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Diversité et immunogénicité des protéines salivaires de Culicidae

T H È S E

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AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse.

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RESUME

Contexte : Les maladies transmises par les moustiques comme le paludisme ou certaines arboviroses sont à l'origine d'une morbidité et mortalité importantes dans les pays (inter)tropicaux. L'augmentation des échanges internationaux peut conduire à la recrudescence des maladies vectorielles dans les pays de l'hémisphère Nord. Actuellement, la vaccination et la chimioprophylaxie protègent d'un petit nombre de maladies transmises par les moustiques mais leur utilisation peut être contraignante et hors de portée des pays pauvres. Eviter la piqûre de moustiques vecteurs en utilisant des mesures antivectorielles personnelles et collectives reste le moyen le plus efficace pour se protéger de ces maladies. Les méthodes actuelles permettant d'évaluer l'efficacité de ces mesures antivectorielles sont lourdes à mettre en œuvre et ne donnent qu'une indication globale du risque, à l'échelle des populations mais pas au niveau individuel. De nombreuses études ont montrées que la salive d'arthropodes pouvait induire une réponse anticorps chez l'hôte qui pourrait être utilisé pour mieux définir l'efficacité de ces mesures de protection antivectorielle. **Objectif :** L'objectif général de notre projet était d'identifier des antigènes salivaires spécifiques d'espèces de moustiques pour évaluer le contact homme/vecteur. **Résultats :** Dans cette optique, nous nous sommes tout d'abord assurés de l'absence de différences intraspécifiques entre différentes colonies de moustiques, une condition indispensable pour pouvoir observer des différences au niveau de l'espèce. Par ailleurs, nous avons mis au point un protocole pour préserver les échantillons salivaires dans des conditions de terrains non optimales où le maintien de la chaîne de froid peut être difficile. A partir de ces expérimentations préliminaires, nous avons évalué la diversité du répertoire protéique salivaire de quatre espèces d'*Anopheles* par différentes approches (analyses *in silico*, protéomiques et immuno-protéomiques), et montré une spécificité de genre et d'espèce aussi bien au niveau protéique qu'antigénique. Enfin, nous avons pu montrer une évolution spatio-temporelle de l'intensité de la réponse anticorps anti-salive ainsi que sa spécificité de genre et d'espèce, chez des individus exposés à différents niveaux à *Aedes caspius.* **Conclusion :** L'ensemble de ces résultats souligne la possibilité de caractériser des antigènes salivaires spécifiques de genre et d'espèces qui peuvent avoir un intérêt pour mesurer le contact hôte/vecteur au niveau individuel, le risque de transmission de maladies vectorielles ou l'efficacité des mesures anti-vectorielles.

Mots clefs : maladies vectorielles, sialome, répertoire protéique, marqueurs d'exposition, protéomique, antigènes salivaires, paludisme.

ABSTRACT

Background: Mosquito-borne diseases such as malaria or some arbovirus diseases are the cause of important morbidity and mortality in (sub)tropical countries. Vector-borne infection can also be introduced in countries of the North hemisphere due to the increase of international exchanges. Vaccination and chemoprophylaxis strategies currently afford protection against a small number of mosquito-borne diseases and their used can be restrictive and out of reach of low income countries. The primary mean to protect individuals from arthropod-borne diseases is thus the prevention of bites from infected arthropods which could be achieved by a combination of personal protective measures and vector control strategies. Methods currently used to assess the effectiveness of such anti-vector measures are not easily applicable and only give a global estimation of the vector exposure. Numerous studies have shown that hematophagous arthropod saliva could induce a specific antibody response in exposed individuals. These antibody responses could be used to assess the effectiveness of anti-vector measures. **Objectives:** The global aim of this study was to identify species-specific mosquito salivary antigens in order to assess the host/vector contact. **Results:** We first verify the lack of intraspecific differences among several mosquito colonies which is essential to further observe potential differences at the species level. Moreover, a convenient storage method was developed to preserve salivary samples in non optimal condition on the field, where a continuous cold environment could be hard to maintain. Based on these preliminary results, we evaluated the salivary gland protein repertory diversity among four *Anopheles* species using complementary approaches (*in silico*, proteomic and immune-proteomic analyses), and we shown a genus and species specificity at the protein and antigen level. At least, a spatio-temporal evolution of anti-saliva antibody responses was shown according to the *Aedes caspius* density using sera of differentially exposed individuals. The specificity of this response was also reported at the genus and species level. **Conclusion:** All together, these results suggest the feasibility to characterize genus and species specific salivary antigens which could be used as immunological markers of exposure to evaluate host/vector contacts, the risk of vector-borne disease transmission or the effectiveness of anti-vector strategies.

Key words : vector-borne disease, sialome, protein repertory, maker of exposure, proteomic, salivary antigens, malaria.

INTRODUCTION

Les maladies à transmission vectorielle constituent un problème de santé publique dans le monde entier et ont un impact important sur l'économie, le bien-être et la survie de la population humaine. Les individus résidant en zones tropicales et intertropicales sont les plus touchés en raison de la présence de nombreux vecteurs impliqués dans la transmission d'agents pathogènes de nature parasitaire, virale ou bactérienne. Ces maladies transmises par des arthropodes hématophages font des millions de morts chaque année sous ces latitudes où vivent les individus les plus pauvres de la planète et l'incidence de ces maladies risque d'augmenter dans un futur proche dû à des changements écologiques et climatiques qui peuvent favoriser le contact homme / vecteurs / pathogènes (Gratz, 1999, Sutherst, 1993, Pherez, 2007, Vora, 2008, Gould *et al.*, 2009). De plus, l'augmentation soutenue des voyages et du commerce international a transformé le monde en une vaste plateforme d'échanges où les vecteurs et les agents infectieux circulent aussi librement que les individus (Tatem et al., 2006). En conséquence, l'émergence des maladies transmises par les arthropodes risque aussi d'augmenter dans les pays de l'hémisphère Nord, déjà touchés à moindre échelle par des maladies vectorielles endémiques (Kurkela et al., 2005, Chastel, 2009, Campbell et al., 2001).

La vaccination et les traitements étiologiques ou préventifs protègent actuellement contre un nombre limité de maladies vectorielles et leur utilisation peut être contraignante ou inaccessible aux populations pauvres. Eviter la piqûre des arthropodes vecteurs reste donc le moyen le plus efficace pour se protéger de ces maladies (Enayati et al., 2009). Il est possible d'éviter les piqûres en utilisant des méthodes de protection individuelle, incluant l'utilisation de répulsifs cutanés, le port de vêtements longs ou l'utilisation de moustiquaires imprégnées d'insecticide. Ces méthodes peuvent être complétées par la lutte anti-vectorielle qui vise à réduire la densité de populations de vecteurs à l'aide de méthodes telles que l'aménagement du territoire, la lutte anti-larvaire ou la

pulvérisation intra-domiciliaires à effet rémanent (WHO, 2006, Alexander *et al.*, 2003, Talbert *et al.*, 1998, Enayati *et al.*, 2009).

L'efficacité de ces méthodes ainsi que la mesure du risque de l'exposition aux maladies vectorielles d'un point de vue global, est actuellement évaluée soit (i) en laboratoire en testant l'efficacité des moustiquaires imprégnées d'insecticides collectées après une période d'utilisation sur le terrain (Malima et al., 2008, Tungu et al., 2010, Pennetier et al., 2007, Kasili et al., 2010), (ii) soit sur le terrain en mesurant l'incidence de cas cliniques ou la mortalité associée aux maladies vectorielles dans des essais cliniques (Curtis *et al.*, 2000, Lengeler, 2004), (iii) ou grâce à des méthodes entomologiques telles que l'échantillonnage de vecteurs par piégeage ou sur appâts humains (Ocampo et al., 2009, Okech et al., 2008, Shaukat et al., 2010). Cependant, ces méthodes sont lourdes à mettre en œuvre et ne donnent qu'une indication globale du risque d'exposition aux maladies vectorielles à l'échelle des populations mais pas au niveau des individus.

En tant que principal interface dans le contact homme / vecteur, la salive d'arthropode peut avoir de nombreuses applications médicales et épidémiologiques, notamment comme marqueurs immunologiques d'exposition aux piqûres d'arthropodes. L'analyse des réponses anticorps de l'hôte contre la salive d'arthropodes pourrait en effet permettre d'évaluer l'exposition individuelle aux vecteurs ainsi que l'efficacité des mesures de protection antivectorielle. L'état des connaissances actuelles sur l'activité pharmacologique et les propriétés immunogéniques des principaux composants salivaires de plusieurs espèces d'arthropodes hématophages d'importance médicale sont exposées dans une revue de la littérature intitulée « **Implication of blood feeding arthropod salivary proteins in host-vector interactions** » présentée en première partie de ce travail.

Revue **A. Fontaine**, I. Diouf, N. Bakkali, D. Missé, F. Pagès, T. Fusai, C. Rogier, L. Almeras, **Implication of blood feeding arthropod salivary proteins in host-vector interactions**, *(en préparation)*

Implication of blood feeding arthropod salivary proteins in host-vector interactions

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C. Rogier, T. Fusai, L. Almeras.

(en préparation)

Implication of blood feeding arthropod salivary proteins in host-vector interactions

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Abstract:

Blood feeding arthropods (BFA) saliva contains an array of anti-hemostatic, anti-inflammatory and immunomodulatory molecules that participate to the success of the blood meal. BFA saliva is also involved in the transmission and the establishment of pathogen into the host and in uncomfortable allergic responses. This review aim to give a comprehensive overview of the pharmacological activity and immunogenic properties of the main salivary proteins characterized in various BFA species. The potential biological and epidemiological applications of these immunogenic salivary molecules will be discussed with an emphasis on their use as biomarker of exposure to BFA bites or vaccine candidates which could improve host protection against vector-borne diseases.

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1. Introduction

Vector-borne diseases are a major health problem worldwide, causing serious impact on the economy and survival of human population living mainly in tropical and sub-tropical countries. In these countries, diseases transmitted by hematophagous arthropods affect more than 300 million individuals each year and are responsible of more than 1 million deaths (Hotez et al., 2009, WHO, 2004, Nahlen et al., 2005, Snow et al., 2005). To a lesser extent, the human population from developed countries is also exposed to a variety of vector-borne pathogens (Kurkela et al., 2005, Chastel, 2009, Campbell et al., 2001). The emergence or reemergence of arthropod-borne diseases in these areas could increase in a near future with climate change and the increasing international trade and travel (Tatem *et al.*, 2006, Vorou *et al.*, 2007, Gould *et al.*, 2009). Arthropod-borne diseases, such as West Nile, Yellow fever, dengue or Chikungunya virus originate from (sub)tropical regions and have become established in the Northern hemisphere (Hubalek, 2008). The ability of local arthropods to transmit emerging pathogens and the global expansion of other vectors, notably the *Aedes albopictus* mosquito, renders temperate countries vulnerable to tropical diseases and thus increases the public health concern (Talbalaghi et al., 2010, Lambrechts et al., 2010). The epidemic of Chikungunya virus in Northeastern Italy in 2007 (Rezza et al., 2007) and the two autochthonous cases of dengue fever in South France in September 2010 (La Ruche et al., 2010) are recent examples of the introduction and local transmission of tropical vector-borne diseases into non-endemic regions. Aside from transmitting diseases, arthropods are also responsible for causing nuisance and uncomfortable allergic responses in human hosts worldwide (Belkaid et al., 2000, Arlian, 2002, Balenghien et al., 2010).

Research into vaccines or prophylactic drugs against pathogens has so far delivered little to protect individuals from many arthropod-borne diseases.

Currently, vaccines are only available for a limited number of pathogens such as the yellow fever virus (Barrett *et al.*, 2009), the Japanese encephalitis virus (Wilder-Smith *et al.*, 2010), the Rift valley fever virus (Ikegami *et al.*, 2009) or the tick-borne encephalitis virus (Rendi-Wagner, 2008). Protection against malaria, the most debilitating arthropod-borne disease, still relies on the use of prophylactic drugs and is hampered by the escalation of drug-resistance (Pradines et al., 2003). Moreover, these prophylactic measures provide no protection against a majority of arthropod-borne diseases and are beyond the reach of low-income countries.

Thus, the primary mean to protect individuals from arthropod-borne diseases is the prevention of bites from infected arthropods. This can be achieved by a combination of personal protective measures and vector control strategies. Personal protective measures include protective clothing, skin repellent, the use of insecticide-treated nets, screening of houses or indoor residual spraying. Area control can be achieved to reduce the abundance of vector populations by eliminating breeding sites (e.g. environmental sanitation or larviciding of water surfaces) or by controlling adult vectors (e.g. spraying of insecticides) (WHO, 2006, Alexander *et al.*, 2003, Talbert *et al.*, 1998, Enayati *et al.*, 2009). These methods has been historically successful in reducing (Nevill et al., 1996, Keiser et al., 2005, Killeen et al., 2007) or eliminating (Killeen et al., 2002, Patterson, 1989) some vector-borne diseases transmission.

Currently, individual exposure to populations of arthropods and by implication, the evaluation of arthropod-borne diseases transmission and the efficiency of anti-vectorial measures, are evaluated by laboratory bioassay tests (*e.g.*, by assessing in laboratory the effectiveness of insecticide treated nets retrieved after an interval in the field) (Malima et al., 2008, Tungu et al., 2010, Pennetier et al., 2007, Kasili et al., 2010), by measuring vector-borne diseases incidence, morbidity or mortality in controlled clinical trials on the field (Curtis *et al.*, 2000, Lengeler, 2004) or by entomological methods (Thavara et al., 2001,

Killeen et al., 2000). Concerning mosquito-borne diseases, the entomological reference method to measure vector density is the human bait catch (Mathenge et al., 2005). Mosquitoes are caught as they land on human volunteers for taking a blood meal. This technique can give a good estimation of the average number of bites per person per day received from one vector species (*i.e.,* Human biting rate). However, in term of execution and supervision, this method is very laborious and is dependent on the collector skills. In addition, the deliberate exposure of human volunteers to vector-borne diseases raised some ethical considerations. This method only gives a global estimation of the vector density as the human biting rate was shown to vary within small geographic areas (Mbogo CM, 2003, Kreuels B, 2008, Orlandi_Pradines et al, 2009). Results from the human bait catch can also be difficultly extrapolated to children. Alternative entomological methods exist to capture medically important BFA, such as carbon dioxide dry ice traps, light traps and odor bait traps (to collect flying dipterans) (Cohnstaedt et al., 2008) or the drag-flag method (to collect ticks) (Carroll *et al.*, 1992). However, these tools do not make differences between anthropophilic and zoophilic vectors. There is thus a need to develop new indicators and methods to evaluate the effectiveness of anti-vectorial strategies at the individual level.

A common feature shared among arthropods vectors of diseases is their habit of feeding on blood. To ensure the success of the blood meal, hematophagous arthropods regurgitate saliva into the host's skin. Saliva contains a cocktail of pharmacologically active components that counteract host haemostasis and inflammatory response (Ribeiro, 1995, Ribeiro *et al.*, 2003). Human antibody responses to the saliva of various hematophagous arthropods have been repeatedly reported (Nascimento et al., 2001, Lane et al., 1999, Barral et al., 2000, Poinsignon et al., 2008b, Orlandi-Pradines et al., 2007, Remoue et al., 2006, Waitayakul et al., 2006, Peng et al., 2004, Wasinpiyamongkol et al.,

2009), suggesting their potential use as immunological tools to evaluate individual exposure to arthropod bites.

The present review is particularly concerned with the current knowledge on antibody responses against blood feeding arthropods (BFA) saliva. Immunogenic salivary proteins of BFA were first studied for their allergenic properties but there is strong evidence about their applications to improve host protection against some vector-borne diseases and for their use as alternative immunological tools to assess individual exposure to BFA bites. An overview of the pharmacological activity of the main salivary proteins characterized in various BFA species will be first presented to give a better understanding of the role of saliva in host's defense, including hemostasis and immune response.

2. Hematophagous arthropods inside the taxonomic hierarchy

Vector-borne diseases of public health importance are transmitted to vertebrates during the blood meal of infected arthropods. Hematophagy has evolved independently from several millions of years (as early as Triassic, 150-200 My before present) within approximately 5 orders, 14 families, 400 genera and more than 14,000 species of blood sucking arthropods worldwide (Ribeiro, 1995, Balashov, 1999, Adams, 1999). This independent evolution conducted to morphological and behavioral differences in hematophagous arthropods taxonomy.

The arthropods can be divided into holometabolous and hemimetabolous groups. Holometabolous arthropods undergo a complete metamorphosis through larvae, pupae and adult stages, with adults differing from immature stages in structure and habits. Blood meal feeding is restricted to adult within this group of arthropods. Among holometabolous arthropods, insects belonging to the Nematocera sub-order, such as mosquitoes (Fam: Culicidae*)*, sand flies (Fam: Psychodidae) or horse flies (Fam: Tabanidae) families are optional blood

feeders, *i.e.*, only females required proteins acquired during a blood meal for their ovarian maturation and egg production. Indeed, feeding on nectar of flowers, plant exudates and fruit juice is a common feature in both sexes and produce energy for flight and basal metabolic needs (Lewis, 1966, Manda et al., 2007). The Nematocera suborder contains several hematophagous species of major medical importance, such as mosquitoes or sand flies. Parasites and virus transmitted by mosquitoes are the cause of the most debilitating and life threatening vector-borne diseases worldwide (Tolle, 2009). Malaria parasites, transmitted to human in tropical and subtropical areas by some *Anopheles* species, kills about one million individuals each year. Dengue and yellow fever viruses, primary transmitted by *Ae. aegypti* also induce a huge burden on human health in tropical areas. Emerging diseases, such as dengue and Chikungunya, threaten temperate regions with the global expansion of the Asian tiger mosquito *Ae. albopictus*. At least, *Culex* mosquitoes were responsible of West Nile virus outbreaks which lead to fatal cases in human worlwide (Diamond, 2009, Zeller *et al.*, 2004). Sand flies (Fam: Psychodidae) of the genus *Phlebotomus* (Old world) and *Lutzomia* (New world) are also Nematoceran arthropods which induce a huge impact on public health by transmitting Leishmaniasis, a disease causing 2 million new cases each year with 12 million people currently infected (WHO, 2010). Other nematoceran species, including black flies (Fam: Simuliidae) and biting midges (Fam: Ceratopogonidae) are also able to transmit diseases of veterinary and human importance (Carpenter et al., 2009, Basanez et al., 2009). Conversely, obligate hematophagy is exhibited in other holometabolous arthropods of both sex such as tsetse flies (Fam: Glossinidae), which transmit Trypanosomes protozoans that cause Human African trypanosomiasis (sleeping sickness) (Table 1) (Fevre et al., 2008).

Hemimetabolous arthropods, undergoing incomplete metamorphosis, typically with immature stages resembling adult in form and habits, such as members of the Reduviidae (bugs) family or Ixodoidae (ticks) superfamily (Prasad, 1987,

Ribeiro *et al.*, 2009) are obligate hematophages. Some triatoma bugs from the Reduviidae family transmit Chagas disease in Latin America (Rassi et al., 2010) and ticks are vectors of numerous bacteria and virus worldwide such as Lyme borreliosis, tularemia or tick-borne rickettsioses (Table 1) (Fritz, 2009).

Additionally, distinct blood feeding behavior could be exhibited between hematophagous arthropods. BFA feed either by introducing their mouthparts directly into a blood vessel in the host skin (solenophagous; *e.g.*, Culicidae (mosquitoes) and Reduviidae (bugs)) or from a blood pool produced by laceration of blood vessels (telmophagous or pool feeders; *e.g,* flies from the Tabanidae and Psychodidae families and Ixodoidae (ticks)). Other important behavior differences exist among BFA, including the host-vector contact rate that can be influenced by the habit to take multiple blood feeding (Wekesa et al., 1997) or by the duration of blood feeding that can range from few minutes for Culicidae (Chadee et al., 2002) to several days for ticks (Krober *et al.*, 2007). Host preferences and the length of the extrinsic incubation period (time between the acquisition of an infectious agent by a vector and its ability to transmit it to other vertebrate host) are further features that may have significant implications on the mechanisms relating to the host defense and diseases transmission (Bellan, 2010, Yakob et al., 2010, Mahande et al., 2007).

3. The role of saliva in blood feeding

During the blood meal, hematophagous insects deliver saliva into the host's skin. This saliva contains a great variety of pharmacologically active molecules which participate to the success of the blood meal. Salivation was shown to have a significant role on the speed of the blood meal acquisition in various mosquito species (Ribeiro *et al.*, 1985, Mellink *et al.*, 1981). In insects belonging to the Nematocera sub-order, the salivary glands of blood feeding female are morphologically larger than those of non-blood feeding male (James, 2003,

Moreira-Ferro et al., 1999). Furthermore, the expression of several secreted salivary proteins was shown to be restricted or enriched in the female mosquito salivary glands (Arca et al., 2007, Lanfrancotti et al., 2002, Arca et al., 1999, Das et al., 2010). All these data strongly suggest that salivary components are essential to overcome host defenses, including hemostasis and immune responses (Ribeiro *et al.*, 2003). In addition, this host immune modulation following blood feeding could favor pathogen establishment.

3.1. Salivary proteins and host's hemostasis

Hemostasis is a host cellular and molecular response that prevents blood loss from a damaged vessel through several redundant processes, such as blood vessel vasoconstriction, formation of a primary platelet plug (primary hemostasis) or its strengthening by blood coagulation (secondary hemostasis) (for review: (Wu *et al.*, 1996, Romney *et al.*, 2009)).

BFA have evolutionarily developed an important molecular diversity of molecules in their saliva in order to prevent these physiological responses. The most potent vasodilators known so far are maxadilan and *Simulium vittatum* erythema protein (SVEP), respectively expressed in the saliva of *Lutzomia longipalpis* sand flies (Fam: Psychodidae) and *Simulium vittatum* black flies (Fam: Simuliidae). These two species are pool feeders which require strong vasodilatory substances to increase blood flow perfusion in superficial regions of the skin. The Old World Psychodidae *Phlebotomus papatasi* expresses adenosine and adenosine monophosphate (5'AMP) vasodilators instead of maxadilan (Ribeiro et al., 1999). Different molecules are used by other BFA species. Mosquitoes induce vasodilation by secreting either tachykinins (rename sialokinins) (Champagne *et al.*, 1994, Beerntsen *et al.*, 1999) or salivary peroxidase which inactivate vasoconstrictor agents (catecholamines and serotonin) released during the hemostatic process (Ribeiro *et al.*, 1993b). Ticks

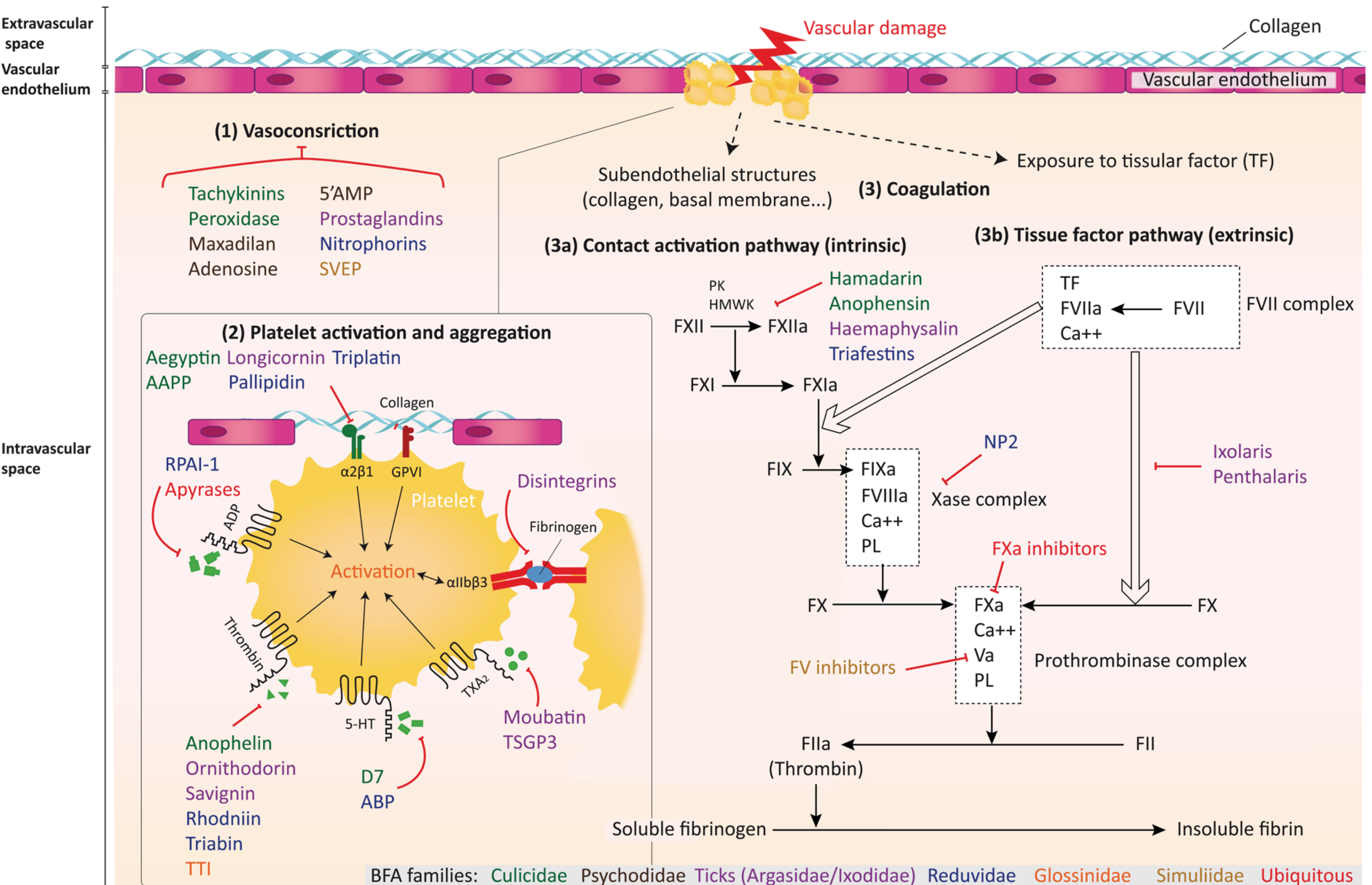

Figure 1

and *Rhodnius prolixus* bugs (Fam: Reduvidae) can induce vascular smooth muscle relaxation by the secretion of prostaglandins and nitrophorins, respectively (Figure 1) (Bowman *et al.*, 1996, Ribeiro *et al.*, 1993a).

Damage to blood vessel endothelium is also accompanied by the activation of platelets which aggregate to form a hemostatic plug using fibrinogen as a connecting agent (Cines *et al.*, 1998, Wu *et al.*, 1996). Saliva of BFA contains numerous compounds which degrade or inhibit platelet aggregation factors (*i.e.*, ADP, thrombin, thromboxane A2, serotonin) in order to prevent the formation of the platelet clot. Apyrase, an enzyme that hydrolyzes ADP released by damaged cells and activated platelets, is ubiquitously found in the saliva of various BFA (Ribeiro et al., 1984, Champagne et al., 1995, Mans et al., 1998, Valenzuela et al., 2001b, Faudry et al., 2004). The *Rhodnius prolixus* aggregation inhibitor 1 (RPAI-1) inhibits platelet aggregation by direct binding to ADP (Francischetti et al., 2000). Other strategies were developed by BFA to overcome platelet aggregation. Some proteins of the D7 family, amine-binding proteins (ABP) or lipocalin family (Moubatin and TSGP3), bind to platelet aggregation effectors (e;g., serotonin (5-HT) or thromboxane A_2 (TXA_2) to reduce their concentration at the feeding site (Andersen *et al.*, 2003, Calvo *et al.*, 2006, Mans *et al.*, 2008c, Mans *et al.*, 2008a, Alvarenga *et al.*, 2010). Others salivary proteins, such as Aegyptin and Anopheline antiplatelet protein (AAPP) (both members of the 30-kDa allergen) (Calvo et al., 2007b, Yoshida et al., 2008), pallipidin (Noeske-Jungblut et al., 1994), triplatin (Morita et al., 2006) and longicornin (Cheng et al., 1999), block platelet adhesion to collagen (by interacting with glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$ receptors on platelets) and subsequent platelet aggregation. Others molecules, named disintegrins inhibit the binding of fibrinogen to αIIbβ3 integrins on platelet (Figure 1) (Mans et al., 2002, Wang et al., 1996, Reddy et al., 2000).

This platelet activation conducts to interactions between platelet surface and coagulation proteins. Through a series of reactions involving several blood

coagulation factors, the coagulation pathway (including the contact activation pathway and tissue factor pathway), is propagated until the formation of thrombin (Figure 1). The latter converts circulating soluble fibrinogen into insoluble fibrin, leading to blood clotting and complete cessation of hemorrhage (Carmeliet, 2001, Levi et al., 2002, Troy, 1988). The formation of this blood clot after the disruption of blood vessel continuity is very brief (between 2 and 6 min) (Mahapatra *et al.*, 2009) and arthropod's blood ingestion take at least several minutes from bite to repletion (Chadee *et al.*, 2002, Daniel *et al.*, 1983). BFA have thus evolved salivary anticoagulants that target specific factors of the blood coagulation cascade, thereby blocking or delaying the clot formation process during the blood meal. As a key molecule in both primary and secondary hemostasis, thrombin is a prime target to overcome host hemostasis and BFA have developed a variety of inhibitors toward its functional domains (Fuentes-Prior et al., 1997). Anophelin (Fam: Culicidae), Rhodniin, Triabin (Fam: Reduviidae), Ornithodorin and savignin (Fam: Argasidae) or the tsetse thrombin inhibitor (TTI) (Fam: Glossinidae) are examples of thrombin inhibitors expressed in the saliva of various BFA families (van de Locht et al., 1996, Nienaber et al., 1999, Friedrich et al., 1993, Cappello et al., 1998, Francischetti et al., 1999). Other anti-coagulation strategies have been reported as the inhibition of the factor Xa active site (Stark *et al.*, 1995, Perez de Leon *et al.*, 1998, Narasimhan *et al.*, 2002, Waxman *et al.*, 1990, Abebe *et al.*, 1994), the inhibition of factor V or VIII activity (Abebe et al., 1996, Pereira et al., 1996), inhibition of the extrinsic coagulation pathway (Ixolaris and Penthalaris**)** (Monteiro et al., 2008, Francischetti et al., 2002, Francischetti et al., 2004), inhibition to the intrinsic Xase complex activity (Nitrophorin 2, NP2) (Isawa et al., 2000) or inhibition of the contact activation pathway (Hamadarin, Anophensin, Haemaphysalin, triafestin-1 and 2) (Isawa et al., 2007a, Isawa et al., 2002, Isawa et al., 2007b). BFA have thus elaborated a variety of salivary components that target several key proteins and pathways of the host

hemostasis. Usually, more than one anti-hemostatic compound is found in the saliva of a single blood-sucking arthropod. Some proteins have more than one anti-hemostatic function and distinct proteins can contribute to the same function (Calvo et al., 2009a). This provides redundancy and reinforces the efficiency of the anti-hemostatic response. Figure 1 give an insight of the major well characterized anti-hemostatic salivary proteins (described in the text above) with their activities and their corresponding organism's family.

3.2. Salivary proteins and host's immunity

Hemostasis is not the only host response encountered by BFA when they take a blood meal. Vertebrate hosts have evolved systems of immune defenses to eliminate foreign organisms in the body which can largely impair BFA blood feeding. Tissue injury causes the immediate onset of acute inflammation and innate immunity, a protective body response that ensures removal of detrimental stimuli, promotes tissue repair and prevents colonization of the damaged tissues by opportunistic pathogens. A hallmark of nematoceran arthropod bites is the apparition of cutaneous pruritic wheal-and-flare reactions that appear at the biting site. Both arms of adaptive immunity (humoral and cell-mediated immunity) are involved in these reactions. Typically, naïve hosts develop no reaction or delayed-type hypersensitivity (DTH, appearing 4-6 h after the bite) when first exposed to bites. DTH skin reactions are mainly mediated by cellular immunity (Reunala et al., 1994b, Reunala et al., 1994c). After repetitive exposure, this reaction usually evolves to immediate-type hypersensitivity (ITH, appearing 10-15 min after the bite) mediated by saliva-specific immunoglobulin E (IgE) (Reunala et al., 1990). Some individuals may then reach the stage of desensitization (no detectable skin reactions) after prolonged and intense exposure (Reunala et al., 1990, Stebbings, 1974). To counteract these immune responses, BFA have developed an array of salivary component.

3.2.1. Inflammation and Innate immunity

Inflammation is characterized by multiple interactions between resident cells of epidermis and dermis, such as endothelial cells, leukocytes, mast cells, neutrophils and platelets which are the first to make contact with arthropods mouthparts as well as their saliva and their potential pathogens. These cells release pro-inflammatory mediators and chemotactic factors such as histamine, macrophage inflammatory proteins-1α (MIP-1α), tumor necrosis factor-α (TNF-α), or leukotriene B_4 (LTB_4) (Canetti et al., 2001, Chakravarti et al., 2007, Ramos et al., 2005) which activate and recruit leukocytes at the site of hemorrhage. This inflammatory response represents an obstacle for the blood meal intake and BFA have evolved an array of molecules which are able to inhibit or scavenge pro-inflammatory agonists. The majority of these molecules were described in saliva of ticks which may be particularly susceptible to inflammation due to their continuous and prolonged blood meal. Tick saliva has the ability to scavenge histamine with monomine, high-affinity histamine-binding proteins (HBPs) and prostaglandins (Paesen *et al.*, 1999, Mans *et al.*, 2008c, Bowman *et al.*, 1996, Hogaboam *et al.*, 1993), serotonin, with monotonin (Mans *et al.*, 2008c), leukotrienes with related lipocalins (AM-33 and TSGP4) (Mans *et al.*, 2008b), or TNF-α with prostaglandins (Bowman et al., 1996, Hogaboam et al., 1993). About the others BFA families fewer data are available in the literature. Phlebotomine saliva inhibits the release of the neutrophil chemotactic mediators MIP-1α, TNF-α and LTB_4 (Carregaro et al., 2008). Bissonnette et al have shown that rat peritoneal mast cells incubated with salivary gland extracts of *Ae. aegypti* inhibited the release of TNF-α impairing their cytotoxicity (Bissonnette et al., 1993). Using several recombinant proteins from the D7 family, Calvo and colleagues reported that some D7 proteins from *An. gambiae* and *Ae. aegypti* could function as scavengers of serotonin and histamine (Calvo et al., 2006) (Figure 2).

Inflammation is also intimately linked to the activity of proteases of the blood coagulation cascade. Coagulation proteases modulate inflammation by the binding to four protease-activated receptors (PARs) which are located at the surface of various vascular cells, including endothelial cells and platelets. PAR-3 and PAR-4 are thrombin receptors whereas PAR-2 can be activated by the tissue factor-FVIIa complex or FXa. PAR-1 can be activated by all of these three coagulation factors or complex. PARs activation is associated with the production of pro-inflammatory cytokines, cell adhesion molecules (ICAMs, E-selectin, P-selectin) and infiltration of leukocytes (Levi *et al.*, 2005, Mackman, 2004). Thus, salivary inhibitors directed against thrombin, FXa or the tissue factor-FVII complex can also counteract host inflammation, highlighting protein redundancy functions (Figure 2).

Ticks have the particularity to remain attached to their hosts for a long period and are therefore more susceptible than other blood sucking arthropods to the host's immune attacks. Thus, in addition to all anti-inflammatory proteins, several salivary inhibitors of the complement system were described in ticks' salivary gland extracts (Lawrie et al., 1999). Isac (*Ixodes scapularis* salivary anti-complement activity) and Salp20 proteins from *Ixodes scapularis* (Fam: Ixodidae), possess a salivary anticomplement activity (Valenzuela et al., 2000, Tyson et al., 2007). Similar functions are exhibited by two salivary anti-complement proteins in *I. ricinus* ticks, named IRAC I and IRAC II (*I. Ricinus* anti-complement) (Daix et al., 2007). The Salp15 salivary protein from *Ixodes ricinus* and *Ixodes scapularis* was reported to inhibit a part of the membrane attack complex of the complement system (Schuijt et al., 2008). An inhibitory action on complement pathways was also observed salivary extracts from phlebotomine *Lutzomyia longipalpis* and *Lutzomyia migonei* (Cavalcante et al., 2003) (Figure 2).

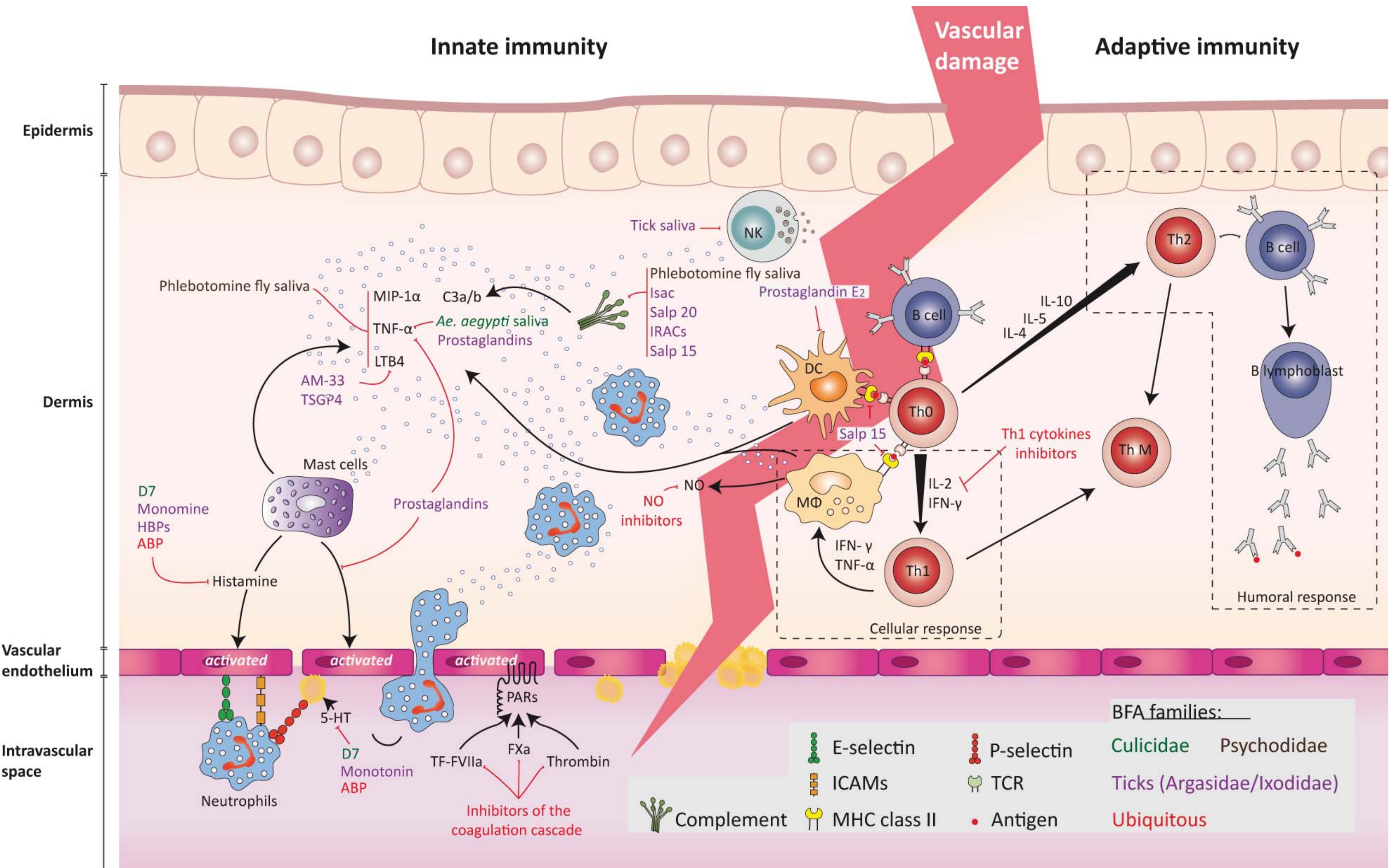

Figure 2

At the end, acute inflammation is a major contributor of the innate immune system and provides cellular leukocytes (e.g., natural killer cells, macrophages, dendritic cells, mast cells, granulocytes) that mediate the first line of defense against invading microorganisms. Innate immunity is immediate and programmed to detect invariant features of invading pathogens called pathogen associated molecular patterns (PAMP) (Nathan, 2002, Iwasaki *et al.*). NK cells and macrophages are involved in killing virus, bacteria and protozoan pathogens after PAMP recognition by their pattern-recognition receptors (PRR). Saliva of Ixodide ticks was reported to decrease or suppress natural killer cells activity as well as nitric oxide (NO) production by macrophages (Kopecky *et al.*, 1998, Kubes *et al.*, 2002, Ferreira *et al.*, 1998). NO production by macrophages is also inhibited by the saliva of *Phlebotomus papatasi* and *Culicoides sonorensis* (Bishop *et al.*, 2006, Waitumbi *et al.*, 1998).

The innate immune system not only acts in controlling infection in a non specific manner but can also influence the type of adaptive immune response. Antigen presenting cells (APC) such as macrophages and dendritic cells (DC) are potent inducers of the adaptative immune response. Indeed, APC capture and process salivary or pathogen antigens at the biting site, migrate into the draining lymph nodes and present them to T lymphocytes, promoting a cell or antibody mediated responses. This transport of salivary antigens was evidenced by the capacity of tick saliva-treated APC, to stimulate proliferation of T lymphocytes obtained from the lymph nodes of tick bite-sensitized animals (Nithiuthai *et al.*, 1985). *Ixodes ricinus* tick saliva and prostaglandin E_2 from the *Ixodes scapularis* tick's saliva was shown to inhibit the maturation and early migration of dendritic cells from inflamed skin to these draining lymph nodes (Sa-Nunes et al., 2007, Skallova et al., 2008). The salivary protein Salp15 from this same species could induce an immunosuppression of DC, adding to its role in inhibiting the complement system (Hovius et al., 2008). *Rhipicephalus sanguineus* tick's saliva also inhibits the differentiation of DC and reduces their population,

greatly impairing the development of an appropriate adaptive immune response by the host (Cavassani et al., 2005).

Given the role of DC as key investigators of adaptive immunity, alteration of their function might delay the establishment of adaptive immune responses, allowing the tick to attach on a host for several days or week and complete their blood meal.

3.2.2. Adaptive immunity

Adding to innate immunity, exposure to BFA saliva may thus trigger the adaptive (or acquired) immune system which is antigen-specific and mediated by B and T lymphocytes (Fearon *et al.*, 1996, Litman *et al.*, 2010). The developments of this immune response by the bitted host constitute another set of barriers to successfully obtain a blood meal and BFA have thus developed salivary immunomodulatory components to counteract this host defense mechanism.

