,7	5,12
Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	22,16	1	0	5	6	39,0	5,44
Q7Q685	AGAP005981-PA OS=Anopheles gambiae GN=AGAP005981 PE=3 SV=4 - [Q7Q685_ANOGA]	19,75	1	6	6	6	44,4	6,68
Q7Q3D8	AGAP007827-PA OS=Anopheles gambiae GN=AGAP007827 PE=3 SV=2 - [Q7Q3D8_ANOGA]	15,01	1	5	5	5	46,6	6,92
Q7Q3V3	AGAP008061-PA OS=Anopheles gambiae GN=AGAP008061 PE=3 SV=3 - [Q7Q3V3_ANOGA]	12,98	1	5	5	5	49,6	7,20
Q5TVM9	AGAP011026-PA OS=Anopheles gambiae GN=AGAP011026 PE=3 SV=1 - [Q5TVM9_ANOGA]	6,67	1	0	3	4	63,4	7,02
Q9UB34	Putative 5'-nucleotidase (Precursor) OS=Anopheles gambiae GN=5Ntd PE=2 SV=1 - [Q9UB34_ANOGA]	6,67	1	0	3	4	63,4	6,89
Q7PQM3	6-phosphogluconate dehydrogenase, decarboxylating OS=Anopheles gambiae GN=AGAP004197 PE=3 SV=4 - [Q7PQM3_ANOGA]	9,13	1	0	3	3	53,1	7,49
Q7PYD5	AGAP001884-PA OS=Anopheles gambiae GN=AgaP_AGAP001884 PE=3 SV=4 - [Q7PYD5_ANOGA]	4,37	1	0	2	2	54,0	8,59
Q7QJ33	Ribosome biogenesis protein WDR12 homolog OS=Anopheles gambiae GN=AGAP007244 PE=3 SV=3 - [WDR12_ANOGA]	4,91	1	2	2	2	47,8	6,87
F5HJK0	AGAP000399-PB OS=Anopheles gambiae GN=AgaP_AGAP000399 PE=4 SV=1 - [F5HJK0_ANOGA]	7,55	1	0	2	2	38,6	8,63
Q7QFB8	AGAP000399-PA OS=Anopheles gambiae GN=AgaP_AGAP000399 PE=4 SV=5 - [Q7QFB8_ANOGA]	7,71	1	0	2	2	38,4	8,00

Protéines F7IW82 et Q2TLV8 identifiées avec les 13 mêmes peptides
ProtéinesQ5TVM9 et Q9UB34 identifiées avec les 3 mêmes peptides
Protéines F5HJK0 et Q7QFB8 identifiées avec les 2 mêmes peptides

Spot 466_Plasmodium

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
PFL1725w	organism=Plasmodium_falciparum_3D7 | product=ATP synthase beta chain, mitochondrial precursor, putative | location=Pf3D7_12:1487072-1488679(+) | length=535	8,60	1	4	4	5	58,4	6,42

Peptides communs entre E3XEC7 (ANODA, identifié dans 450 et 466) et PFL1725w mais 1 peptide spécifique également pour PFL1725w. Moins de peptides identfiés chez PFL1725w

Spot 905

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
Q7Q3F6	AGAP007852-PA (Fragment) OS=Anopheles gambiae GN=AGAP007852 PE=4 SV=4 - [Q7Q3F6_ANOGA]	29,77	1	8	18	37	85,2	8,53
Q16KR4	Aconitase, mitochondrial OS=Aedes aegypti GN=AAEL012897 PE=4 SV=1 - [Q16KR4_AEDAE]	18,78	1	0	12	18	85,7	8,44
Q17EL3	Aconitase, mitochondrial OS=Aedes aegypti GN=AAEL003734 PE=4 SV=1 - [Q17EL3_AEDAE]	18,43	1	0	12	18	87,3	8,44
Q7Q3A1	AGAP007784-PA OS=Anopheles gambiae GN=AGAP007784 PE=3 SV=4 - [Q7Q3A1_ANOGA]	6,87	1	4	4	5	82,8	9,29
Q9TW03	Apyrase (Precursor) OS=Anopheles gambiae GN=apy PE=2 SV=1 - [Q9TW03_ANOGA]	10,77	1	5	5	5	61,7	8,69
Q7QC97	AGAP002518-PA OS=Anopheles gambiae GN=AgaP_AGAP002518 PE=3 SV=4 - [Q7QC97_ANOGA]	3,79	1	2	2	2	86,2	7,36
Q7PUR8	AGAP001826-PA OS=Anopheles gambiae GN=AgaP_AGAP001826 PE=4 SV=5 - [Q7PUR8_ANOGA]	0,98	1	2	2	2	371,0	7,96