Salivary gland extracts of *Ae. aegypti* mosquito and *Rhipicephalus sanguineus* ticks were shown to suppress antigen-stimulated proliferation of CD4+ Th cells in murine models (Wasserman *et al.*, 2004, Ferreira *et al.*, 1998). The Salp 15 salivary protein of *Ixodes scapularis* ticks interferes with TCR signaling in CD4+ T-cells which results to a specific repression of their activation (Anguita et al., 2002). This same tick species also suppress T-cell proliferation by secreting a salivary protein that sequesters IL-2 (Gillespie et al., 2001). IL-2 sequestration can affect the proliferation of both naïve T-cell and Th1 cells without altering Th2 cells expansion after antigen stimulation (Romagnani, 1997). As a generalized pattern, salivary gland extracts from distinct BFA families were shown to inhibit Th1 cytokines secretion such as IFN-γ and IL-2 (Mbow *et al.*, 1998, Wasserman *et al.*, 2004, Cross *et al.*, 1994, Ramachandra *et al.*, 1992, Urioste *et al.*, 1994), promoting the development of a antibody-

mediated Th2 response. The polarization of the host immune response toward a Th2 response was indeed observed in various BFA species (Schoeler *et al.*, 1999, Mejri *et al.*, 2007, Mbow *et al.*, 1998, Caljon *et al.*, 2006).

All these data support that saliva components can perturb immune response at the biting site. This immunomodulatory phenomenon can orchestrate the development of the acquired immune response, favouring antibody production mediated by Th2 response to a detriment of Th1 cell-mediated response which could be deleterious for BFA and their pathogens.

3.3. Salivary proteins and enhancement of vector-borne pathogens infection.

The immuno-modulatory property of saliva was shown to favor the transmission and the establishment of some vector-borne pathogens which are transported into host skin via saliva.

Titus and Ribeiro were the first to describe the enhancing effect of sand fly salivary gland extracts on cutaneous leishmaniasis when coinoculated with *Leishmania* promastigotes. Mice which have been injected with *Leishmania* parasite concomitantly with small amount of salivary gland proteins developed larger lesions and harbored more parasites than controls (Titus *et al.*, 1988). These findings were further reported for other *Leishmania* and sand flies species (Theodos *et al.*, 1991, Bezerra *et al.*, 2001). The injection of Triatominae bugs saliva into mouse skin in the presence of *Trypanosoma cruzi* parasites was reported to induce an up-to-six fold blood parasitaemia (Mesquita et al., 2008) and saliva of ixodid ticks was shown to potentiate the transmission of Thogoto virus (Jones et al., 1992). Interestingly, enhancement of Thogoto virus infection was only observed when salivary gland extracts derived from metastriate ixodid ticks and not with prostriate ixodid ticks, argasid ticks or mosquito saliva (Jones et al., 1992). These results highlight the strong specificity of the vector/pathogen interaction that is needed in order to potentiate this enhancing transmission

effect and suggest that this effect can be due to few proteins specific to the BFA species or genus.

In *Lutzomia longipalpis* sand fly, the vasodilator maxadilan was reported to be the principal salivary molecule responsible of this enhanced parasite transmission as it exacerbate infection with *Leishmania major* to the same degree as whole saliva (Morris et al., 2001). *Borrelia burgdorferi*, the spirochetal agent of Lyme disease was shown to use the Salp15 protein expressed in *Ixodes scapularis* tick saliva to enhance its transmission and survival within the vertebrate host. The spirochaetes pathogen specifically up-regulates the expression of Salp15 and associate with it in order to be protected from borreliacidal effects induced by antibody-mediated killing (Ramamoorthi et al., 2005). This enhanced infection induced by saliva seems to be a widespread phenomenon as it was reported in various BFA species and for various virus, bacterial or parasite pathogens (Jones et al., 1992, Mesquita et al., 2008, Thangamani et al., 2010, Rocha et al., 2004). Mosquito saliva was reported to accelerated and amplified several virus infections such as West Nile virus (Schneider et al., 2006), La Crosse virus (Osorio et al., 1996) or Cache-valley virus (Edwards et al., 1998). All these data suggest that BFA vectors are not simple "flying or crawling" syringes but rather play a dynamic role in the host/vector/pathogen relationship. Indeed, increase infectivity was observed when pathogens were delivered to the host by BFA bites compared to a delivery by syringe in various studies (Vaughan *et al.*, 1999, Limesand *et al.*, 2000, Osorio *et al.*, 1996, Gillan *et al.*, 2004). As a result, the development of vaccines against BFA salivary components could be a promising approach to reduce the morbidity of vector-borne diseases in exposed individuals.

4. Salivary proteins and host antibody response: immunological tools in sight?

A large number of studies have demonstrated the production of human and animal antibodies against the salivary gland components of different BFA families of major health importance. Immunogenic salivary proteins were first studied for their allergenic properties but there is strong evidence about their applications to improve host protection against some vector-borne diseases (*i.e.,* vaccination strategy) and for their use as alternative immunological tools to assess individual exposure to BFA bites (*i.e.,* host/vector contacts).

4.1. Saliva of BFA and allergy

Since the middle thirties, numerous studies have described host immediate-type hypersensitivity (ITH) reactions in response to the bite of various BFA families, such as Psychodidae (Theodor, 1935), Culicidae (Dubin *et al.*, 1948, McKiel *et al.*, 1961, Wilson *et al.*, 1965) or Glossinidae (Ellis et al., 1986). This ITH skin reaction, also known as type I hypersensitivity, is now widely accepted to be an allergic reaction which involves the production of IgE antibodies in response to specific salivary allergens (Reunala et al., 1994b). The subsequent exposure to the same allergens cross-link IgE antibodies bound to receptor on mast cell and basophiles that reside in tissues and triggers the release of chemical mediators of inflammation (Janeway *et al.*, 2001, Reisman, 1993, Anand *et al.*, 2010, Kambayashi *et al.*, 2007).

Several studies have attempted to characterize allergens involves in ITH by using different techniques (*e.g.,* skin testing, RAST, ELISA, Immunoblot) and different allergen preparations in a purpose to develop diagnosis tools and treatments of allergic reactions (Shen et al., 1989, Chapman et al., 1986). Chapman and colleagues have compared the allergenic potency of whole body, thoracic and abdominal hemolymph and salivary glands from *Triatoma protracta* (reduviid bug) by RAST inhibition (Chapman et al., 1986).

They demonstrated that allergens were concentrated in salivary glands. Similar results were observed for the *Ixodes holocyclus* tick (Gauci et al., 1988). Recently, Wongkamchai and colleagues have further evidenced that major allergens are present in saliva, followed by salivary gland extracts and whole body extracts from four mosquito species (Wongkamchai et al., 2010).

The collect of saliva is however tedious, time consuming and constitute a major drawback in the widespread use of salivary components to test or treat BFA-induced allergic reactions. The synthetic production of BFA saliva allergens is thus a promising alternative strategy to elaborate safe and highly standardized allergens in large scale production to improve specific diagnosis and immunotherapy for patients allergic to BFA bites. In that purpose, several investigators have characterized major salivary allergen in various BFA species and produced them in recombinant forms. A panel of studies using immunoblot method have revealed a number of salivary proteins detected by IgE antibodies of individuals with skin hypersensitivity to BFA bites, including mosquitoes (Brummer-Korvenkontio *et al.*, 1994, Brummer-Korvenkontio *et al.*, 1997, Cornelie *et al.*, 2007, Jeon *et al.*, 2001, Peng *et al.*, 1997, Shen *et al.*, 1989), ticks (Gauci et al., 1988, Parmar et al., 1996) or reduviid bugs (Chapman et al., 1986, Marshall et al., 1986). Some of these salivary allergens are now well characterized in various BFA species. Peng and colleagues have produced three recombinant *Aedes aegypti* salivary allergens corresponding to a 68 kDa salivary apyrase (rAed a1), a 37-kDa protein belonging to the D7 family (rAed a2) and a 30 kDa salivary gland allergen (rAed a3) (Peng et al., 2001, Peng et al., 2006) recognized by IgE of mosquito-allergic individuals. Recombinant procalin, a 20 kDa salivary protein member of the lipocalin family, was found to be the major salivary allergen of the *Triatoma protracta* reduviid bug saliva (Paddock et al., 2001). Arg r 1 (17 kDa), another recombinant salivary protein of the lipocalin family was reported to bind specific IgE in allergic patient and to be the major allergen responsible for anaphylactic reactions caused by the bites of

the *Argas reflexus* tick (Hilger et al., 2005). The Der-p2 (15.6 kDa) allergen-like protein and the tsetse Antigen 5 (TAg5, 28.9 kDa) characterized respectively in the *Ixodes ricinus* tick and Glosina morsitans morsitans saliva also elicit IgE in allergic patients (Horackova et al., 2010, Caljon et al., 2009). Collectively, these works shown a low sensitivity of these recombinant proteins to detect allergic individual, which could be attributed to the small amount and low diversity of allergen compared to whole saliva. The identification of species-shared antigens and the combination of synthetic allergens might enhance this sensitivity. BFA whole body extracts are currently practiced to treat allergy (Ariano *et al.*, 2004, Beaudouin *et al.*, 2001). Thus, synthetic allergens may improve desensitization protocols and overcome the lack of standardizations in allergen immunotherapy (Wongkamchai et al., 2010, Bircher, 2005).

4.2. Saliva of BFA and vaccines

It was suggested that salivary components described to enhance infection, could be effective vaccine candidates to reduce the morbidity of vector-borne diseases in exposed individuals. This hypothesis was strengthen by the discovery that pre-exposure mice to salivary gland extracts of *Phlebotomus papatasii* abrogate the size of dermal lesions and reduces loads of *Leishmania major* parasites in tissue (Belkaid et al., 1998). The history of exposure to uninfected bites was reported to protect against several vector-borne infections, including Lyme borreliosis (Wikel et al., 1997) and tularemia (Bell et al., 1979) in animals pre-exposed to tick bites. A pre-exposition of mice to *Anopheles stephensi* bites could protect them from rodent malaria (Donovan et al., 2007), but these results are controversial (Kebaier et al., 2010). These protecting effects might be partly due to the development of host immunity against vector salivary proteins which were described to enhance pathogen establishment.

Based on these reflections, Morris and colleagues have tested the potential of *Lutzomia longipalpis* maxadilan as a vaccine candidate to protect mice against

Leishmania infection (Morris et al., 2001). Mice vaccinated with synthetic maxadilan were highly resistant to infection, as evidenced by smaller cutaneous lesions, and shorter healing period compared to controls. As maxadilan is only expressed in new world Psychodidae from the genus *Lutzomia* (Ribeiro et al., 1999), it cannot confer protection against *Leishmania* infection transmitted by Old world sand flies from the genus *Phlebotomus*. However, similar protection was observed in mice vaccinated with SP15, a 15 kD salivary protein from the Old world *P. papatasi* sand fly (Oliveira et al., 2008, Valenzuela et al., 2001a). The vaccination of mice with a plasmid containing the *sp15* gene conducted to a long lasting protection against *Leishmania* infection (up to 3 months) (Valenzuela et al., 2001a). Other DNA vaccine constructed with the *ljm19* gene expressed in salivary glands of *Lu. Longipalpis* sand fly was also reported to protect against the fatal outcome of visceral leishmaniasis caused by *Leishmania infantum chagasi* in hamsters (Gomes et al., 2008).

The potential of the Salp 15 salivary ticks proteins as vaccine candidate to protect host against Lyme borreliosis was also investigated by Dai and colleagues (Dai et al., 2009). Mice immunized with recombinant Salp15 proteins were partially protected from spirochetes transmitted by *I. scapularis* ticks. Interestingly, the authors observed that co-immunization with Salp15 and OspA (a *Borrelia burgdorferi* outer-surface protein (Steere et al., 1998)) exert a better protection against *B. burgdorferi* than either of these two candidates when used alone.

These results support that a saliva-based vaccine approach is conceivable for *Leishmania* sand fly and Lyme disease vectors. Protection conferred by saliva-based vaccines might results from the spatial overlapping of both pathogen and salivary antigens at the biting site which can enhance the host immune response in a local area. To our knowledge, no vaccine candidates have been developed against salivary proteins from others BFA. The characterization of salivary proteins that potentiates pathogen infection in others BFA species might be

interesting candidates to develop saliva-based vaccine to improve host protection against the fatal outcome of vector-borne diseases.

Other vaccine strategies using salivary proteins were undertaken in order to reduce the host/vector contact by avoiding blood intake or diminishing the duration of the blood meal, particularly in ticks (Prevot et al., 2007, Garcia-Varas et al., 2009, Mulenga et al., 1999). Additionally, vaccine candidates targeting gut or body BFA antigens were also developed and shown a good protective efficiency, but these approaches are beyond the scope of this review and supplementary details can be found in other works (de la Fuente *et al.*, 2007, Labuda *et al.*, 2006, de la Fuente *et al.*, 2003).

4.3 Saliva of BFA and exposure markers

The absence of antibody response against saliva from mosquitoes or Culicoides midges in sera of children (Reunala et al., 1994a) or horses (Wilson et al., 2001) living in Iceland, a country exempt from these two biting arthropods, adding to the apparition of IgG antibody responses in animals or humans following exposure to BFA bites (Wilson et al., 2001, Peng et al., 1996, Brummer-Korvenkontio et al., 1994, Abdel-Naser et al., 2006) are strong arguments suggesting that acquisition of antibody response against BFA saliva is exposure dependant. Based on these observations, several investigators have assessed the relevance of immunogenic saliva proteins as an immunological marker of exposure to BFA bites. In this aim, it was necessary to evaluate if arthropod saliva displayed some properties inherent to immunological markers, including the ability to reflect exposure, the half-life of the antibody response or the specificity of these antibody responses at the genus or species levels. The relevance of these tools also has to be assessed in clinical trials on the field, and efforts must be further deployed to obtained highly sensitive and reproducible tools.

4.3.1. Relationship between anti-saliva IgG responses and BFA exposure

To evaluate host/vectors contact, it is necessary to take into account factors which can influence individual exposure to BFA bites, such as seasonal changes (*e.g.*, hot/cold seasons or wet/dry seasons), ecological environments (wetland or urban), human behavior (anti-vectorial protection or human activity).

Using sera from 1059 Canadian blood donors sampled before and after the summer mosquito exposure peak, Peng and colleagues have shown a significant increase of IgG antibody levels against *Aedes vexans* saliva after the summer peak exposure (Peng et al., 2002). Similar observations were reported for individuals living in Finnish Lapland (Palosuo et al., 1997), in South of France (Fontaine et al., 2011) and in Africa (Remoue et al., 2007) exposed to various mosquito species. These works demonstrated that levels of serological immune responses are influenced by seasons corresponding to variations of exposure level to BFA bites.

However, individual activities which can modify host/vectors contacts were also reported to be detectable using serological analysis. Effectively, Schwartz and colleagues were the first to describe a correlation between tick exposure and the level of IgG antibody specific for *Ixodes damini* tick saliva in outdoor workers (in New Jersey, E.U.) (Schwartz et al., 1990). A significant decrease of the IgG anti-tick saliva levels was observed in absence of tick exposure during few months (from October to January) (Schwartz et al., 1990). Additionally, Orlandi-Pradines and colleagues have evaluated the consequences of a transient exposure to *Anopheles gambiae* and *Aedes aegypti* mosquitoes in French travelers during a five month journey in tropical Africa on anti-saliva IgG responses (Orlandi-Pradines et al., 2007). This study reported that several travelers from areas free of *An. gambiae* and *Ae. aegypti* mosquitoes were able to develop an antibody response against saliva from these two unrelated mosquitoes. Moreover, this IgG response quickly (*i.e.,* two months) wane after

exposure and return to pre-exposure levels. Thus, transient exposure (*e.g.,* seasons or travel into endemic areas) to BFA bites seems sufficient for developing an IgG response against arthropod saliva. This IgG antibody response was shown to appear rapidly and to be short lived. These different properties of IgG responses are highly interesting and promising for further serological evaluation of individual exposure to BFA bites. Additionally, IgM antibodies levels directed against *Triatoma infestans* saliva were shown to be detectable as soon as one day after a single challenge with these triatomine bugs and were shown to decrease even more rapidly (18 days) than IgG in chicken (Schwarz et al., 2010). These IgM responses were shown to be sensitive to detect bug exposure but were not exposure dependent. These results highlight the potential use of the short persistence of IgM responses in complement of IgG responses as an indicator of recent BFA exposure.

In addition to measure kinetic variations to BFA bites, some studies have demonstrated that IgG levels against BFA saliva could be used to discriminate individuals geographically exposed to distinct BFA density. In a recent study, we compared antibody responses of southeast French individuals living in three areas with a distinct ecological environment and by implication, a distinct *Ae. caspius* mosquito density (Fontaine et al., submitted). We observed a significant increase in the antibody responses according to spatial *Ae. caspius* density. This correlation between anti-saliva IgG response and BFA densities was also reported for other mosquito species (Waitayakul et al., 2006, Remoue et al., 2007) and *Glossina* (Poinsignon et al., 2008b) in tropical countries.

In a recent clinical assay, Drame and colleagues have confirmed the validity of *An. gambiae* crude saliva as an immunological marker to assess the efficiency of insecticide-treated nets (ITNs) in a malaria hypo-endemic transmission area (Drame et al., 2010). They measure anti-saliva IgG levels, blood parasitaemia and vector densities before and after the introduction of ITNs. A significant decrease in the anti-saliva IgG response was observed after the introduction of

ITNs. This diminution of antibody response was associated with a drop in parasite load but not with vector densities as measure by standard entomological methods (light traps). This study demonstrated that anti-saliva antibody responses could be a more efficient tool than classical entomological methods to give an estimation of the real intensity of BFA bites in individuals living in areas where vector densities are very low.

Thus, variations of the IgG antibody level appeared to be correlated with BFA density which was dependant of several factors, such as seasons, ecological environments, individual activities or the level of anti-vectorial protection. The observed link between anti-saliva IgG responses and BFA exposure as well as the waning of these antibody responses after a period of non-exposure are in favor of the potential use of immunogenic saliva as an immunological marker of exposure.

4.3.2. Diversity and specificity of salivary proteins

As some areas can exhibit a high biodiversity in term of BFA species (Toprak *et al.*, 2007, Balenghien *et al.*, 2006), a high level of specificity is therefore necessary to assess individual exposure by immunological test based on BFA saliva. Several studies have reported diverse degree of cross-reactivity between different BFA species, ranging from low (Wheeler et al., 1991, Trevejo et al., 2005) to high species-specificity (Marshall *et al.*, 1986, Rohousova *et al.*, 2005, Volf *et al.*, 2001, Pinnas *et al.*, 1986). The presence of cross-reactivity was often described in related species (Wheeler et al., 1991, Trevejo et al., 2005) suggesting that this phenomenon can occur in closely related protein sequences.

The specificity of the saliva based immunological test is a prerequisite to assess individual exposure to a specific genus or species of arthropods. An important step forward the knowledge of salivary proteins diversity in the Arthropoda phylum was the cataloguing of salivary gland proteins expressed and secreted in

several species of BFA. The recent elucidation of the genome of major blood sucking arthropods (Arensburger *et al.,* Holt *et al.*, 2002, Mongin *et al.*, 2004, Nene *et al.*, 2007, Hill *et al.*, 2005) adding to an increasing transcriptomic and proteomic work on salivary gland, enabled to identify salivary molecules in various hematophagous arthropods. To date, the transcription repertoire (name sialotranscriptome) of at least 30 different species of BFA was described, revealing a number of both ubiquitous and specific protein throughout the taxonomic hierarchy (Ribeiro et al., 2010, Calvo et al., 2010, Anatriello et al., 2010, Alves-Silva et al., 2010, Calvo et al., 2009b, Santos et al., 2007, Arca et al., 2007, Arca et al., 2005). The independent evolution of hematophagous arthropods and host immune pressure over the salivary products conducted to a diversity of pharmacological molecules even among different genera within a same family (Ribeiro et al., 2010, Calvo et al., 2007a). An insight of the taxonomic variability at the protein sequence level of some BFA salivary proteins is shown on figure 3. The homology/diversity of salivary proteins can be observed at two levels: (i) Homologous salivary proteins can be conserved at different taxonomic levels from genus to the entire arthropod phylum and (ii) the distribution of percentage identity of homologous proteins inside each taxonomic level is highly variable. This supports the existence of numerous candidates which can be used to assess individual exposure to specific BFA. Interestingly, studies on BFA saliva use generally inbred laboratory strains. However, for a same species, it exist distinct colonies coming from arthropods collected in the field which differed in their origins and laboratory colonization histories. In order to evaluate sialome divergence which could occur following the rearing of BFA until several decades (*e.g.,* mosquitoes) under laboratory conditions, the sialomes (*i.e.,* saliva and salivary gland) of three mosquito colonies, (*i.e. Ae. aegypti* colonies *Rockefeller, PAEA* and *Formosus*), were compared using 1D SDS-PAGE (Almeras et al., 2010, Almeras et al., 2009).

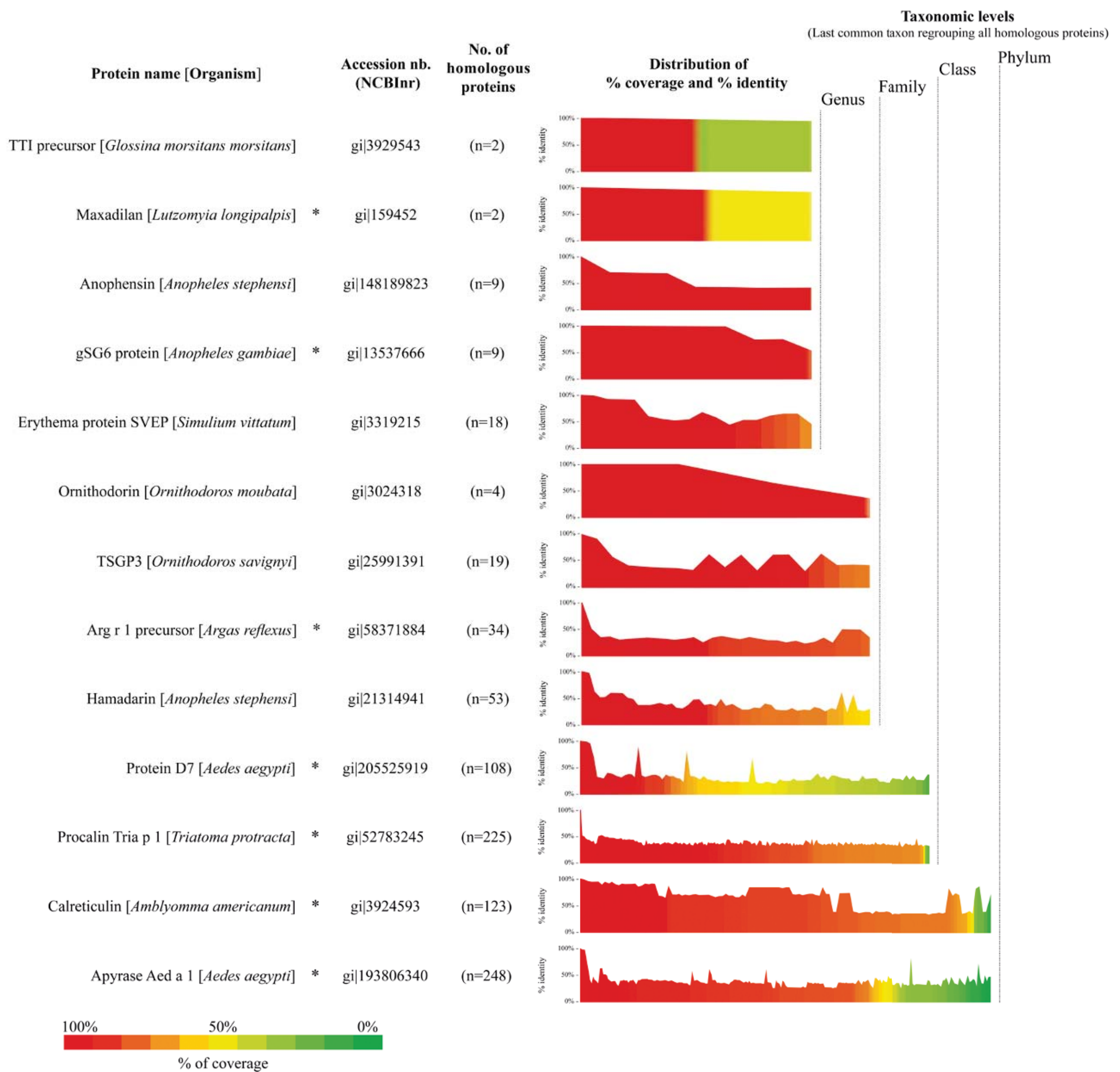

Figure 3

At the saliva and salivary gland levels, no significant differences were detected between these three colonies, suggesting that expression of salivary proteins is highly conserved across populations. But these data could not exclude that a long history of laboratory rearing could have induced a homogenization of salivary protein repertoires, which may differ from their field counterparts. This conserved repertory of salivary proteins at the species level is essential to develop anti-saliva based immunological tools to assess individual exposure to different BFA colonies settled in various areas throughout the world.

4.3.3. Synthetic salivary components as immunological markers of exposure

Host IgG responses against crude BFA saliva or salivary gland extracts have thus proved their efficiency to evaluate the effectiveness of anti-vector strategies. However, sampling of BFA saliva or salivary gland extracts is laborious, time consuming and samples preparation may vary due to a lack of standardization. It is also known that the salivary protein content of BFA can vary according to the age or diets and this may lead to further discrepancy in results and findings among different studies (Choumet et al., 2007, Prates et al., 2008). Differences have also been described according to sex and age in phlebotomine sandflies (Volf et al., 2000). Thus, a gain of sensitivity, specificity and reproducibility could be obtained by the identification of specific immunogenic salivary proteins or peptides and their production in recombinant form.

Poinsignon and colleagues have evaluated the immunogenicity of the recombinant *Anopheles gambiae* salivary gland protein 6 (gSG6), a small salivary protein highly conserved in the *Anopheles* genus (Lombardo et al., 2009), as immunological markers of exposure (Poinsignon et al., 2008a). The recombinant protein was observed to react with IgG antibody of children exposed to the bite of *An. gambiae*. BLAST analysis (NCBInr, NIH, Bethesda,

Nov 15th, 2010) reveals that homologous proteins of gSG6 protein can only be found in 8 *Anopheles* species so far, suggesting its specificity at the *Anopheles* genus (Figure 3). Recombinant gSG6 proteins should be thus used to measure individual exposure to a wide number of species in the *Anopheles* genus. Recently, the use of a gSG6 derived peptide has shown its efficiency to evaluate human exposure to *An. funestus* (Poinsignon et al., 2010). Human hosts exposed to *Amblyomma americanum* and *Dermacentor variabilis* ticks were also shown to develop a specific IgG response against a recombinant calreticulin (rTC) protein isolated from the salivary glands of the *A. americanum* tick (Sanders et al., 1998). The use of recombinant salivary proteins which are highly conserved between several related vectors species could be useful to assess the risk of disease transmission in individuals living in area where vector species diversities is not well characterized.

Detection and selection of highly specific peptides inside the whole salivary protein sequence could further increase the specificity of such immunological markers and reduce production costs. In order to optimize the specificity of the gSG6 biomarker, Poinsignon and colleagues have designed a gSG6-based peptide sequence (gSG6-P1) according to its predicted immunogenic properties (Poinsignon et al., 2008a). A positive association between the anti-gSG6-P1 IgG responses and the level of exposure was observed in individuals exposed to *An. gambiae* bites. This peptide was also detected in individuals exposed to a very low number of the malaria vector bites, suggesting its potential to reveal *An. gambiae* exposure in context where classical entomological methods would be difficulty achieved (*i.e.,* urban areas, altitude, travelers) (Poinsignon et al., 2009).

Interestingly, anti-rTC antibody seropositivity has higher specificity but lower sensitivity than antibodies directed against the whole saliva, to detect individuals exposed to tick (Sanders et al., 1999). The use of a single recombinant salivary protein to assess individual exposure to tick bites may explain this lack of

sensitivity. Indeed, the use of two recombinant proteins (named LJM17 and LJM11) was report to be more effective and sensitive than whole saliva to estimate the level of exposure to *Lutzomia longipalpis* sand fly, a vector of *Leishmania* parasites (Teixeira et al., 2010, Souza et al., 2010).

Recombinant proteins which have been primary produced for their biological properties or their role in allergic responses, such as rSVEP (Cupp et al., 1998), maxadilan (Jackson et al., 1996), anophensin (Isawa et al., 2007a), the tsetse thrombin inhibitor (Cappello et al., 1998) or Arg r 1(Hilger et al., 2005) and rAed a1, 2 and 3 (Peng et al., 2001, Peng et al., 2006), could be also considered as potential marker of exposure candidates. Some of these proteins appeared to be relatively specific to the vector family or genus (Figure 3) and might be promising epidemiological markers of vector exposure. Recently, Ribeiro and colleagues have cataloged conserved salivary proteins at different taxonomic levels in the Nematocera suborder from which some other candidates might emerge (Ribeiro et al., 2010).

Taken together, all these data support the use of immunogenic salivary components as new tools to identify individuals at risk of vector-borne diseases and to monitor BFA population and anti-vector intervention strategies. A gain of sensitivity and specificity could be achieved by the selection and production of recombinant antigens or peptides which do not share sequence homology with other BFA species. Such synthetic product might be used on large cohort studies using high-throughput methods such as Luminex technology. Such multiplex assays are cost and time effective and were proven to be useful strategies for the detection of serum antibodies directed against infectious pathogens (Pickering et al., 2002, Lal et al., 2004) and to evaluate individual exposure to vector borne diseases (Ambrosino et al., 2010).

5. Conclusion

In order to facilitate their blood meals, BFA have elaborated a diversity of salivary component which have essential roles to counteract host hemostatic defenses. Aside from these pharmacological activities, salivary components can modulate host immunity at the biting site and induce an immune environment which is favorable for pathogen transmission. This immuno-modulation is associated with the production of specific antibody responses. Since the 1980s, several studies have investigated antibody responses of vertebrate hosts against salivary proteins in the first attempt to treat uncomfortable allergic reactions to BFA bites. However, the immunogenic properties of some salivary proteins can further be used as vaccine candidates to improve host protection against some vector-borne diseases. Finally, salivary proteins could be used to assess individual exposure to various BFA species. These immunological markers could represent tools that might give a relevant estimation of vector/host contacts, effectiveness of various control or surveillance programs, and estimation of the pathogen transmission risk in complement to methods currently available. A gain of sensitivity, specificity and reproducibility could be obtained by the identification of species specific immunogenic salivary peptides or the combination of several recombinant salivary proteins.

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Figure legends

Figure 1: Schematic representation of arthropod's salivary proteins acting on primary (vasoconstriction and formation of the primary platelet plug) and secondary (formation of fibrin through the coagulation cascade) hemostasis. Hematophagous arthropods induce injuries to vascular endothelium when probing for a blood meal. The initial event of this vascular damage is vasoconstriction (1) which retards extravascular blood loss and enhance adhesion of platelets to exposed subendothelial collagen. This adhesion activates platelets (2) and causes the release of platelet activation agonists (Adenosine diphosphate (ADP), Thrombin, Thromboxane A_2 (TXA_2), serotonin (5-HT)) as well as platelet membrane integrin receptor αIIbβ3. Fibrinogen binds to this receptor and crosslink platelets to form a platelet plug. The blood coagulation cascade (3) is then initiated to strengthen the platelet plug with fibrin at the site of injury. The coagulation cascade is separated into two pathways converging into a common pathway. The contact activation pathway (intrinsic) involves high-molecular weight kininogen (HMWK), prekallikrein (PK), factor XII, factor XI, factor IX (3a) and the tissue factor pathway (extrinsic) involves the tissue factor and factor VII complex (3b). Both pathways lead to the activation of factor X. The common pathway leads to the generation of thrombin from prothrombin and the ultimate production of insoluble fibrin from fibrinogen. Blood feeding arthropods (BFA) have evolved anti-hemostatic salivary proteins that inhibit specific agonist and factors of platelet aggregation and the blood coagulation cascade. The known action of some BFA proteins is indicated as well as their corresponding organism's family. Salivary protein affiliation to BFA families is indicated by color as represented on the bottom right corner legend).

Figure 2: Schematic representation of arthropod's salivary proteins involves in the modulation of innate and adaptive immunity. Protective immunity against BFA involves both innate and adaptive immunity. Cells involves in the innate response, such as neutrophils, mast cells, macrophages (MΦ) or Natural Killer (NK) cells, are the first line of defense against antigens secreted by BFA. Once activated, these cells released molecules (*e.g.*, macrophage inflammatory proteins -1 α (MIP-1α), tumor necrosis factor- α (TNF- α) or leukotriene B_4 (LB_4) that initiate the inflammation process, a protective body response that ensures removal of detrimental stimuli and promotes tissue repair. This local inflammation can further be triggered by the activation of complement which have chemotactic and inflammatory properties. Endothelial cells can be activated by the binding of factors of the coagulation cascade to PAR receptors, this conduct to an over-expression of surface adhesive molecules (ICAMs, E-selectin) which participate to neutrophil migration. Activated platelets are also involved in neutrophil migration by expressing P-selectin on their surface. BFA have evolved an array of salivary components which are able to inhibit or scavenge these pro-inflammatory agonists. Cells from the innate immune response participate to the induction of the adaptive immune response. Antigen presenting cells, such as dendritic cells (DC) and macrophages are able to migrate to the lymph nodes where they interact with naïve CD4+ helper T lymphocytes (Th0 cells) via the interplay of their T cell receptors (TCR) and major histocompatibility complex (MHC) class II proteins. Th0 cells have the potential to proliferate and to differentiate into two distinct lineages of effectors cells: Th1 and Th2 cells, depending on the cytokines environment. Memory T helper (Th M) cells which can improve the quality of the response to a subsequent exposure by developing more efficient memory capacity over time are also produced. In a general pattern, BFA saliva down-regulate the expression of Th1 cytokines (such as IL-2) modulating the adaptive immune response to an antibody mediated Th2 response. The action of saliva or salivary proteins is indicated on the figure as well as their corresponding organism's family. Salivary protein affiliation to BFA families is indicated by color as represented on the bottom right corner legend).

Figure 3: Protein sequence diversity of BFA salivary proteins. Sequences of 13 salivary proteins of blood sucking arthropods that have been produced in recombinant form (as described in the present review) were submitted to BLAST analysis on the non-redundant protein database (NCBInr, NIH, Bethesda). The blastp program was used with default parameters excepting the following: search was done on the *Arthropoda* taxonomic level (taxid: 6656, Nov 15th, 2010, 12,289,957 sequences), the E-value threshold has been changed to a setting of 1 in order to recover only hits with highest significance on the overall protein sequence, and the hit-list size was set up to 5000 proteins. The number of homologous proteins with a score superior to 40 and their respective percentage of coverage and identity were recovered for all query proteins and sorted according to their increasing percentage of coverage. The number of homologous proteins is indicated into brackets (this includes the query sequence) and distributions of both percentage of coverage (bar graph with a colored scale) and percentage of identity (line profile above each bar graph) are represented. Proteins are grouped according to taxonomic level of the last common taxon regrouping their corresponding homologous proteins. For graphical convenience, subclass, class and infraclass as well as superfamily, family and subfamily taxonomic levels were grouped into class and family, respectively. Salivary proteins which have been reported to be targeted by an immune response are indicated by an asterisk (*).

Table 1: Prevalence and the mortality of major vector-borne diseases.

BFA Vectors			Diseases	Prevalence (x1000 per year)	Mortality (x1000)	Ref.
Order	**Family**	**Genius**				
Diptera	Culicidae	*Anopheles*	Malaria,	247,000	1,000	WHO
		Culex	Lymphatic filariasis	-	-	
			West Nile disease	-	-	
			Japanese encephalitis	-	-	
		Aedes	Yellow fever	200	30	WHO
			Chikungunya	-	-	
			Dengue	50,000	22	WHO
	Psychodidae	*Phlebotomus* *Lutzomyia*	Leishmaniasis	2,000	50	WHO
	Glossinidae	*Glossina*	Human African Trypanosomiasis	30	-	WHO
	Simulidae	*Simulium*	Onchocerciasis	-	-	
	Tabanidae	*Tabanus*	Loiasis	-	-	
Hemiptera	Reduviidae	*Triatoma* *Rhodnius*	Chagas disease	10,000	10	WHO
Ixodida	Ixodidae	*Amblyomma*	Rickettsiosis	-	-	
			Tularemia	-	-	
		Ixodes	Lyme disease	-	-	
			Babesiosis	-	-	
	Argasidae	*Ornithodoros*	Relapsing fever	-	-	

The taxonomic classification of the major hematophagous arthropod vectors is given with their corresponding diseases. The prevalence and mortality per year of these diseases is indicated according to World Health Organization availability on web site (http://www.who.int/mediacentre/factsheets/en/).

OBJECTIFS DE CE PROJET

Dans ce contexte, l'objectif principal de ce projet était d'évaluer la possibilité d'utiliser la réponse anticorps anti-salive de moustiques pour mesurer l'exposition à des espèces spécifiques de moustiques vecteurs ainsi que d'identifier des candidats protéiques salivaires pouvant être utilisés comme marqueurs d'exposition à ces vecteurs. Dans le cadre de cette thèse, nous nous sommes intéressés principalement à trois genres de moustiques (Diptera : Culicidae), *Anopheles, Aedes*, et *Culex* qui sont responsables de la majeure partie de la mortalité et morbidité imputables aux maladies transmissibles en transmettant respectivement le paludisme (Culicidae : *Anopheles*) et diverses arboviroses telles que la dengue, la fièvre jaune, le Chikungunya (Culicidae : *Aedes*), West Nile ou l'encéphalite de Saint Louis (Culicidae : *Culex*).

Préalablement à l'évaluation de marqueurs d'exposition aux piqûres de moustiques spécifiques de genres ou espèces, il nous semblait indispensable de contrôler plusieurs facteurs tels que :

- L'homogénéité du protéome salivaire entre colonies d'une même espèce de moustiques et l'optimisation de protocoles de conservation d'échantillons biologiques en conditions non optimales (conditions de terrain).
- L'évaluation de la diversité du protéome salivaire entre plusieurs espèces de moustiques d'un même genre
- La relation entre l'exposition aux piqûres et la réponse sérologique anti-salive au niveau quantitatif (densité de moustiques / niveau de réponse IgG) et qualitatif (spécificité de la réponse).

Enfin, nous discuterons des perspectives qu'ouvrent l'ensemble de ces résultats sur le développement d'outils immunologiques qui pourraient permettre de mesurer le niveau d'exposition à différents genres ou espèces de moustiques, dont les applications peuvent être multiples telles que : tester l'efficacité des mesures de protection, évaluer le risque de transmission de maladies, diagnostiquer ou traiter les allergies associées aux piqûres ainsi que dans la protection de l'hôte contre certaines maladies vectorielles en tant que candidats vaccins. Ces travaux pourraient par ailleurs être transposés à d'autres arthropodes hématophages.

Homogénéité du protéome salivaires entre colonies d'une même espèce de moustiques et optimisation de protocoles de conservation d'échantillons biologiques en non optimales (conditions de terrain).

Avant de rechercher des marqueurs immunologiques d'exposition aux piqûres de moustiques, notamment chez les voyageurs, il était nécessaire de vérifier s'il était possible de détecter une réponse anticorps spécifique et de mesurer sa cinétique d'apparition et de disparition chez des populations transitoirement exposées à différentes espèces de moustiques. C'est ainsi qu'il a été montré dans une étude préliminaire effectuée au sein de notre laboratoire, que des militaires français exposés aux moustiques du genre *Anopheles* et *Aedes* pendant un séjour de 5 mois en Afrique inter tropicale, pouvaient développer une réponse anticorps vis-à-vis des antigènes de la salive d'*An. gambiae* et *Ae. aegypti*. L'analyse cinétique de la réponse anticorps anti-salive a permis de mettre en évidence une augmentation et diminution de cette réponse en relation avec l'exposition aux piqûres. De plus, cette réponse anticorps anti-salive semble être spécifique tout au moins du genre (Orlandi-Pradines et al., 2007). Une protéine antigénique (gSG6) a été identifiée comme potentiel marqueur d'exposition au genre *Anopheles*, puis validé par nos collaborateurs (Poinsignon et al., 2008, Poinsignon et al., 2010). Cette étude initiale indiquait qu'il était possible d'évaluer le contact homme/vecteur mais également d'identifier des protéines salivaires antigéniques spécifiques du genre et peut être même d'espèce de moustiques.

Il existe plusieurs colonies de moustiques d'une même espèce maintenues en élevage dans différents laboratoires à travers le monde. Une colonie peut être définie comme un groupe de moustiques d'une même espèce qui a été collecté sur le terrain parfois loin de son lieu d'origine et qui est maintenu en laboratoire

pendant plusieurs générations. La plupart des travaux réalisés dans les laboratoires sur l'étude de la salive ou des glandes salivaires de moustiques sont effectués à partir de différentes colonies de moustiques d'élevages. Ainsi, préalablement à la caractérisation de marqueurs spécifiques d'espèces, il était indispensable de s'assurer de l'homogénéité du protéome salivaire au sein d'une même espèce en vérifiant l'absence de différences entre plusieurs colonies. Dans ce but, nous avons comparé la composition du répertoire protéique salivaire de trois colonies de l'espèce *Aedes aegypti* (*Rockefeller, Formosus* et *PAEA*) prélevées dans la nature à différents endroits et à différents temps puis élevés en laboratoires. Les résultats de cette étude sont présentés dans l'**article N.1**.

Cependant, la collecte de la salive est un travail long et laborieux avec des rendements faibles (de 20 à 75 ng de protéines par moustique, (Remoue et al., 2006)). De ce fait, de nombreuses équipes utilisent le prélèvement de glandes salivaires qui ont l'avantage d'être relativement plus facile à collecter et de contenir plus de matériel protéique (de 0.5 à 1 ug par moustique) (Waitayakul et al., 2006). La comparaison du protéome de glandes salivaires a ainsi été effectuée entre les mêmes colonies d'*Ae. aegypti* que précédemment (Rockefeller, Formosus et PAEA) afin de vérifier son homogénéité au sein de l'espèce. Les résultats de ce travail sont présentés dans l'article N. 2:

Article N.1 L. Almeras , E. Orlandi-Pradines, **A. Fontaine**, C. Villard, E. Boucomont, L.de Senneville, M. Baragatti, A. Pascual, B. Pradines, N. Corre-Catelin, F. Pages, P. Reiter, C. Rogier, T. Fusai, **Sialome individuality between *Aedes aegypti* colonies**, Vector-Borne and Zoonotic Diseases, 2009, Oct;9(5):531-41

Article N.2 L. Almeras, **A. Fontaine**, M. Belghazi, S. Bourdon, E. Boucomont-Chapeaublanc, E. Orlandi-Pradines, M. Baragatti, N. Corre-Catelin, P. Reiter, B. Pradines, T. Fusai, C. Rogier, **Salivary gland protein repertoire from *Aedes aegypti* mosquitoes**, Vector-Borne Zoonotic Diseases, 2010, May;10(4):391-402

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Sialome Individuality Between *Aedes aegypti* Colonies

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Abstract

Aedes aegypti is responsible for the transmission of arboviruses. The Yellow Fever, Dengue and Chikungunya viruses are transmitted to the vertebrate host by injection of infected saliva during the blood meal of its vectors. Saliva contains different components with various biochemical activities; anti-hemostatic, angiogenic, inflammatory, and immunomodulatory. This work compares the sialomes of three *Ae. aegypti* colonies (*Rockefeller*, *PAEA*, and *Formosus*), where the repertoire of salivary proteins from these colonies was analyzed by a proteomic approach. This study indicated that major proteins were detectable in the three colonies. However, differences in the abundance of some saliva proteins have been observed between the three *Ae. aegypti* colonies.