Peptides communs entre Q7Q3F6 et Q16KR4/Q17EL3 (AEDAE) mais peptides spécifiques également pour chaque protéine. Moins de peptides identfiés chez Q16KR4/Q17EL3 (AEDAE).

Spot 1193

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	22,04	1	0	7	14	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	23,06	1	0	7	14	47,1	7,80
Q6JEK5	Bacteria responsive protein 1 OS=Anopheles gambiae PE=2 SV=1 - [Q6JEK5_ANOGA]	5,37	1	0	2	3	49,6	7,20
Q7Q3V3	AGAP008061-PA OS=Anopheles gambiae GN=AGAP008061 PE=3 SV=3 - [Q7Q3V3_ANOGA]	5,37	1	0	2	3	49,6	7,20
Q7PQM3	6-phosphogluconate dehydrogenase, decarboxylating OS=Anopheles gambiae GN=AGAP004197 PE=3 SV=4 - [Q7PQM3_ANOGA]	6,85	1	0	2	2	53,1	7,49
Q7PYD5	AGAP001884-PA OS=Anopheles gambiae GN=AgaP_AGAP001884 PE=3 SV=4 - [Q7PYD5_ANOGA]	4,57	1	0	2	2	54,0	8,59

Protéines F7IW82 et Q2TLV8 identifiées avec les 7 mêmes peptides

Protéines Q6JEK5 et Q7Q3V3 identifiées avec les 2 mêmes peptides

Spot 1230

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
F7IW82	AGAP000610-PA OS=Anopheles gambiae GN=AgaP_AGAP000610 PE=4 SV=1 - [F7IW82_ANOGA]	34,34	1	0	11	21	49,2	8,60
Q2TLV8	SAGLIN OS=Anopheles gambiae PE=4 SV=1 - [Q2TLV8_ANOGA]	35,92	1	0	11	21	47,1	7,80
Q7PQM3	6-phosphogluconate dehydrogenase, decarboxylating OS=Anopheles gambiae GN=AGAP004197 PE=3 SV=4 - [Q7PQM3_ANOGA]	18,46	1	7	7	8	53,1	7,49
Q7PYD5	AGAP001884-PA OS=Anopheles gambiae GN=AgaP_AGAP001884 PE=3 SV=4 - [Q7PYD5_ANOGA]	18,09	1	7	7	7	54,0	8,59

Q7Q7K5	AGAP011515-PA OS=Anopheles gambiae GN=AGAP011515 PE=3 SV=4 - [Q7Q7K5_ANOGA]	13,35	1	0	3	3	39,0	5,44

Protéines F7IW82 et Q2TLV8 identifiées avec les 11 mêmes peptides

Spot 307

Accession	Description	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	MW [kDa]	calc. pI
A7UVK8	AGAP002076-PA OS=Anopheles gambiae GN=AgaP_AGAP002076 PE=4 SV=2 - [A7UVK8_ANOGA]	28,66	1	9	15	21	71,3	5,47
Q5TTG1	V-type proton ATPase catalytic subunit A OS=Anopheles gambiae GN=Vha68-2 PE=3 SV=1 - [VATA_ANOGA]	21,17	1	9	10	16	68,2	5,39
Q7Q7Y8	AGAP004944-PA OS=Anopheles gambiae GN=AGAP004944 PE=3 SV=3 - [Q7Q7Y8_ANOGA]	26,58	1	5	12	15	70,5	5,45
Q7PQK5	AGAP004192-PA OS=Anopheles gambiae GN=AGAP004192 PE=3 SV=3 - [Q7PQK5_ANOGA]	10,17	1	2	6	8	72,7	5,30
A0NCC6	AGAP000526-PA OS=Anopheles gambiae GN=AgaP_AGAP000526 PE=4 SV=2 - [A0NCC6_ANOGA]	15,70	1	6	6	8	48,1	5,90
Q7Q4E8	AGAP008364-PA OS=Anopheles gambiae GN=TEP15 PE=4 SV=3 - [Q7Q4E8_ANOGA]	5,35	1	6	6	6	163,5	5,96
F5HJA6	AGAP003785-PC OS=Anopheles gambiae GN=AgaP_AGAP003785 PE=3 SV=1 - [F5HJA6_ANOGA]	12,54	1	5	5	6	69,3	5,41
Q7PFH8	AGAP010876-PA (Fragment) OS=Anopheles gambiae GN=AGAP010876 PE=3 SV=4 - [Q7PFH8_ANOGA]	8,11	1	4	4	4	69,3	5,67
Q7PYT9	AGAP002102-PA OS=Anopheles gambiae GN=AGAP002102 PE=4 SV=3 - [Q7PYT9_ANOGA]	3,71	1	2	2	2	67,2	6,00
Q7PJV2	AGAP010147-PA OS=Anopheles gambiae GN=AGAP010147 PE=4 SV=3 - [Q7PJV2_ANOGA]	1,17	1	0	2	2	224,2	5,76
F5HLG8	AGAP004504-PB OS=Anopheles gambiae GN=AgaP_AGAP004504 PE=4 SV=1 - [F5HLG8_ANOGA]	2,84	1	0	2	2	145,6	5,27
Q7PQ67	AGAP004504-PA OS=Anopheles gambiae GN=AgaP_AGAP004504 PE=4 SV=5 - [Q7PQ67_ANOGA]	5,62	1	0	2	2	70,0	5,88

Peptides communs entre A7UVK8 (ANODA), Q7Q7Y8 et Q7PQK5 mais peptides spécifiques également pour chaque protéine.
Moins de peptides identfiés chez Q7Q7Y8 et Q7PQK5).

ProtéinesF5HLG8 et Q7PQ67 identifiées avec les 2 mêmes peptides

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WHO: Word Health Organisation

RDT: Rapid Diagnostic Test

ACT: Artemisin-based Combination Therapies

Ab: Antibody / antibodies

CSP: Circumsporozoite Protein

EPI: Expanded Program on Immunization

AMA-1: Apical Membrane Antigen-1

IGS: Intergenic Spacer

ITN: Insecticide Treated Net

LLIN: Long Lasting Insecticide Treated Net

IRS: Indoor Residual Spraying

ITPS: Insecticide Treated Plastic Sheeting

TBV: Transmission Blocking Vaccine

EIR: Entomological Inoculation Rate

HLC: Human Landing Catching

MSP: Merozoite Surface Protein

Identification and validation of new immune-epidemiological biomarkers for evaluating the human exposure to *Anopheles* malaria vectors