Key Words: *Aedes aegypti*—saliva—proteomic.

Introduction

Several parasitic and viral diseases are transmitted through bites of arthropod vectors during their blood feeding. Dengue is an important disease caused by an arbovirus, resulting in human illnesses ranging from asymptomatic infections to a fatal disease characterized by hemorrhage or shock (De Paula and Fonseca, 2004). The *Aedes aegypti* and *Aedes albopictus* mosquitoes are responsible for dengue virus (DV) transmission in tropical and subtropical regions (Philip Samuel and Tyagi, 2006; Fontenille and Toto, 2001). These vectors acquire the DV during a blood meal from a human being. After a replication cycle inside the mosquito's body, viruses reach the salivary gland epithelium and may be transmitted in a subsequent blood meal (Monath, 1994). During this meal, mosquitoes secrete complex mixtures containing anti-hemostatic, anti-angiogenic, inflammatory, and immunomodulatory compounds (Ribeiro, 1995).

Previous studies have suggested that mosquito saliva could potentiate dengue infection by modulating the host T cell response (Wanasen et al., 2004; Zeidner et al., 1999). Conversely, others have demonstrated that the proinflammatory environment induced by saliva components could protect the host from pathogen establishment (Kamhawi et al., 2000). Ader et al. described a reduction of DV infectivity on dendritic cells in the presence of *Ae. aegypti* saliva (Ader et al., 2004). This protective phenomenon was accentuated by a pre-sensitization of dendritic cells with mosquito saliva, suggesting that preexposure to uninfected mosquito saliva might be beneficial for the host. The saliva factors that limit viral uptake might therefore become novel vaccine candidates. The sandfly *Phlebotomus papatasi* salivary protein SP15, which affects the immune response of the host to *Leishmania major*, was used for the development of a vaccine preventing infection in a mouse model (Valenzuela et al., 2001). Molecular studies of mosquito saliva components may enable a more detailed understanding of their involvement in pathogen transmission. Therefore, there has been an increase in the interest in characterizing the compounds that are secreted during the blood feeding. Several groups have developed methods to collect salivary glands and have analyzed their components (Kalume et al., 2005; Choumet et al., 2007). However, only few laboratories have investigated mosquito saliva components, because a limited amount of saliva can be obtained from mosquitoes (Remoue et al., 2006; Choumet et al., 2007; Orlandi-pradines et al., 2007).

As mentioned by Ribeiro, the so-called "magic potions" are composed of a wide range of components and considerable variation exists in composition between species (Ribeiro and Francischetti, 2003). Differences have also been de-

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scribed between colonies from the same species of *Phlebotomus sp*. (Volf et al., 2000). Progress over the past two decades in the sequencing of arthropod genomes (Lawson et al., 2006; Nene et al., 2007) provides the opportunity to study and compare salivary compositional diversity by proteomic approaches. However, the majority of the proteins identified have unknown functions (Ribeiro and Francischetti, 2003; Valenzuela et al., 2003).

The majority of the studies on mosquito saliva have been performed on highly inbred laboratory strains that have been maintained under laboratory conditions for several decades, which may differ substantially from their field counterparts. Divergence is also expected to occur between mosquito populations of different laboratory strains that may have different rearing conditions and/or have originated from different parental strains. In order to know if it is possible to compare results from studies on saliva between laboratories working on the same species, but not the same colony, the sialomes of three colonies from the same species, *i.e. Ae. aegypti* colonies *Rockefeller*, *PAEA* and *Formosus*, reared in strictly the same conditions in one laboratory of Institut Pasteur, were compared. These colonies were not exposed to recent variations in field/environmental conditions and were not experimentally infected by arboviruses. Comparative saliva protein patterns between the three colonies indicated a global profile of homogeneity and some quantitative divergences. The repertoire of salivary proteins from these colonies was analyzed using a proteomic approach.

Materials and Methods

Mosquitoes and saliva collection

Three colonies of *Ae. aegypti* mosquitoes, which differed in their origins and laboratory colonization histories, were used (Table 1). Adult *Ae. aegypti* from *Formosus*, *Rockefeller*, and *PAEA* colonies were reared at the Institut Pasteur (Paris) and maintained under strictly identical standard conditions: 26°C and 60% humidity. Saliva from adult mosquito females was collected as described by F. Remoue et al. (Remoue et al., 2006) in two independent experiments (A and B, with a two-month interval), in which the mosquitoes from the three colonies were handled at the same time in strictly identical conditions. Saliva was pooled by colonies into a microcentrifuge tube on ice and then stored at −80°C until needed.

Briefly, 10-day-old uninfected *Ae. aegypti* females (bred in an insectary) were sedated with CO_2 and their legs and wings were removed. Mosquitoes were selected two days after their first blood feeding on rabbit blood maintained at 37°C. The proboscis of each mosquito was placed in a conventional plastic pipette tip containing 10 μl of saliva buffer (10 mM Hepes, 150 mM NaCl, 5 mM EDTA, pH 7.2), which had been previously fixed on a glass slide by adhesive cellotape. Salivation was increased by topical application of 0.5 μl of 0.25% malathion (BDH, Poole, UK) in acetone to the thorax region. After one hour of salivation at room temperature, the liquid in the tip was collected and pooled with other saliva from mosquitoes of the same colony. The protein concentration of each sample was determined by the Lowry method (DC Protein assay Kit, Bio-Rad) according to the manufacturer's instructions.

SDS-PAGE

Saliva samples from the two experiments (A and B) and the three colonies were handled at the same time under the same conditions. Prior to electrophoresis, mosquito saliva samples were desalted and concentrated by precipitation with acetone (Sigma, St Louis, MI). Saliva samples were resuspended in a Tris buffer containing 5% SDS (Sigma). The samples were reduced with 1% (w/v) dithiothreitol (Sigma) and boiled for 5 min before protein separation by 12% SDS-PAGE. Gels were either stained with silver nitrate (Sigma) as previously described (Almeras et al., 2007) or with Sypro Ruby (Bio-Rad) according to the manufacturer's protocol. Stained gels were digitalized using Typhoon™ Trio Image scanner (GE Healthcare UK) and saliva densitometry profiles were analyzed using ImageQuant™ TL software (GE Healthcare UK). Background subtraction was automatically performed and the densitometry profiles were normalized to take into account global differences. Relative abundance of proteins in each band was estimated by dividing the area under the curve of the pick corresponding to the band by the sum total of the areas under the curves for all of the bands.

In-gel tryptic digest

Excised bands from Sypro Ruby stained gels were prepared as described previously by Shevchenko et al. (Shevchenko et al., 1996). Briefly, proteins were digested overnight at 37°C with sequencing-grade trypsin (12.5 μg/mL; Promega Madison, WI, USA) in 50 mM NH_4HCO_3 (Sigma). The resulting peptides were extracted with 25 mM NH_4HCO_3 for 15 min, dehydrated with acetonitrile (ACN) (Sigma), incubated with 5% acid formic (Sigma) for 15 min under agitation, then dehydrated with ACN, and finally completely dried using a SpeedVac. Samples were then stored at −20°C before analysis by mass spectrometry (MS).

MS analysis

For MS analysis, a LCQ DecaXPplus (ThermoFinnigan, San Jose, CA) ion trap was used. Nano-liquid separation of peptides was carried out using an Ettan MDLC chromatographic system (GE Healthcare) in high throughput configuration. Ten microliters of the digest were first trapped on a zorbax 300SB-C18 5 × 0.3 mm column and eluted at a flow rate of approximately 200 nL/min on a zorbax 300SB-C18,

TABLE 1. CHARACTERISTICS OF *AE. AEGYPTI* COLONIES

Ae. aegypti *colonies*	*Origin*	*Collection date (Length of time in laboratory—years)*
Formosus	Senegal (Kedougou)	2002 (5)
Rockefeller	Antilles	~1950 (~50)
PAEA	Tahiti (Papeete)	1987 (20)

3.5 μm, 150 $\times$ 0.075 mm by a linear gradient of eluant B (0.1% Formic acid, 84% ACN) in eluant A (1% Formic acid). Chromatographic system was piloted by Unicorn 5.01 software (GE Healthcare). MS measurements were performed using a LCQTM Deca XP Plus ion trap mass spectrometer (ThermoFinnigan) equipped with a LCQTM nanospray ionization source. A spray voltage of 1.8 kV was applied to the liquid junction via an in-union high voltage contact coupled to a silicaTip emitter (New Objective). Operation of the mass spectrometer was fully automated during the entire procedure using Excalibur 1.3 data system (ThermoFinnigan). Continuous cycles of one full scan (m/z 500 to 1700) followed by three data-dependent MS/MS measurements at 35% normalized collision energy were performed. MS/MS measurements were allowed for the three most intense precursor ions with a maximum rejection time limit of 1 min. All MS/MS spectra were sequence database searched using Bioworks 3.1 (ThermoFinnigan) or Mascot software. The MS/MS spectra were searched against the non-redundant *Ae. aegypti* database (NCBInr and Vectorbase). The following search parameters were used: precursor-ion mass tolerance of 1.2 Da, fragment ion tolerance of 0.6 Da with methionine oxidation and cysteine carboxyamidomethylation specified as differential modifications, and a maximum of one missed cleavage site allowed.

Statistical analysis

Differences in the relative abundance of each protein between the colonies were analyzed, taking into account the hypothetical differences between gels or experiments using two-way variance component analysis (ANOVA), with the colony as a factor and the gel or the experiment as a co-factor. To take into account that multiple tests were performed, we considered significant p-values after the Bonferoni correction: $p < 0.05/33 = 0.0015$. All statistical analyses were performed with SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Comparative analysis of salivary protein profiles from Ae. aegypti colonies

One dimensional SDS-PAGE separation of saliva from *Ae. aegytpti* colonies yielded protein profiles with numerous bands of molecular weights ranging from 15 to 250 kDa (Figure 1A).

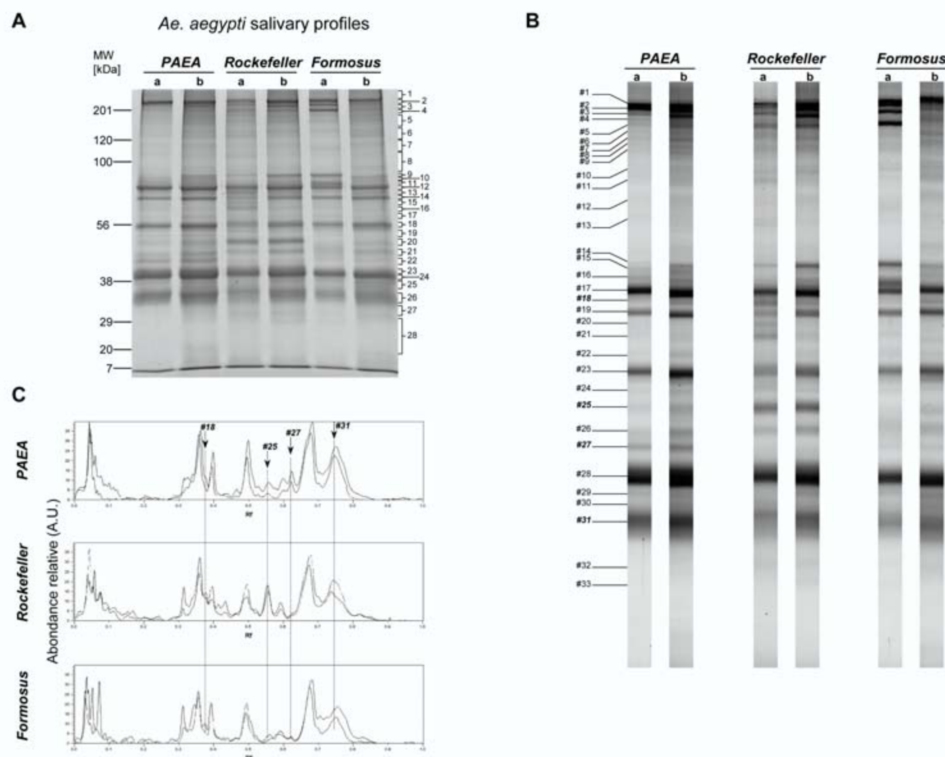

FIG. 1. Comparative salivary protein profiles of three *Ae. aegypti* colonies. (A) Salivary proteins collected from *Formosus, Rockefeller* and *PAEA Ae. aegypti* colonies were separated on 12% SDS-PAGE gels and stained with Sypro Ruby. Saliva collection was performed in two independent experiments at two-month intervals, which are denoted "*a*" and "*b*". *Ae. aegypti* colonies are indicated for each well at the top of the gel. Numbers at the right side of the gel correspond to the 28 bands excised for further analysis by mass spectrometry. The band identity is listed in Table 3. Standard molecular masses are indicated at the left side. (B) Salivary protein profiles of the three colonies were analyzed with the Image Quant TL software. Thirty-three bands were detected with the software and are noted at the left side of the protein profiles. Bold numbers correspond to significant differences in protein band intensity between colonies (ANOVA tests, see Table 2). (C) Schematic representation of normalized densitometric protein profiles of salivary proteins from the three *Ae. aegypti* colonies are presented. For each colony, protein profiles from two independent experiments are superimposed. As indicated in (B), significant differences in protein band intensity between colonies are indicated by arrows with the same numbers and dashed lines. MW: molecular weight. kDa: kiloDalton. A.U.: arbitrary units. Rf: relative front of migration.

More than 30 protein bands could be detected on the Sypro Ruby stained gel for each colony; however, these individual bands presented a wide dynamic concentration range (bands differed by their intensities). For the *Ae. aegypti PAEA* colony, six predominant bands were observed in saliva (band numbers #1, #17, #19, #23, #28, and #31; Figure 1B).

Although saliva protein profiles from the three *Ae. aegypti* colonies appeared globally superimposable, some bands seemed to be quantitatively different between colonies. Before comparing protein band intensity between the three *Ae. aegypti* colonies, it was necessary to take into account variations associated with the time of saliva collection (experimental effect) and with the gel effect on protein migration. Two gels were analyzed under the same conditions as the gel presented in Figure 1A (data not shown). Densitometric scanning and normalization of the gels were performed, allowing an accurate comparison of the saliva protein profiles between colonies. The relative abundance of each band for each colony, experiment, and gel are presented in Table 2 and Figure 1C for gel 1. Comparison of saliva protein profiles from *Ae. aegypti* colonies collected at two time points indicated that no significant differences could be observed from one experiment to the next. Comparison of saliva protein profiles from *Ae. aegypti* colonies collected at each time point, but loaded on two distinct gels, also indicated no significant differences, except for band number #6 ($p < 0.0015$; ANOVA).

Statistical tests (ANOVA) were performed to determine bands that differed by their intensity between colonies, taking into account gel (p-value in the column "Ctrl1" of Table 2) or experimental (p-value in the column "Ctrl2" of Table 2) effects. The relative abundances of four bands (#18, #25, #27, and #31) differed notably between colonies (ANOVA, $p < 0.02$, $p < 3.14\ 10^{e-6}$, $p < 4\ 10^{e-4}$, and $p < 0.01$, respectively, Figure 1B & 1C, Table 2). Among these four bands, bands #25 and #27 in the *Rockefeller* and *PAEA* colonies, respectively, had at least eight fold and five fold changes compared to the other colonies, and the abundances of these two protein bands (e.g. #25 and #27) differed significantly according to the colonies (with Bonferoni correction, $p < 0.0015$).

Identification of Ae. aegypti salivary proteins expressed at significantly different levels between colonies

For sialome analysis, 28 bands numbered in Figure 1A were excised and submitted to trypsin digestion before peptide mixture analysis by MS (LCQ DecaXPplus). Each protein band was analyzed twice and, when possible, these protein bands were collected from two different *Ae. aegypti* colonies. All 28 bands excised allowed the identification of at least one protein, corresponding to 104 proteins identified (Table 3). As expected, several proteins could be identified in each band excised, such as bands 11 and 25 that contained nine and four proteins, respectively (Table 3). Inversely, the same protein was also detected in several excised bands, such as apyrase (gi|157113141, identified in bands 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, and 19) or D7 protein (gi|157113329, identified in bands 22, 23, 24, 25, 26, and 27). Indeed, the 104 proteins identified correspond to 44 distinct proteins according to their NCBI numbers (Table 3).

The four detected bands presenting significant differences in protein relative abundance all contain several proteins. Three proteins were identified in band #18, corresponding to two apyrases (gi|157113127, gi|157113141) and an adenosine deaminase (gi|157110767). In band #25, two proteins were identified, one corresponding to a serpin family protein (gi|94469320) and the other one to a matrix protein (gi|157109437). Two proteins were identified in band #27, including a D7 protein (gi|157113329). In band #31, four proteins were identified, including a variant of a 30 kDa allergen protein (gi|94468552) and a venom allergen (gi|157110207).

Discussion

This study compared for the first time the sialome between three *Ae. aegypti* colonies collected in different areas of the world and reared under strictly identical laboratory conditions during variable periods; from a few years for the *Formosus* colony to a half-century for the *Rockefeller* colony. Salivary profile analysis indicated that major proteins are detectable in all three colonies tested. This suggests that, despite differences in origin and variable rearing time, these colonies conserved their own genus' characteristics. However, these communities of protein expression could be also attributed to the long history of laboratory rearing, which could decrease their individuality and explain the reproducibility of the protein profiles. Mosquitoes reared under laboratory conditions are less subject to environmental pressures, such as microbial exposure or climate and nutrition, and this reduction in environmental selection may result in a diversity decrease (Aguilar et al., 2005; Norris et al., 2001). A salivary protein profile comparison of these colonies to their counterparts collected in the field could be interesting, in order to determine if inbred populations present a similar protein profile with field mosquitoes and to evaluate the relevance of working on these colonies.

We have shown that differences in protein abundance for each band could not be attributed to gel or experimental conditions. There was little or no difference in the abundance of proteins between experiments or between gels for the same colony. These differences were generally not significant and were taken into account for testing the differences between colonies using ANOVA. Differences in the relative abundance of four protein bands have been observed between these three *Ae. aegypti* colonies. These proteins were identified by MS and correspond, as expected, to secreted proteins. D7 proteins was detected in the saliva of the three colonies, but a member of D7 protein family (gi|157113329, bands #27 and #31) appeared significantly up-regulated in the *PAEA* colony compared to the two other colonies. The D7 family is among the most abundant salivary proteins and is widespread in several bloodsucking dipteran species (Valenzuela et al., 2002), including *Aedes* (James et al., 1991), *Anopheles* (Arca et al., 1999), and *Culex* (Valenzuela et al., 2002) mosquitoes. They are distantly related to the odorant-binding superfamily, and as such, these salivary proteins were also shown to bind biogenic amines (*e.g.* serotinin, histamine, and norepinephrine) and could interfere with the haemostatic and inflammatory responses (Calvo et al., 2006).

A protein of the serpin family (band #25) was significantly increased in saliva from the *Rockefeller* colony. Members of the serpin family are the principal anticoagulant regulator of the blood coagulation cascade protease (Gettins, 2002) and

Table 2. Statistical Analysis of Protein Band Expression with Significant Differences Between Colonies

Band numbers	Ae. aegypti															ANOVA test (p-value)	
	PAEA					*Rockefeller*					*Formosus*						
	Gel 1		*Gel 2*			*Gel 1*		*Gel 2*			*Gel 1*		*Gel 2*				
	a	*b*	*a*	*b*	*mean*	*a*	*b*	*a*	*b*	*mean*	*a*	*b*	*a*	*b*	*mean*	*Ctrl1*	*Ctrl2*
#1	7.65	6.13	12.28	6.67	8.18	4.72	6.29	6.40	4.86	5.57	5.43	5.34	8.36	9.55	7.17	0.183	0.275
#2	6.41	4.45	6.36	3.36	5.15	4.55	6.26	2.37	5.36	4.63	9.58	6.17	3.91	2.45	5.53	0.803	0.860
#3	1.76	2.88	2.34	4.01	2.75	3.04	4.87	3.67	6.55	4.53	6.21	1.66	10.15	2.67	5.17	0.391	0.423
#4	0.21	1.57	0.57	1.91	1.06	0.31	1.34	1.20	1.30	1.04	0.44	0.56	4.09	0.64	1.43	0.855	0.880
#5	0.35	1.07	0.64	0.50	0.64	2.57	2.36	4.55	2.66	3.03	9.24	1.35	10.76	1.55	5.72	0.127	0.055
#6	0.20	0.46	0.61	1.24	0.63	0.63	0.51	2.13	1.31	1.14	0.38	0.91	1.83	1.62	1.18	0.088	0.458
#7	0.04	0.28	0.40	0.63	0.34	0.36	0.34	1.23	0.84	0.69	0.31	0.68	0.65	1.52	0.79	0.119	0.324
#8	0.14	0.54	0.46	0.63	0.44	0.68	0.18	1.13	0.57	0.64	0.57	0.35	0.70	1.04	0.67	0.416	0.584
#9	0.08	0.30	0.51	1.46	0.59	0.07	0.30	0.76	1.30	0.61	0.71	0.77	1.31	2.19	1.25	0.057	0.251
#10	0.08	0.22	0.50	0.50	0.33	0.44	0.45	0.99	0.72	0.65	0.19	0.94	0.22	1.17	0.63	0.326	0.347
#11	0.03	0.31	0.15	0.91	0.35	1.24	0.25	0.33	0.92	0.68	0.03	1.95	0.35	1.32	0.91	0.513	0.398
#12	0.06	0.42	0.01	0.16	0.16	0.09	0.47	0.10	0.15	0.20	0.46	1.37	0.83	0.11	0.69	0.096	0.112
#13	0.15	0.18	0.15	0.25	0.18	0.83	0.11	0.39	0.22	0.39	0.13	0.21	0.31	0.25	0.22	0.365	0.317
#14	0.13	1.28	0.27	1.58	0.82	1.27	3.52	0.76	2.62	2.04	6.33	2.22	5.09	1.98	3.90	0.064	0.067
#15	0.35	1.22	0.19	1.91	0.92	0.00	0.55	0.03	0.37	0.24	0.83	0.47	1.16	0.26	0.68	0.288	0.239
#16	0.51	1.07	1.90	2.48	1.49	0.93	1.38	1.11	0.58	1.00	7.90	1.53	7.02	1.05	4.37	0.136	0.082
#17	12.00	11.6	10.71	7.10	10.36	12.92	9.63	9.19	8.13	9.97	8.17	9.45	5.63	7.64	7.72	0.058	0.205
#18	**2.09**	**1.67**	**0.10**	**0.69**	**1.14**	**4.01**	**1.18**	**3.80**	**3.33**	**3.08**	**0.09**	**1.40**	**0.08**	**0.63**	**0.55**	**0.020**	**0.021**
#19	5.63	6.40	4.71	5.18	5.48	1.81	4.18	3.42	4.32	3.43	5.94	7.22	3.57	6.95	5.92	0.053	0.011
#20	0.05	0.01	0.06	0.15	0.07	0.73	0.02	0.02	0.16	0.23	0.01	0.11	0.01	0.01	0.03	0.391	0.399
#21	0.09	0.31	0.02	0.24	0.16	2.79	0.70	0.13	0.97	1.15	0.40	0.36	0.20	0.08	0.26	0.117	0.157
#22	0.18	0.86	0.08	0.25	0.34	0.08	0.26	0.04	0.01	0.10	0.51	0.25	0.30	0.01	0.27	0.301	0.419
#23	11.06	12.79	9.80	12.58	11.56	5.69	8.72	5.04	7.63	6.77	9.96	13.34	6.17	13.63	10.77	0.050	0.002
#24	0.22	0.28	0.11	0.15	0.19	0.68	0.26	0.16	0.73	0.46	0.16	0.97	0.02	0.87	0.50	0.418	0.306
#25	**0.80**	**1.60**	**0.70**	**1.72**	**1.20**	**10.88**	**8.39**	**11.61**	**9.12**	**10.00**	**0.01**	**1.55**	**0.05**	**1.33**	**0.74**	**1.97 10^{-6}**	**3.14 10^{-6}**
#26	0.63	1.67	0.78	1.18	1.06	2.97	3.03	1.32	2.50	2.45	0.18	2.90	0.74	2.08	1.47	0.143	0.042
#27	**2.03**	**2.21**	**1.45**	**1.43**	**1.78**	**0.77**	**0.05**	**0.15**	**0.14**	**0.28**	**0.33**	**0.66**	**0.27**	**0.11**	**0.34**	**2.97 10^{-5}**	**4.07 10^{-4}**
#28	27.43	23.61	28.24	27.23	26.63	28.92	20.91	26.91	22.85	24.90	20.54	23.10	18.24	25.87	21.94	0.178	0.173
#29	0.23	0.07	0.01	0.06	0.09	0.74	0.27	0.45	0.21	0.42	0.42	0.56	0.03	0.84	0.46	0.137	0.153
#30	1.48	0.11	0.51	0.40	0.63	0.10	0.39	1.13	0.06	0.2	0.00	0.38	0.47	0.72	0.39	0.778	0.762
#31	**17.87**	**14.36**	**15.27**	**13.28**	**15.19**	**4.77**	**12.44**	**7.41**	**7.64**	**8.07**	**4.48**	**11.03**	**6.97**	**9.27**	**7.94**	**0.010**	**0.007**
#32	0.01	0.01	0.10	0.06	0.05	0.39	0.22	1.74	0.93	0.82	0.03	0.22	0.19	0.56	0.25	0.033	0.088
#33	0.02	0.01	0.04	0.06	0.03	0.01	0.21	0.31	0.97	0.37	0.02	0.03	0.38	0.04	0.12	0.155	0.239

Note: Relative abundances of proteins estimated by the area under the curve of a band divided by the summed areas under the curves over all of the bands for two independent experiments "a" and "b" and loaded on two distinct gels, denoted 1 and 2 (gel 1 corresponds to the gel presented in Figure 1A). Colony-specific means are estimated. Differences between colonies tested (*p*-value) by two-way variance component analysis, taking into account the hypothetical differences between gels (Ctrl1) or experiments (Ctrl2), are indicated. Protein band expression with significant differences between colonies are indicated in bold.

TABLE 3. PROTEINS IDENTIFIED FROM THE DIFFERENTIAL 1-D SDS-PAGE PROFILES BETWEEN *AE. AEGYPTI* COLONIES

Band number excised	*Band number detected*	*Protein–name*	*Accession no.[a)]*	*Coverage (%)*	*MS/MS peptide sequences*	*Theor.*	*Observ.*	*Significance (Mascot score)*
1	/	conserved hypothetical protein [Aedes aegypti]	gi 157124742	3	10	367.24	/	455.8
		conserved hypothetical protein [Aedes aegypti]	gi 157124820	1	4	379.83	/	241.9
		SGS1 [Aedes aegypti]	gi 66727481	6	17	345.66	/	830.1
2	#1	conserved hypothetical protein [Aedes aegypti]	gi 157124742	2	6	367.24	220.3	257.6
	#2	conserved hypothetical protein [Aedes aegypti]	gi 157124820	12	32	379.83	215.9	1367.7
		SGS1 [Aedes aegypti]	gi 66727481	19	46	345.66		2405.5
3	#3	conserved hypothetical protein [Aedes aegypti]	gi 157124742	22	66	367.24		2903.5
		conserved hypothetical protein [Aedes aegypti]	gi 157124820	5	17	379.83	210.9	768.9
		SGS1 [Aedes aegypti]	gi 66727481	16	43	345.66		1882.0
4	#4	conserved hypothetical protein [Aedes aegypti]	gi 157124742	15	41	367.24		1842.2
		conserved hypothetical protein [Aedes aegypti]	gi 157124820	4	12	379.83		527.2
		conserved hypothetical protein [Aedes aegypti]	gi 157126144	15	25	252.73	201.6	1185.9
		vitellogenin-C [Aedes aegypti]	gi 37528871	4	8	241.91	194.3	319.7
		vitellogenin-B [Aedes aegypti]	gi 37528873	17	28	249.25		1303.1
		SGS1 [Aedes aegypti]	gi 66828491	9	21	345.66		938.6
5	#6	conserved hypothetical protein [Aedes aegypti]	gi 157124820	13	38	379.83		1780.8
		SGS1 [Aedes aegypti]	gi 66828491	18	46	345.66	177.4	2063.9
	#7	conserved hypothetical protein [Aedes aegypti]	gi 157124742	3	11	367.24	168.8	438.8
		apyrase, putative [Aedes aegypti]	gi 157113141*	10	5	62.71		261.3
6	#8	conserved hypothetical protein [Aedes aegypti]	gi 157124820	9	25	379.83		1162.9
		conserved hypothetical protein [Aedes aegypti]	gi 157124742	4	12	367.24		517.5
		SGS1 [Aedes aegypti]	gi 66828491	15	37	345.66	158.5	1788.0
	#9	hypothetical protein AaeL_AAEL006138 [Aedes aegypti]	gi 157112385	2	4	249.27	137.5	158.9
		apyrase, putative [Aedes aegypti]	gi 157113141*	15	6	62.71		246.7
		serine protease inhibitor (serpin-4), putative [Aedes aegypti]	gi 157131308*	11	3	47.75		140.8
7	#10	conserved hypothetical protein [Aedes aegypti]	gi 157124820	8	25	379.83		1038.9
		conserved hypothetical protein [Aedes aegypti]	gi 157124742	1	6	367.24	129.6	206.5
	#11	SGS1 [Aedes aegypti]	gi 66828491	9	24	345.66	121.6	1121.1
		apyrase, putative [Aedes aegypti]	gi 157113141*	10	5	62.71		229.0
		serine protease inhibitor (serpin-4), putative [Aedes aegypti]	gi 157131306*	15	6	49.63		284.8
8	#12	conserved hypothetical protein [Aedes aegypti]	gi 157124820	2	5	379.83		250.1
		conserved hypothetical protein [Aedes aegypti]	gi 157124742	3	8	367.24	114.1	404.8
	#13	SGS1 [Aedes aegypti]	gi 66828491	5	12	345.66	104.9	471.9
		apyrase, putative [Aedes aegypti]	gi 157113141*	8	3	62.71		84.9
9	#14	apolipophorin II [Aedes aegypti]	gi 2746729	28	9	42.41		401.4
		conserved hypothetical protein [Aedes aegypti]	gi 157124820	1	4	379.83		149.9
		conserved hypothetical protein [Aedes aegypti]	gi 157124818	1	4	379.72	92.1	157.2
		conserved hypothetical protein [Aedes aegypti]	gi 157124742	6	18	367.24		841.7
10	#15	transferrin precursor [Aedes aegypti]	gi 2645497	7	4	70.56		134.8
		putative secreted protein [Aedes aegypti]	gi 18568302*	12	6	64.36	90.0	271.4
		apyrase, putative [Aedes aegypti]	gi 157113141*	5	2	62.71		123.9
11	#16	hypothetical protein AaeL_AAEL006138 [Aedes aegypti]	gi 157112385	4	10	249.27		339.0
		vitellogenin-C [Aedes aegypti]	gi 37528871	2	5	241.91		167.0

		tep3 [Aedes aegypti]	gi	157119257	3	4	162.33		173.3
		prophenolixidase [Aedes aegypti]	gi	157136180	5	3	78.25	86.4	79.8
		transferrin precursor [Aedes aegypti]	gi	18091765	25	14	70.57		600.9
		putative secreted protein [Aedes aegypti]	gi	18568300*	5	3	66.17		116.4
		putative secreted protein [Aedes aegypti]	gi	18568302*	12	6	64.36		214.3
		apyrase, putative [Aedes aegypti]	gi	157113141*	16	6	62.71		290.2
		apolipophorin II [Aedes aegypti]	gi	2746729	13	4	42.06		135.0
12	#17	conserved hypothetical protein [Aedes aegypti]	gi	157126144	1	4	252.73		180.4
		vitellogenin-B [Aedes aegypti]	gi	37528873	1	4	249.25		152.7
		vitellogenin-C [Aedes aegypti]	gi	37528871	1	4	241.91		174.3
		Quiescin-sulfhydryl oxidase4, putative [Aedes aegypti]	gi	157131250	3	2	73.99	82.3	69.6
		transferrin precursor [Aedes aegypti]	gi	15081255	11	7	70.32		281.9
		apyrase, putative [Aedes aegypti]	gi	157113141*	22	10	62.71		528.4
		5′-nucleotidase [Aedes aegypti]	gi	94469274*	11	5	61.20		237.1
13	**#18**	**salivary apyrase, putative [Aedes aegypti]**	**gi**	**157113127***	**7**	**4**	**63.19**		**146.7**
		apyrase, putative [Aedes aegypti]	**gi**	**157113141***	**15**	**6**	**62.71**	**79.9**	**270.1**
		adenosine deaminase[Aedes aegypti]	**gi**	**157110767***	**6**	**2**	**59.83**		**102.2**
14	#19	apyrase, putative [Aedes aegypti]	gi	157113141*	5	2	62.71	74.5	114.1
		adenosine deaminase[Aedes aegypti]	gi	157110767*	31	13	59.83		635.7
15	#20	apyrase, putative [Aedes aegypti]	gi	157113141*	5	2	62.71	70.2	81.4
16	#21	apyrase, putative [Aedes aegypti]	gi	157113141*	8	3	62.71	66.3	139.1
17	#22	apyrase, putative [Aedes aegypti]	gi	157113141*	14	6	62.71	61.1	252.5
		salivary serpin[Aedes aegypti]	gi	94469320*	14	4	47.76		218.2
18	#23	apyrase, putative [Aedes aegypti]	gi	157113141*	3	2	62.71		110.9
		salivary anti Fxa serpin [Aedes aegypti]	gi	94468358*	23	8	47.87	56.0	469.3
		SERPIN1 protein precursor, putative [Aedes aegypti]	gi	157135059*	16	5	47.00		309.8
19	#24	apyrase, putative [Aedes aegypti]	gi	157113141*	7	3	62.71		119.1
		serine protease inhibitor (serpin-4), putative [Aedes aegypti]	gi	157131306*	18	6	49.63	51.5	383.2
		putative serpin [Aedes aegypti]	gi	18568304*	19	7	47.08		351.3
		Gelsolin precursor [Aedes aegypti]	gi	157112200	19	6	43.02		276.4
20	**#25**	**salivary serpin [Aedes aegypti]**	**gi**	**94469320***	**6**	**2**	**47.76**	**48.0**	**107.1**
		fibrinogen and fibronectin [Aedes aegypti]	**gi**	**157109437**	**15**	**3**	**33.61**		**180**
21	#26	serine protease inhibitor (serpin-4), putative [Aedes aegypti]	gi	157131310*	10	3	41.80	195.8	
		SERPIN1 protein precursor, putative [Aedes aegypti]	gi	157135061*	13	5	41.23	44.4	196.2
		inosin-uridine preferring nucleoside hydrolase [Aedes aegypti]	gi	157113517	18	7	37.91		284.9
		angiopoietin-like protein [Aedes aegypti]	gi	18568324*	7	2	34.03		69.3
22	**#27**	**D7 protein, putative [Aedes aegypti]**	**gi**	**157113329***	**9**	**3**	**36.87**	**42.3**	**120.1**
		angiopoietin-like protein variant [Aedes aegypti]	**gi**	**94468352***	**14**	**3**	**33.40**		**152.9**
23	#28	long form D7Bclu 1 salivary protein [Aedes aegypti]	gi	16225992*	16	6	38.57		208.4
		D7 protein, putative [Aedes aegypti]	gi	157113329*	15	5	36.87	39.3	224.5
		putative 34 kDa family secreted salivary protein [Aedes aegypti]	gi	94468336*	11	3	35.69		107.0
		angiopoietin-like protein variant [Aedes aegypti]	gi	94468352*	17	4	33.40		143.6
24	#28	long form D7Bclu 1 salivary protein [Aedes aegypti]	gi	16225992*	8	3	38.57		139.4
		D7 protein, putative [Aedes aegypti]	gi	157113329*	9	3	36.87		140.7
		putative 34 kDa secreted protein [Aedes aegypti]	gi	18568296*	15	4	36.15		251.4
		putative 34 kDa family secreted salivary protein [Aedes aegypti]	gi	94468336*	12	3	35.69	39.3	209.8
		angiopoietin-like protein [Aedes aegypti]	gi	94468352*	8	3	33.40		111.1
		30 kDa salivary gland allergen variant 3 [Aedes aegypti]	gi	94468552*	8	2	27.63		80.1

(*continued*)

Table 3. Proteins Identified from the Differential 1-D SDS-PAGE Profiles Between *Ae. aegypti* Colonies (Cont'd)

Band number excised	*Band number detected*	*Protein–name*	*Accession no.*[a)]	*Coverage (%)*	*MS/MS peptide sequences*	*Theor.*	*Observ.*	*Significance (Mascot score)*
25	#29	long form D7Bclu 1 salivary protein [Aedes aegypti]	gi|16225992*	13	5	38.57		187.0
	#30	D7 protein, putative [Aedes aegypti]	gi|157113329*	9	3	36.87	38.0	139.9
		30 kDa salivary gland allergen variant 3 [Aedes aegypti]	gi|94468552*	8	2	27.63	36.5	107.1
		putative 19.6 kDa secreted protein [Aedes aegypti]	gi|61742031*	12	2	21.77		155.9
26	**#31**	**D7 protein, putative [Aedes aegypti]**	**gi|157113329***	**9**	**3**	**36.87**		**114.2**
		venom allergen[Aedes aegypti]	**gi|157110207***	**8**	**1**	**28.87**		**72.2**
		30 kDa salivary gland allergen variant 3 [Aedes aegypti]	**gi|94468552***	**15**	**4**	**27.6**	**35.5**	**189.7**
		conserved hypothetical protein [Aedes aegypti]	**gi|157167432**	**15**	**3**	**23.56**		**198.0**
27	#32	D7 protein, putative [Aedes aegypti]	gi|157113329*	9	3	36.87		114.2
		30 kDa salivary gland allergen variant 3 [Aedes aegypti]	gi|94468552*	15	4	27.63	31.3	189.7
		putative 19.6 kDa secreted protein [Aedes aegypti]	gi|61742031*	16	3	21.77		198.0
28	#33	conserved hypothetical protein [Aedes aegypti]	gi|108877762	15	2	17.14	28.8	88.76
		putative 14.5 kDa salivary protein [Aedes aegypti]	gi|94468650*	21	2	17.21	15.0	83.7

Note: Proteins were identified using a LCQ DecaXPplus mass spectrometer, following in-gel trypsin digestion. the band name excised and detected corresponds to the same numbers as indicated in Figure 1A and 1B, respectively. The identities of the bands, their [a)] NCBI accession numbers, the theoretical and observed *MW* values, as well as the number of peptide sequences, the corresponding percentage sequence coverage, and the Macot score are listed for MS/MS analysis (protein scores greqater than 53 are significant [$p < 0.05$]). Protein band intensity with significant differences between colonies is indicated in bold (see Table 2). Genes encoding putative secretory products are indicated by asterisks "*" (Ribeiro et al., 2007; Valenzuela et al, 2003).

were shown to have a protective role during ookinete invasion at the midgut level of *Anopheles* mosquitoes (Abraham et al., 2005; Danielli et al., 2003). Correspondingly, increased expression of this protein in the *Rockefeller* mosquito colony could facilitate its blood meal.

Apyrase proteins (gi|157113127, gi|157113141) and adenosine deaminase (gi|157110767), identified in band #18, were found more abundantly in the *Rockefeller* colony compared to the two other colonies. Apyrases were implicated in dephosphorylation of ADP and ATP but not AMP (Ribeiro et al., 1984), which could inhibit platelet aggregation and prevent neutrophil activation (Sun et al., 2006). Ribeiro et al. (Ribeiro et al., 2001) demonstrated that adenosine deaminase was secreted by *Ae. aegypti* during its blood meal. This enzyme has been implicated in the hydrolysis of adenosine into inosine and ammonia. Inosine has been described to inhibit the production of inflammatory cytokines (Hasko et al., 2000). Indeed, an abundance of these enzymes at the feeding site could be beneficial for blood feeding. Additionally, comparative analysis between several mosquitoes species indicated an inverse correlation between salivary apyrase activity and probing time (Ribeiro el al, 1985; Ribeiro 2000; Boisson et al, 2006). These studies suggest that the *Rockefeller* colony could be less efficient in the biting process.

For the first time, differences in the amount of saliva proteins were described between three *Ae. aegyptis* colonies using a proteomic approach. These results should be confirmed by more biological replicates or another analysis method such as capillary electrophoresis using fewer biological samples. Recently, protein expression profiling from a single cell was performed using multidimensional capillary electrophoresis (Gao and Zhang, 2008).

Detection of the same protein at variable molecular weights has already been described in the salivary gland of mosquitoes (Choumet et al., 2007). Ribeiro et al. (Ribeiro et al., 2007), identified, on two-dimensional gel electrophoresis of *Ae. aegypti* salivary glands, multiple isoforms of the same proteins (e.g., D7 protein family, 30 kDa antigen, angiopoietin-like protein-2). In the present study, many proteins were found in several bands, including those identified in bands presenting differences in relative abundance between *Ae. aegypti* colonies. It could be hypothesized that these secreted proteins may be subjected to protein processing phenomenon such as proteolytic cleavage and/or post-translational modifications during the transit from salivary gland cells to saliva. Indeed, the differences in the amount of saliva proteins could be attributed to protein maturation phenomenon, instead of variations in levels of protein synthesis. However, the consequence of such modifications on their activity remains unknown.

This is the first time that the salivary protein repertoires from several *Ae aegypti* colonies have been analyzed and compared using a proteomic approach. Recently, we analyzed the *Aedes* sialome, allowing the identification of 10 salivary proteins, eight of which have also been identified in the present study (Orlandi-pradines et al., 2007). The enhancement of the number of proteins identified in the present study could be attributed to sample preparation, which included direct desalting by acetone precipitation without a lyophilization step.

Investigators prefer to use salivary gland extracts instead of saliva for proteomic analysis, due to the difficulties to collect large quantities of saliva. The vast majority of the saliva components discovered until now was identified in proteins extracted from salivary glands, by protein/peptide sequencing and gene cloning (Kalume et al., 2005; Ribeiro et al., 2007; Valenzuela et al., 2002). In 2002, sequencing of the salivary gland cDNA library from *Ae. aegypti* mosquitoes allowed the description of 31 novel protein sequences with putative secretion signals (Valenzuela et al., 2002). More recently, Ribeiro et al. analyzed a set of expressed sequence tags from cDNA libraries of *Ae. aegypti* salivary gland mosquitoes (Ribeiro et al., 2007). A putative secretion signal was identified in the sequence of 136 transcripts. In the present study, the identification of 44 distinct proteins from *Ae. aegypti* saliva confirms the secretion of numerous proteins already predicted by the sialotranscriptome analysis from *Ae. aegypti* salivary glands (Valenzuela et al., 2002; Ribeiro et al., 2007). Among these, we also identified several members of the D7 family (gi|157113329, gi|16225992), serpin family (gi|157135059, gi|94468358, gi|94469320, gi|18568304), enzymes such as apyrases and nucleotidases (gi|157113127, gi|157113141, gi|94469274) or serine proteases (gi|157131306, gi|157131308, gi|157131310), and immunity-related proteins (gi|18568324, gi|94468352). However, sGS1, a protein which has been described as expressed in the salivary gland and not predicted as secreted, was found in the sialomes analyzed in the present study. This protein has been described to be a sporozoite receptor and localized in the basal lamina of salivary glands (Korochkina et al., 2006). No rational explanation could be made to explain the identification of this protein in the saliva component. However, this protein appears to be secreted because (i) samples of saliva were obtained without any salivary gland damage during the collection process, and (ii) sGS1 family members were previously identified in saliva obtained from *Ae. aegypti* and *Anopheles gambiae* species in independent experiments (Orlandi-Pradines, 2008). The analysis of transcripts and proteomes differ in their methodology, but several proteins were identified by both approaches. Indeed, numerous proteins predicted by the transcript analysis are confirmed to actually be present inside the saliva from *Aedes* mosquitoes.

Saliva, as compared to salivary glands, is composed exclusively of secreted proteins which are injected during mosquito blood feeding. These proteins are exposed to the immune system and may trigger an antibody response that could be used as markers of exposure to mosquito bites (Remoue et al., 2006, Orlandi-pradines et al., 2007). Analysis of the salivary protein profiles from the three mosquito colonies present a good global reproducibility, suggesting that saliva proteins obtained from mosquitoes of the same colony reared and salivated under the same standard laboratory conditions are similar. The differences observed between colonies of the same species were quantitative instead of qualitative, since no specific protein was exclusively expressed in one colony.

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Disclosure Statement

No competing interests exist.

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Salivary gland protein repertoire from *Aedes aegypti* mosquitoes

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ORIGINAL ARTICLE

Salivary Gland Protein Repertoire from *Aedes aegypti* Mosquitoes

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Abstract

Diseases caused by arthropod-borne viruses are a significant threat to the health of human and animal populations throughout the world. Better knowledge of the molecules synthesized in the salivary gland and saliva of hematophagous arthropods could be of use for improving the control of pathogen transmission. Recently, a sialome analysis of three *Aedes aegypti* mosquito colonies (*PAEA*, *Rockefeller*, and *Formosus*) carried out in our laboratory allowed us to identify 44 saliva proteins. Of these secreted proteins, none was exclusively expressed in one colony, suggesting that expression of salivary proteins is highly conserved across populations. In another study, we reported that some of these salivary proteins could be used as the genus-specific markers for travelers' exposure to mosquito vectors. Here, comparison of salivary gland protein profiles between these same three *Ae. aegypti* colonies was performed using the one-dimensional SDS-PAGE difference gel electrophoresis method. As observed at the saliva level, no significant differences were detected between these three colonies. The salivary gland protein repertoire from the *Ae. aegypti* mosquito was analyzed using a proteomic approach. One hundred and twenty proteins were identified in these salivary glands representing the largest description of the *Ae. aegypti* salivary gland protein catalog. We succeeded in identifying 15 secreted proteins, some of which have already been reported as being involved in blood feeding. A comparison of the proteins identified between the salivary glands and the sialome is discussed.

Key Words: *Ae. aegypti*—Proteome—Salivary glands.

Introduction

Mosquitoes from the *Aedes* genus (e.g., *Aedes aegypti* and *Aedes albopictus*) transmit arboviruses such as the Dengue, Yellow fever, or Chikungunya viruses to humans, causing significant morbidity and mortality throughout the world (Halstead 1988, Monath 1994, James 1996). Few vaccines or antiviral agents are available, and ways of controlling the transmission of these pathogens are needed. Pathogen transmission can occur in two ways during blood feeding: (i) when an adult female mosquito ingests infected blood from humans or (ii) via salivation from an infected mosquito to humans. This phenomenon requires interactions between virus and host salivary gland cells through cell receptors (Yazi Mendoza et al. 2002, Salas-Benito et al. 2007). Salivary glands are used as reservoirs for the viruses that are injected during blood meals (Beerntsen et al. 2000). The salivary glands of arthropods thus play a key function in virus transmission.

At the bite site, mosquitoes inject salivary proteins, which are produced in the salivary glands and facilitate blood meals. These salivary proteins counteract vertebrate hemostasis (platelet aggregation, blood clotting, and vasoconstriction), and may modulate the human immune response (Ribeiro 1995, Ribeiro and Francischetti 2003). The regurgitated proteins may also induce an antibody response against the salivary antigens of arthropods in people living in endemic areas (Gillespie et al. 2000, Remoue et al. 2006, 2007), or in travelers transiently exposed to vectors in tropical areas (Orlandi-Pradines et al. 2007). This antibody response could be used to distinguish *Anopheles* sp. from *Aedes* sp. exposure

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(Orlandi-Pradines et al. 2007). Mosquito saliva could be used as an epidemiological marker for evaluating individual human exposure to mosquito bites. Taken together, these data suggest that better knowledge of saliva and salivary gland components is an important issue for understanding the mechanisms of blood feeding and virus transmission, as well as for evaluating human exposure to vectors.

Recently, we compared the relative abundance of saliva proteins collected from three *Ae. aegypti* colonies (Almeras et al. 2008). We observed that protein profiles were superimposed, despite significant variations in the relative abundance of two protein bands. For the first time, based on a proteomic approach, 44 distinct proteins were identified in the saliva collected from *Ae. aegypti* mosquitoes (Orlandi-Pradines et al. 2007, Almeras et al. 2008). These observations will serve as a basis for future work to determine the possible role of these proteins in blood feeding success and viral transmission, or their use as antigenic epidemiological markers to measure human exposure to *Aedes* mosquitoes.

However, collecting mosquito saliva is extremely time consuming and labor intensive, so most research groups prefer to work on mosquito salivary glands. To investigate the mosquito salivary protein repertoire, the salivary-gland cDNA library from adult females was randomly sequenced, and analyzing the transcripts made it possible to predict the protein secreted. Sialotranscriptome from several mosquito species such as *Anopheles gambiae* (Arca et al. 1999, 2005, Francischetti et al. 2002, Calvo et al. 2006b), *Anopheles stephensi* (Valenzuela et al. 2003), *Anopheles darlingi* (Calvo et al. 2004), *Anopheles funestus* (Calvo et al. 2007), *Anopheles dirus B* (Diptera: Culicidae) (Jariyapan et al. 2006), *Ae. aegypti* (Valenzuela et al. 2002, Ribeiro et al. 2007), *Ae. albopictus* (Arca et al. 2007), or *Culex pipiens quinquefasciatus* (Ribeiro et al. 2004), and *C. pipiens pallens* (Chen et al. 2007) were performed. These descriptions of salivary gland transcripts play a part in improving our knowledge of the components of saliva and the discovery of new pharmacological agents. However, very few data are available regarding the proteins genuinely expressed in the salivary glands of *Ae. aegypti* mosquitoes. Despite the description of 614 transcripts in the salivary glands of *Ae. aegypti*, only 24 proteins have been identified by mass spectrometry (MS) (Ribeiro et al. 2007).

The objectives of the present study were (i) to compare the salivary gland protein profiles from three *Ae. aegypti* colonies (*Rockefeller*, *PAEA*, and *Formosus*) that are maintained under highly inbred laboratory rearing using one-dimensional SDS-PAGE difference gel electrophoresis (1D DIGE), and (ii) to investigate the salivary gland protein repertoire from these colonies by means of a proteomic approach. Finally, a comparison of the proteins identified between the salivary glands and saliva will be discussed.

Materials and Methods

Mosquitoes and salivary glands extraction

Three populations of 10-day-old uninfected *Ae. aegypti* mosquitoes, which differed in their origins and laboratory colonization histories, were used in this study (Table 1). Adult female *Ae. aegypti* from *Formosus*, *Rockefeller*, and *PAEA* colonies were reared at the Institut Pasteur (Paris) and maintained under strictly identical standard conditions: 26°C and 60% humidity. Mosquitoes were selected 2 days after their first blood feeding on rabbit blood maintained at 37°C. The salivary glands from adult mosquito females were dissected using a fine entomological needle under a stereomicroscope at 4× magnification, in two independent experiments (at 2-month intervals), in which the mosquitoes from the three colonies were handled at the same time under strictly identical conditions. The salivary glands from each experiment were pooled by colony into a microcentrifuge tube on ice in phosphate-buffered saline and then stored frozen at −20°C until needed.

Table 1. Characteristics of the *Aedes aegypti* Colonies

Ae. aegypti *colonies*	*Origin*	*Collection date (length of time in laboratory, years)*
Formosus	Senegal (Kedougou)	2002 (5)
Rockefeller	Caribbean	~1950 (~50)
PAEA	Tahiti (Papeete)	1987 (20)

CyDye labeling and SDS-PAGE

The salivary glands were disrupted by ultrasonication (Vibracell 72412; Bioblock Scientific, Illkirch, France) for 5 min on ice at maximum amplitude. Salivary gland homogenates (SGH) were centrifuged for 15 min at 16,100 *g*, and the protein concentration of the supernatant was determined in duplicate using the Lowry method (DC Protein assay Kit; Bio-Rad) according to the manufacturer's instructions. Salivary gland proteins were then concentrated by precipitation with acetone (Sigma, St. Louis, MI), and were suspended in a buffer containing 8 M urea (Sigma), 2 M thiourea (Sigma), 4% (w/v) CHAPS (Sigma), and 30 mM Tris (Sigma), adjusted to pH 8.5 to obtain a protein concentration adjusted to 2.5 μg/μL.

The salivary gland proteins were then minimally labeled with CyDye according to the manufacturer's recommended protocols. Briefly, proteins (25 μg) were labeled with 200 pmol of either cyanine 5 (Cy5) or Cy3 or Cy2 (GE Healthcare, UK), freshly dissolved in anhydrous DMF (Sigma), and incubated on ice for 30 min in the dark. The reaction was quenched with 1 μL of free lysine (10 nM; Sigma) by incubation of 10 min on ice. Cy5, Cy3, and Cy2 labeled samples were then pooled, and an equal volume of 2×Tris buffer was added containing 5% (w/v) SDS (Sigma). For protein separation, 15 μg of pooled labeled samples reduced with 1% (w/v) dithiothreitol (Sigma) was loaded per lane on to a 10% SDS-PAGE.

Image analysis

After electrophoresis, the gels with CyDye-labeled proteins were scanned three times with a Typhoon™ Trio Image scanner (GE Healthcare), each time at different excitation wavelengths (Cy3, 580 BP 30/green [532 nm]; Cy5, 670 BP 30/red [633 nm]; Cy2, 520 BP 40/blue [488]). Prescans were performed to adjust the photomultiplier tube voltage to obtain images with a maximum intensity of 60,000 to 80,000 U. Images of salivary gland profiles were further analyzed using ImageQuant™ TL software (GE Healthcare). Background subtraction was performed and the densitometry profiles were normalized to take into account global differences. Relative abundance in proteins from each band was estimated

by dividing the area under the curve of the peak corresponding to the band by the sum total of the areas under the curves for all the bands. The gels were then stained with Sypro Ruby (Bio-Rad) according to the manufacturer's protocol.

In-gel tryptic digest

Excised bands from the Sypro Ruby–stained gels were prepared as described previously by Almeras et al. (2008). Samples were then stored at −20°C before their analysis with MS.

MS analysis

The resulting peptides were extracted from the gel and analyzed by nanoscale capillary liquid chromatography-tandem MS (nano LC-MS/MS). Purification and analysis were performed on a C18 capillary column using a CapLC system (Waters, Milford, MA) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Ultima; Waters). Chromatographic separations were conducted on a reversed-phased capillary column (Atlantis™ dC18, 3 μm, 75 μm×150 mm Nano Ease™; Waters) with a 180–200 nL/min flow. The gradient profile consisted in a linear gradient from 95% A (H_2O, 0.1% HCOOH) to 60% B (80% acetonitrile, 0.1% HCOOH) in 60 min followed by a linear gradient to 95% B in 10 min. Mass data acquisitions were piloted by MassLynx 4.0 software using automatic switching between MS and MS/MS modes. The internal parameters of Q-TOF were set as follows. The electrospray capillary voltage was set to 3.2 kV, the cone voltage was set to 30 V, and the source temperature was set to 80°C. The MS survey scan was m/z 400–1300 with a scan time of 1 s and an interscan time of 0.1 s. When the intensity of a peak rose above a threshold of 15 counts, tandem mass spectra were acquired. Normalized collision energies for peptide fragmentation were set using the charge-state recognition files for +2 and +3 peptide ions. The scan range for MS/MS acquisition was from m/z 50 to 1500 with a scan time of 1 s and an interscan time of 0.1 s. Fragmentation was performed using argon as the collision gas and with the collision energy profile optimized for various mass ranges and charge of precursor ions. Mass data collected during a nano LC-MS/MS analysis were processed using ProteinLynx Global Server 2.2 software (Waters) with the following parameters: no background subtraction, smooth 3/2 Savitzky Golay and no deisotoping to generate peak lists in the micromass pkl format. Pkl files were then fed into a local search engine Mascot Daemon v2.2.2 (Matrix Science, London, UK). The data were screened against the National Center for Biotechnology Information nonredundant protein database (January 10, 2008) with other *Metazoa* (273,686 sequences) as a taxonomy setup. Search parameters allowed for one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine; precursor and product ion mass error tolerance was <0.2 Da. All identified proteins had a Mascot score greater than 67, corresponding to a statistically significant ($p < 0.05$) confident identification.

Statistical analysis

Differences in the relative abundance of each protein band between the colonies were analyzed taking into account the hypothetical differences between gels or experiments using two-way analysis of variance, with the colony as a factor and the gel or the experiment as a cofactor. To take into account that multiple tests performed, we considered significant p-values after the Bonferroni correction: $p < 0.05/30 \approx 0.0017$. All statistical analyses were done with SAS software version 9.1 (SAS Institute, Cary, NC).

Results and Discussion

The most frequent strategy employed to study the molecules expressed in the salivary glands of mosquitoes encompasses either random sequencing of clones from salivary gland cDNA libraries or uses a signal trapping method for specific isolation of cDNA-encoding proteins with signal peptides (Arca et al. 2002, Francischetti et al. 2002, Lanfrancotti et al. 2002, Valenzuela et al. 2003, Calvo et al. 2004). Although emerging evidence suggests that transcriptome profiling is necessary, this approach seems to be insufficient for comprehensive delineation of biological systems. It is possible that some transcripts identified might not be expressed at the protein level (Mounsey et al. 2002). Conversely, if a protein is able to be identified inside a tissue, the corresponding transcript can be automatically designated as a protein-coding region. Therefore, in addition to monitoring gene expression at the transcriptional level, large-scale analysis of the proteome is also important for the understanding of the cellular, metabolic, and regulatory networks in living organisms or specific tissues.

In this study, we carried out a proteomic analysis of salivary gland proteins from three *Ae. aegypti* colonies, and compared protein identifications between the salivary glands and sialomes from these mosquitoes. Good reproducibility of salivary gland protein profiles was observed between colonies, and more than 100 distinct proteins were successfully identified.

Comparative analysis of salivary gland protein profiles from *Ae. aegypti* colonies

Recently, we performed a sialome (i.e., proteins present in saliva) protein profile comparison between three *Ae. aegypti* mosquito colonies (*PAEA*, *Rockefeller*, and *Formosus*) reared under identical laboratory conditions (Almeras et al. 2008). Despite significant quantitative differences for only two protein bands, salivary profile analysis indicated that major proteins were detectable in the three colonies. These data suggested that *Ae. aegypti* colonies conserved their own species characteristics. In this study, we compared the salivary gland protein profiles from these same three *Ae. aegypti* colonies. A 1D DIGE method was chosen to increase the accuracy of this comparison, providing high sensitivity and reproducibility between experiments. DIGE technology, using sample multiplexing and the linear dynamic range of fluorescent protein labeling, allows small differences to be accurately detected and quantified with statistical confidence, rather than conventional one-sample-per-lane techniques (Chakravarti et al. 2005, Timms and Cramer 2008).

SGH from *PAEA*, *Rockefeller*, and *Formosus* colonies were thus, respectively, labeled with Cy5, Cy3, and Cy2, followed by 1D SDS-PAGE separation of the salivary glands. Protein profiles analyzed with Image Quant TL software made it possible to detect numerous bands with molecular weights

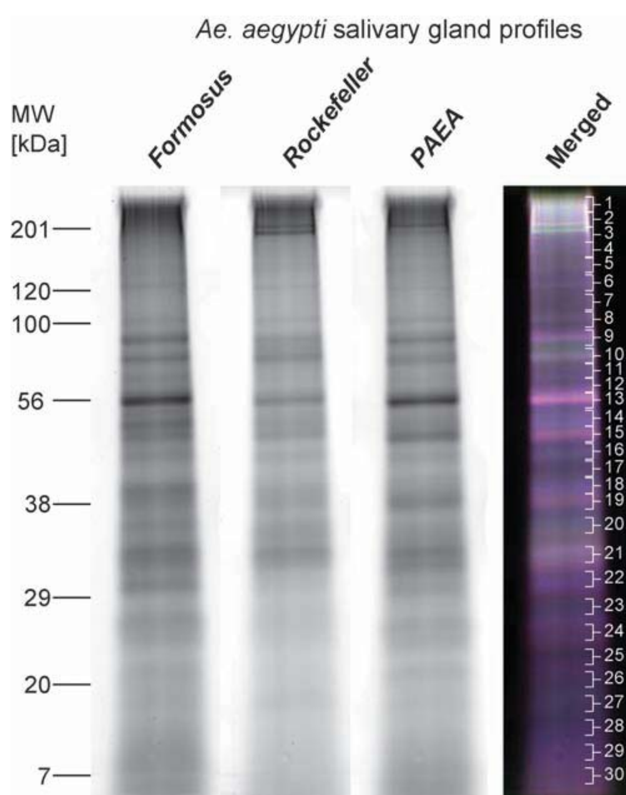

FIG. 1. Comparative salivary gland protein profiles of three *Aedes aegypti* colonies. Salivary gland proteins collected from *Formosus*, *Rockefeller*, and *PAEA Ae. aegypti* colonies were, respectively, labeled with cyanine 2 (Cy2), Cy3, and Cy5, before being mixed and separated on 10% SDS-PAGE gels. Salivary gland protein profiles from each *Ae. aegypti* colony, and the merged protein profiles are indicated at the top of the gel. The numbers on the right side of the gel correspond to the 30 bands excised for further analysis by mass spectrometry. Band identity is listed in Table 2. Standard molecular masses are indicated on the left side. MW, molecular weight.

ranging from about 7 to 225 kDa (Fig. 1). Thirty protein bands could be detected on the gel for each colony; however, these individual bands presented a wide dynamic concentration range (i.e., the bands differed in intensity; Fig. 1). For the three *Ae. aegypti* colonies, seven predominant bands were observed (band numbers 9, 10, 13, 15, 19, 21, and 22; Fig. 1).

Before comparing protein profiles between colonies, the variations associated with the sample collection (experimental effect) and with protein migration (gel effect) were assessed. Two gels were then performed under the same conditions as the gel presented in Figure 1. A pool of labeled samples collected at two time points (as described in the Materials and Methods section) were loaded on each gel (data not shown). A densitometric scan and normalization of the gels were performed, giving an accurate comparison of the salivary gland protein profiles between colonies. Statistical tests (analysis of variance) were performed to determine bands that differed in intensity between colonies, taking into account gel or experimental effects as described previously (Almeras et al. 2008). No statistically significant difference was detected between the three *Ae. aegypti* colonies. Several hypotheses could explain this result: first, as observed previously in the sialome (Almeras et al. 2008), the three colonies conserved their own species characteristics limiting protein expression variations between these mosquito colonies; second, despite the high sensitivity and reproducibility of CyDye protein labeling, the 1D DIGE technique is insufficient for detecting significant protein variations (perhaps additional experiments with a better protein separation method [such as 2D DIGE] could be performed to verify protein pattern reproducibility between these three colonies); third, as observed in a previous *Ae. aegypti* sialome analysis (Almeras et al. 2008), a long history of laboratory rearing could have limited environmental pressures and induced a homogenization of salivary gland protein repertoires. To determine if inbred rearing has induced a decrease in protein colony singularity, a comparison of salivary gland protein profiles between these colonies and their counterparts collected in the field would have to be performed. In summary, this analysis shows that despite variations in band intensity, no statistically significant differences were noted between the three colonies, suggesting low variability in protein expression as observed at the saliva level.

Identification of Ae. aegypti *salivary gland proteins*

Thirty bands numbered in Figure 1 were excised and submitted to trypsin digestion before an analysis of peptide mixture by MS (LC-MS/MS) for identification. Each protein band was analyzed twice. Only the protein band numbered 30 failed to be identified by MS. All the other bands excised allowed the identification of at least one protein, corresponding to 164 proteins identified (Table 2). As expected, several proteins could be identified in each excised band, such as bands 8 and 21, which contain 7 and 5 proteins, respectively (Table 2). Inversely, the same protein was also detected in several excised bands, such as malate dehydrogenase (gi|108875864, identified in bands 20, 21, and 22) or ADP, ATP carrier protein (gi|108872852, identified in bands 11, 22, 23, and 24). The 164 proteins identified effectively correspond to 120 distinct proteins according to their NCBI numbers (Table 2).

The proteins identified were classified according to their known or predicted cellular localization and biological function (Fig. 2). Although more than 50% of the proteins identified were assigned in the cytoplasmic and mitochondrial compartments, 15 proteins were classified as secreted salivary proteins representing the third group in terms of the number of proteins identified (12.5%). Of these secreted proteins, 11 (9.2%) were involved in mosquito blood-feeding. Some of these secreted proteins have been described as modulating host immune response (members of the D7 family (gi|108877769 and gi|108877768) (Calvo et al. 2006a), adenosine deaminase (gi|108878609) (Hasko et al. 2000), apyrase (gi|108877845) (Sun et al. 2006), purine hydrolase (gi|18568280), and 30 kDa allergen (gi|94468546, gi|94468552, and gi|18568322) (Ribeiro et al. 2007). Members of the serpin family (gi|108881841 and gi|18568304) have been implicated in the regulation of blood coagulation (Gettins 2002). The secreted ferritin G subunit (gi|108876699) has an antioxidant function and could be used to store iron (Dunkov

Table 2. Identified Proteins from Salivary Gland Profiles of the *Aedes aegypti* Colonies

Band number excised	*Protein name*	*Accession no.*[a]	*Molecular weight (kDa)*		*Coverage (%)*	*Number of MS/MS peptide sequences identified*	*Significance (Mascot score)*
			Theoretical	*Observed*			
1	Myosin heavy chain, nonmuscle or smooth muscle (*Ae. aegypti*)	gi|108878633	222.35	225	20	35	2028.06
	SGS1 (*Ae. aegypti*)[b]	gi|66828491	346.51		5	15	733.86
	Conserved hypothetical protein (*Ae. aegypti*)	gi|108873896	369.18		4	13	643.09
	na+/k+ atpase alpha subunit (*Ae. aegypti*)	gi|108871576	111.86		4	3	239.84
	Actin (*Ae. aegypti*)[d]	gi|108879763	42.15		12	4	172.45
2	Myosin heavy chain, nonmuscle or smooth muscle (*Ae. aegypti*)	gi|108878633	222.35	211	14	22	1256.12
	Conserved hypothetical protein (*Ae. aegypti*)	gi|108873896	369.18		5	16	705.79
3	Myosin heavy chain, nonmuscle or smooth muscle (*Ae. aegypti*)	gi|108878633	222.35	201	36	63	3664.63
	Vitellogenin-B (*Ae. aegypti*)[b]	gi|37528873	250.39		4	8	389.27
	Glutamate synthase (*Ae. aegypti*)	gi|108868750	231.67		2	4	145.47
4	Myosin heavy chain, nonmuscle or smooth muscle (*Ae. aegypti*)	gi|108878633	222.35	168	9	18	1106.45
	Mitochondrial ATP synthase alpha subunit (*Ae. aegypti*)	gi|94468442	59.52		7	3	151.76
	Actin (*Ae. aegypti*)[d]	gi|108879764	42.05		7	3	138.12
5	Myosin heavy chain, nonmuscle or smooth muscle (*Ae. aegypti*)	gi|108878633	222.35	141	10	21	1005.36
	Pyruvate carboxylase (*Ae. aegypti*)	gi|550486	132.85		6	7	312.53
	na+/k+ atpase alpha subunit (*Ae. aegypti*)	gi|108871577	111.91		5	4	311.51
	Conserved hypothetical protein (*Ae. aegypti*)	gi|108879668	71.58		10	6	289.44
	Stretchin-mlck (*Ae. aegypti*)	gi|108876801	80.19		6	4	262.23
	Pupal-specific flight muscle actin (*Ae. aegypti*)	gi|41393662	41.84		18	6	242.55
	Fructose-bisphosphate aldolase (*Ae. aegypti*)	gi|108878478	39.97		13	4	226.9
6	na+/k+ atpase alpha subunit (*Ae. aegypti*)	gi|108871577	111.91	124	19	15	1105.81
	2-Oxoglutarate dehydrogenase (*Ae. aegypti*)	gi|108877402	115.04		19	17	842.19
	Paramyosin, long form (*Ae. aegypti*)	gi|108872770	103.39		13	11	592.78
	Calcium-transporting atpase sarcoplasmic/endoplasmic reticulum type (calcium pump) (*Ae. aegypti*)	gi|108877602	110.55		11	10	540.78
	Myosin heavy chain, nonmuscle or smooth muscle (*Ae. aegypti*)	gi|108878633	222.35		2	4	258.77
7	Glycogen phosphorylase (*Ae. aegypti*)	gi|108884025	97.35	111	20	17	784.28
	Alpha-actinin (*Ae. aegypti*)	gi|108876788	104.20		12	10	612.73
	Elongation factor 2 (*Ae. aegypti*)	gi|12667408	95.31		9	8	335.54
	Spermatogenesis associated factor (*Ae. aegypti*)	gi|108873203	89.43		9	7	275.19
	Dynamin (*Ae. aegypti*)	gi|108876817	94.24		6	4	243.88
	Actin (*Ae. aegypti*)[d]	gi|108879763	42.15		12	4	220.54
	Paramyosin, long form (*Ae. aegypti*)	gi|108872770	103.39		4	4	199.61

(*continued*)

Table 2. (Continued).

Band number excised	Protein name	Accession no.[a]	Molecular weight (kDa)		Coverage (%)	Number of MS/MS peptide sequences identified	Significance (Mascot score)
			Theoretical	Observed			
8	Aconitase, mitochondrial (*Ae. aegypti*)	gi|108870681	86.41	102	56	31	1736.69
	Glutamate semialdehyde dehydrogenase (*Ae. aegypti*)	gi|108877298	87.53		20	14	766.36
	Heat shock protein (*Ae. aegypti*)	gi|108868694	81.82		10	7	354.58
	Heat shock cognate 70 (*Ae. aegypti*)[d]	gi|94468818	72.35		10	6	284.42
	Conserved hypothetical protein (*Ae. aegypti*)	gi|108873896	369.18		1	3	216.65
	Conserved hypothetical protein (*Ae. aegypti*)	gi|108873839	81.32		10	7	218.96
	Myosin heavy chain, nonmuscle or smooth muscle (*Ae. aegypti*)	gi|108878534	222.99		2	2	159.16
9	Conserved hypothetical protein (*Ae. aegypti*)	gi|108873896	369.18	93	6	18	1169.56
	NADH-ubiquinone oxidoreductase (*Ae. aegypti*)	gi|108871047	80.11		18	10	611.62
	3-Hydroxyacyl-coa dehyrogenase (*Ae. aegypti*)	gi|108873690	82.87		9	6	324.3
	Heat shock cognate 70 (*Ae. aegypti*)[d]	gi|94468966	71.38		12	7	357.15
	Moesin/ezrin/radixin (*Ae. aegypti*)	gi|108876119	69.13		9	6	272.3
	Glycerol-3-phosphate dehydrogenase (*Ae. aegypti*)	gi|108880571	81.65		7	5	265.87
	Tropomyosin invertebrate (*Ae. aegypti*)	gi|108881790	29.54		15	4	207.76
10	Transferrin precursor (*Ae. aegypti*)[b]	gi|2645497	71.70	81	32	18	942.53
	Apyrase, putative (*Ae. aegypti*)[b–d]	gi|108877845	63.17		23	11	547.61
	Proline oxidase (*Ae. aegypti*)	gi|108870085	44.60		21	8	408.27
	Tropomyosin invertebrate (*Ae. aegypti*)	gi|108881789	32.75		18	5	292.78
	Vitellogenin-C (*Ae. aegypti*)[b]	gi|37528871	243.28		3	6	273.14
	Succinate dehydrogenase (*Ae. aegypti*)	gi|108873156	72.80		8	5	230.48
11	Chaperonin-60kD, ch60 (*Ae. aegypti*)	gi|108872102	61.15	72	14	7	307.99
	Transketolase (*Ae. aegypti*)	gi|108879967	68.46		11	6	285.15
	Bifunctional purine biosynthesis protein (*Ae. aegypti*)	gi|108870755	64.64		7	4	185.01
	Proline oxidase (*Ae. aegypti*)	gi|108870085	44.60		8	3	173.47
	Apyrase, putative (*Ae. aegypti*)[b–d]	gi|108877845	63.17		5	2	110.22
	Lamin (*Ae. aegypti*)	gi|108881054	64.01		4	3	100.61
	Failed axon connections protein, putative (*Ae. aegypti*)	gi|108881007	34.51		12	3	98.32
	Cytochrome P450 (*Ae. aegypti*)	gi|108877345	62.65		5	2	95.91
	Actin (*Ae. aegypti*)[d]	gi|108879764	42.05		5	2	86.32
	adp,atp carrier protein (*Ae. aegypti*)	gi|108872852	28.85		9	2	73.15
12	Pyrroline-5-carboxylate dehydrogenase (*Ae. aegypti*)	gi|108878912	63.63	66	27	14	666.26
	Chaperonin-60kD, ch60 (*Ae. aegypti*)	gi|108872102	61.15		19	9	526.56
	Malic enzyme (*Ae. aegypti*)	gi|108883625	72.55		10	6	320.16
	Adenosine deaminase (*Ae. aegypti*)[b,d]	gi|108878609	59.94		17	7	331.37
	Pyruvate kinase (*Ae. aegypti*)	gi|108868621	58.04		18	7	305.23
	α-tubulin (*Ae. aegypti*)	gi|94468850	50.56		11	4	198.02
	Actin (*Ae. aegypti*)[d]	gi|108879764	42.05		9	3	191.16

(*continued*)

Table 2. (Continued).

Band number excised	Protein name	Accession no.[a]	Molecular weight (kDa)		Coverage (%)	Number of MS/MS peptide sequences identified	Significance (Mascot score)
			Theoretical	Observed			
13	Mitochondrial ATP synthase alpha subunit (*Ae. aegypti*)	gi|94468442	59.52	57	62	26	1626.05
	ATP synthase beta subunit (*Ae. aegypti*)	gi|108881686	53.87		55	19	1251.26
	β-4 tubulin (*Ae. aegypti*)	gi|111035022	50.59		39	12	670.51
14	ATP synthase beta subunit (*Ae. aegypti*)	gi|108881105	53.94	51	35	13	844.37
	Mitochondrial ATP synthase alpha subunit (*Ae. aegypti*)	gi|94468442	59.52		25	13	777.66
	Enolase (*Ae. aegypti*)	gi|108882996	46.87		28	9	546.64
	Phosphoglycerate kinase (*Ae. aegypti*)	gi|18091771	44.05		25	10	503.85
	Pupal-specific flight muscle actin (*Ae. aegypti*)	gi|41393662	41.84		31	9	502.26
	Mitochondrial processing peptidase beta subunit (*Ae. aegypti*)	gi|108878872	52.84		18	8	487.53
	Serine protease inhibitor (serpin-4), putative (*Ae. aegypti*)[b]	gi|108881841	50.03		23	9	484.3
	Translation elongation factor EF-1 alpha/Tu (*Ae. aegypti*)	gi|94468780	50.78		25	10	458.31
	Succinyl-coa synthetase beta chain (*Ae. aegypti*)	gi|108871926	48.64		16	7	420.09
	Putative serpin (*Ae. aegypti*)[b,d]	gi|18568304	47.31		17	6	320.72
	Aspartate ammonia lyase (*Ae. aegypti*)	gi|108875839	54.78		14	5	308.42
	Actin (*Ae. aegypti*)[d]	gi|108879764	42.05		17	5	260.23
	Imaginal disc growth factor (*Ae. aegypti*)	gi|108882601	48.28		10	4	239.51
15	Ubiquinol-cytochrome c reductase complex core protein (*Ae. aegypti*)	gi|108879066	45.88	45	33	11	780.32
	Pupal-specific flight muscle actin (*Ae. aegypti*)	gi|41393662	41.84		36	10	686.74
	Citrate synthase (*Ae. aegypti*)	gi|108881547	51.85		29	12	577.39
	Fructose-bisphosphate aldolase (*Ae. aegypti*)	gi|108878478	39.97		21	6	400.63
	Creatine kinase (*Ae. aegypti*)	gi|94468822	40.19		29	8	351.2
16	Fructose-bisphosphate aldolase (*Ae. aegypti*)	gi|108878479	39.55	41	48	14	954.41
	Creatine kinase (*Ae. aegypti*)	gi|94468822	40.19		51	14	832.83
	Isocitrate dehydrogenase (*Ae. aegypti*)	gi|108884328	44.04		32	10	535.44
	Pyruvate dehydrogenase (*Ae. aegypti*)	gi|108869893	42.80		10	4	211.72
	d-3-phosphoglycerate dehydrogenase (*Ae. aegypti*)	gi|108878977	35.72		15	4	184.92
	Sodium/potassium-dependent atpase beta-2 subunit (*Ae. aegypti*)	gi|108872996	36.37		22	5	174.87
	Putative purine hydrolase (*Ae. aegypti*)	gi|18568280	38.16		7	3	123.13
	Tropomyosin invertebrate (*Ae. aegypti*)	gi|108881790	29.54		8	2	84.51
17	Tropomyosin invertebrate (*Ae. aegypti*)	gi|108881788	31.03	40	31	9	396.92
	ATP synthase beta subunit (*Ae. aegypti*)	gi|108881105	53.94		6	3	181.52

(*continued*)

Table 2. (Continued).

Band number excised	Protein name	Accession no.[a]	Molecular weight (kDa)		Coverage (%)	Number of MS/MS peptide sequences identified	Significance (Mascot score)
			Theoretical	Observed			
18	Glycerol-3-phosphate dehydrogenase (*Ae. aegypti*)	gi|108883108	39.44	39	34	9	614.75
	Tropomyosin invertebrate (*Ae. aegypti*)	gi|108881786	32.47		29	9	477.96
	D7 protein, putative (*Ae. aegypti*)[b–d]	gi|108877769	37.44		14	5	210.98
19	ATP synthase beta subunit (*Ae. aegypti*)	gi|108881105	53.94	38	27	11	687.94
	D7 protein, putative (*Ae. aegypti*)[b–d]	gi|108877769	37.44		28	10	435.98
	Tropomyosin invertebrate (*Ae. aegypti*)	gi|108881783	32.61		24	7	415.68
	Glycerol-3-phosphate dehydrogenase (*Ae. aegypti*)	gi|108883107	39.83		19	5	261.53
20	D7 protein, putative (*Ae. aegypti*)[b–d]	gi|108877768	39.17	36	6	2	88.63
	Malate dehydrogenase (*Ae. aegypti*)	gi|108875864	45.04		19	7	427.58
	Electron transport oxidoreductase (*Ae. aegypti*)	gi|108869776	34.44		12	3	207.17
	F0F1-type ATP synthase b subunit (*Ae. aegypti*)[d]	gi|94468834	53.93		6	3	177.09
	Putative 34 kDa secreted protein (*Ae. aegypti*)[b,d]	gi|18568296	36.38		10	3	172.42
	Succinyl-CoA synthetase alpha subunit (*Ae. aegypti*)	gi|94468890	34.79		9	3	163.5
	Prohibitin (*Ae. aegypti*)	gi|108871326	33.12		12	4	141.06
	Tropomyosin invertebrate (*Ae. aegypti*)	gi|108881790	29.54		12	3	116.27
21	Malate dehydrogenase (*Ae. aegypti*)	gi|108875864	45.04	34	37	13	768.73
	14-3-3 protein sigma, gamma, zeta, beta/alpha (*Ae. aegypti*)	gi|108872598	29.60		21	5	271.81
	Vacuolar ATP synthase subunit E (*Ae. aegypti*)	gi|94469084	25.72		17	4	191.63
	Succinyl-CoA synthetase alpha subunit (*Ae. aegypti*)	gi|94468890	34.80		11	3	183.81
	Mitochondrial porin (*Ae. aegypti*)	gi|94468842	30.79		16	4	173.1
22	14-3-3 protein sigma, gamma, zeta, beta/alpha (*Ae. aegypti*)	gi|108872598	29.60	31	41	9	550.16
	ATP synthase gamma subunit (*Ae. aegypti*)	gi|108875130	33.01		31	8	499.79
	Multifunctional 14-3-3 family chaperone (*Ae. aegypti*)	gi|94468884	28.32		33	7	443.26
	Mitochondrial porin (*Ae. aegypti*)	gi|94468842	30.79		29	7	363.65
	Putative 30 kDa allergen-like protein (*Ae. aegypti*)[b–d]	gi|18568322	23.79		23	5	239.35
	adp,atp carrier protein (*Ae. aegypti*)	gi|108872852	28.85		19	5	172.44
	30 kDa salivary gland allergen variant 3 (*Ae. aegypti*)[b,d]	gi|94468552	27.91		11	3	167.84
	ADP/ATP translocase (*Ae. aegypti*)	gi|94468376	33.21		14	4	166.24
	Vacuolar ATP synthase subunit E (*Ae. aegypti*)	gi|94469084	25.72		20	4	151.98
	Malate dehydrogenase (*Ae. aegypti*)	gi|108875864	45.04		7	3	149.5

(*continued*)

Table 2. (Continued).

Band number excised	Protein name	Accession no.[a]	Molecular weight (kDa) Theoretical	Molecular weight (kDa) Observed	Coverage (%)	Number of MS/MS peptide sequences identified	Significance (Mascot score)
23	Phosphoglycerate mutase (*Ae. aegypti*)	gi|108878150	28.59	28	40	9	562.34
	ADP/ATP translocase (*Ae. aegypti*)	gi|94468376	33.21		31	10	441.23
	Multifunctional 14-3-3 family chaperone (*Ae. aegypti*)	gi|94468884	28.32		34	8	433.29
	adp, atp carrier protein (*Ae. aegypti*)	gi|108872852	28.85		29	8	369.73
	30 kDa salivary gland allergen variant 2 (*Ae. aegypti*)[b,c]	gi|94468546	29.42		17	5	276.02
	Electron transfer flavoprotein beta-subunit (*Ae. aegypti*)	gi|108879274	22.90		22	5	204.1
	Putative 30 kDa allergen-like protein (*Ae. aegypti*)[b–d]	gi|18568322	23.79		15	3	173.73
	Conserved hypothetical protein (*Ae. aegypti*)	gi|108873666	23.63		12	3	163.2
	NADH-ubiquinone oxidoreductase 24 kda subunit (*Ae. aegypti*)	gi|108878789	23.08		18	3	116.94
24	Triosephosphate isomerase (*Ae. aegypti*)	gi|108882001	26.70	26	32	6	340.44
	Peroxiredoxin 6, prx-6 (*Ae. aegypti*)	gi|108882310	25.10		29	5	210.96
	Secreted ferritin G subunit precursor, putative (*Ae. aegypti*)	gi|108876699	17.68		27	3	186.04
	Creatine kinase (*Ae. aegypti*)	gi|94468822	40.19		6	2	108.69
	Mitochondrial ATP synthase alpha subunit (*Ae. aegypti*)	gi|94468442	59.52		4	2	105.66
	Conserved hypothetical protein (*Ae. aegypti*)	gi|108883588	21.79		14	2	104.93
	adp, atp carrier protein (*Ae. aegypti*)	gi|108872852	28.85		9	2	102.97
	Troponin i (*Ae. aegypti*)	gi|108872887	24.66		9	2	95.02
	Ribosomal protein S5 (*Ae. aegypti*)	gi|94468378	24.79		6	1	85.13
25	Ferritin subunit 1, putative (*Ae. aegypti*)	gi|108876700	23.74	23	31	6	313.14
	Adenylate kinase isoenzyme (*Ae. aegypti*)	gi|108874575	22.57		22	4	257.7
	Glutathione s-transferase (*Ae. aegypti*)	gi|108871931	23.26		23	4	196.64
	40S ribosomal protein S8 (*Ae. aegypti*)	gi|94468438	23.40		11	2	122.15
26	Hypothetical protein AaeL_AAEL004249 (*Ae. aegypti*)	gi|157105256	17.24	21	26	3	146.76
	Myosin light chain 1, putative (*Ae. aegypti*)	gi|157167811	18.15		15	2	108.88
	Calcium-binding protein, putative (*Ae. aegypti*)	gi|157119961	22.12		10	3	94.79
27	Myosin light chain 1, putative (*Ae. aegypti*)	gi|157167807	18.30	17	29	4	228.82
	Superoxide dismutase (*Ae. aegypti*)	gi|157127037	15.62		18	2	129.98
	Histone H2B (*Ae. aegypti*)	gi|157137739	13.86		25	3	124.59
	Cytochrome c oxidase subunit iv (*Ae. aegypti*)	gi|157108935	46.92		8	3	119.64
	Histone H4 (*Ae. aegypti*)	gi|157138406	11.40		21	2	106.07

(*continued*)

Table 2. (Continued).

Band number excised	Protein name	Accession no.[a]	Molecular weight (kDa) Theoretical	Molecular weight (kDa) Observed	Coverage (%)	Number of MS/MS peptide sequences identified	Significance (Mascot score)
28	Histone H4 (*Ae. aegypti*)	gi|157138406	11.40	14	40	4	195.63
	Myosin light chain 1, putative (*Ae. aegypti*)	gi|157167807	18.30		22	3	163.55
	Mitochondrial NADH-ubuiquinone oxidoreductase 13 kDa-B subunit (*Ae. aegypti*)	gi|94469096	13.87		13	2	116.65
29	Cytochrome c oxidase, subunit VB, putative (*Ae. aegypti*)	gi|157119566	14.01	11	20	2	83.15
30	n.i.			7			

The proteins were identified by mass spectrometry after in-gel trypsin digestion. The band name corresponds to the same numbers as indicated in Fig. 1.