Malaria is a major public health problem in tropical and subtropical areas. Morbidity and mortality are mainly due to *Plasmodium falciparum* transmitted to human individuals by the bite of female *Anopheles* mosquitoes. In order to orientate appropriate strategies for malaria elimination and for a better evaluation of the efficacy of control methods, the indicators measuring the risk of transmission should be more sensitive. It has been shown that the human antibody response against *Anopheles* salivary proteins/peptides represents a biomarker of exposure to mosquito bites and could be an indicator of malaria transmission. However, this tool must be optimized. This work has thus two objectives: i) to validate the salivary protein cE5 as biomarker of exposure to *Anopheles* bites and as an indicator for evaluating the efficacy of vector control strategy, and 2) to identify new salivary proteins as a candidate biomarker only specific to human exposure to infective bites of *Anopheles*.
First, we demonstrated that the IgG antibody response to cE5 protein could be an indicator of human-vector contact, complementary and very sensitive, measuring the human exposure to *Anopheles* bites and a tool evaluating the short-term efficacy of insecticide treated nets. Subsequently, the proteomic methods, 2D - DIGE and mass spectrometry, allowed to identify five salivary proteins (gSG6, gSG1b, TRIO, SG5 and the long form D7) which are overexpressed in the salivary glands of *An . gambiae* infected by wild *P. falciparum*. Peptides for each protein, identified in silico, appear antigenic in individuals exposed to *Anopheles* bites, after the evaluation by the epitope mapping technique.
Altogether, this work is not only the first step to optimize this immuno-epidemiological tool assessing the human-vector contact, but also demonstrates the possibility to define a new biomarker specific to the infective bites of *Anopheles*.

Key words: malaria, biomarker, salivary proteins/peptides, *Anopheles gambiae*, *Plasmodium falciparum*, antibody response, proteomic

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Identification et validation de nouveaux bio-marqueurs immuno-épidémiologiques pour évaluer l'exposition humaine aux piqûres d'Anophèles, vecteurs de paludisme

Le paludisme constitue un problème majeur de santé publique en zone tropicale et subtropicale. La morbidité ainsi que la mortalité sont principalement dû au parasite *Plasmodium falciparum* transmis à l'homme par la piqûre de moustiques femelle du genre *Anopheles*. Dans le but d'orienter au mieux les stratégies d'élimination du paludisme et d'une meilleure évaluation de l'efficacité des méthodes de lutte, les indicateurs mesurant le risque de transmission doivent être plus sensibles. Il a été montré que la réponse anticorps humaine contre des protéines/peptides salivaires d'*Anopheles* représente un bio-marqueur d'exposition aux piqûres de moustiques et pouvait être un indicateur de la transmission du paludisme. Toutefois cet outil doit être optimisé. Ce travail a ainsi un double objectif : i) valider la protéine salivaire CE5 comme bio-marqueur d'exposition aux piqûres d'*Anopheles* et comme indicateur évaluant l'efficacité de stratégie de lutte anti-vectorielle, et 2) identifier de nouvelles protéines salivaires comme candidat bio-marqueur spécifique à l'exposition de l'homme aux seules piqûres infectantes d'*Anopheles*.
Tout d'abord, nous avons démontré que la réponse anticorps IgG contre la protéine CE5 pourrait être un indicateur du contact homme-vecteur, complémentaire et très sensible, en mesurant l'exposition de l'homme aux piqûres d'*Anopheles* et un outil évaluant l'efficacité, à court terme, des moustiquaires imprégnées d'insecticide. Par la suite, les méthodes de protéomique 2D-DIGE et de spectrométrie de masse ont permis d'identifier cinq protéines salivaires (gSG6 , gSG1b , TRIO , SG5 et la forme longue D7) qui sont surexprimées dans les glandes salivaires d'*An. gambiae* infectées par *P. falciparum.* Des peptides de chaque protéine, définis *in silico*, apparaissent antigéniques chez des individus exposés aux piqûres d'*Anopheles*, après évaluation par la technique d'épitope mapping.
L'ensemble de ces travaux est non seulement une première étape pour optimiser cet outil immuno-épidémiologique évaluant le contact homme-vecteur mais démontre également la possibilité de définir un nouveau bio-marqueur qui serait spécifique des piqûres infectantes d'*Anopheles*.

Mots clés : paludisme, bio-marqueur, protéines/peptides salivaire, *Anopheles gambiae*, *Plasmodium falciparum*, réponse anticorps, protéomique