The identities of the bands, their [a]NCBi accession numbers, the theoretical and observed molecular weight values, as well as the number of peptide sequences, the corresponding percentage sequence coverage and the Mascot score are listed for MS/MS analysis (Protein scores greater than 67 were considered to be significant ($p < 0.05$)).

Proteins identified previously in *Ae. aegypti* saliva or salivary glands by [b]Almeras et al. (2008), [c]Valenzuela et al. (2002), and [d]Ribeiro et al. (2007).

n.i., no identification; MS, mass spectrometry.

et al. 2002). For the other secreted proteins (e.g., vitellogenin-C [gi|37528871], vitellogenin-B [gi|37528873], SGS1 [gi|66828491], and 34 kDa secreted protein [gi|18568296]), their functions in mosquito saliva are unknown (Ribeiro et al. 2007).

Numerous proteins identified previously in saliva were not found in the salivary glands (Almeras et al. 2008). This could be attributed to the presence of housekeeping proteins in the salivary glands that could mask the detection of the secreted proteins present in smaller quantities. Effectively, numerous proteins involved in cytoskeleton maintenance, transporter function, or nuclear regulation have been characterized by MS analysis (Table 2). However, most of the secreted proteins identified here corresponded to proteins that were detected as predominant bands in the saliva (Almeras et al. 2008). Taken together, these data suggest that only proteins expressed at high levels in *Ae. aegypti* saliva are also detected in their salivary glands. One secreted protein (e.g., ferritin G subunit, gi|108876699), not detected in saliva, was identified in the salivary glands.

It is interesting to note that certain secreted proteins identified in both the saliva and salivary glands of the same *Ae. aegypti* colonies were detected at several molecular weights (Table 2) (Almeras et al. 2008). This observation was more frequently found in the saliva than in the salivary glands. It was the case for D7 protein (detected in three bands in SGH vs. seven bands in the saliva) and the SGS1 protein (one vs. eight bands). These differences could be attributed to maturation phenomena, including posttranslational modifications, which could occur during the transit of the secreted proteins from salivary gland cells to saliva, as evoked previously (Almeras et al. 2008).

Few studies have investigated *Ae. aegypti* salivary gland composition at the protein level. As a result, comparisons of our results with those of others is limited. Valenzuela et al. (2002) identified 10 aminoterminal sequences obtained by Edman degradation of 1D SDS-PAGE separated salivary gland proteins. Ribeiro et al. (2007), using 2D gel electrophoresis, succeeded in identifying 24 salivary gland proteins. Several of these proteins, such as apyrase, members of the D7 protein family, and certain salivary allergens, were also identified in the present study (Table 2). This indicates that

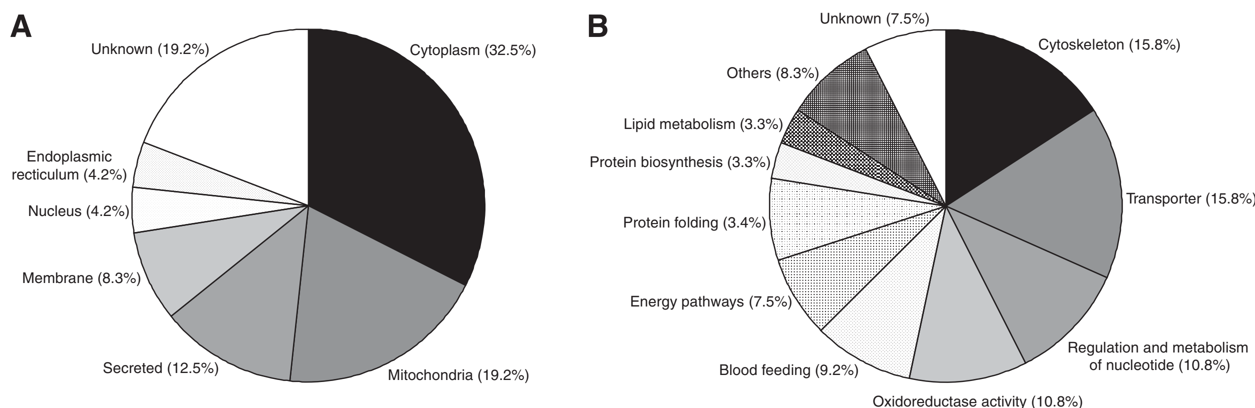

FIG. 2. Pie chart representing the proteins identified by mass spectrometry in the salivary glands of *Ae. aegypti* mosquitoes ($n = 120$). The proteins have been classified according to their cellular localization (**A**) and biological function (**B**). The percentage of proteins associated with each part of the pie chart is indicated in parentheses.

only the more abundant proteins were found in these different studies. Interestingly, of the 120 salivary gland proteins identified, 106 were described at the protein level for the first time (Table 2). This proteomic approach confirms the translation of several transcripts predicted until now only by means of the cDNA base strategy (Valenzuela et al. 2002, Ribeiro et al. 2007).

The salivary gland protein repertoire from other mosquito species and genera using proteomic approaches has been performed for *Ae. albopictus* (Arca et al. 2007), *An. gambiae* (Kalume et al. 2005, Choumet et al. 2007), *An. stephensi* (Valenzuela et al. 2003), and *Culex pipiens quinquefasciatus* (Ribeiro et al. 2004). Regardless of the method (i.e., Edman degradation or MS), the number of secreted proteins identified in the salivary glands in the present and previous studies did not exceed 20, while more than 40 secreted proteins were identified by MS in the saliva of *Ae. aegypti* (Almeras et al. 2008). This suggests that secreted salivary proteins should be screened for preferentially in saliva rather than in the salivary glands.

Concluding remarks

Genome sequencing has made it conceivable to develop proteomic analysis of several mosquito species (Holt et al. 2002, Lawson et al. 2007, Nene et al. 2007). In this study, to investigate the salivary gland protein repertoire of adult female *Ae. aegypti* mosquitoes, a proteomic analysis was performed. One hundred and twenty proteins were identified in these salivary glands, representing the largest description of the *Ae. aegypti* salivary gland protein catalog. Finally, this paper plays a part in identifying 15 secreted proteins, some of which have already been reported to be involved in bloodfeeding. Some of these secreted proteins could be used as antigenic markers for the serological estimation of human exposure to *Ae. aegypti* mosquitoes.

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Disclosure Statement

No competing financial interests exist.

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Ces résultats indiquent que le sialome (protéome de la salive et des glandes salivaires) est relativement homogène entre différentes colonies de moustiques d'une même espèce.

Dans la première étude réalisée sur la salive, 44 protéines salivaires ont été identifiées et l'ensemble des protéines identifiées étaient présentes dans les trois colonies. La comparaison des protéomes salivaires n'a pas permis de mettre en évidence de différences qualitatives mais certaines protéines salivaires présentaient des niveaux d'expression variables selon les colonies.

La deuxième étude a permis d'identifier un total de 120 protéines dans les glandes salivaires des trois colonies d'*Ae. aegypti*, parmi lesquelles 12% sont des protéines sécrétées. Ces protéines secrétées correspondent aux protéines majoritaires retrouvées dans la salive de l'étude citée ci-dessus. L'analyse comparative des profils protéiques des glandes salivaires n'a pas permis de mettre en évidence de différences statistiquement significatives entre colonies, reflétant une homogénéité des répertoires protéiques au sein de l'espèce.

Toutefois, les recherches sur le développement de marqueurs immunologiques basés sur la salive de moustiques peuvent être entravées par l'accès à certaines espèces de moustiques non disponibles en laboratoire. De telles problématiques peuvent se poser lorsque l'on souhaite travailler sur une espèce eurygame[1] qui ne s'accouple pas naturellement dans les cages d'insectariums ou lorsque l'espèce n'est tout simplement pas disponible parce qu'aucun laboratoire ne s'est intéressé à la collecter et à la maintenir en élevage. L'élevage dans des laboratoires non sécurisés de certaines espèces vectrices à fort potentiel de colonisation, comme *Aedes albopictus*, peut aussi poser des problèmes d'ordre éthiques et écologiques. Tous ces facteurs sont des limites à la production de masse de moustiques, qui est nécessaire pour ce type de recherches expérimentales.

[1] Eurygame : Se dit de moustiques qui exigent de grands espaces pour s'accoupler.

La collecte de glandes salivaires sur le terrain représente une alternative pouvant pallier ce problème. Il est en effet possible de prélever les moustiques directement dans leurs habitats. Dans de telles conditions, plusieurs méthodes peuvent être utilisées pour avoir accès à une grande quantité de moustiques, comme la capture sur appâts humains ou la collecte de larves en vue de les faire émerger sous cages. Habituellement, la collecte de glandes salivaires se fait en respectant la chaîne du froid pour éviter toutes dégradations ou modifications protéiques pouvant perturber la recherche de bio-marqueurs d'exposition (Arca et al., 1999, Almeras et al., 2010).

Cependant, le maintien des échantillons collectés au froid peut être parfois difficile sur le terrain, notamment lors du transport en zones tropicales dû à l'isolement de certains sites.

Afin d'améliorer la préservation des échantillons salivaires dans de telles conditions, l'efficacité de différents milieux de collecte a été évaluée dans des conditions de terrains (dissection et stockage pendant 5 jours à température ambiante) sur la préservation des glandes salivaires d'*Ae. aegypti* et d'*An. gambiae* en vue d'une utilisation par des approches immunologiques (ELISA), ou biochimique (SDS-PAGE, Western blot) qui seront utilisées pour détecter de potentiels marqueurs immunologiques d'expositions Nos résultats sont présentés dans l'article N.3.

Article N.3 A. Fontaine, A. Pascual, I. Diouf, N. Bakkali, S. Bourdon, T. Fusai, C. Rogier, L. Almeras, **Mosquito salivary gland protein preservation in the field for immunological and biochemical analysis,** Parasite & vectors *(sous presse)*

ARTICLE N.3

Mosquito salivary gland protein preservation in the field for immunological and biochemical analysis

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Parasites & Vectors *(sous presse)*

Mosquito salivary gland protein preservation in the field for immunological and biochemical analysis.

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Keywords: mosquito, salivary gland, protein preservation, ELISA, immunoblot.

Abstract

Mosquito salivary proteins are involved in several biological processes that facilitate their blood feeding and have also been reported to elicit an IgG response in vertebrates. A growing number of studies have focused on this immunological response for its potential use as a biological marker of exposure to arthropod bites. As mosquito saliva collection is extremely laborious and inefficient, most research groups prefer to work on mosquito salivary glands (SGs). Thus, SG protein integrity is a critical factor in obtaining meaningful data from immunological and biochemical analysis. Current methodologies rely on an immediate freezing of SGs after their collection. However, the maintenance of samples in a frozen environment can be hard to achieve in field conditions. In this study, SG proteins from two mosquito species (*Aedes aegypti* and *Anopheles gambiae s.s.*) stored in different media for 5 days at either +4°C or room temperature (RT) were evaluated at the quantitative (*i.e.*, ELISA) and qualitative (*i.e.*, SDS-PAGE and immunoblotting) levels. Our results indicated that PBS medium supplemented with an anti-protease cocktail seems to be the best buffer to preserve SG antigens for 5 days at +4°C for ELISA analysis. Conversely, cell-lysis buffer (Urea-Thiourea-CHAPS-Tris) was best at preventing protein degradation both at +4°C and RT for further qualitative analysis. These convenient storage methods provide an alternative to freezing and are expected to be applicable to other biological samples collected in the field.

Mosquitoes are responsible for a wide range of important diseases that cause morbidity and mortality in tropical and temperate regions [1, 2]. Pathogen transmission occurs during the blood-feeding of infected mosquitoes, concomitant with salivary protein release [3]. Analysis of salivary mosquito contents using transcriptomic and proteomic tools [4-6] have revealed a panel of salivary molecules with anti-hemostatic and immune-modulatory properties which facilitate blood meals by counteracting host's defences [6, 7]. It was repeatedly demonstrated that mosquito salivary proteins could also elicit a host IgG response in natural conditions [8-10]. Thus, the potential use of these antigenic proteins as epidemiological markers for evaluating individual human exposure level to specific mosquito species is a major research area. Additionally, the identification of such vector-borne immunogenic proteins can lead to a panel of promising applications such as the evaluation of anti-mosquito strategies effectiveness, the mapping of new infestation areas, the estimation of disease transmission risk or the development of vaccines protecting the host against the transmission and establishment of pathogens [11, 12]. As our aim is to identify biological markers of individual exposure to arthropod bites using correspondent antigenic materials, it was necessary to develop a convenient protocol to collect and preserve biological samples in the field.

The most common method used to obtain salivary proteins is salivary gland (SG) dissection [13-15]. Mosquito SGs contain a cocktail of enzymes and active proteins necessary for their blood-feeding that could alter salivary protein integrity [3, 16]. To avoid protein degradation, SGs are generally collected on ice and stored at or below -20°C until needed [15, 17]. However, maintaining samples in a frozen environment can be hard to achieve in field conditions.

Although hundreds of mosquito species have been reared in laboratories, relatively few have been continuously maintained through several generations in a caged environment [18]. Furthermore, continuous mass rearing is a tremendous task that requires particular skills and time and is also subject to

biosafety considerations. Thus, to avoid the risk of mosquito settlement by bringing larvae outside their natural habitat in an area exempt of this species, collection of SG in the field appears more reasonable. However, SG dissection currently necessitates a large number of living mosquitoes *in situ* in close proximity to a freezer system to prevent protein degradation. Yet, in inter-tropical areas, mainly in sub-Saharan regions, some villages are highly isolated and frozen apparatus are not always available. Additionally, transport of SGE until a freezer system could take several hours and a continuous sample freezing could be hazardous. Therefore, alternative and convenient procedures need to be developed to preserve biological materials when a continuously cold environment would be hard to maintain. To this end, the preservation of SG proteins from two mosquito species (*Anopheles gambiae s.s* and *Aedes aegypti*) in different storage mediums and temperature conditions over 5 days have been evaluated quantitatively (*i.e.*, ELISA) and qualitatively (*i.e.*, SDS-PAGE and immunoblotting).

SGs from non-blood fed, 5-8 day-old adult *An. gambiae s.s.* (Kisumu strain [19]) and *Ae. aegypti* (Bora-Bora strain [20]) female mosquitoes bred in a laboratory under standard conditions (*i.e.,* 26°C and 60% humidity) at the "Institut de Recherche pour le Développement" (IRD, Montpellier) were dissected under a stereomicroscope, as previously described [15]. For each species, a total of 30 pairs of SGs were pooled and stored in each buffer and temperature condition. Briefly, samples were collected and stored 5 days at + 4°C or RT (about 21°C) either in a Phosphate Buffered Saline buffer supplemented with an anti-protease cocktail (one tablet of Complete, EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA) in 5 ml of PBS, PBSpi buffer), or in a cell lysis buffer (8 M urea (Sigma), 2 M thiourea (Sigma), 4% (w/v) CHAPS (Sigma) and 30 mM Tris (Sigma) adjusted to pH 8.5). After 5 days storage at + 4°C or RT, all the samples were preserved at -20°C until needed. As reference, 30 pairs of SGs from each

species were collected in two independent replicates and placed on ice in PBS followed by freezing at -20°C [15].
Before testing the protein preservation conditions, the quantity of SG proteins collected was estimated for each sample. As protein degradation could occur in the different conditions tested, 2 pools of 30 pairs of SG for each mosquito species were collected in 2 independent experiments and conserved in reference conditions on ice in PBS. These pooled samples were then used for protein concentration measurements by the Lowry method (DC Protein assay Kit, Bio-Rad) according to the manufacturer's instructions. Protein concentration of *An. gambiae s.s.* and *Ae. Aegypti* samples were estimated at an average of 18.1 ± 2.0 µg **(**mean ± standard deviation**)** and 25.0 ± 2.4 µg per tube of 30 SG pairs, respectively.
SGs preserved in each condition were then disrupted by ultrasonication for 5 min on ice. Each sample was split into two equal quantities and precipitated with cold acetone (Sigma). One protein sample was suspended in bicarbonate buffer 0.1 M (pH 9.6) at 2 µg/mL, suitable for ELISA procedure, and the other was suspended in cell lysis buffer at 2.5 µg/µL, suitable for biochemical analysis [15]. To avoid several freeze-thaw cycles of the SGE samples and sera, ELISA and immunoblot experiments were run in parallel. Sera from 5 individuals (3 Senegalese and 2 Gabonese) regularly exposed to *An. gambiae* and *Ae. aegypti* mosquito bites and sera from 2 non-exposed French individuals who had not travelled abroad for the past 5 years were selected for this study. The protocol was approved by the ethical committee of Marseille (France) and by the Senegal National Ethics Committee (Dakar, Senegal). The informed consent of each participant was obtained at the beginning of the study, after a thorough explanation of its purpose.
ELISA analyses were performed as previously described [21]. Each serum (diluted at 1/50) was tested in duplicate and in control wells without SG extracts. IgG antibody levels are reported as adjusted OD (aOD), calculated for

each serum as a mean OD value with SG extracts minus the OD value of the control wells.

A high IgG antibody response against *An. gambiae* SGs stored in the reference condition was observed by ELISA for exposed individuals (mean aOD ± standard deviation: 0.73 ± 0.21), in contrast to non-exposed individuals (0.15 ± 0.03). Comparable IgG antibody responses were obtained against *Ae. aegypti* SGs stored in the reference condition (0.75 ± 0.29 and 0.06 ± 0.01 for exposed and non-exposed individuals respectively; Figure 1A). These positive sera were considered suitable for the evaluation of the SGs' antigenicity.

The aOD of IgG antibody against SGs of both mosquito species from exposed individuals differed according to storage conditions. For *An. gambiae* samples, the highest mean aOD from exposed individuals was observed for SGs collected in PBSpi (0.97 ± 0.26), and to a lesser extent in cell lysis buffer (0.69 ± 0.30), when maintained for 5 days at +4°C. However, for samples collected in these same buffers (*i.e.,* PBSpi or cell lysis buffer) but stored at RT, a decrease in aOD was observed (0.46 ± 0.21 and 0.64 ± 0.22 for samples collected in PBSpi and cell lysis buffer respectively; Figure 1A). For *Ae. aegypti* samples, only SGs collected in PBSpi and stored at +4°C showed an aOD from exposed individuals (0.77 ± 0.29) similar to that detected under the reference preservation condition (0.75 ± 0.29; Figure 1B). The aOD corresponding to IgG response against *An. gambiae* and *Ae. aegypti* SGs from non-exposed individuals was lower than the aOD detected with sera from exposed individuals. The aOD obtained with non-exposed sera against *An. gambiae* SGs preserved in cell lysis buffers at RT (0.33± 0.01) was unexpectedly higher than that obtained with *An. gambiae* SGs preserved in the other conditions. For *An. gambiae* SGs preservation conditions, significant increase and decrease (Wilcoxon signed rank test) of aOD from exposed individual sera were observed between reference sample and PBSpi at +4°C ($p=0.03$) or PBSpi at

RT (*p=0.03*), respectively. For *Ae. aegypti* SGs preservation conditions, significant decreases (Wilcoxon signed rank test) of aOD from exposed individual sera were observed between reference sample and PBSpi at RT (*p=0.03*) or cell lysis buffer at RT (*p=0.03*) (Figure 1). Collectively, these results indicated that PBSpi at +4°C appeared to be the most efficient medium for preserving SG antigenicity during a 5-day storage period for further analysis by ELISA.

SG preservation was further evaluated by biochemical analysis including comparison of protein profiles by SDS-PAGE and immune response by immunoblots. For each preservation condition, 10 µg of SG protein was minimally labeled with CyDye as previously described [15, 22] and separated by 12% SDS-PAGE (BioRad, Hercules, USA). Protein profiles were then analyzed using the ImageQuant™ TL software (GE Healthcare, UK), as previously described [23].

For *An. gambiae,* the diversity of protein bands, compared to the frozen reference, was independent of the preservation conditions used (Figure 2A), but large band intensity variations were observed dependent on the preservation conditions. For the same band, protein abundance between PBSpi RT and cell lysis buffer at 4°C could vary up to 11-fold (Figure 2B). Protein profiles with higher band diversity and intensity were obtained for samples preserved in cell lysis buffer either at +4°C or RT.

To further assess the consequences of sample preservation on the antigenic repertory, gels were transferred onto a nitrocellulose membrane (GE Healthcare) by semidry blotting [24] and further incubated with human pool sera from exposed individuals (n=5, diluted at 1/100) and revealed mouse anti-human Fcγ/IgG horseradish peroxidase (HRP) conjugated antibody (1/5 000, Beckman Coulter, USA) using an ECL Plus detection system (GE Healthcare). In accordance with SDS-PAGE analysis, immunoblots indicated that the antigenic repertoire appears better preserved in cell lysis

buffer both at + 4°C or RT. In fact, numerous antigenic bands were detected only under this last preservation condition (Figure 2C). The intensity and diversity of antigenic profiles were most intense after cell lysis preservation when compared to the other conditions. Similar results were obtained for *Ae. aegypti* SG samples (Figure 3).

Surprisingly, in the reference condition, the low quality of protein and immune profiles suggested that protein degradation could occur in samples left at 4°C during SG collection periods, but the addition of a protease inhibitor cocktail could counteract this deleterious phenomenon. Interestingly, in *An. gambiae* samples, also the band at 70 kDa, reported as a major antigen [25], was detected of in all preservation conditions on the SDS-PAGE, this band was not recognized by the pool sera, in the reference and PBSpi at RT conditions. The non-detection of this band could be reasonably attributed to an under detection limit of the immunoblot by this pool sera. Effectively, the band at 70 kDa is largely less abundant in the reference and PBSpi at RT conditions compared to three other conditions (PBSpi +4°C, cell lysis buffer +4°C or RT) accordingly to their corresponding densitometric protein profiles.

Although cell lysis buffer seems to be the best condition to preserve protein integrity for biochemical analysis at both + 4°C and RT, the lower aOD detected by ELISA from samples in these conditions could be attributed to the reagents from the cell lysis buffer (despite the acetone precipitation) interfering with the ELISA reaction, rather than a degradation of protein antigenicity. Effectively, Godfrin and collaborators demonstrate that high concentrations of CHAPS, Urea and Thiourea inhibit antigen binding to microplate surface and could also disturb antigen recognition by the specific antibodies in ELISA [26]. In addition, the combination of detergent and chaotropic agents in the cell lysis buffer induces protein denaturation leading to the loss of conformational epitopes [27]. The better conservation of these conformational epitopes in PBS is a supplementary argument to explain differences observed between

preservation conditions in ELISA. Conversely, in immunoblots, epitopes recognized are mainly sequential due to reduction and denaturation of proteins, which could explain the disparate results obtained between the ELISA reactions and the SDS-PAGE or immunoblots.

To summarize, the cell lysis buffer solution seems to prevent protein degradation and preserve antigenicity at +4°C and also at RT. Nevertheless, despite cleaning the samples by acetone treatment, traces of this buffer could disrupt the ELISA experiments. In this specific case, protein preservation under PBSpi appeared to be more efficient to preserve SG antigenic proteins after 5 days of storage at +4°C. These convenient storage methods provide an alternative to freezing, which is hard to achieve under field conditions, and are expected to be applicable to biological samples in many systems.

Abbreviations:

IgG: Immunoglobulin G.

RT: Room temperature.

PBS: Phosphate Buffered Saline.

PBSpi: Phosphate Buffered Saline supplemented with a protease inhibitor cocktail.

EDTA: ethylenediaminetetraacetic acid.

ELISA: Enzyme-Linked ImmunoSorbent Assay.

HRP: Horseradish peroxidase.

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Author contribution

Conceived and designed the experiments: FA, AL, FT and RC. Performed the experiments: FA, PA and BS. Analyzed the data: FA, PA, AL and RC. Contributed reagents/materials/analysis tools: BS, DI and BN. Wrote the paper: FA, AL and RC.

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The authors have declared no conflict of interest.

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Figure legends

Figure 1: Scatter plot graphs of human IgG responses from exposed (E) and non-exposed (NE) individuals against *An. gambiae* (A) and *Ae. aegypti* (B) salivary gland (SG) protein extracts according to different preservation conditions by Enzyme-Linked ImmunoSorbent Assay (ELISA). Antibody responses are represented by aOD: mean OD value of wells with salivary antigen minus mean OD value of wells without salivary antigen. Each point shows the aOD value for a single individual. Horizontal bars show medians. Differences between the reference sample (salivary glands collected on ice and store fresh at -20°C) and other preservation conditions were tested using Wilcoxon signed-rank test. *p*-values are indicated only when significant differences were observed. Ref. S: reference sample; CBL: cell lysis buffer.

Figure 2: (A) Comparative *An. gambiae* salivary gland (SG) protein profiles between different preservation conditions. Salivary gland proteins were separated on 12% SDS-PAGE gels. Each protein profile corresponds to a distinct preservation condition. Lane 1: SG dissected on ice and stored at -20°C in PBS (reference); lane 2: SG dissected at RT and stored 5 days at 4°C in PBS containing protease inhibitor cocktail (PBSpi); lane 3: SG dissected at RT and stored 5 days at RT in PBSpi; lane 4: SG dissected at RT and stored 5 days at 4°C in cell lysis buffer; lane 5: SG dissected at RT and stored 5 days at RT in cell lysis buffer. Standard molecular weights (MW) are indicated at the left side in kilodaltons (kDa). (B) Schematic representations of densitometric protein profiles from the 5 salivary gland preservation conditions. The line color corresponds to the colored box used at the top of each protein profile. The arrow head indicates the band that was used for abundance comparison. A.U.: Arbitrary Unit. R.f.: Relative front of migration. (C) IgG immune profiles against *An. gambiae* salivary gland proteins using the pooled sera from exposed individuals. The immunoblots were performed by transferring the SDS-PAGE gel shown in (A) onto a nitrocellulose membrane. Antigenic bands detected only in samples preserved in cell lysis buffer are indicated with an asterisk (*).

Figure 3: (A) Comparative *Ae. aegypti* salivary gland (SG) protein profiles between different preservation conditions. Salivary gland proteins were separated on 12% SDS-PAGE gels. Each protein profile corresponds to a distinct preservation condition. Lane 1: SG dissected on ice and stored at -20°C in PBS (reference); lane 2: SG dissected at RT and stored 5 days at 4°C in PBS containing a protease inhibitor cocktail (PBSpi); lane 3: SG dissected at RT and stored 5 days at RT in PBSpi; lane 4: SG dissected at RT and stored 5 days at 4°C in cell lysis buffer; lane 5: SG dissected at RT and stored 5 days at RT in cell lysis buffer. Standard molecular weights (MW) are indicated at the left side in kilodaltons (kDa). (B) Schematic representations of densitometric protein profiles from the 5 salivary gland preservation conditions. The line color corresponds to the colored box used at the top of each protein profile. A.U.: Arbitrary Unit. R.f.: Relative front of migration. (C) IgG immune profiles against *Ae. aegypti* salivary gland proteins using the pooled sera from exposed individuals. The immunoblots were performed by transferring the SDS-PAGE gel shown in (A) onto a nitrocellulose membrane. Antigenic bands detected only in samples preserved in cell lysis buffer are indicated with an asterisk (*).

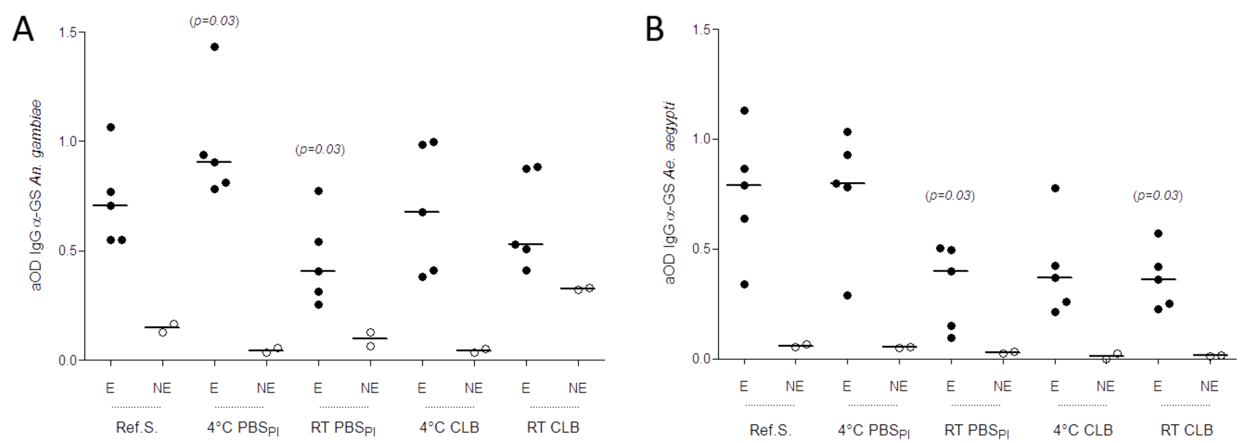

Figure 1

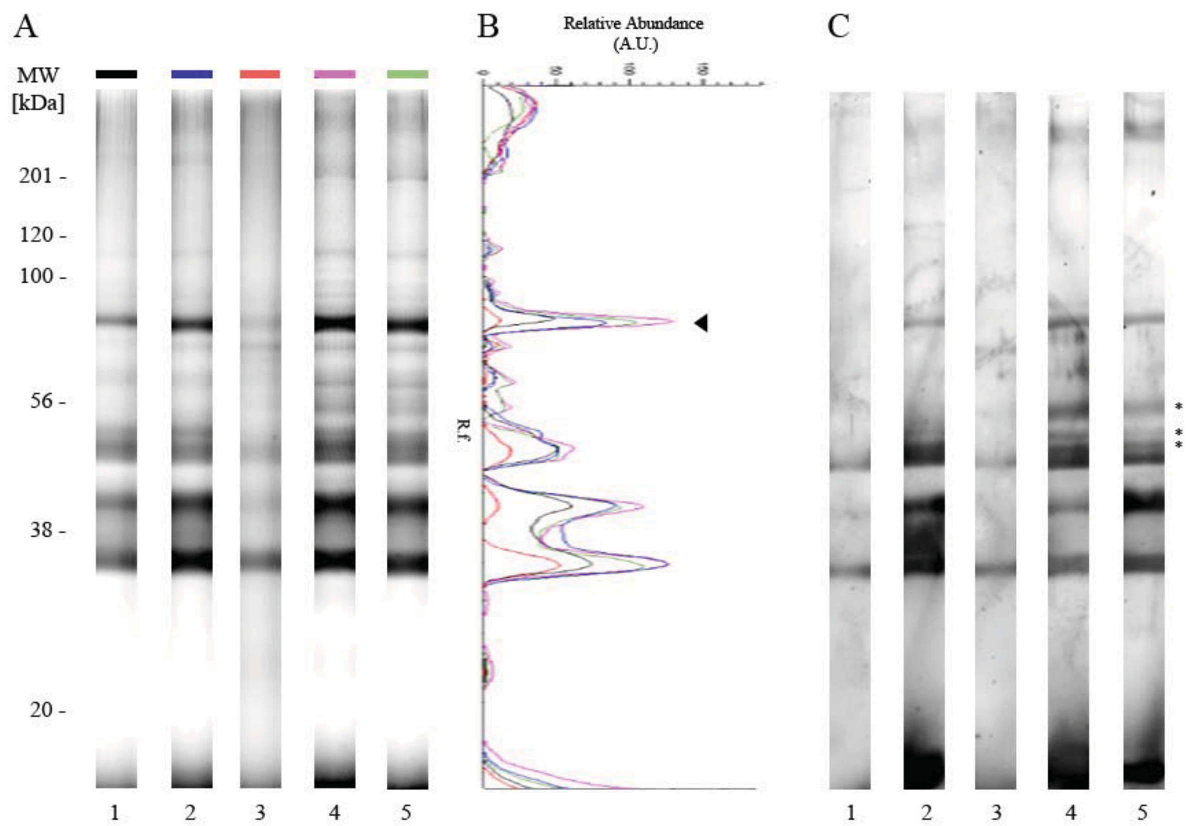

Figure 2

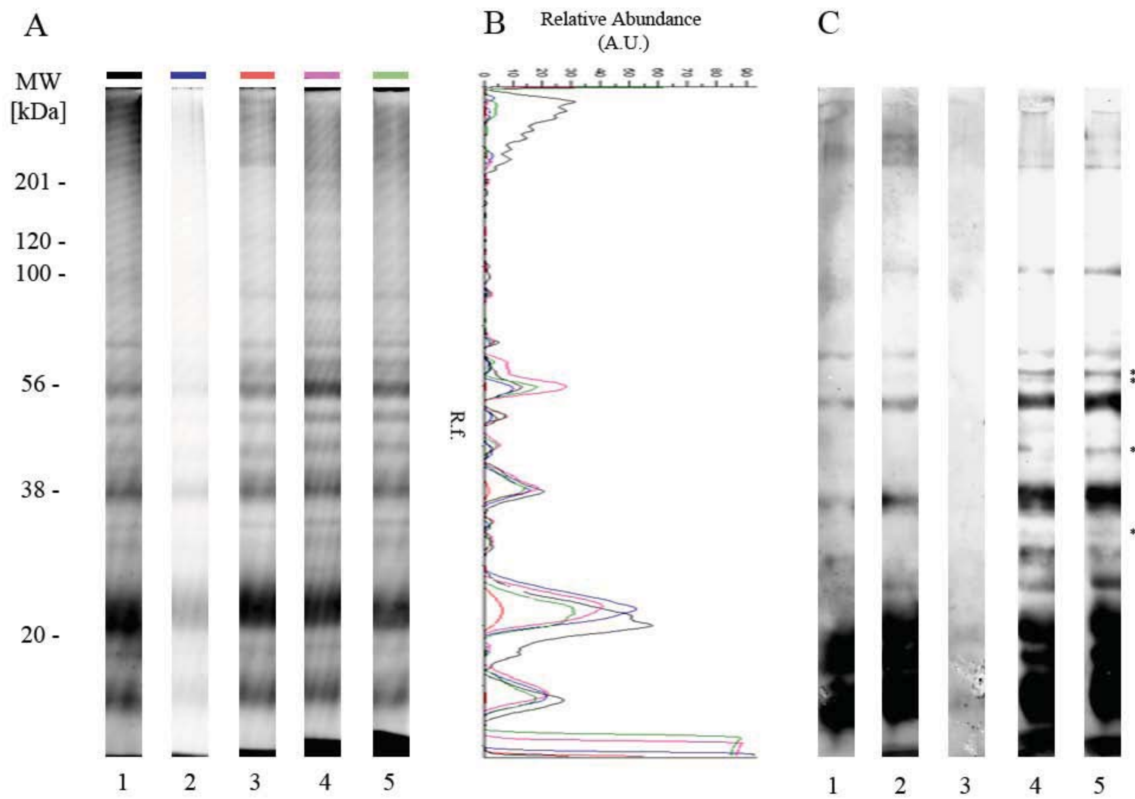

Figure 3

Cette recherche de solutions de préservation alternatives au maintien d'échantillons dans la chaîne du froid à été initié suite à des problèmes rencontrés par notre équipe lors de la collecte de glandes salivaires sur le terrain, et notemment en Afrique. Une mission a été réalisée au Sénégal en septembre 2008 pour avoir accés à certaines espèces de moustiques qui n'étaient pas disponibles en laboratoire ou à des moustiques prélevés sur le terrain pour pouvoir évaluer l'homogénéité du protéome salivaire entre des moustiques sauvages et d'autres élevés en laboratoire. Dans cette optique, nous avons dû rejoindre des villages éloignés des grandes villes de plusieurs heures de voitures, tels que Dielmo (Fontenille *et al.*, 1997a) et N'Diop (Fontenille *et al.*, 1997b). Dans ces endroits il peut être difficile de conserver une température basse, notemment pendant le transport des échantillons.

Dans ce travail, nous avons montré qu'un milieu PBS supplémenté d'un cocktail concentré d'anti-protéases pouvait préserver convenablement les échantillons de glandes salivaires à 4°C en vue d'une utilisation par ELISA. Par contre, un tampon de lyse constitué d'urée/thiourée/chaps s'est montré le plus efficace à 4°C et température ambiante pour préserver les échantillons en vue d'analyses qualitatives, telles que par exemple la détection de bandes antigéniques par western-blot. Ces méthodes de préservation relativement simples et peu coûteuses peuvent être une alternative convaincante à la conservation à basse température.

Ces méthodes alternatives de préservations d'échantillons salivaires ont été réalisées dans le but d'identifier des marqueurs immunologiques d'exposition spécifiques à des genres et/ou espèces de moustiques qui permetrons à terme,

d'évaluer l'efficacité des méthodes de luttes anti-vectorielle ou le risque de transmission de pathogènes.
Ces méthodes de conservation devraient pouvoir aussi être utilisées sur des échantillons de glandes salivaires d'autres espèces vectrices, comme les glossines, les phlébotomes, les punaises ou les tiques. Il est aussi possible d'envisager l'utilité de ces méthodes dans la conservation de protéines d'agents pathogènes présents dans les glandes salivaires dans la perspective de les analyser ultérieurement. Ceci pourrait permettre de déterminer les niveaux de risques de transmission sur un site d'étude.

L'évaluation de la diversité du protéome salivaire entre plusieurs espèces de moustiques d'un même genre

L'accès à de grandes quantités d'échantillons salivaires est donc une contrainte majeure en vue de leur utilisation comme marqueurs immunologiques d'exposition. Des problèmes de temps, de moyens, de logistiques ou de disponibilité de personnel formé à la dissection peuvent constituer un obstacle au développement de tels outils et leur utilisation à grande échelle.

La production d'antigènes salivaires synthétiques est une alternative à l'utilisation d'échantillons « natifs » qui a l'avantage de pouvoir s'affranchir de ces problèmes. De plus, l'utilisation de protéines synthétiques comme marqueurs d'exposition peut améliorer la reproductibilité de ces outils immunologiques et peuvent être plus facilement utilisables pour évaluer l'exposition à diverses espèces d'arthropodes hématophages grâce à des méthodes multiplex, telles que la technologie Luminex™ qui permet de tester plusieurs dizaines d'antigènes différents en une fois avec peu de sérum (Ambrosino et al., 2010).

De plus, des travaux effectués sur la salive ou les glandes salivaires de moustiques ont montrés que des réactions croisées pouvaient avoir lieu entre différents genres ou espèces (Peng *et al.*, 1997, Trevejo *et al.*, 2005). La présence de ces réactions croisées peut s'expliquer par une exposition simultanée à plusieurs arthropodes hématophages ou à l'existence d'antigènes conservés entre des genres ou familles d'arthropodes. Des études transcriptomiques (Arca et al., 2007, Arca et al., 2005, Calvo et al., 2004, Calvo et al., 2010, Valenzuela et al., 2003) et le séquençage du génome des principaux moustiques d'importance médicale (Holt et al., 2002, Nene et al., 2007) ont mis en évidence une grande diversité de protéines salivaires, avec des familles conservées entre genres et familles d'arthropodes, mais aussi des protéines spécifiques de genres, voir d'espèces (Ribeiro et al., 2010, Calvo et al., 2007). La sélection de candidats spécifiques de genres ou d'espèces sur la base de leurs séquences protéiques ou de leur expression sélective dans diverses espèces en vue de leur production de façon recombinante peut permettre d'augmenter la sensibilité et la spécificité des tests immunologiques d'exposition (Souza et al., 2010, Teixeira et al., 2010).

Dans cette optique, nous avons tenté de déterminer des protéines spécifiques du genre ou d'espèces d'*Anopheles* en comparant par plusieurs approches (analyses *in silico*, 1D-SDS-PAGE et iTRAQ® (isobaric Tag Relative and Absolute Quantitation)) le protéome salivaire de quatre espèces vectrices du paludisme chez l'homme : *An. gambiae s.s.*, *An. arabiensis*, *An. stephensi* et *An. albimanus*. Ce travail qui est encore en préparation, fera l'objet de l'**article N.4**.

Article N.4 **A. Fontaine**, S. Briolant, S. Buffet, M. Belghazi, C. Villard, M. Pophillat, S. Bourdon, T. Fusai, C. Rogier, L. Almeras, **Comparative analysis of the salivary gland proteome of four *Anopheles* species,** *(en preparation)*

Comparative analysis of the salivary gland proteome of four *Anopheles* species

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(en préparation)

Salivary gland proteome comparison between four *Anopheles* species

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Abstract

Background: Previous studies reported that *Anopheles* salivary proteins could be used as biological markers of individual bite exposure. Thus, a better knowledge of the degree of similarity and diversity between salivary proteomes from various *Anopheles* vector species is necessary to highlight genus- and/or species-specific proteins. **Objective / methods:** In this aim, salivary gland protein repertoire (sialomes) from four *Anopheles* species (*An. gambiae s.s., An. arabiensis*, *An. stephensi* and *An. albimanus*) were compared using *in-silico* analysis and complementary proteomic approaches: one-dimensional SDS-PAGE (1-D SDS-PAGE) coupled to mass spectrometry identification and isobaric Tag for Relative and Absolute Quantitation (iTRAQ™). Diversity of immunogenic salivary gland proteins was also assessed by Immunoblot. **Results:** A computational hierarchical clustering of salivary gland proteins sequences recovered from Uniprot KB database have shown a similar salivary protein repertoire between *An. gambiae s.s*. and *An. arabiensis*, two species belonging to the same *An. gambiae s.l.* complex as well as specific salivary proteins in others species. Proteomic analysis confirmed the low proteome heterogeneity observed between the *An. gambiae* and *An. arabiensis* and have revealed a more diversified salivary protein repertoire between the other species **Conclusion:** Collectively, all these data repertoire for the first time, *Anopheles* secreted salivary proteins genus and/or species specific. The involvement of such proteins as candidates for genus- or species-specific serological biomarkers of individual exposure to *Anopheles* bites will be discussed.

Introduction

Some anthropophilic mosquitoes from the *Anopheles* genus are vectors of *Plasmodium* parasites, causal agents of malaria in tropical and sub-tropical countries. This major vector-borne disease affects yearly more than 300 millions of individuals and leads to 1.1 - 2.7 million deaths, mainly in Africa (Snow et al., 2005, WHO, 2000). Among the approximately 470 *Anopheles* species indexed worldwide (http://www.mosquitocatalog.org), 34 species colonizing different ecoclimatic settings and areas around the world are considered as main vectors of the five *Plasmodium* parasite species (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*) responsible of human malaria (Kiszewski *et al.*, 2004, McKenzie *et al.*, 1999, White, 2008). Together with *An. funestus*, mosquitoes from the *An. gambiae s.l.* complex (*An. gambiae s.s.* and *An. arabiensis*) are primary vectors of *P. falciparum* malaria in sub-Saharan Africa, where 80% of malaria mortality and morbidity occurs (Annan *et al.*, 2007, Trape *et al.*, 1996). Among other anopheline vectors of medical importance, *An. stephensi* play an important role in urban malaria transmission in the Indo-Pakistan subcontinent (Rowland et al., 2002, Hati, 1997) and *Anopheles albimanus* is a primary vector of malaria in Central America and some areas of South America (Gutierrez et al., 2009, Gutierrez et al., 2008).

Due to the lack of licensed malaria vaccine (Crompton et al., 2010, Rogier et al., 2006) and the increasing spread of drug resistance, vector control remains the most effective method to protect individuals from arthropod-borne diseases (Enayati et al., 2009). Prevention of arthropods infective bites can be achieved either by personal protective measures, such as insecticide-treated nets (ITNs), skin repellent or by vector control strategies by reducing vector populations (*e.g.:* eliminating breeding sites, indoor residual spraying) (WHO, 2006, Alexander *et al.*, 2003, Talbert *et al.*, 1998, Enayati *et al.*, 2009).

Currently, exposure of individuals to populations of mosquitoes and by implication, the effectiveness of these anti-vectorial measures are mainly

evaluated by entomological methods (Ocampo et al., 2009, Okech et al., 2008, Shaukat et al., 2010). These methods involved the estimation of human biting rate (HBR: average number of bites per person per unit of time received from one species) which is an expression of the density of a mosquito species relative to that of man (Garrett-Jones, 1964). HBR can be estimated by human bait collections and is a component of numerous index of malaria transmission such as the vectorial capacity or the entomological inoculation rate (EIR) (Shaukat et al., 2010, Lardeux et al., 2007, Smith et al., 2009). However, HBR can vary significantly within small geographic areas (Mbogo et al., 2003, Kreuels et al., 2008, Orlandi-Pradines et al., 2009) due to the heterogeneity of the human population such as differential attractiveness to mosquitoes (Qiu et al., 2006), differential proximity to breeding sites (Staedke et al., 2003) or other environmental and socioeconomic factors. Furthermore, ethical considerations can argue against the human bait catch using adult volunteers in endemic countries. These methods are thus mainly applicable to the population level but are poorly efficient to evaluate the heterogeneity of individual exposure. Some of them can be further hampered by ethical limitations (*e.g.*, deliberate exposure of individuals to vector-borne diseases).

While obtaining a blood meal, mosquitoes inject saliva into the host's skin. This saliva contains a cocktail of pharmacologically active components that counteract host haemostasis and inflammatory response to ensure the success of the blood meal (Ribeiro, 1995, Ribeiro *et al.*, 2003) . The regurgitated salivary proteins of arthropods were reported to induce an antibody response among people living in endemic areas (Cornelie et al., 2007, Poinsignon et al., 2008, Remoue et al., 2007, Remoue et al., 2006, Barral et al., 2000) or in travellers transiently exposed to vectors in tropical areas (Orlandi-Pradines et al., 2007). This antibody response was described as being short lived and link to the level of exposure (Remoue et al., 2006, Nascimento et al., 2001, Orlandi-Pradines et al., 2007, Palosuo et al., 1997), suggesting the potential use of arthropod saliva

as an immunological marker to evaluate individual exposure to arthropod bites. Recently, Drame and colleagues have shown the efficiency of *An. gambiae* saliva to assess the intensity of this vector exposure and to evaluate malaria vector-control strategies (Drame et al., 2010).

Several studies have observed various degree of specificity in the anti-saliva antibody response (Peng et al., 2006, Brummer-Korvenkontio et al., 1997, Jeon et al., 2001) which could be link to the degree of protein sequences similarities among the Culicidae family. An important step forward the knowledge of salivary proteins diversity in Culicidae was the cataloguing of salivary gland proteins expressed in several mosquito species. The recent elucidation of the genome from mosquitoes of major health importance (Holt et al., 2002, Nene et al., 2007), adding to an increasing transcriptomic and proteomic works on their salivary gland extracts (Arca et al., 2007, Arca et al., 2005, Calvo et al., 2004, Calvo et al., 2009, Calvo et al., 2010, Ribeiro et al., 2004, Valenzuela et al., 2003, Ribeiro et al., 2007), enabled to identify ubiquitous and conserved salivary molecules in various hematophagous arthropods families and genus (Ribeiro et al., 2010). The molecular sialome diversity among different mosquito species from a same genus is less documented, but some immunological studies have observed species specific anti-saliva antibody responses in other arthropods (Volf *et al.*, 2001, Rohousova *et al.*, 2005). Thus, the identification of corresponding mosquito species specific antigens could be used as candidates to develop immunological markers of exposure.

In this aim, salivary gland protein repertoire from four *Anopheles* species (*An. gambiae s.s., An. arabiensis*, *An. stephensi* and *An. albimanus*) were compared using *in-silico* analysis and complementary proteomic approaches: 1-D SDS-PAGE coupled to MS identification and iTRAQ®. Diversity of immunogenic salivary gland proteins was also assessed by Immunoblot. Collectively, all these data repertoried for the first time genus and/or species specific *Anopheles* secreted salivary proteins. The involvement of such proteins

as candidates of genus- or species-specific serological biomarkers of exposure to *Anopheles* bites will be discussed.

Materials and methods

Sera samples

Sera from 5 individuals living in the Senegalese village of Dielmo (13°45'N, 16°25'W), sampled in March 1995 were used in this study. These individuals were exposed to high malaria levels (about 200 infective bites/person/per year) with *An. gambiae, An. arabiensis* and *An. funestus* as principal vectors (Fontenille *et al.*, 1997a, Fontenille *et al.*, 1997b). The protocol was approved by the ethical committee of Marseille (France) and by the Senegal National Ethics Committee (Dakar, Senegal). The informed consent of each participant was obtained at the beginning of the study, after a thorough explanation of its purpose.

Salivary protein sequences retrieval and sequence clustering

All salivary protein sequences were retrieved in FASTA format from the online non-redundant universal Protein knowledgebase (UniProtKB, (Bairoch et al., 2005)) under the taxonomies *An. gambiae* [7165], *An. arabiensis* [7173], *An. stephensi* [30069] and *An. albimanus* [7167], using the search term "salivary" in any fields of their description text. Signal peptides were predicted by submission of the protein sequences to the SignalP server 3.0 (Bendtsen et al., 2004), allowing the identification of putative secreted proteins.

All putative secreted sequences were merged in a single FASTA file and submitted to the CD-HIT server (at http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi) (Huang et al.) for hierarchical clustering (H-CD-HIT) as describe elsewhere (Li et al., 2008). Briefly, the program performs clustering three times in succession with decreasing similarity thresholds. Short sequences were allowed to be clustered with a longer sequence if it was completely contained within it. First, clustering start with the input dataset at a high identity threshold (≥ 90%). The longest sequence becomes the representative of the first cluster. Then, each remaining sequence is compared to the representatives of all

existing clusters. If the predefined similarity threshold is met, the sequence is grouped into the most similar cluster. Otherwise, a new cluster is defined with that sequence as the representative. The last two steps of the hierarchical clustering (≥ 50% and ≥ 30% similarity threshold) start with representatives of the previous clustering runs and the whole process produces a hierarchical structure. The percentage identities in the two first steps are calculated by counting the numbers of identical peptides between two protein sequences. The last step (≥ 30% similarity threshold) was performed with PSI-CD-HIT that uses pairwise sequence alignment (BLAST) to calculate similarities. More details can be found from the user's guide (www.bioinformatics.org/cd-hit/cd-hit-user-guide.pdf).

Mosquitoes and salivary glands extraction

Uninfected 5-day-old adult females of the *An. gambiae sensu stricto*, *An. arabiensis*, *An. stephensi* and *An. albimanus* species were used in this study. *An. stephensi* and *An. albimanus* species were reared at the Institut Pasteur (Centre de production et infection des *Anopheles*, Paris). *An. gambiae s.s.* was reared at the Institut de Recherche pour le Développement (laboratoire de Lutte contre les Insectes Nuisibles, Montpellier). *An. arabiensis* species was collected on the field at the larvae stage at Dakar (Senegal) at the end of the rainy season in September 2008 (Machault et al., 2009). After emergence, adult mosquitoes were reared at the Institut de Recherche pour le Développement (Dakar). A random sample of *An. arabiensis* was identified by polymerase chain reaction (PCR) at the species level after salivary gland dissection (Fontenille *et al.*, 1997a). All mosquitoes used in the experiments were maintained under identical standard conditions: 26°C and 60% humidity, took no blood meals and were maintained on a diet of 10% syrup solution. The salivary glands from adult mosquito females were dissected under a stereomicroscope at 4X magnification. The salivary glands from each experiment were pooled by strains into a

microcentrifuge tube on ice in phosphate-buffered saline (PBS) and then stored frozen at –20°C until needed.

Sample preparation

Salivary glands were disrupted by ultrasonication (Vibracell 72412, Bioblock Scientific, Illkirch, France) for 5 min on ice at maximum amplitude. The concentration of salivary gland homogenates was determined in duplicate by Lowry method (DC Protein assay Kit, Bio-Rad) according to the manufacturer's instructions. Salivary gland proteins were then concentrated by precipitation with acetone (Sigma, St Louis, MI), and were suspended in a UTC buffer containing 8M urea (Sigma), 2M thiourea (Sigma), 4% (w/v) CHAPS (Sigma) and 30 mM Tris (Sigma), adjusted to pH 8.5 in order to obtain a protein concentration adjusted to 2.5µg/µL.

One-dimensional electrophoresis (SDS-PAGE)

For each species, 20 ug of salivary gland proteins were separated onto a 12% SDS-PAGE in a PROTEAN II xi or Mini (BioRad, Hercules, USA). A broad range molecular weight marker (BioRad) was loaded on each gel. Gels were stained with Sypro Ruby (BioRad) according to the manufacturer's protocol and digitalized using the Typhoon™ Trio Image scanner (GE Healthcare, UK). Salivary glands densitometry profiles were analyzed using the ImageQuant™ TL software (GE Healthcare, UK). Background subtraction was performed and the densitometry profiles were normalized to take into account global differences. Relative abundance in protein from each band was estimated by dividing the area under the curve of the peak corresponding to the band by the sum of the areas under the curves of all bands.

Western blotting

For each species, 15 ug of salivary gland proteins were separated onto a 12% SDS-PAGE as previously described. Gels were then transferred to a nitrocellulose membrane (0.45-µm, Amersham Pharmacia, Saclay, France) by semidry blotting (0.8 mA per cm²) (Towbin et al., 1992). Blots were saturated with 5% w/v non-fat dried milk, and were carried out with a pool sera from 5 individuals exposed to *Anopheles gambiae s.l.* (Ndiop, Senegal). These sera were chosen blindly to avoid bias in the analysis. Pool sera was diluted at 1/50 in phosphate buffer saline (PBS) containing 0.2% v/v tween-20 with 5% w/v non-fat dried milk. After an overnight incubation, blots were incubated with anti-human Fcγ/IgG FITC conjugated antibody 1/1000 (Sigma, St Louis, MI). Immunoblots were directly digitalized using a Typhoon™ Trio Image scanner (GE Healthcare) and densitometric analysis of IgG immune profiles was performed using ImageQuant™ TL software (GE Healthcare), as previously described (Almeras et al., 2010).

Protein band excision and in-gel digestion

Based on ImageQuant™ TL analysis, protein bands of interest were excised using Shimadzu Biotech Xcise System (Champs sur Marne, France). Protein bands were digested overnight at 37°C with sequencing-grade trypsin (12.5 µg/mL; Promega Madison, WI, USA) in 50 mM NH_4HCO_3 (Sigma). The resulting peptides were extracted with 25 mM NH_4HCO_3 for 15 min, dehydrated with acetonitrile (ACN) (Sigma), incubated with 5% acid formic (Sigma) for 15 min under agitation, then dehydrated with ACN, and finally completely dried using a SpeedVac. Samples were then stored at -20°C before analysis by mass spectrometry (MS).

Mass spectrometry analysis for proteins from SDS-PAGE

For MS analysis, a LCQ DecaXPplus (ThermoFinnigan, San Jose, CA) ion trap was used. Nano-liquid separation of peptides was carried out using an Ettan MDLC chromatographic system (GE Healthcare) in high throughput configuration. Ten microliters of the digest were first trapped on a zorbax 300SB-C18 5 x 0.3 mm column and eluted at a flow rate of approximately 200 nl/min on a zorbax 300SB-C18, 3.5 µm, 150 x 0.075 mm by a linear gradient of eluant B (0.1% Formic acid, 84% ACN) in eluant A (1% Formic acid). The chromatographic system was piloted by the Unicorn 5.01 software (GE Healthcare). MS measurements were done on a LCQTM Deca XP Plus ion trap mass spectrometer (ThermoFinnigan) equipped with a LCQTM nanospray ionization source. A spray voltage of 1.8 kV was applied to the liquid junction via an in-union high voltage contact coupled to a silicaTip emitter (New Objective). Operation of the mass spectrometer was fully automated during the entire procedure using the Excalibur 1.3 data system (ThermoFinnigan). Continuous cycles of one full scan (m/z 500 to 1700) followed by three data-dependent MS/MS measurements at 35% normalized collision energy were done. MS/MS measurements were allowed for the three most intense precursor ions with a maximum rejection time limit of 1 min.

MS data analysis for proteins from SDS-PAGE

All MS/MS spectra were sequence database searched using the Bioworks 3.1 (ThermoFinnigan) or Mascot software v.2.2.2. The MS/MS spectra were searched against the non-redundant protein database (NCBInr, NIH, Bethesda) of *An. gambiae* [7165], *An. arabiensis* [7173], *An. stephensi* [30069] and *An. albimanus* [7167] together (July 27th, 2009, 16677 sequences). The following search parameters were used: precursor-ion mass tolerance of 0.8 Da, fragment ion tolerance of 0.8 Da with methionine oxidation and cysteine carboxyamidomethylation specified as differential modifications, and a

maximum of one missed cleavage site allowed. Each identified peptide had a Mascot score greater than 31, corresponding to a statistically significant ($P < 0.05$) confident identification. For single peptide-based identification, in addition to Mascot score significance, were considered only peptide sequence with at least six consecutive amino acids detected on MS spectra. These validation criteria were added in order to limit the number of false positive matches without missing real proteins of interest.

iTRAQ labeling and strong cation exchange fractionation

Protein samples used for iTRAQ labeling were those prepared during the sample preparation procedure. In order to remove urea from the protein samples, 10 µg of salivary gland proteins from each mosquito species was precipitated in acetone for 2 h at – 20 °C, centrifuged for 15 min at 16 000 × *g*, resuspended in 50 µL of 4% (w/v) CHAPS and precipitated again in acetone. Then, the samples were dissolved in 20 µL of dissolution buffer and reduced, alkylated, trypsin-digested and labeled using the iTRAQ reagents four-plex kit according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The resulting peptide solutions from *An. gambiae*, *An. arabiensis, An. stephensi* and *An. albimanus* samples were labeled with iTRAQ114, iTRAQ115, iTRAQ116, iTRAQ117, respectively, and incubated at room temperature for 1 h. Labeled peptides were then pooled and acidified by mixing with the cation buffer load iTRAQ reagent for a total volume of 1 ml. The peptide mixture was subsequently fractionated by strong cation exchange (SCX) chromatography. Briefly, iTRAQ-labeled peptides were loaded onto a PolySULFOETHYL A™ column 100 mm × 2.1 mm, containing 5-µm particles with a 200-Å pore size (PolyLC Inc., Columbia, MD, USA) which was previously washed with buffer A (10 mM KH_2PO_4 and 25% v/v ACN, pH 3.0). The peptides were eluted at a flow rate of 200 µl/min with a gradient of 0-60% buffer B (10 mM KH_2PO_4, 500 mM KCl and 25% v/v ACN, pH 3.0) for 30 min, followed by a holding step

at 60% buffer B for 3 min and a ramping up to 100% buffer B for 15 min. The elution was monitored by absorbance at 214 and 280 nm, and a total of 12 fractions were collected.

Nano LC-MS/MS analysis

Each fraction of iTRAQ-labeled sample was dried using a Speedvac, reconstituted in 12 µl of buffer (1% v/v formic acid in H_2O) and analyzed by nano LC-MS/MS. Purification and analysis of iTRAQ-labeled peptides were performed as described earlier in "MS data analysis" with minor modifications. After on line desalting of the sample for 20 min, the elution profile consisted of a linear gradient from 95% A (H_2O, 0.1% HCOOH) to 60% B (80% ACN, 0.1% HCOOH) for 130 min followed by a linear gradient to 95% B for 10 min. Normalized collision energies for peptide fragmentation were set using the charge-state recognition files for +2, +3 and +4 peptide ions with the collision energy profile increased by 20% compared to the optimized profile used without iTRAQ labeling. This modified profile gave a good, but not complete, fragmentation of the iTRAQ molecule and sufficient intensity of the reporter ions to perform quantification of the peptides.

Data processing and analysis

Mascot distiller software (v2.1.1, Matrix Science, London, United Kingdom) was used to convert MassLynx.raw MS/MS data files into mascot generic files (mgf). The default parameters were used except for the following: in MS processing, peak half width was fixed to 0.1; in MS/MS processing, precursor charge was used as a maximum; in time domain, the minimum precursor mass was fixed at 400, the maximum precursor mass was fixed at 1,700 and the precursor m/z tolerance was 0.1; and in peak picking, the max peak m/z was 10,000. For protein identification, .mgf data files were searched against the non-redundant protein database (NCBInr, NIH, Bethesda) of *An. gambiae, An. arabiensis, An. stephensi* and *An. albimanus* together (March 20th, 2009,

15745 sequences) using the MASCOT algorithm (v2.1.0, Matrix Science, London, United Kingdom). MS/MS data files were searched together to produce a merged output using the "merge MS/MS files into single search" option. Search parameters for peptide and MS/MS mass tolerance were ± 0.8 Da and ± 0.8 Da, respectively; one missed cleavage made from the trypsin digest was allowed. Fixed modifications were iTRAQ (K), iTRAQ (N-terminal) and MMTS (C), and variable modifications were oxidation (M) and iTRAQ (Y). Peptides were considered identified if their MASCOT individual ion score was higher than the MASCOT identity scores ($P < 0.01$). A false discovery rate of 0% was obtained when the decoy database search was performed against a randomized decoy database created by MASCOT using identical search parameters and validation criteria. For protein quantization, data analysis was performed with Multi-Q 1.6.1.1. as previously described (Han et al., 2008, Lin et al., 2006). The MassLynx.raw data files from the Q-TOF Ultima (Waters, MA, USA) were previously converted into mzXML format by the massWolf program (http:// tools.proteomecenter.org). After the data conversion, an automated quantization of peptide abundance was performed by MultiQ software using selected, unique iTRAQ-labeled peptides with confident MS/MS identification (MASCOT score≥ 30) and detected signature ions (m/z = 114, 115, 116, 117). For the detector dynamic range filter, signature peaks with ion counts higher than 1,000 counts or lower than 30 counts were filtered out by Multi-Q. To calculate average protein ratios, the ratios of quantified, unique iTRAQ non-degenerated peptides were weighted according to their peak intensities to minimize the standard deviation and automatically normalized by Multi-Q. The final protein quantization results were exported to an output file in the .CSV (comma separated values) data format. Each quantified protein was manually validated. Geometric means of the ratios of *An. arabiensis*, *An. stephensi* and *An. albimanus* / *An. gambiae ss* (iTRAQ115, 116, 117 / iTRAQ114) and the standard deviation were calculated.

Results

Hierarchical clustering of secreted protein sequences of female Anopheles.

In order to have an insight into the homology and diversity among secreted female *Anopheles* salivary proteins, a computational analysis was performed on protein sequences deposited online in Uniprot KB database. These protein sequences were mainly submitted by authors of high-throughput sialotranscriptomic studies (Arca et al., 1999, Arca et al., 2005, Valenzuela et al., 2003) or were predicted based on the genome annotation of the sequenced PEST (Pink Eye Standard) strain of *An. gambiae* (Holt et al., 2002, Mongin et al., 2004). A total of 143 salivary gland protein sequences were recovered from the non-redundant Protein knowledgebase (UniProtKB) on the 4 *Anopheles* species (July 10th, 2010). The computational analysis was only performed on secreted female salivary gland protein sequences in order to identify paralogous (*i.e.*, homologous intra-species protein derived from a gene duplication event) and orthologous (*i.e.*, homologous inter-species protein derived from a speciation event) proteins that are injected into the human host during mosquito blood meals. Among all these protein sequences, 27 were removed from the analysis, 3 which were identified in male *An. gambiae* salivary glands (Calvo et al., 2006) and 24 which were not predicted to be secreted proteins based on the prediction of a signal peptide. Thus, a total of 116 salivary proteins were considered for further analysis. Protein sequences were either predicted (24%) or evidenced at transcript level (76%). The number of protein sequences was very different between the 4 *Anopheles* species (65, 5, 41 and 5 protein sequences for *An. gambiae*, *An. arabiensis*, *An. stephensi* and *An. albimanus*, respectively). The clustering was performed using CD-HIT program (Huang et al., 2010) based on full-length sequence similarities. Three clustering steps were performed one after another at different similarity threshold (≥ 90%, ≥ 50% and ≥ 30% identity). This agglomerative hierarchical clustering approach is reported to maximize the quality of clustering (Li et al., 2008) and produce a tree like

structure (Figure 1) which is useful to assess the level of identity between proteins.

The first clustering step (≥ 90% identity threshold) identified 95 clusters from the original 116 protein sequences. Among these 95 clusters 77 corresponded to non-redundant (NR) protein sequences (*i.e.*, sequences which were not grouped with other sequences over a specified similarity threshold) and 18 were composed of at least two protein sequences (mean number of proteins per cluster ± 95% CI of the mean: 2.17 ± 0.19). These 18 clusters were almost exclusively composed of paralogous sequences excepted for the 5 *An. arabiensis* protein sequences which were all clusterised with *An. gambiae* sequences (cluster 0, 1, 2, 7, 15) (Figure 1 and Supplementary table 1).

The second and third clustering steps (≥ 50% and ≥ 30% identity threshold) identified 75 and 56 clusters, respectively. The ≥ 50% identity threshold step resulted in 55 NR protein sequences and 20 clusters composed of at least two protein sequences (mean number of proteins per cluster 3.05 ± 0.87). An increased number of clusters composed of orthologous protein sequences can be observed compared to the first step clustering (10 with mixed *An. gambiae* and *An. stephensi* protein sequences alone and 4 with mixed *An. gambiae*, *An. arabiensis* and *An. stephensi* protein sequences). *An. albimanus* protein sequences did not clustered with other *Anopheles* species at this step. The final identity threshold step resulted in 22 clusters composed of at least two protein sequences (mean number: 3.73 ± 1.44). All but two clusters were composed of orthologous protein sequences (15 with mixed *An. gambiae* and *An. stephensi* protein sequences alone, 3 with mixed *An. gambiae*, *An. arabiensis* and *An. stephensi* protein sequences alone, and 2 with *An. gambiae*, *An. stephensi* and *An. albimanus* protein sequences). A total of 34 NR proteins composed of 21 (62%) *An. gambiae* protein sequences, 11 (32%) *An. stephensi* protein sequences and 2 (6%) *An. albimanus* protein sequences were observed (Figure 1 and Supplementary table 1).

Analysis of salivary gland protein repertoire from four Anopheles species by SDS-PAGE.

A low number of protein sequences were available in online protein database for both *An. arabiensis* and *An. albimanus* species and the vast majority of protein sequences were inferred from cDNA and DNA sequences. Thus, a proteomic analysis was carried out in order to confirm the existence of predicted protein, but also to detect homologous proteins among the four *Anopheles* species, and to evaluate diversity among these four mosquito species.

One dimensional SDS-PAGE separation of salivary gland protein of laboratory strains of *An. gambiae*, *An. stephensi* and *An. albimanus* and salivary gland from wild *An. arabiensis* mosquitoes was performed. Numerous protein bands of molecular weights ranging from about 30 to 200 kDa were observed (Figure 2A). A total of 23, 25, 24 and 22 individualized protein bands were detected for *An. gambiae*, *An. arabiensis*, *An. stephensi* and *An. albimanus*, respectively. A densitometric scanning of the gels was performed to visualize the relative abundance of each band for each species pattern (Figure 2B). The salivary gland protein profiles from *An. gambiae* and *An. arabiensis* appeared similar with principally slight differences on band intensities.

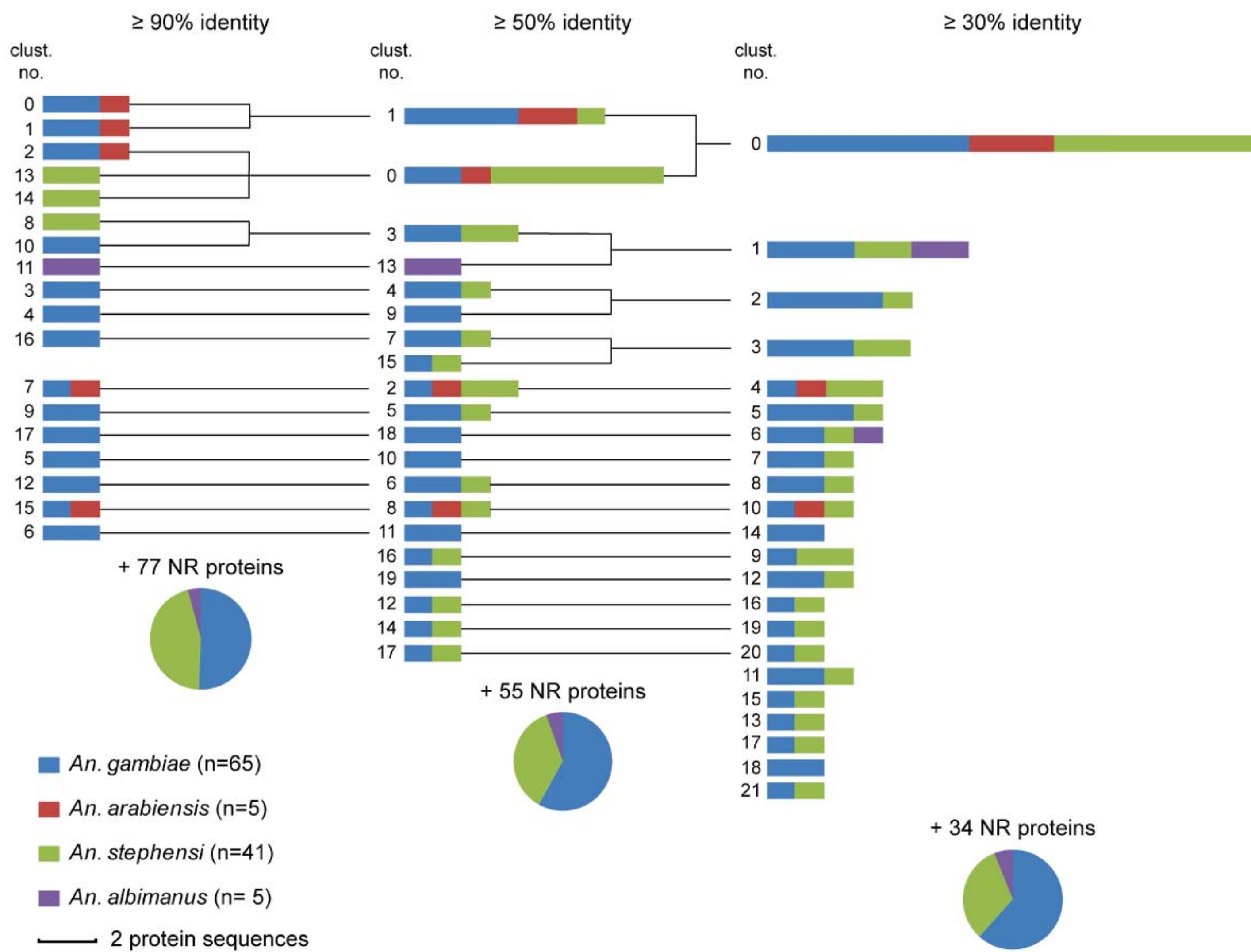

Figure 1

Conversely, the salivary gland protein profil of *An. stephensi* and *An. albimanus* were different between them and were highly distinct from *An. gambiae* and *An. arabiensis* profiles at the qualitative (*i.e.,* MW band sizes) and quantitative (*i.e.,* band intensities) levels.
The gel was submitted to 25 slices per track and submitted to trypsin digestion before an analysis of the peptide mixture by mass spectrometry (MS). Protein identification is under process and the results will be included in the manuscript as soon data will be available.

Analysis of salivary gland protein repertoire from four Anopheles species by iTRAQ®

The protein detection and identification from SDS-PAGE band patterns can be hampered by the presence of highly abundant proteins which can mask the detection of low abundance proteins. A complementary gel-free based proteomic technique, taking into account protein identity and expression level (*i.e.,* isobaric tags for relative and absolute quantification, iTRAQ®), was thus used to have a more comprehensive view of the *Anopheles* salivary gland proteome. Peptides generated from trypsin digests of proteins extracted from each *Anopheles* salivary glands were labeled with isobaric tags to determine the relative abundance of proteins identified by MS.

A total of 59 unique proteins were identified with 12 proteins identified as *An. gambiae*, 3 as *An. arabiensis*, 8 as *An. stephensi*, 2 as *An. albimanus* and 34 identified as *An. gambiae str. PEST* (*i.e.*, predicted proteins base on genome annotation) (Table 1).

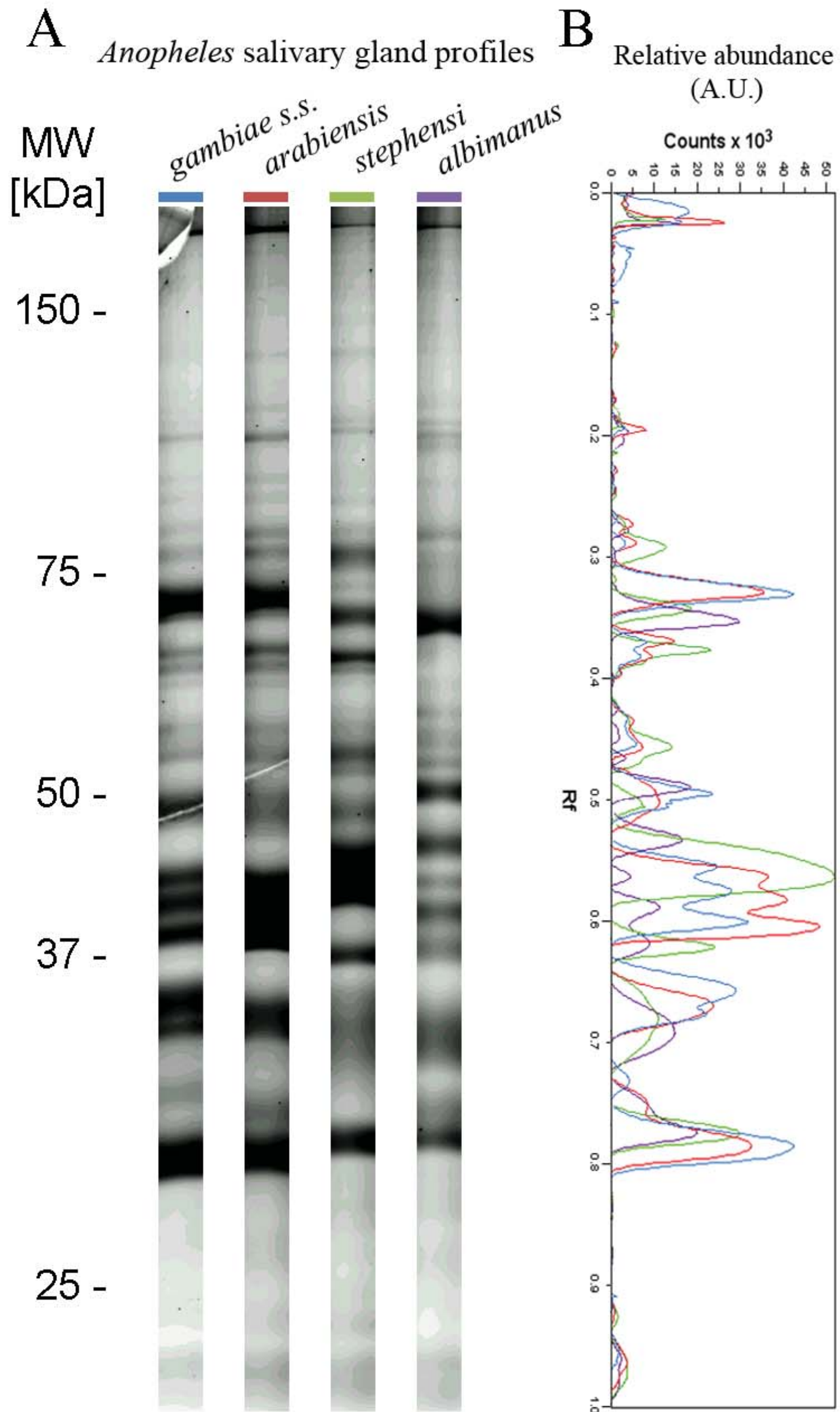

Figure 2

Among these proteins, 53 were quantified with at least one non-degenerated peptide. Based on signal peptide prediction, 32 of these proteins were predicted to be secreted. The reporter ion peaks mean ratio (± 95% confident interval) of all quantified proteins from *An. arabiensis*, *An. stephensi* and *An. albimanus*, normalized on *An. gambiae* were 1.14 ± 0.23 (secreted: 1 ± 0.23; others: 1.36 ± 0.47), 4.66 ± 2.59 (secreted: 6.72 ± 4.22; others: 1.52 ± 0.383) and 1.36 ± 0.86 (secreted: 1.58 ± 1.43; others: 1.02 ± 0.28), respectively. An arbitrary 3 fold-change cutoff (ratios≥ 3 or ≤ 0.33) was applied for all iTRAQ ratios to select with confidence differentially expressed proteins. Among both secreted and quantified proteins, this filtering measure resulted in a final set of 2 differentially expressed proteins in *An. arabiensis* versus *An. gambiae* (1 increased and 1 decreased proteins), 23 differentially expressed proteins in *An. stephensi* versus *An. gambiae* (9 increased and 14 decreased proteins) and 17 differentially expressed proteins in *An. albimanus* versus *An. gambiae* (3 increased and 14 decreased proteins). Conversely, 30, 9, and 15 secreted proteins were not differentially expressed in *An. arabiensis*, *An. stephensi* and *An. albimanus*, respectively, compared to *An. gambiae* (Table 2).

Antigenic diversity of Anopheles salivary gland proteins

Protein diversity between the four *Anopheles* species was also evaluated at the antigenic level. IgG antibody responses of a pool sera from 5 Senegalese individuals exposed to *An. gambiae s.l* (*An. gambiae* and *An. arabiensis* species) were compared using immunoblot against salivary gland extracts from *An. gambiae .s.s, An. arabiensis, An. stephensi* and *An. albimanus* (Figure 3). Superimposition of densitometric IgG profiles indicated that immune responses were distinct against the four mosquito salivary gland extracts at the qualitative (singularity of IgG profiles), and quantitative (band intensities) levels (Figure 3B and 3C).

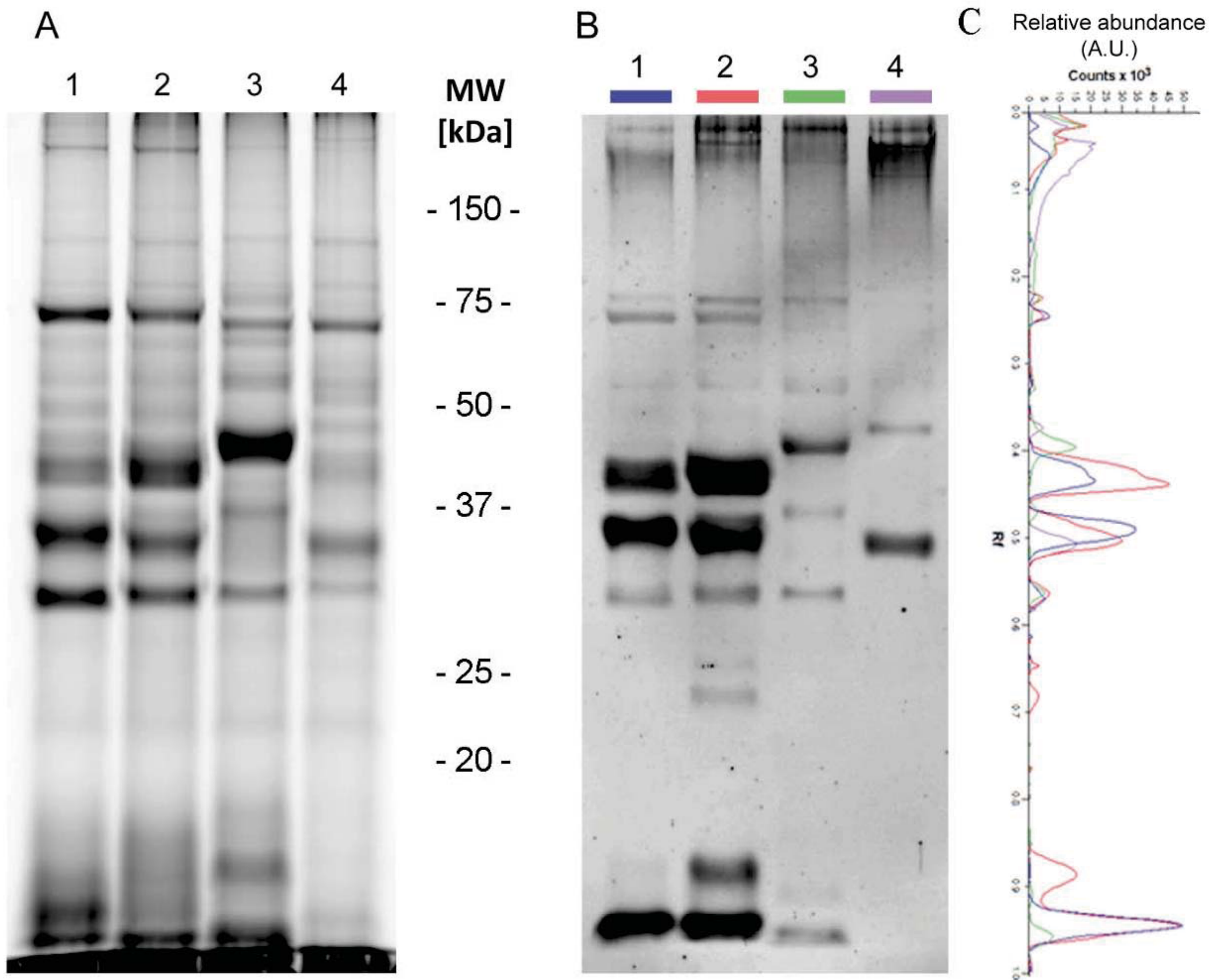

Figure 3

Most antigenic bands were shared between *An. gambiae s.s.* and *An. arabiensis* excepting for 3 of them arround 26, 23 and 10 kDa which were only exhibited on *An. arabiensis* salivary gland extracts. The comparison of protein profiles between species of *An. gambiae s.l* did not present detectable difference at the corresponding molecular weight (Figure 3A). However, 3 major antigenic bands arround 40, 35 and less than 5 kDa were recognized by pool sera only in salivary gland extracts from *An. gambiae s.s.* and *An. arabiensis*. The 35 kDA and 40 kDa antigenic bands appear to be specific to *An. gambiae s.l* . The antigenic band less than 5 kDa was also detected in *An. stephensi* with 7.2 fold less intensity. Adding to these antigenic bands, a less intense one located at 70 kDa was only recognized in the *An. gambiae s.l.* complex.

Discussion

In the purpose to identify species specific salivary proteins which could be considered as potential immunological markers of exposure to the bite from distinct *Anopheles* species, we compared salivary gland protein repertoires among four *Anopheles* species. These *Anopheles* species were selected based on their availability and their significance as major vectors of human malaria in different areas of the world. The genus *Anopheles* is subdivided into seven subgenera, including *Cellia* Theobald (tropical areas of the Afrotropical, Oriental, and Australasian regions) and *Nyssorhynchus* Blanchard (neotropical region in South America) (Mohanty et al., 2009). In our selected species, *An. gambiae*, *An. arabiensis* and *An. stephensi* belong to the *Cellia* subgenera, whereas *Anopheles albimanus* belong to the *Nyssorhynchus* one. The phylogenetic relationships among these four *Anopheles* species was carried out using multiple alignment of the conserved cytochrome oxidase II (COII) protein sequence in all species (Mohanty et al., 2009). The degrees of phylogenetic relationships exhibited within these four species are concordant to their taxonomic classification status (Supplementary figure 1). The *Anopheles* genus

exhibit an individualized clade compared to the *Aedes* genus used at outgroup. The *An. gambiae s.s.* and *An. arabiensis* grouped as closely related species, and the *Nyssorhynchus* species phylogenetically distant to the *Cellia* subgenera.

Based on these observations, we expected to characterize both conserved and specific salivary proteins among the salivary protein repertoire of these species. Comparatives analyses of sialotranscriptomes between different *Anopheles* species have revealed significant divergence of salivary proteins (Calvo et al., 2009, Calvo et al., 2004, Calvo et al., 2007). Moreover, a lack of cross-reactivity in antibody responses against salivary proteins of several species from a same genus was reported for several hematophagous arthropods (*i.e.,* Reduviid bugs and Phlebotomine sand flies) (Rohousova *et al.*, 2005, Volf *et al.*, 2001, Marshall *et al.*, 1986, Pinnas *et al.*, 1986). Thus, the identification of secreted salivary protein(s) specifically associated to a mosquito species at the expression level and/or antigenic level seems possible.

In order to have an insight into the salivary proteins diversity among these *Anopheles* species, a comparison of salivary proteins sequences retrieved from database was performed. Despite the low number of sequences available for some *Anopheles* species, hierachical clustering has evidenced that some secreted salivary gland protein families are conserved between these *Anopheles* species, including the short form D7 family, the 30 kDa / GE-rich protein family or apyrase / 5'nucleotidase family. However, members of these protein families split into individual or paralogous clusters with the increasing percentage identity threshold, highlighting diversities between *Anopheles* species. This computational analysis was hindered by incomplete salivary protein database available for some *Anopheles* species. Nevertheless, few differences between the sialome of *An. arabiensis* and *An. gambiae* were revealed. Others salivary proteins, which did not match to any homologous proteins at low identity threshold (30%), appeared highly specific to *Anopheles* species (*i.e., An. gambiae s.l.*, *An. stephensi* or *An. albimanus*). Interpretations of these

results have to take into account that *An. gambiae* is the only *Anopheles* species that have been sequenced so far.

To overcome database availability limitations, the comparison of salivary protein profiles from these 4 *Anopheles* species was undertaken using SDS-PAGE. A strong protein profile similarity was observed between the two closely related *Anopheles* species belonging to the *Anopheles gambiae s.l.* complexe, although one was collected in the field and the other one came from a continuous laboratory rearing. This low diversity of protein profiles is coherent with our previous *in silico* analysis and suggest that laboratory rearing seems not modify dramatically protein profile expression at the qualitative and quantitative level. Moreover, in previous studies, we already observed that protein repertoires from three *Aedes aegypti* colonies, originated from different areas and reared under laboratory conditions during variable periods presented a low variability at the saliva and salivary gland levels (Almeras *et al.*, 2010, Almeras *et al.*, 2009). Conversely, the singularity of protein profiles between *An. gambiae s.l., An. stephensi* and *An. albimanus*, supports the existence of specific salivary proteins among these species. A further systematic identification of the entire protein bands from these four *Anopheles* species could confirm protein diversities.

To complete the protein repertoire diversity overview between these four *Anophele* species, a gel-free comparative proteomic approach (iTRAQ) was performed. Few differences were detected between *Anopheles* species belonging to the *An. gambiae s.l.* complex, accordingly to *in silico* and protein profile comparisons. Conversely, eight out of nine secreted salivary proteins more abundantly expressed in the sialome of *An. stephensi*, were identified as *An. stephensi.* Three and 14 salivary proteins were shown to be up- and down-expressed respectively, in *An. albimanus* compared to *An. gambiae*. The low number of salivary proteins identified as *An. arabiensis* or *An. albimanus* could be attributed to the highly restricted salivary protein database size for these last

two species. However, compared to housekeeping salivary proteins, secreted proteins from *An. stephensi* and *An. albimanus* shown a higher diversity compared to *An. gambiae*. Similar observations were reported on another *Anopheles* species, suggesting that evolution of mosquito salivary-secreted proteins occurs at a faster pace than housekeeping proteins (Calvo et al., 2009, Calvo et al., 2004).

At least, as our aim is to estimate host/vector contact based on species specific antibody response, we analyzed antigenic protein profiles for these four *Anopheles* using pool sera from individuals exposed to both *An. gambiae s.s.* and *An. arabiensis* species only. Effectively, the five Senegalese individuals living in Dielmo village were exposed mainly to these two last *Anopheles* species, as previously reported (Ambrosino *et al.*, 2010{Fontenille, 1997 #1530)}. As expected, more intense antigenic bands were detected against salivary gland extracts from *Anopheles gambiae s.l.,* suggesting a relationship between specificity of exposure and antibody response level. This specificity of antibody response was already reported at the genus level ((Orlandi-Pradines *et al.*, 2007) and also at the species level (*i.e.,* between three *Aedes* species) for individuals living in the south of France (Fontaine *et al.*, submitted). In addition, the detection of supplementary antigenic bands exclusively in *An. arabiensis*, underlined that despite the low diversity observed at the molecular (*in silico* analysis) and at the expression (SDS-PAGE, iTRAQ) levels between *An. gambiae s.s.* and *An. arabiensis*, antigenic diversity could occurred. These results suggest the possibility to distinct specific exposure from very close mosquito species. However, further analysis is necessary to exclude cross-reactivity. Moreover, the antigenic band at 70 kDa detected only in *An. gambiae s.l.* was previously shown to be specific to this complex (Cornelie et al., 2007). Some antigenic protein bands recognized in all *Anopheles* species indicated a conservation of antigen inside the genus (*i.e.,* shared-antigen). Such antigenic proteins could be considered as potential candidate markers of exposure at the

genus level. Nevertheless, the presence of singulars antigenic bands against non-endemic mosquitoes (*i.e., An. stephensi* and *An. albimanus*) is in favor of cross-reactivities.

All together, these results have shown that sialomes diversity can be observed at the molecular, expression and antigenic levels between species from a same genus of Culicidae. This first description gives confidence for future characterization of immunological markers of exposure to specific *Anopheles* species. The development of a serological method based on specific antibody response against malaria vectors salivary proteins could be a complementary tool to entomological methods to assess human exposure to *Anopheles* bites. Such biomarkers could be useful to evaluate the protective effiency of antivectorial devices and the risk of pathogen transmission. However, the antigenic cross-reactivity against non-endemic mosquitoes underlined the challenge for this purpose.

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Conflicts of interest statement

The authors declared no conflict of interest concerning the work in this paper.

Author contribution

Conceived and designed the experiments: AL, FA, RC. Performed the experiments: FA, AL, BM, VC, PM. Analyzed the data: FA, AL, BuS, BrS. Contributed reagents/materials/analysis tools: BN, DI, BoS, FT, GS. Wrote the paper: FA, AL, RC.

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Figure legends

Figure 1: Hierarchical clustering of secreted protein sequence from female *Anopheles* salivary proteins. Three clustering steps were performed one after another at different similarity threshold (≥ 90%, ≥ 50% and ≥ 30% identity) producing a hierarchical structure. The repartition of proteins from *Anopheles* species into clusters of more than 2 protein sequences are represented by stacked bars and non-redundant (NR) protein sequences by pie charts according to the legend. The number of secreted salivary protein sequences recovered for each species from the UniprotKB online database is indicated into brackets. The bar at the bottom indicate the length of a stacked bar corresponding to 2 protein sequences.

Figure 2: Salivary gland protein profiles between four *Anopheles* species. Salivary gland proteins collected from *An. gambiae*, *An. arabiensis*, *An. stephensi* and *An. albimanus* were separated on 12% SDS-PAGE gels and stained with Sypro Ruby. *Anopheles* species corresponding to each well are indicated at the top of the gel. Standard molecular masses are indicated at the left side. (B) Schematic representations of densitometric protein profiles of salivary gland proteins from the four *Anopheles* species are presented. Major differences of protein band expression between species are indicated by arrows. MW: molecular weight. kDa: kiloDalton. R.A.: Relative abundance; A.U.: arbitrary units; Rf: relative front of migration.

Figure 3: Specificity of the IgG response between mosquito species using western blotting. Fifteen micrograms of each salivary gland extracts of *An. gambiae s.s.* (1), *An. arabiensis* (2), *An.stephensi* (3) and *An. albimanus* (4) were loaded per well, separated by 12% SDS-PAGE and blotted onto a nitrocellulose membrane. IgG immune profiles from a pool sera of 5 Senegalese

individuals exposed to *An. gambiae s.l.* were tested by immunoblotting experiments (A). Protein profile of salivary gland extracts separated by SDS-PAGE before their transfer on nitrocellulose membrane. (B) Antigenic profiles revealed by the pool of sera against each species. (C) Schematic representation of normalized densitometric antigenic profiles of salivary gland extracts from the four mosquito species. Species are indicated by the same colors at the top of each immunoblot profile. MW: molecular weight, kDa: kiloDalton, Rf: relative front of migration. A.U.: Arbitrary Unit.

Table 1: Differentially expressed proteins in *An. arabiensis*, *An. stephensi* and *An. albimanus* species compared to *An. gambiae* (iTRAQ®). Identified proteins are represented with their NCBI accession numbers, their name, the *Anopheles* species in which they were identified, their theoretical mass weight (MW), their signal peptide prediction based on the SignalP Neural Network (NN) (Y: secreted proteins, N: non-secreted proteins), their number of quantified peptides and their Mascot score. Ratio 1: *An. arabiensis* versus *An. gambiae*, ratio 2: *An. stephensi* versus *An. gambiae*, ratio 3: *An. albimanus* versus *An. gambiae*. AGA: *An. gambiae*, AGA*: *An. gambiae str. PEST,* AAR: *An. arabiensis*, AST: *An. stephensi*, AAL: *An. albimanus*.

Supplementary Figure 1: Evolutionary relationships of the four *Anopheles* species using the cytochrome oxidase II (COII) protein sequences. The cytochrome oxidase II (COII) gene sequence was shown to be valuable in estimating phylogenetic relationships of *Anopheles* (Mohanty et al., 2009). Multiple sequence alignment of COII proteins was carried out with Clustal W 1.7 multiple sequence alignment program (Thompson et al., 1994). The *Aedes aegypti* sequence was taken as an outgroup. The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al.*, 1987). The bootstrap consensus tree inferred from 10000 replicates is taken to represent the

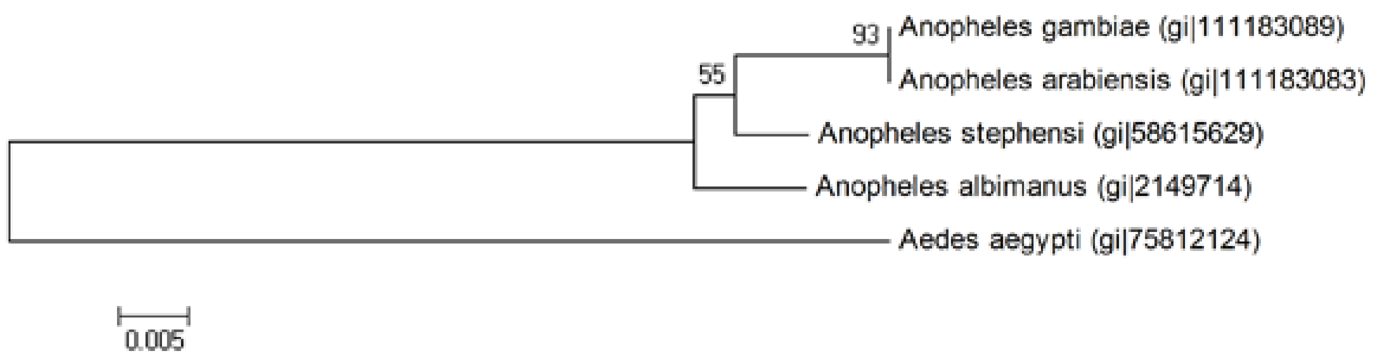
evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Supplementary Table 1: Hierarchical clustering of secreted salivary gland proteins of *An. gambiae*, *An. arabiensis*, *An. stephensi* and *An. albimanus* species. A three step clustering was performed at ≥ 90%, ≥ 50% and ≥ 30% identity threshold with the H-CD-HIT server. Clusters are sorted into protein families. The NCBI and UniProt KB accession number is indicated for each protein. * indicate the representative protein sequence of each cluster and the percentage identity between each protein sequence of a cluster and the representative is given. Protein in bold are new clusterised proteins at each identity threshold. AGA: *An. gambiae*, AAR: *An. arabiensis*, AST: *An. stephensi*, AAL: *An. albimanus*.

Table 1

Accession No.	Protein name	Organisms	Predicted MW [kDa]	SignalP (NN)	No. quant. peptides	Mascot Score	Ratio1 ± Std1	Ratio2 ± Std2	Ratio3 ± Std3
Secreted proteins (n=32)									
gi|15718081	D7 protein	AST	36.4	Y	9	139	0.54 ± 0.44	18.72 ± 10.69	1.85 ± 1.64
gi|158288615	AGAP000610-PA	AGA*	47.1	Y	7	67	0.53 ± 0.28	0.26 ± 0.27	0.21 ± 0.22
gi|37201975	GE rich salivary gland protein	AST	28.5	Y	6	164	0.64 ± 0.34	36.35 ± 18.92	3.49 ± 2.01
gi|21314941	contact-activation-inhibitor protein hamadarin	AST	18.7	Y	6	58	1.21 ± 0.42	21.71 ± 8.05	1.15 ± 0.55
gi|4539761	salivary peroxidase	AAL	65.4	Y	6	175	1.21 ± 1.17	1.36 ± 0.89	22.2 ± 13.3
gi|190576759	anti-platelet protein	AGA	26.9	Y	5	151	0.40 ± 0.26	0.11 ± 0.1	0.13 ± 0.11
gi|58377530	AGAP011026-PA	AGA*	63.4	Y	5	160	0.50 ± 0.16	0.16 ± 0.22	0.15 ± 0.25
gi|148189823	anophensin	AST	16.2	Y	5	170	1.31 ± 0.91	32.83 ± 21.31	1.21 ± 0.62
gi|29501376	short D7-4 salivary protein precursor	AST	18.4	Y	4	251	0.93 ± 0.67	26.51 ± 13.22	1.67 ± 0.83
gi|29501528	TRIO salivary gland protein precursor	AST	43.7	Y	4	56	1.03 ± 0.37	26.07 ± 13.55	1.34 ± 0.68
gi|118783568	AGAP004192-PA	AGA*	72.7	Y	4	84	1.61 ± 0.49	1.37 ± 0.32	1.56 ± 0.55
gi|13537666	gSG6 protein	AGA	13.1	Y	3	60	0.22 ± 0.06	0.04 ± 0.04	0.04 ± 0.05
gi|16225968	short form D7r3 salivary protein	AAR	19.9	Y	3	118	0.34 ± 0.24	0.16 ± 0.15	0.31 ± 0.3
gi|58389663	AGAP008282-PA	AGA*	18.5	Y	3	657	0.50 ± 0.11	0.04 ± 0.09	0.04 ± 0.11
gi|34556108	putative gVAG protein precursor	AGA	28.9	Y	3	183	0.64 ± 0.26	0.12 ± 0.17	0.08 ± 0.2
gi|58385309	AGAP004551-PA	AGA*	26.8	Y	3	59	1.15 ± 0.28	1.90 ± 1.2	0.33 ± 0.18
gi|158300147	AGAP012407-PA	AGA*	53.1	Y	3	61	1.44 ± 0.36	1.97 ± 0.82	1.42 ± 0.22
gi|4127309	hypothetical protein	AGA	13.5	Y	3	101	1.63 ± 1.08	0.87 ± 0.32	0.21 ± 0.29
gi|158296846	AGAP008279-PA	AGA*	36.8	Y	2	150	0.57 ± 0.16	0.14 ± 0.49	0.10 ± 0.24
gi|30267888	secretion protein gp65	AAL	43.4	Y	2	123	0.94 ± 0.66	0.70 ± 0.51	6.54 ± 1.25
gi|13537664	gSG1b protein	AGA	43.6	Y	2	84	0.93 ± 0.51	0.12 ± 0.03	0.14 ± 0.04
gi|27372911	salivary apyrase	AST	64.2	Y	2	65	1.07 ± 0.19	32.17 ± 23.29	1.08 ± 0.23
gi|58389748	AGAP008216-PA	AGA*	16.3	Y	2	142	3.19 ± 0.79	0.10 ± 0.45	0.12 ± 0.32
gi|18873404	hypothetical protein	AGA	46.5	Y	1	47	0.37 ± 0.1	0.71 ± 0.47	0.37 ± 0.23
gi|3378531	D7r2 protein	AGA	16.5	Y	1	401	0.45 ± 0	0.23 ± 0	0.75 ± 0
gi|4538887	D7-related 1 protein	AGA	18.7	Y	1	161	0.47 ± 0	0.40 ± 0	0.51 ± 0
gi|31209291	AGAP004334-PA	AGA*	38.2	Y	1	64	0.70 ± 0	0.57 ± 0	0.36 ± 0
gi|13537668	gSG7 protein	AGA	16.3	Y	1	133	0.92 ± 0.24	0.09 ± 0.4	0.04 ± 0.09
gi|29501532	hyp37.3 putative secreted salivary gland protein	AST	39.1	Y	1	90	1.30 ± 0.46	4.75 ± 0.26	1.10 ± 0.19

Accession No.	Protein name	Organisms	Predicted MW [kDa]	SignalP (NN)	No. quant. peptides	Mascot Score	Ratio1 ± Std1	Ratio2 ± Std2	Ratio3 ± Std3
gi|58382307	AGAP003025-PA	AGA*	11.3	Y	1	62	1.18 ± 0	3.99 ± 0	1.35 ± 0
gi|18389917	TRIO protein	AGA	43.7	Y	1	180	1.62 ± 0.80	0.28 ± 0.43	0.26 ± 0.29
gi|16225961	short form D7r1 salivary protein	AAR	18.6	Y	1	182	2.41 ± 0	0.32 ± 0	0.43 ± 0
Housekeeping proteins (n=4)									
gi|18389895	salivary gland 1-like 3 protein	AGA	31.0	N	7	262	1.79 ± 0.81	0.21 ± 0.25	0.17 ± 0.13
gi|158429477	Crystal Structure Of Anopheles Gambiae D7r4-Tryptamine Complex	AGA	17.1	N	6	63	0.86 ± 0.71	0.57 ± 0.49	0.38 ± 0.32
Unknown proteins (n=19)									
gi|158299190	AGAP010147-PA	AGA*	224.17	N	12	395	0.61 ± 0.25	1.96 ± 0.79	1.37 ± 0.38
gi|58385536	AGAP005134-PA	AGA*	59.41	N	5	96	1.36 ± 0.93	2.19 ± 0.55	1.53 ± 0.33
gi|118792669	AGAP012081-PA	AGA*	22.7	N	3	66	0.62 ± 0.13	1.26 ± 0.44	0.79 ± 0.34
gi|58374842	AGAP012894-PA	AGA*	13.8	N	3	60	0.99 ± 0.26	1.87 ± 0.59	1.15 ± 0.29
gi|158295212	AGAP006037-PA	AGA*	40.2	N	3	49	3.62 ± 2.58	3.01 ± 2.28	1.44 ± 1.64
gi|158287848	AGAP010957-PA	AGA*	17.2	N	2	52	0.50 ± 0.12	0.51 ± 0.12	0.81 ± 0.17
gi|158301594	AGAP001797-PA	AGA*	78.7	N	2	158	0.89 ± 0.38	1.98 ± 0.8	1.82 ± 0.71
gi|58391523	AGAP009623-PA	AGA*	35.4	N	2	77	1.14 ± 0.2	1.33 ± 1.65	0.95 ± 0.68
gi|158285710	AGAP007406-PA	AGA*	50.3	N	2	44	1.92 ± 0.72	2.29 ± 1.01	1.55 ± 0.04
gi|31202411	AGAP009537-PA	AGA*	11.77	N	2	50	1.58 ± 0.8	2.18 ± 0.79	2.45 ± 1.95
gi|158301478	AGAP001903-PA	AGA*	35.36	N	2	45	1.97 ± 0.94	1.07 ± 0.23	1.31 ± 0.81
gi|158301772	AGAP001676-PA	AGA*	41.52	N	1	150	0.48 ± 0	0.90 ± 0	1.29 ± 0
gi|158300600	AGAP012048-PA	AGA*	51.51	N	1	48	0.55 ± 0	1.14 ± 0	0.68 ± 0
gi|158301878	AGAP001569-PA	AGA*	18.22	N	1	52	0.57 ± 0	0.21 ± 0	0.29 ± 0
gi|58390364	AGAP007827-PA	AGA*	46.6	N	1	45	0.59 ± 0	2.15 ± 0	1.42 ± 0
gi|118789559	AGAP007966-PA	AGA*	32.8	N	1	80	0.74 ± 0	1.53 ± 0	1.03 ± 0
gi|158289809	AGAP010735-PA	AGA*	139.2	N	1	46	1.22 ± 0	3.02 ± 0	0 ± 0
gi|158290384	AGAP002919-PB	AGA*	46.7	N	1	68	2.10 ± 0	0.59 ± 0	0.21 ± 0
gi|158297658	AGAP011454-PA	AGA*	169.6	N	1	44	4.40 ± 0.36	2.02 ± 0.78	0.79 ± 0.13
non quantified proteins (n=6)									
gi|118778689	AGAP006958-PA	AGA*	82.0	N	0	50	N/A	N/A	N/A
gi|158289742	AGAP010691-PA	AGA*	74.0	N	0	50	N/A	N/A	N/A
gi|158293925	AGAP011514-PA	AGA*	25.7	N	0	147	N/A	N/A	N/A
gi|16225958	long form D7 salivary protein	AAR	35.7	Y	0	43	N/A	N/A	N/A
gi|31222545	AGAP008278-PA	AGA*	35.6	Y	0	43	N/A	N/A	N/A
gi|57966984	AGAP003541-PA	AGA*	50.4	N	0	44	N/A	N/A	N/A



Supplementary figure 1

Cluster no.	Access. Nbr gi|number	Access. Nbr	≥ 90% identity Protein name	Organism	Length	% identity ≥ 90%
Short form D7 family						
>Cluster 0						
0	4538891	Q9UB32	D7-related 3 protein	AGA	169	96%
1	3378529	O76816	D7r3 protein (Fragment)	AGA	156	93%
2	16225968	Q95V97	Short form D7r3 salivary protein	AAR	181	*
>Cluster 1						
0	4538889	Q9UB31	AGAP008282-PA (D7-related 2 protein)	AGA	168	*
1	3378531	O76815	D7r2 protein (Fragment)	AGA	150	99%
2	16225965	Q95V98	Short form D7r2 salivary protein	AAR	168	99%
>Cluster 2						
0	4538887	Q9UB30	D7-related 1 protein	AGA	165	*
1	4127333	O97414	D7r1 protein (Fragment)	AGA	111	100%
2	16225961	Q95V99	Short form D7r1 salivary protein	AAR	165	98%
>Cluster 13						
0	15718083	Q95P12	D7-related 1 protein	AST	166	*
1	16225971	Q95V96	Short form D7 salivary protein	AST	166	96%
>Cluster 14						
0	16225980	Q95V94	Short form D7clu5 salivary protein	AST	166	*
1	21314941	Q8MTP1	Contact-activation-inhibitor protein hamadarin	AST	166	96%
30 kDa allergen/GE rich family/anti-platelet family						
>Cluster 8						
0	83016745	Q7YT37	Anti-platelet aggregation protein (GE rich salivary gland protein)	AST	269	*
1	29501380	Q86M95	GE rich salivary gland protein	AST	269	99%
>Cluster 10						
0	190576759	B3VDI7	Anti-platelet protein	AGA	252	*
1	190576761	B3VDI8	Anti-platelet protein (Fragment)	AGA	224	99%
>Cluster 11						
0	71389019	Q45TQ1	Salivary gland protein	AAL	240	*
1	71389021	Q45TQ0	Salivary gland protein	AAL	240	99%
Apyrase / 5'-nucleotidase family						
>Cluster 3						
0	3378558	O76813	5'-nucleotidase (Fragment)	AGA	98	100%
1	4582528	Q9UB34	Putative 5'-nucleotidase (EC 3.1.3.5)	AGA	570	*
>Cluster 4						
0	4582524	Q9TW03	Putative apyrase (EC 3.6.1.5)	AGA	557	*
1	4127329	O97412	Apyrase (EC 3.6.1.5) (Fragment)	AGA	81	100%
SG2 family						
>Cluster 16						
0	4210617	O97408	SG2 protein	AGA	114	*
1	4127303	Q7JMV5	GSG2 protein (Fragment)	AGA	111	100%
Long form D7 family						
>Cluster 7						
0	18389891	Q8WR35	D7 protein long form	AGA	311	*
1	16225958	Q95VA0	Long form D7 salivary protein	AAR	311	98%
Antigen 5/gVAG family						
>Cluster 9						

0	34556108	O97413	Putative gVAG protein	AGA	260	*
1	18389883	Q8WR39	Antigen 5-related 1 protein	AGA	178	97%
Miscellaneous (1)						
>Cluster 17						
0	4127344	O97416	CE5 protein (Fragment)	AGA	101	*
1	3378535	O76817	F1 protein (Fragment)	AGA	73	98%
Miscellaneous (2)						
>Cluster 5						
0	56809867	Q2TLV8	AGAP000610-PA (SAGLIN)	AGA	412	*
1	3378533	O76814	D3 protein (Fragment)	AGA	94	98%
SG3 family						
>Cluster 12						
0	4210619	O97409	SG3 protein	AGA	189	*
1	4127305	Q7JMV4	GSG3 protein (Fragment)	AGA	36	100%
SG6 family						
>Cluster 15						
0	13537666	Q9BIH5	GSG6 protein	AGA	115	*
1	225572575	C1K7K0	GSG6 salivary protein	AAR	115	98%
SG1 family (2)						
>Cluster 6						
0	4127301	Q9UB33	GSG1 protein (Fragment)	AGA	153	99%
1	4210615	O97407	SG1 protein	AGA	401	*
NR proteins						
>Cluster 18	4539761	Q9XYP9	Salivary peroxidase	AAL	591	*
>Cluster 19	27372911	Q8I6Q2	Salivary apyrase	AST	575	*
>Cluster 20	27372903	Q8I6Q6	Putative 53.7 kDa salivary protein	AST	516	*
>Cluster 21	27372929	Q8I6P3	Putative salivary protein SG1B	AST	415	*
>Cluster 22	18389889	Q8WR36	Calreticulin	AGA	406	*
>Cluster 23	29501536	Q86M90	SG1D salivary protein	AST	404	*
>Cluster 24	18873404	O97415	Putative uncharacterized protein	AGA	401	*
>Cluster 25	29501528	Q86M94	TRIO salivary gland protein	AST	393	*
>Cluster 26	18389917	Q8WR22	TRIO protein (Fragment)	AGA	391	*
>Cluster 27	30267888	Q86CT5	Secretion protein gp65	AAL	386	*
>Cluster 28	13537664	Q9BIH6	GSG1b protein	AGA	385	*
>Cluster 29	27372941	Q8I6N7	Putative salivary protein SG1C	AST	383	*
>Cluster 30	29501532	Q86M92	Hyp37.3 putative secreted salivary gland protein	AST	351	*
>Cluster 31	13537662	Q9BIH7	AGAP004334-PA (GSG5 protein)	AGA	332	*
>Cluster 32	27372933	Q8I6P1	Putative salivary lipase	AST	317	*
>Cluster 33	16225974	Q95NY5	D7 protein (Long form D7clu2 salivary protein)	AST	315	*
>Cluster 34	29501532	Q52P96	AGAP001988-PA (Hyp37.7-like)	AGA	267	*
>Cluster 35	27372895	Q8I6R0	Salivary antigen-5 related protein	AST	259	*
>Cluster 36	18389885	Q8WR38	Antigen 5-related 2 protein	AGA	257	*
>Cluster 37	18378723	Q8WQZ0	Amylase (Fragment)	AGA	226	*
>Cluster 38	27372919	Q8I6P8	Putative 19.1 kDa salivary protein SG3	AST	199	*
>Cluster 39	13537672	Q9BIH2	AGAP003841-PA (Putative uncharacterized protein gSG10)	AGA	195	*
>Cluster 40	18389879	Q8WR41	30 kDa protein	AGA	182	*
>Cluster 41	27372939	Q8I6N8	Putative salivary protein SG1A	AST	179	*
>Cluster 42	18389893	Q8WR34	Mucin-like protein (Fragment)	AGA	178	*
>Cluster 43	27372897	Q8I6Q9	Hypothetical salivary protein 16	AST	171	*
>Cluster 44	13537660	Q9BIH8	GSG2-like protein	AGA	168	*

>Cluster 45	29501376	Q86M97	Short D7-4 salivary protein	AST	168	*
>Cluster 46	13509404	Q9BII2	Putative uncharacterized protein (Fragment)	AGA	166	*
>Cluster 47	18378603	Q8WR17	D7-related 5 protein	AGA	166	*
>Cluster 48	17016228	Q9BIH3	D7-related 4 protein (D7r4 protein)	AGA	165	*
>Cluster 49	16225977	Q95V95	Short form D7clu4 salivary protein	AST	164	*
>Cluster 50	27372927	Q8I6P4	Putative 13.4 kDa salivary protein	AST	164	*
>Cluster 51	18389881	Q8WR40	Selenoprotein (Fragment)	AGA	163	*
>Cluster 52	27372915	Q8I6Q0	Putative salivary protein D7B	AST	161	*
>Cluster 53	27372923	Q8I6P6	Putative 13.5 kDa salivary protein	AST	156	*
>Cluster 54	29501378	Q86M96	Short D7-5 salivary protein	AST	151	*
>Cluster 55	13537668	Q9BIH4	GSG7 protein	AGA	145	*
>Cluster 56	27372909	Q8I6Q3	Putative salivary protein gSG7	AST	142	*
>Cluster 57	148189823	A5HUP6	Anophensin	AST	142	*
>Cluster 58	34303827	Q86M91	Lysozyme (Salivary lysozyme) (EC 3.2.1.17)	AST	141	*
>Cluster 59	894206	Q17005	Lysozyme c-1 (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase)	AGA	140	*
>Cluster 60	27372935	Q8I6P0	Putative 13.3 kDa salivary protein	AST	140	*
>Cluster 61	27372901	Q8I6Q7	Hypothetical salivary protein SG2A	AST	129	*
>Cluster 62	13537674	Q9BIH1	AGAP010647-PA (GSG8 protein) (Fragment)	AGA	128	*
>Cluster 63	27372925	Q8I6P5	Putative 11.9 kDa salivary protein	AST	128	*
>Cluster 64	4127309	O97411	Putative uncharacterized protein (Fragment)	AGA	122	*
>Cluster 65	27372907	Q8I6Q4	Putative salivary protein SG2B	AST	116	*
>Cluster 66	29501530	Q86M93	GSG6 salivary gland protein	AST	114	*
>Cluster 67	116247566	A0A114	Putative salivary secreted peptide	AGA	105	*
>Cluster 68	13509402	Q9BII3	Putative uncharacterized protein (Fragment)	AGA	104	*
>Cluster 69	27372921	Q8I6P7	Salivary anti-thrombin anophelin	AST	101	*
>Cluster 70	27372931	Q8I6P2	Putative salivary protein hyp10	AST	99	*
>Cluster 71	124244265	A2TJI3	Salivary defensin	AST	96	*
>Cluster 72	27372899	Q8I6Q8	Hypothetical salivary protein 8.2	AST	93	*
>Cluster 73	18389905	Q8WR28	Putative uncharacterized protein	AGA	92	*
>Cluster 74	18389915	Q8WR23	Putative uncharacterized protein	AGA	91	*
>Cluster 75	13509406	Q9BII1	GSG1a protein (Fragment)	AGA	90	*
>Cluster 76	18389901	Q8WR30	Putative uncharacterized protein	AGA	90	*
>Cluster 77	27372893	Q8I6R1	Putative salivary protein hyp12	AST	90	*
>Cluster 78	62546223	Q52P95	AGAP006495-PA (Hyp6.2)	AGA	85	*
>Cluster 79	27372905	Q8I6Q5	Putative salivary secreted serine protease inhibitor	AST	84	*
>Cluster 80	62546225	Q52P94	AGAP007195-PA (Hyp6.3)	AGA	83	*
>Cluster 81	6851157	Q9NJS1	Salivary anti-thrombin peptide anophelin	AAL	83	*
>Cluster 82	4127307	O97410	Putative uncharacterized protein (Fragment)	AGA	81	*
>Cluster 83	18389911	Q8WR25	AGAP000152-PA (Putative uncharacterized protein)	AGA	78	*
>Cluster 84	27372913	Q8I6Q1	5 kDa salivary peptide hyp15	AST	78	*
>Cluster 85	13537676	Q9BIH0	GSG9 protein (Fragment)	AGA	76	*
>Cluster 86	27372917	Q8I6P9	Putative 6.3 kDa salivary peptide	AST	70	*
>Cluster 87	161610988	A9QVW3	Putative defensin 5	AGA	68	*
>Cluster 88	62546227	Q52P93	AGAP004836-PA (Hyp3.5)	AGA	57	*
>Cluster 89	18389903	Q8WR29	Putative uncharacterized protein	AGA	56	*
>Cluster 90	18389907	Q8WR27	Putative uncharacterized protein	AGA	56	*
>Cluster 91	18389913	Q8WR24	Putative uncharacterized protein	AGA	48	*
>Cluster 92	18389909	Q8WR26	Putative uncharacterized protein	AGA	46	*
>Cluster 93	17026155	Q8WSY2	Putative uncharacterized protein (Fragment)	AGA	34	*
>Cluster 94	17026157	Q8WSY1	Putative uncharacterized protein (Fragment)	AGA	22	*

Cluster no.	Access. Nbr gi|number	Access. Nbr	≥ 50% identity Protein name	Organism	Length	% identity ≥ 50%	% identity ≥ 90%
Short form D7 family							
>Cluster 1							
0	4538889	Q9UB31	AGAP008282-PA (D7-related 2 protein)	AGA	168	61%	
1	3378531	O76815	D7r2 protein (Fragment)	AGA	150	61%	99%
2	16225965	Q95V98	Short form D7r2 salivary protein	AAR	168	61%	99%
3	**29501376**	**Q86M97**	**Short D7-4 salivary protein**	**AST**	**168**	**62%**	
4	4538891	Q9UB32	D7-related 3 protein	AGA	169	96%	
5	3378529	O76816	D7r3 protein (Fragment)	AGA	156	93%	
6	16225968	Q95V97	Short form D7r3 salivary protein	AAR	181	*	
>Cluster 0							
0	4538887	Q9UB30	D7-related 1 protein	AGA	165	58%	
1	4127333	O97414	D7r1 protein (Fragment)	AGA	111	58%	100%
2	16225961	Q95V99	Short form D7r1 salivary protein	AAR	165	58%	98%
3	**16225977**	**Q95V95**	**Short form D7clu4 salivary protein**	**AST**	**164**	**70%**	
4	**29501378**	**Q86M96**	**Short D7-5 salivary protein**	**AST**	**151**	**80%**	
5	15718083	Q95P12	D7-related 1 protein	AST	166	*	
6	16225971	Q95V96	Short form D7 salivary protein	AST	166	96%	
7	16225980	Q95V94	Short form D7clu5 salivary protein	AST	166	50%	
8	21314941	Q8MTP1	Contact-activation-inhibitor protein hamadarin	AST	166	50%	96%
30 kDa allergen/GE rich family/anti-platelet family							
>Cluster 3							
0	190576759	B3VDI7	Anti-platelet protein	AGA	252	62%	
1	190576761	B3VDI8	Anti-platelet protein (Fragment)	AGA	224	62%	99%
2	83016745	Q7YT37	Anti-platelet aggregation protein (GE rich salivary gland protein)	AST	269	*	
3	29501380	Q86M95	GE rich salivary gland protein	AST	269	99%	
>Cluster 13							
0	71389019	Q45TQ1	Salivary gland protein	AAL	240	*	
1	71389021	Q45TQ0	Salivary gland protein	AAL	240	99%	
Apyrase / 5'-nucleotidase family							
>Cluster 4							
0	3378558	O76813	5'-nucleotidase (Fragment)	AGA	98	78%	100%
1	4582528	Q9UB34	Putative 5'-nucleotidase (EC 3.1.3.5)	AGA	570	78%	
2	**27372911**	**Q8I6Q2**	**Salivary apyrase**	**AST**	**575**	*	
>Cluster 9							
0	4582524	Q9TW03	Putative apyrase (EC 3.6.1.5)	AGA	557	*	
1	4127329	O97412	Apyrase (EC 3.6.1.5) (Fragment)	AGA	81	100%	
SG2 family							
>Cluster 7							
0	4210617	O97408	SG2 protein	AGA	114	67%	
1	4127303	Q7JMV5	GSG2 protein (Fragment)	AGA	111	67%	100%
2	**27372907**	**Q8I6Q4**	**Putative salivary protein SG2B**	**AST**	**116**	*	
>Cluster 15							
0	**13537660**	**Q9BIH8**	**GSG2-like protein**	**AGA**	**168**	*	
1	**27372901**	**Q8I6Q7**	**Hypothetical salivary protein SG2A**	**AST**	**129**	**53%**	
Long form D7 family							
>Cluster 2							

0	18389891	Q8WR35	D7 protein long form	AGA	311	61%	
1	16225958	Q95VA0	Long form D7 salivary protein	AAR	311	61%	98%
2	**16225974**	**Q95NY5**	**D7 protein (Long form D7clu2 salivary protein)**	**AST**	**315**	*	
3	**27372915**	**Q8I6Q0**	**Putative salivary protein D7B**	**AST**	**161**	**68%**	
Antigen 5/gVAG family							
>Cluster 5							
0	34556108	O97413	Putative gVAG protein	AGA	260	*	
1	18389883	Q8WR39	Antigen 5-related 1 protein	AGA	178	97%	
2	**27372895**	**Q8I6R0**	**Salivary antigen-5 related protein**	**AST**	**259**	**84%**	
Miscellaneous (1)							
>Cluster 18							
0	4127344	O97416	CE5 protein (Fragment)	AGA	101	*	
1	3378535	O76817	F1 protein (Fragment)	AGA	73	98%	
Miscellaneous (2)							
>Cluster 10							
0	56809867	Q2TLV8	AGAP000610-PA (SAGLIN)	AGA	412	*	
1	3378533	O76814	D3 protein (Fragment)	AGA	94	98%	
SG3 family							
>Cluster 6							
0	4210619	O97409	SG3 protein	AGA	189	67%	
1	4127305	Q7JMV4	GSG3 protein (Fragment)	AGA	36	67%	100%
2	**27372919**	**Q8I6P8**	**Putative 19.1 kDa salivary protein SG3**	**AST**	**199**	*	
SG6 family							
>Cluster 8							
0	13537666	Q9BIH5	GSG6 protein	AGA	115	*	
1	225572575	C1K7K0	GSG6 salivary protein	AAR	115	98%	
2	**29501530**	**Q86M93**	**GSG6 salivary gland protein**	**AST**	**114**	**77%**	
SG1 family (2)							
>Cluster 11							
0	4127301	Q9UB33	GSG1 protein (Fragment)	AGA	153	99%	
1	4210615	O97407	SG1 protein	AGA	401	*	
SG7 family							
>Cluster 16							
0	**13537668**	**Q9BIH4**	**GSG7 protein**	**AGA**	**145**	*	
1	**148189823**	**A5HUP6**	**Anophensin**	**AST**	**142**	**68%**	
Hypothetical or uncharacterized proteins (2)							
>Cluster 19							
0	**18389911**	**Q8WR25**	**AGAP000152-PA (Putative uncharacterized protein)**	**AGA**	**78**	**55%**	
1	**18389915**	**Q8WR23**	**Putative uncharacterized protein**	**AGA**	**91**	*	
SG1 family (1)							
>Cluster 12							
0	**13537664**	**Q9BIH6**	**GSG1b protein**	**AGA**	**385**	*	
1	**27372941**	**Q8I6N7**	**Putative salivary protein SG1C**	**AST**	**383**	**56%**	
Miscellaneous (3)							
>Cluster 14							
0	**18389893**	**Q8WR34**	**Mucin-like protein (Fragment)**	**AGA**	**178**	*	
1	**27372923**	**Q8I6P6**	**Putative 13.5 kDa salivary protein**	**AST**	**156**	**63%**	
Lysosyme family							
>Cluster 17							
0	**894206**	**Q17005**	**Lysozyme c-1 (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase)**	**AGA**	**140**	**85%**	

1	**34303827**	**Q86M91**	**Lysozyme (Salivary lysozyme) (EC 3.2.1.17)**	**AST**	**141**	*
NR proteins						
>Cluster 20	4539761	Q9XYP9	Salivary peroxidase	AAL	591	*
>Cluster 21	27372903	Q8I6Q6	Putative 53.7 kDa salivary protein	AST	516	*
>Cluster 22	27372929	Q8I6P3	Putative salivary protein SG1B	AST	415	*
>Cluster 23	18389889	Q8WR36	Calreticulin	AGA	406	*
>Cluster 24	29501536	Q86M90	SG1D salivary protein	AST	404	*
>Cluster 25	18873404	O97415	Putative uncharacterized protein	AGA	401	*
>Cluster 26	29501528	Q86M94	TRIO salivary gland protein	AST	393	*
>Cluster 27	18389917	Q8WR22	TRIO protein (Fragment)	AGA	391	*
>Cluster 28	30267888	Q86CT5	Secretion protein gp65	AAL	386	*
>Cluster 29	29501532	Q86M92	Hyp37.3 putative secreted salivary gland protein	AST	351	*
>Cluster 30	13537662	Q9BIH7	AGAP004334-PA (GSG5 protein)	AGA	332	*
>Cluster 31	27372933	Q8I6P1	Putative salivary lipase	AST	317	*
>Cluster 32	29501532	Q52P96	AGAP001988-PA (Hyp37.7-like)	AGA	267	*
>Cluster 33	18389885	Q8WR38	Antigen 5-related 2 protein	AGA	257	*
>Cluster 34	18378723	Q8WQZ0	Amylase (Fragment)	AGA	226	*
>Cluster 35	13537672	Q9BIH2	AGAP003841-PA (Putative uncharacterized protein gSG10)	AGA	195	*
>Cluster 36	18389879	Q8WR41	30 kDa protein	AGA	182	*
>Cluster 37	27372939	Q8I6N8	Putative salivary protein SG1A	AST	179	*
>Cluster 38	27372897	Q8I6Q9	Hypothetical salivary protein 16	AST	171	*
>Cluster 39	13509404	Q9BII2	Putative uncharacterized protein (Fragment)	AGA	166	*
>Cluster 40	18378603	Q8WR17	D7-related 5 protein	AGA	166	*
>Cluster 41	17016228	Q9BIH3	D7-related 4 protein (D7r4 protein)	AGA	165	*
>Cluster 42	27372927	Q8I6P4	Putative 13.4 kDa salivary protein	AST	164	*
>Cluster 43	18389881	Q8WR40	Selenoprotein (Fragment)	AGA	163	*
>Cluster 44	27372909	Q8I6Q3	Putative salivary protein gSG7	AST	142	*
>Cluster 45	27372935	Q8I6P0	Putative 13.3 kDa salivary protein	AST	140	*
>Cluster 46	13537674	Q9BIH1	AGAP010647-PA (GSG8 protein) (Fragment)	AGA	128	*
>Cluster 47	27372925	Q8I6P5	Putative 11.9 kDa salivary protein	AST	128	*
>Cluster 48	4127309	O97411	Putative uncharacterized protein (Fragment)	AGA	122	*
>Cluster 49	116247566	A0A114	Putative salivary secreted peptide	AGA	105	*
>Cluster 50	13509402	Q9BII3	Putative uncharacterized protein (Fragment)	AGA	104	*
>Cluster 51	27372921	Q8I6P7	Salivary anti-thrombin anophelin	AST	101	*
>Cluster 52	27372931	Q8I6P2	Putative salivary protein hyp10	AST	99	*
>Cluster 53	124244265	A2TJI3	Salivary defensin	AST	96	*
>Cluster 54	27372899	Q8I6Q8	Hypothetical salivary protein 8.2	AST	93	*
>Cluster 55	18389905	Q8WR28	Putative uncharacterized protein	AGA	92	*
>Cluster 56	13509406	Q9BII1	GSG1a protein (Fragment)	AGA	90	*
>Cluster 57	18389901	Q8WR30	Putative uncharacterized protein	AGA	90	*
>Cluster 58	27372893	Q8I6R1	Putative salivary protein hyp12	AST	90	*
>Cluster 59	62546223	Q52P95	AGAP006495-PA (Hyp6.2)	AGA	85	*
>Cluster 60	27372905	Q8I6Q5	Putative salivary secreted serine protease inhibitor	AST	84	*
>Cluster 61	62546225	Q52P94	AGAP007195-PA (Hyp6.3)	AGA	83	*
>Cluster 62	6851157	Q9NJS1	Salivary anti-thrombin peptide anophelin	AAL	83	*
>Cluster 63	4127307	O97410	Putative uncharacterized protein (Fragment)	AGA	81	*
>Cluster 64	27372913	Q8I6Q1	5 kDa salivary peptide hyp15	AST	78	*
>Cluster 65	13537676	Q9BIH0	GSG9 protein (Fragment)	AGA	76	*
>Cluster 66	27372917	Q8I6P9	Putative 6.3 kDa salivary peptide	AST	70	*
>Cluster 67	161610988	A9QVW3	Putative defensin 5	AGA	68	*

>Cluster 68	62546227	Q52P93	AGAP004836-PA (Hyp3.5)	AGA	57	*
>Cluster 69	18389903	Q8WR29	Putative uncharacterized protein	AGA	56	*
>Cluster 70	18389907	Q8WR27	Putative uncharacterized protein	AGA	56	*
>Cluster 71	18389913	Q8WR24	Putative uncharacterized protein	AGA	48	*
>Cluster 72	18389909	Q8WR26	Putative uncharacterized protein	AGA	46	*
>Cluster 73	17026155	Q8WSY2	Putative uncharacterized protein (Fragment)	AGA	34	*
>Cluster 74	17026157	Q8WSY1	Putative uncharacterized protein (Fragment)	AGA	22	*

Cluster no.	Access. Nbr gi|number	Access. Nbr	≥ 30% identity Protein name	Organism	Length	% identity ≥ 30%	≥ 50%	≥ 90%
Short form D7 family								
>Cluster 0								
0	4538889	Q9UB31	AGAP008282-PA (D7-related 2 protein)	AGA	168	61%		
1	3378531	O76815	D7r2 protein (Fragment)	AGA	150	61%	99%	
2	16225965	Q95V98	Short form D7r2 salivary protein	AAR	168	61%	99%	
3	29501376	Q86M97	Short D7-4 salivary protein	AST	168	62%		
4	4538891	Q9UB32	D7-related 3 protein	AGA	169	96%		
5	3378529	O76816	D7r3 protein (Fragment)	AGA	156	93%		
6	16225968	Q95V97	Short form D7r3 salivary protein	AAR	181	*		
7	4538887	Q9UB30	D7-related 1 protein	AGA	165	31%/87%	58%	
8	4127333	O97414	D7r1 protein (Fragment)	AGA	111	31%/87%	58%	100%
9	16225961	Q95V99	Short form D7r1 salivary protein	AAR	165	31%/87%	58%	98%
10	16225977	Q95V95	Short form D7clu4 salivary protein	AST	164	31%/87%	70%	
11	29501378	Q86M96	Short D7-5 salivary protein	AST	151	31%/87%	80%	
12	15718083	Q95P12	D7-related 1 protein	AST	166	31%/87%		
13	16225971	Q95V96	Short form D7 salivary protein	AST	166	31%/87%	96%	
14	16225980	Q95V94	Short form D7clu5 salivary protein	AST	166	31%/87%	50%	
15	21314941	Q8MTP1	Contact-activation-inhibitor protein hamadarin	AST	166	31%/87%	50%	96%
16	**17016228**	**Q9BIH3**	**D7-related 4 protein (D7r4 protein)**	**AGA**	**165**	**35%/92%**		
30 kDa allergen/GE rich family/anti-platelet family								
>Cluster 1								
0	190576759	B3VDI7	Anti-platelet protein	AGA	252	62%		
1	190576761	B3VDI8	Anti-platelet protein (Fragment)	AGA	224	62%	99%	
2	83016745	Q7YT37	Anti-platelet aggregation protein (GE rich salivary gland protein)	AST	269	*		
3	29501380	Q86M95	GE rich salivary gland protein	AST	269	99%		
4	71389019	Q45TQ1	Salivary gland protein	AAL	240	55%/99%		
5	71389021	Q45TQ0	Salivary gland protein	AAL	240	55%/99%	99%	
6	**18389879**	**Q8WR41**	**30 kDa protein**	**AGA**	**182**	**46%/85%**		
Apyrase / 5'-nucleotidase family								
>Cluster 2								
0	3378558	O76813	5'-nucleotidase (Fragment)	AGA	98	78%	100%	
1	4582528	Q9UB34	Putative 5'-nucleotidase (EC 3.1.3.5)	AGA	570	78%		
2	27372911	Q8I6Q2	Salivary apyrase	AST	575	*		
3	4582524	Q9TW03	Putative apyrase (EC 3.6.1.5)	AGA	557	45%/93%		
4	4127329	O97412	Apyrase (EC 3.6.1.5) (Fragment)	AGA	81	45%/93%	100%	
SG2 family								
>Cluster 3								
0	13537660	Q9BIH8	GSG2-like protein	AGA	168	*		
1	27372901	Q8I6Q7	Hypothetical salivary protein SG2A	AST	129	53%		
2	4210617	O97408	SG2 protein	AGA	114	42%/100%	67%	
3	4127303	Q7JMV5	GSG2 protein (Fragment)	AGA	111	42%/100%	67%	100%
4	27372907	Q8I6Q4	Putative salivary protein SG2B	AST	116	42%/100%		
Long form D7 family								
>Cluster 4								
0	18389891	Q8WR35	D7 protein long form	AGA	311	61%		
1	16225958	Q95VA0	Long form D7 salivary protein	AAR	311	61%	98%	
2	16225974	Q95NY5	D7 protein (Long form D7clu2 salivary protein)	AST	315	*		

3	27372915	Q8I6Q0	Putative salivary protein D7B	AST	161	68%	
Antigen 5/gVAG family							
>Cluster 5							
0	34556108	O97413	Putative gVAG protein	AGA	260	*	
1	18389883	Q8WR39	Antigen 5-related 1 protein	AGA	178	97%	
2	27372895	Q8I6R0	Salivary antigen-5 related protein	AST	259	84%	
3	**18389885**	**Q8WR38**	**Antigen 5-related 2 protein**	**AGA**	**257**	**49%/97%**	
Miscellaneous (1)							
>Cluster 6							
0	4127344	O97416	CE5 protein (Fragment)	AGA	101	*	
1	3378535	O76817	F1 protein (Fragment)	AGA	73	98%	
2	**6851157**	**Q9NJS1**	**Salivary anti-thrombin peptide anophelin**	**AAL**	**83**	**39%/90%**	
3	**27372921**	**Q8I6P7**	**Salivary anti-thrombin anophelin**	**AST**	**101**	**48%/100%**	
Miscellaneous (2)							
>Cluster 7							
0	**27372929**	**Q8I6P3**	**Putative salivary protein SG1B**	**AST**	**415**	*****	
1	56809867	Q2TLV8	AGAP000610-PA (SAGLIN)	AGA	412	47%/93%	
2	3378533	O76814	D3 protein (Fragment)	AGA	94	47%/93%	98%
SG3 family							
>Cluster 8							
0	4210619	O97409	SG3 protein	AGA	189	67%	
1	4127305	Q7JMV4	GSG3 protein (Fragment)	AGA	36	67%/100%	
2	27372919	Q8I6P8	Putative 19.1 kDa salivary protein SG3	AST	199	*	
SG6 family							
>Cluster 10							
0	13537666	Q9BIH5	GSG6 protein	AGA	115	*	
1	225572575	C1K7K0	GSG6 salivary protein	AAR	115	98%	
2	29501530	Q86M93	GSG6 salivary gland protein	AST	114	77%	
SG1 family (2)							
>Cluster 14							
0	4127301	Q9UB33	GSG1 protein (Fragment)	AGA	153	99%	
1	4210615	O97407	SG1 protein	AGA	401	*	
SG7 family							
>Cluster 9							
0	13537668	Q9BIH4	GSG7 protein	AGA	145	*	
1	148189823	A5HUP6	Anophensin	AST	142	68%	
2	**27372909**	**Q8I6Q3**	**Putative salivary protein gSG7**	**AST**	**142**	**41%/97%**	
Hypothetical or uncharacterized proteins (2)							
>Cluster 12							
0	18389911	Q8WR25	AGAP000152-PA (Putative uncharacterized protein)	AGA	78	55%	
1	18389915	Q8WR23	Putative uncharacterized protein	AGA	91	*	
2	**27372913**	**Q8I6Q1**	**5 kDa salivary peptide hyp15**	**AST**	**78**	**41%/98%**	
SG1 family (1)							
>Cluster 16							
0	13537664	Q9BIH6	GSG1b protein	AGA	385	*	
1	27372941	Q8I6N7	Putative salivary protein SG1C	AST	383	56%	
Miscellaneous (3)							
>Cluster 19							
0	18389893	Q8WR34	Mucin-like protein (Fragment)	AGA	178	*	
1	27372923	Q8I6P6	Putative 13.5 kDa salivary protein	AST	156	63%	

Lysosyme family						
>Cluster 20						
0	894206	Q17005	Lysozyme c-1 (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase)	AGA	140	85%
1	34303827	Q86M91	Lysozyme (Salivary lysozyme) (EC 3.2.1.17)	AST	141	*
Hypothetical or uncharacterized proteins (1)						
>Cluster 11						
0	**27372931**	**Q8I6P2**	**Putative salivary protein hyp10**	**AST**	**99**	*
1	**18389905**	**Q8WR28**	**Putative uncharacterized protein**	**AGA**	**92**	**34%/92%**
2	**18389901**	**Q8WR30**	**Putative uncharacterized protein**	**AGA**	**90**	**50%/96%**
Trio family						
>Cluster 15						
0	**29501528**	**Q86M94**	**TRIO salivary gland protein**	**AST**	**393**	*
1	**18389917**	**Q8WR22**	**TRIO protein (Fragment)**	**AGA**	**391**	**46%/97%**
SG1 family (3)						
>Cluster 13						
0	**29501536**	**Q86M90**	**SG1D salivary protein**	**AST**	**404**	*
1	**18873404**	**O97415**	**Putative uncharacterized protein**	**AGA**	**401**	**46%/100%**
Hypothetical proteins (1)						
>Cluster 17						
0	**29501532**	**Q86M92**	**Hyp37.3 putative secreted salivary gland protein**	**AST**	**351**	*
1	**29501532**	**Q52P96**	**AGAP001988-PA (Hyp37.7-like)**	**AGA**	**267**	**38%/88%**
SG9/SG10 family						
>Cluster 18						
0	**13537672**	**Q9BIH2**	**AGAP003841-PA (Putative uncharacterized protein gSG10)**	**AGA**	**195**	*
1	**13537676**	**Q9BIH0**	**GSG9 protein (Fragment)**	**AGA**	**76**	**40%/93%**
Hypothetical proteins (2)						
>Cluster 21						
0	**27372899**	**Q8I6Q8**	**Hypothetical salivary protein 8.2**	**AST**	**93**	*
1	**4127307**	**O97410**	**Putative uncharacterized protein (Fragment)**	**AGA**	**81**	**44%/98%**
NR proteins						
>Cluster 22	4539761	Q9XYP9	Salivary peroxidase	AAL	591	*
>Cluster 23	27372903	Q8I6Q6	Putative 53.7 kDa salivary protein	AST	516	*
>Cluster 24	18389889	Q8WR36	Calreticulin	AGA	406	*
>Cluster 25	30267888	Q86CT5	Secretion protein gp65	AAL	386	*
>Cluster 26	13537662	Q9BIH7	AGAP004334-PA (GSG5 protein)	AGA	332	*
>Cluster 27	27372933	Q8I6P1	Putative salivary lipase	AST	317	*
>Cluster 28	18378723	Q8WQZ0	Amylase (Fragment)	AGA	226	*
>Cluster 29	27372939	Q8I6N8	Putative salivary protein SG1A	AST	179	*
>Cluster 30	27372897	Q8I6Q9	Hypothetical salivary protein 16	AST	171	*
>Cluster 31	13509404	Q9BII2	Putative uncharacterized protein (Fragment)	AGA	166	*
>Cluster 32	18378603	Q8WR17	D7-related 5 protein	AGA	166	*
>Cluster 33	27372927	Q8I6P4	Putative 13.4 kDa salivary protein	AST	164	*
>Cluster 34	18389881	Q8WR40	Selenoprotein (Fragment)	AGA	163	*
>Cluster 35	27372935	Q8I6P0	Putative 13.3 kDa salivary protein	AST	140	*
>Cluster 36	13537674	Q9BIH1	AGAP010647-PA (GSG8 protein) (Fragment)	AGA	128	*
>Cluster 37	27372925	Q8I6P5	Putative 11.9 kDa salivary protein	AST	128	*
>Cluster 38	4127309	O97411	Putative uncharacterized protein (Fragment)	AGA	122	*
>Cluster 39	116247566	A0A114	Putative salivary secreted peptide	AGA	105	*
>Cluster 40	13509402	Q9BII3	Putative uncharacterized protein (Fragment)	AGA	104	*
>Cluster 41	124244265	A2TJI3	Salivary defensin	AST	96	*

>Cluster 42	13509406	Q9BII1	GSG1a protein (Fragment)	AGA	90	*
>Cluster 43	27372893	Q8I6R1	Putative salivary protein hyp12	AST	90	*
>Cluster 44	62546223	Q52P95	AGAP006495-PA (Hyp6.2)	AGA	85	*
>Cluster 45	27372905	Q8I6Q5	Putative salivary secreted serine protease inhibitor	AST	84	*
>Cluster 46	62546225	Q52P94	AGAP007195-PA (Hyp6.3)	AGA	83	*
>Cluster 47	27372917	Q8I6P9	Putative 6.3 kDa salivary peptide	AST	70	*
>Cluster 48	161610988	A9QVW3	Putative defensin 5	AGA	68	*
>Cluster 49	62546227	Q52P93	AGAP004836-PA (Hyp3.5)	AGA	57	*
>Cluster 50	18389903	Q8WR29	Putative uncharacterized protein	AGA	56	*
>Cluster 51	18389907	Q8WR27	Putative uncharacterized protein	AGA	56	*
>Cluster 52	18389913	Q8WR24	Putative uncharacterized protein	AGA	48	*
>Cluster 53	18389909	Q8WR26	Putative uncharacterized protein	AGA	46	*
>Cluster 54	17026155	Q8WSY2	Putative uncharacterized protein (Fragment)	AGA	34	*
>Cluster 55	17026157	Q8WSY1	Putative uncharacterized protein (Fragment)	AGA	22	*

L'ensemble des analyses comparatives (*in silico* et protéomiques) ont permis de montrer une forte homogénéité entre les protéomes salivaires d'*An. gambiae* et *An. arabiensis*, deux espèces appartenant au même complexe d'espèces (*An. gambiae sensus lato*). De plus, ces approches ont mis en évidence des répertoires protéiques singuliers entre ce complexe et les autres espèces d'*Anopheles*, suggérant l'existence de protéines salivaires spécifiques qui pourraient être utilisées comme marqueurs d'expositions. Enfin, la spécificité de certaines de ces protéines pour mesurer l'exposition au complexe *An. gambiae s.l.* a été mise en évidence par Western blot. En effet, malgré l'existence de faibles réactions croisées, le sérum d'individus exposés au complexe *An. gambiae s.l.* réagit sur quelques bandes protéiques spécifiques avec une forte intensité. Une fois les analyses terminées, nous espérons pouvoir sélectionner des candidats marqueurs immunologiques d'exposition basés sur leur spécificité de séquences et de leurs antigénicités. Ce travail donne des résultats préliminaires optimistes sur la possibilité de détecter des marqueurs immunologiques spécifiques d'espèces au sein d'un même genre de moustique.

Relation entre l'exposition aux piqûres et la réponse sérologique anti-salive au niveau quantitatif (densité de moustiques/niveau de réponse IgG) et qualitatif (spécificité de la réponse)

L'exposition aux moustiques peut donc induire des réponses anticorps spécifiques dirigées contre la salive ou des extraits de glandes salivaires de moustiques. Des travaux antérieurs réalisés sur plusieurs espèces de moustiques ont montrés que le niveau de ces réponses anticorps pouvait être associé au niveau d'exposition à leur piqûres (Remoue et al., 2006, Waitayakul et al., 2006, Palosuo et al., 1997). De plus, une spécificité au niveau de l'espèce a déjà été observée en comparant la réactivité antigénique sur la salive de plusieurs espèces de moustiques (Brummer-Korvenkontio et al., 1997).

Pour confirmer ces observations et afin de mieux caractériser l'évolution de cette réponse anticorps en fonction de la densité saisonnière et géographique de moustiques, nous avons réalisé une étude comparative sur trois populations d'individus exposés à *Aedes caspius* à différents niveaux : exposition faible ou nulle pour les habitants de Marseille, intermédiaire pour les habitants de Fos-sur-mer et importante pour les habitants de Camargue. Ces différences géographiques d'expositions ont été étayées par des mesures entomologiques dans chacun des sites d'étude. De plus, des prélèvements sanguins ont été réalisés chez ces individus avant (Février 2007), pendant (Septembre 2007) et après (Janvier 2008) le pic d'agressivité de ce moustique pour évaluer la cinétique de cette réponse. Enfin, la spécificité de genre et d'espèce de cette réponse anticorps anti-salive a été évaluée en utilisant les sérums de ces trois populations sur des extraits de glandes salivaires de moustiques du même genre (*Aedes aegypti* et *Aedes albopictus*) qui n'étaient pas présents sur le lieu de l'étude et sur des glandes salivaires de *Culex pipiens*, un moustique présent sur les trois sites. Ce travail fait l'objet de l'article N.5 qui a été soumis pour publication :

Article N.5 **A. Fontaine**, A. Pascual, E. Orlandi-Pradines, S. Bourdon, N. Bakkali, I. Diouf, T. Coffinet, F. Gardella, F. Mouchet, G. L'Ambert, G. Lacour, F. Remoue, F. Pages, T. Fusaï, C. Rogier, L. Almeras **Relationship between exposure to vector bites and antibody responses against mosquito salivary gland extracts,** *(soumis)*

ARTICLE N.5

Relationship between exposure to vector bites and antibody responses against mosquito salivary gland extracts

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Relationship between exposure to vector bites and antibody responses against mosquito salivary gland extracts

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Abstract

Background: Mosquito-borne diseases are a major health problem worldwide and cause important morbidity and mortality not only in tropical areas but also in temperate regions. Serological responses against mosquito saliva proteins may be useful in estimating an individual's exposure to arthropod vector bites. The objective of this study was to determine whether the IgG response level is related to mosquito density and to assess the genus and/or species specificity of this response.

Methodology/Principal findings: The antibody levels of southeast French individuals living in three areas with a distinct ecological environment and by implication, a distinct *Aedes caspius* mosquito density were compared by ELISA analysis on several mosquito salivary gland extracts. A significant increase in the antibody responses was observed based on seasonal and spatial *Ae. caspius* density. Moreover, low IgG reactivities were observed against salivary gland extracts of non-endemic mosquitoes (*Aedes aegypti* and *Aedes albopictus*). Salivary gland antigens from *Culex pipiens* were used as mosquito genus antigenic control. These results suggested that antibody responses seemed to be specific to the mosquito genus and species.

Conclusion/Significance: Collectively, these data confirmed that human antibody responses may be used to assess individual exposure to species-specific mosquito bites. The specific immune response against a mosquito or other hematophagous arthropods' saliva antigens may be used to assess exposure level, the efficiency of anti-vector strategies, the identification of new infestation areas and also to estimate the risk of vector-borne disease transmission.

Author Summary

Camargue is an extensive wetland in southeast France, which provides abundant breeding sites for mosquitoes. The high mosquito density is a nuisance for people and animals and is a potential threat to human health because some of these mosquitoes are potential vectors of disease. Antibody responses against mosquito saliva proteins have been shown to be useful in estimating the transient exposure of individuals to arthropod vector bites. To determine whether the IgG response level is related to mosquito density and whether this response is genus and/or species specific, we compared the antibody levels against mosquito salivary gland extracts from individuals living in three areas of southeast France. These areas were Camargue, Fos-sur-mer and Marseille; these three areas have distinct ecological environments and distinct *Aedes caspius* mosquito densities. A significant increase in the antibody responses during the mosquito peak exposure season was observed in the three sites according to *Ae. caspius* density. Moreover, antibody responses seemed to be specific to mosquito genus and species. Collectively, these data confirmed that human antibody responses may be used to assess an individual's exposure to species-specific mosquito bites.

Introduction

Mosquito-borne diseases are a major health problem worldwide, and they cause important morbidity and mortality not only in tropical areas but also in temperate regions. The European population is exposed to a variety of mosquito-borne pathogens. Outbreaks of mosquito-borne diseases of human importance occurred on a mass scale in Europe in the last century. These outbreaks included the following: Dengue virus in Greece in 1928 [1], West Nile virus in Camargue in 1962 [2] and in Romania in 1996 [3], Sindbis virus in Finland in 2002 [4] and more recently, Chikungunya virus in Italy [5].

The evaluation of arthropod-borne disease transmission in the human population is based on entomological methods. However, these methods are not adapted to consider the differences found in a population, including the differential attractiveness to mosquito [6] or other environmental and socioeconomic factors that induce important variations in individual exposure to vector bites. The use of immunologically based techniques to estimate an individual's exposure to arthropod vector bites, such as those from mosquitoes, ticks, sand flies and Glossina, has been described by several studies [7-11]. The saliva of hematophagous arthropods contains a complex mixture of biologically-active proteins. These proteins may modify hemostatic responses and induce both cellular immunity and specific antibodies [12,13]. Antibody responses against mosquito salivary proteins were primarily studied for their role in allergic responses and numerous salivary allergens were detected with a wide range of molecular weight in various mosquito species [14,15]. The majority of these IgE-targeted proteins were also reported to react with IgG antibodies in both allergic and non-allergic individuals [16-19]. As described previously, mosquito salivary gland extracts can induce an IgG antibody response in people living in endemic areas [20-22] and in travelers transiently exposed to vectors in tropical areas [23]. Thus, a specific immune response against mosquito saliva antigens

could be used by control and surveillance programs to estimate the exposure level and also identify new infestation areas [24].

Mosquito densities and species diversity can be influenced by the surrounding landscape, even in restricted areas [25,26]. The Mediterranean coast of southern France presents areas with distinct demographic and ecological conditions. Located in the Rhone River delta, Camargue is the main wetland of southern France. Water pools, marshes and irrigated fields cover most of its 150,000 hectares [27]. The human population is distributed betweens towns, hamlets and isolated houses [28,29]. The ecology of Marseille city, an urban and dry area located approximately 30 km away, is in sharp contrast to that of Camargue. Fos-sur-mer is a town located between Camargue and Marseille, and the town has a mixed residential and agricultural landscape. For the purposes of this study, we chose three distinct ecological environments of mosquito development.

Aedes caspius is a Paleartic species that is well adapted to a swampy environment. It tolerates varying levels of salinity in larval breeding sites, and its larval development is linked with the alternating dry and flooding seasons in the areas where eggs are laid [30]. In Camargue, *Ae. caspius* is well adapted to this environment and is one of the most abundant anthropophilic mosquito species that is active from March to November [31,32]. After abundant rainfalls, the massive and synchronous adult population emerges and becomes a nuisance [27]. *Ae. caspius* was suspected to be involved in the 1993 Rift Valley fever outbreak in Egypt [33]. In the laboratory, this mosquito demonstrated the ability to transmit the Rift Valley fever virus and the Chikungunya virus [34,35]. Despite the low vector competence of these two viruses, *Ae. caspius* should be considered a potential vector due to its abundance and its high anthropophilic characteristic. Interestingly, *Ae. aegypti* and *Ae. albopictus* vectors of

arboviruses (*e.g.,* yellow fever, dengue or chikungunya viruses) were not endemic in the study area at the time of the present work.

Thus, we proposed to assess whether the exposure to different densities and/or species of mosquitoes throughout the year could influence the antibody response against mosquito salivary gland antigens. To accomplish this, we tested the IgG response against *Ae. caspius* salivary gland antigens of individuals living in three southern French areas (Camargue, Fos-sur-mer and Marseille) with distinct ecological environments at three time points (February 2007, September 2007 and January 2008). We evaluated the IgG responses against salivary gland antigens from *Culex pipiens, Aedes albopictus* and *Aedes aegypti* as controls. The temporal and spatial evolution of the IgG responses according to mosquito species will be discussed.

Materials and methods

Ethics statement

All participants gave their written informed consent, and the Marseille-2 Ethical Committee approved the protocol (N°2006-A00581-50).

Study sites

The study was conducted in the Provence-Alpes-Côte d'Azur (PACA) area in southeastern France. Three study sites were chosen in PACA: (i) Camargue, a large wetland area located inside the Rhone River delta; (ii) Fos-sur-mer, a town with 14 000 inhabitants (population density: 151 inhabitants/km²) and located approximately 15 km from the Camargue area border; and (iii) Marseille, a city with approximately 852 400 inhabitants and located approximately 30 km from Fos-sur-Mer (Figure 1).

Studied population

Volunteers were recruited from Camargue (n = 41, 54% male, mean age ± SD: 45.7 ± 11.3, Caucasians), Fos-sur-mer (n = 26, 42% male, mean age ± SD: 51.5 ± 11, Caucasians) and Marseille (n = 38, 47% male, mean age ± SD: 40.3 ± 12.2, Caucasians). For each individual, blood samples were collected by venous puncture at three different time points: February 2007, September 2007 and January 2008. Sera were obtained through centrifugation of the blood samples and were stored at -20°C. Eligible participants were those who did not travel in the six months prior to and during the study to countries or areas that are endemic for *Ae. aegypti* and *Ae. albopictus* mosquitoes.

Mosquitoes and salivary gland extraction

Adult female *Ae. caspius*, *Cx pipiens*, *Ae. aegytpi* and *Ae. albopictus* mosquitoes were used in this study. *Ae. caspius* and *Cx. pipiens* species were collected in the field at the larvae stage in Camargue from August to September 2009, and the mosquitoes were reared in an insectarium. The *Ae. albopictus* mosquito colony came from the Alpes-Maritimes area and were bred in a laboratory at the Entente Interdépartementale pour la Démoustication (EID) Méditerranée (Cagnes-sur-Mer). *Ae. aegypti* mosquitoes came from the Bora-Bora reference colony, which was bred in a laboratory at the Institut de Recherche pour le Développement (*Montpellier)*. All these mosquitoes were maintained under identical standard conditions: 26°C and 60% humidity. All mosquitoes took no blood meals and were maintained on a diet of 10% syrup solution. The salivary glands from 5- to 8-day old adult female mosquito were dissected on ice in phosphate-buffered saline (PBS) under a stereomicroscope. The salivary glands were pooled by strains into a microcentrifuge tube and were then stored frozen at –20°C until needed. Salivary glands were disrupted by ultrasonication (Vibracell 72412, Bioblock Scientific, Illkirch, France) for 5 min on ice at maximum amplitude. The concentration of salivary gland homogenates was

determined in duplicate by the Lowry method (DC Protein assay Kit, Bio-Rad) according to the manufacturer's instructions. Salivary gland proteins were then suspended in 0.1 M (pH 9.6) bicarbonate buffer to obtain a protein concentration of 1 µg/µL.

ELISA

The sera were tested by ELISA for the presence of IgG antibodies that bind to salivary gland proteins. Microtiter Immunoplates Maxisorp (Nunc, Denmark) were coated with 2 µg/ml (50 µl/well) of either *Ae. caspius*, *Cx. pipiens*, *Ae. albopictus* or *Ae. aegypti* salivary gland extracts diluted in 0.1 M bicarbonate buffer (pH 9.6) overnight at 4°C. Three washes were done with 250 µL of PBS (pH 7.4, Sigma Co., USA) plus 0.05% Tween-20 (Sigma Co., USA) between each incubation. Plates were blocked for 2 h at 37°C with 200 µL of blocking solution buffer consisting of PBS, 0.05% Tween and 5% skimmed milk (Beckton, Dickinson Bioscience, USA). Serum diluted 1:50 in blocking buffer was added (50 µl/well) and incubated at 37°C for 1 h. Fifty microliters of horse radish peroxidase (HRP)-conjugated rabbit anti-human IgG (1:10,000, Invitrogen, Rockville, USA) diluted in the blocking buffer were incubated for 1 h at 37°C. Enzyme activity was detected by incubation with 50 µl of tetramethylbenzidine substrate (KPL, USA) for 10 min at room temperature. The reaction was stopped using 50 µl of 1 M H_2SO_4. The optical density (OD) at 450 nm was determined with a microplate reader (Versa Max® Turnable Multiplate Reader, Molecular Devices, UK). Each serum sample was tested in duplicate and in control wells without salivary gland extracts. The levels of IgG antibodies were expressed as adjusted OD (aOD), which was calculated for each serum sample as the mean OD value with salivary gland extracts minus the OD value of the control wells, i.e., without salivary gland extracts. Individual variations in the IgG antibody responses were assessed according to the OD differences (ΔOD) between pairs of sera collected throughout the year. To

consider pertinent ΔOD between pairs of sera, an arbitrary threshold of 0.1 ΔOD was defined [36].

SDS-PAGE and western blot

Prior electrophoresis, 15 ug of mosquito salivary extracts from *Ae. caspius, Cx. pipiens, Ae. aegypti* and *Ae. albopictus*, were suspended in a Tris buffer containing 5% (w/v) SDS (Sigma). Each sample was reduced with 1 % (w/v) dithiothreitol (Sigma), boiled for 5 min, before to a protein separation by 12% SDS-PAGE in a Mini PROTEAN II (Bio-Rad, Hercules, CA, USA). Gels were transferred to a nitrocellulose membrane (0.45-µm, Amersham Pharmacia, Saclay, France) by semidry blotting (0.8 mA per cm^2) [37]. Blots were saturated with 5% w/v non-fat dried milk, and were carried out with a pool sera from 5 individuals living in Camargue sampled in September 2007. These sera were chosen blindly to avoid bias in the analysis. Pool sera was diluted at 1/100 in phosphate buffer saline (PBS) containing 0.2% v/v tween-20 with 5% w/v non-fat dried milk. After an overnight incubation, blots were incubated with anti-human Fcγ/IgG FITC conjugated antibody 1/1000 (Sigma, St Louis, MI). Immunoblots were directly digitalized using a Typhoon™ Trio Image scanner (GE Healthcare) and densitometric analysis of IgG immune profiles was performed using ImageQuant™ TL software (GE Healthcare), as previously described [38].

Statistical analyses

After verifying that values in each group did not assume a Gaussian distribution, the Kruskal-Wallis, Mann-Whitney and Wilcoxon matched-pairs signed-rank tests and Spearman's rank correlation coefficient were computed when appropriate with STATA version 9.0 (Stata-Corp, USA). All differences were considered significant at $p < 0.05$.

Results

Kinetics of IgG antibody responses against Ae. caspius salivary gland antigens (*Aec*SGA) *from individuals living in distinct ecological environments.*

First, we determined whether mosquito density, linked to the ecological environment and season (spatial and temporal evolution), could influence the IgG responses against mosquito salivary gland antigens. Thus, the IgG responses against *Aec*SGA were assessed in individuals living in Camargue, Fos-sur-mer or Marseille at three time points: February 2007 (T1) and January 2008 (T3), which corresponded to periods outside of the *Ae. caspius* exposure peak, and September 2007 (T2), which corresponded to the *Ae. caspius* exposure peak period [31]. Independent of the sampling time (*i.e.*, T1, T2 or T3), the IgG antibody responses against *Aec*SGA among the sites were significantly different (Kruskal-Wallis test, Table S1). Independent of the site, the IgG response against *Aec*SGA increased significantly from T1 to T2 and decreased significantly from T2 to T3 (Figure 2, Wilcoxon matched pairs test, Table S1). These variations were gradually decreased in individuals living at the Camargue site (*e.g.,* the mean change in Δ_{T2-T1}OD was +0.26, with 95% confident interval, (95% CI) from +0.14 to 0.38) compared to those living in Marseille city (*e.g.,* +0.05 [0.01 to 0.09]), with an intermediate variation for those living in Fos-sur-mer (*e.g.,* +0.13 [0.07 to 0.18]). The mean changes Δ_{T2-T1}OD between the Camargue and Fos-sur-mer or Camargue and Marseille sites were significantly different (p=0.019 and p<0.0001, respectively, Mann-Whitney test). No significant differences (Mann-Whitney test, *ns*) were observed when comparing the mean change Δ_{T2-T1}OD between the Fos-sur-mer and Marseille sites. In contrast, outside of the exposure peak of mosquito bites (*i.e.,* T1 and T3) at the three sites, the antibody response against *Aec*SGA returned to the background level at each site (Camargue, +0.04 [-0.06 to 0.14]; Fos-sur-mer, +0.09 [-0.13 to -0.04]; Marseille, +0.01 [-0.02 to 0.04]). Variations in the

IgG antibody responses detected between T3 and T1 were considered unchanged (mean $\Delta_{T3\text{-}T1}OD < 0.1$).

Kinetics of IgG antibody responses against Cx. pipiens salivary gland antigens (*Cxp*SGA) *from individuals living in distinct ecological environments.*

The IgG responses against *Cxp*SGA were assessed using the same sera as that used for the *Aec*SGA assay. A high inter-individual heterogeneity in the antibody responses was observed at all time points and from all sites (Figure 3). Independent of the sampling time (*i.e.*, T1, T2 or T3), no significant difference was observed in the IgG antibody responses against *Cxp*SGA between the sites (Kruskal-Wallis test, Table S1). With regard to the kinetics, despite the statistically significant variation in the IgG antibody response against *Cxp*SGA detected between some of the time points at the three sites, these variations were weak (Table S1). The IgG antibody variations for the Camargue and Fos-sur-mer sites were considered unchanged (below 0.1 ΔOD), and this finding indicated a global stability of IgG responses against *Cxp*SGA throughout the year. In Marseille city, the variations of IgG responses could reach a maximum of 0.15 (0.21 to 0.08).

Kinetics of antibody responses against Ae. albopictus (AealSGA) and Ae. aegypti salivary gland antigens (AeaeSGA) in individuals living in distinct ecological environments.

To estimate the specificity of the IgG response against *Aec*SGA, the same sera were assessed for IgGs against salivary gland extracts of two *Aedes* genus mosquitoes (*i.e., Ae. albopictus* and *Ae. aegypti*) that were not endemic in the study area until 2008 [31,39]. Independent of the sampling time (*i.e.*, T1, T2 or T3), no significant difference was observed when IgG antibody responses against *Aeal*SGA or *Aeae*SGA were compared between sites (Kruskal-Wallis

test, Table S1). With regard to the kinetics analysis, despite the statistically significant variations in IgG antibody response against *Aeal*SGA or *Aeae*SGA detected between some of the time points at the three sites, these variations were below 0.1 ΔOD and were considered unchanged.

Specificity of the IgG response between mosquito species.

To estimate the cross-reactivity level of the IgG response against the salivary glands between two mosquito species at T2 (September 2007) at the three sites, a Spearman's rank correlation coefficient (rho) test was used, and the corresponding *p*-values were determined (Table 1). Significant positive coefficient correlations (rho>0.42; $p<0.0083$) were observed within the *Aedes* genus at the three sites and mainly for IgG responses against non-prevalent mosquitoes (*i.e.*, *albopictus* and *aegypti*). Conversely, no significant correlation was observed between the IgG responses against *Cxp*SGA and those against the three other *Aedes* species at the three sites, except for the Fos-sur-mer site, where a significant positive coefficient correlation (rho=0.43; $p=0.0079$) was detected between the IgG responses against *Cxp*SGA and *Aec*SGA (Table 1).
Additionally, IgG antibody responses of pool sera from 5 individuals living in Camargue sampled in September 2007 were compared using western blot against salivary gland extracts from *Ae. caspius, Cx. pipiens, Ae. aegypti* and *Ae. albopictus* (Figure 5A). Superimposition of densitometric IgG antigenic profiles indicated that immune responses were clearly distinct against the four mosquito salivary gland extracts at the qualitative (singularity of IgG profiles), and quantitative (band intensities) levels (Figure 5B). In *Aec*SGA, one antigenic band around 42 kDa appeared particularly antigenic. Despite an antigenic band detected at the same molecular weight (42 kDa) in *Aeal*SGA with this pool sera, the corresponding band intensity was 11.5-fold less intense compared to those observed against *Aec*SGA. However, one minor antigenic band around 70 kDa seems detected by pool sera in the four mosquito species. Collectively, these

data support the theory of a specific IgG immune response according to mosquito species exposure bites and salivary gland extracts used.

Discussion

Numerous studies reported that mosquitoes' salivary components could induce an antibody response in humans under natural conditions [10,21,22,40]. Here, we analyzed human antibody responses against *Aec*SGA according to spatial (environment) and temporal (seasons) variations in the level of *Ae. caspius* mosquito exposure. The specificity of the IgG response was also estimated at the genus and species levels.

The Mediterranean coast of southern France includes areas with distinct demographic and ecological conditions, which greatly influence the dispersion and composition of the mosquito fauna. Thus, three sites were selected in this area on the basis of environmental patterns influencing *Ae. caspius* density: the Camargue area, Fos-sur-mer town and Marseille city. In the Camargue wetlands, *Ae. caspius* is well adapted to the rural and swampy environment where it encounters favorable climatic and biotope conditions [27,30]. *Ae. caspius* is the most abundant mammophilic species in Camargue, and its blood sucking activity causes human indisposition during the warm season [32,41]. As a vexatious pest, *Ae. caspius* is the focus of mosquito control campaigns in this area [42]. With regard to Fos-sur-mer and Marseille, we lack comprehensive data on the mosquito species diversity and density. However, Marseille city has an urban habitat that is more suitable to *Cx. pipiens* mosquitoes than rural mosquitoes, such as *Ae. Caspius,* due to breeding site characteristics and mosquito biology [27,43]. Fos-sur-mer, a town located between the Camargue area and Marseille, has an intermediary environment, and *Ae. caspius* mosquitoes were reported to be present. A mosquito collection was conducted in July 2007 with the use of carbon dioxide dry ice traps, and the collection indicated a decreasing mosquito density gradient from Camargue to Marseille.

Cx. pipiens and *Ae. caspius* were the most abundant mosquitoes in Camargue (31% and 29%, respectively), as previously described [31,39]. *Ae. caspius* mosquitoes were captured at the Fos-sur-mer site (21%), but none were found in Marseille (Table S2). Thus, Camargue, Fos-sur-mer and Marseille were considered sites with high, medium and very low levels of exposure to *Ae. caspius* bites, respectively.

In the present study, we showed that the IgG antibody responses against *Aec*SGA evolved in accordance with *Ae. caspius* density, which is influenced by both seasonal changes and the ecological environment. Effectively, the variations in the IgG antibody levels against *Aec*SGA between T2 (peak exposure) and T1 were approximately 2- and 4-fold higher in Camargue than in Fos-sur-mer and Marseille, respectively. The association of anti-salivary gland IgG levels with the intensity of exposure to mosquito bites had already been reported by Remoue and colleagues for *Anopheles gambiae* [21], and together, our results suggest that anti-saliva IgG antibodies may be used as a biologic marker of mosquito exposure. Recently, Drame and colleagues have confirmed the potential of *An. gambiae* saliva as an immunological marker to assess the risk of malaria transmission and the efficiency of antivectorial strategies in a malaria-endemic area [24]. Furthermore, the level of human antibody responses to blood feeding arthropods was shown to be associated with the risk of vector-borne diseases transmission. These data suggest that antibody responses to vector saliva might be used as marker of risks for vector-borne diseases transmission in endemic areas.

During sampling times outside the *Ae. caspius* exposure peaks (T1 and T3), the average IgG response against *Aec*SGA did not pertinently changed, suggesting a short-lived IgG response. A decrease in IgG response against salivary antigens after a period of non-exposure was described for outdoor workers exposed to ticks [44] and for travelers transiently exposed to *An. gambiae* and *Ae. aegypti* mosquitoes [23]. Seasonal variations in the levels of anti-mosquito saliva

antibodies were also reported in young children exposed to *An. gambiae* [7,21]. The transient anti-saliva IgG response may be useful to assess mosquito exposure and could thus provide new tools to evaluate anti-vector strategies or to monitor vector populations [12]. It is interesting to note that baseline IgG levels against *Aec*SGA were significantly and pertinently more important in Camargue and Fos-sur-mer than in Marseille. Repeated seasonal exposure to *Ae. caspius* seemed favorable in maintaining a high baseline IgG level throughout the year. To test this hypothesis, a comparison of the kinetics of the IgG response against *Aec*SGA, collected outside *Ae. caspius* exposure peak (cold season), between individuals living for a long time (*i.e.,* 5 years at least) in Camargue and newcomers could be performed. Further studies may also analyze the kinetics of the antibody response in children born in Camargue during childhood. An increase in the IgG response against salivary gland antigens according to age has already been described in children ages 6 weeks to 5 years living in Senegal [21]. Collectively, these data suggested that to determine the prevalence of seroreactivity against *Aec*SGA, several parameters should be considered, including mosquito density or environment, historical mosquito exposure (time spent in a particular area), and individual behavior (*e.g.,* outdoor/indoor activities, use of mosquito nets or repellents).

To evaluate the specificity of this IgG antibody response, the same sera were first tested against *Cxp*SGA. Our mosquito capture performed in July 2007 indicated that the *Cx. pipiens* species was present at all sites with decreasing densities from Camargue to Marseille. In contrast to the IgG response observed against *Aec*SGA, the IgG levels against *Cxp*SGA were considered unchanged spatially and temporally, with the exception of the levels from Marseille. Individuals from Marseille presented a significant and pertinent increase in the IgG levels against *Cxp*SGA after the peak of exposure. These results indicated that unlike the responses against *Ae. caspius*, the IgG responses against *Cxp*SGA seemed to be not associated with the decreasing density of *Cx. pipiens*

from Camargue to Marseille. This phenomenon could be attributed to a distinct *Cx. pipiens* behavior, which could occur between sites. Effectively, the temperate *Culex pipiens* Linné species can be divided into two different biological forms: *Culex pipiens pipiens* (*Cx. p. pipiens*) and *Culex pipiens molestus* (*Cx. p. molestus*) [45,46]. These two subspecies are relatively morphologically similar but have different physiological and behavioral traits [47,48]. In contrast to the rural *Cx. p. pipiens*, the urban *Cx. p. molestus* is anthropophilic [48], breeds in underground urban habitats, is able to lay its first batch of eggs without a blood meal, do not hibernate and can mate in confined spaces [39,46]. In Camargue, *Culex pipiens* L. mosquitoes were found in high density in bird-baited traps compared with horse or human baited-traps, suggesting that the non-anthropophilic form of *Culex pipiens* L. dominate in this rural area [31,39]. This species is a moderately efficient laboratory West Nile virus vector, but it is considered in southern France to be a main vector in the dry area and a secondary vector in the wetlands such as Camargue [23,27]. Although sparse entomological data are available on Marseille, the anthropophilic *Cx. p. molestus* form is very likely to occur there, which could be an explanation for the pertinent IgG increase during the warm season. Nevertheless, a continuous exposure or too-short period of non-exposure to *Cx. pipiens* bites throughout the year could limit the IgG baseline feedback and could explain the moderate IgG seasonal variations [48,49]. Collectively, these data showed that IgG antibody responses against *Aec*SGA and *Cxp*SGA evolved differently according to site and season, suggesting a specificity of the serological response against *Aec*SGA.

Cross-reactivity was evaluated using correlation tests between the IgG levels against *Aec*SGA and *Cxp*SGA at the exposure peak. Differences in the results were obtained according to the location. For Camargue and Marseille, the absence of a significant correlation between the IgG levels against *Aec*SGA and *Cxp*SGA corresponded to the low antigen cross-reactivity between these two

species, which belonged to different genus. Conversely, for Fos-sur-mer, a significant positive correlation was detected between these two species. Because both species are present at this site, the correlation could be more attributable to dual exposure to *Ae. caspius* and *Cx. pipiens* than to antigen cross-reactivity. Thus, our results are in favor of a specific genus IgG response.

It is interesting to note that the IgG response heterogeneity (*i.e.,* between individuals from the same area) observed in *Cx. pipiens* and *Ae. caspius to* a lesser extent might reflect the heterogeneous exposure to mosquito bites due to individual behaviors (*e.g.,* outdoor/indoor activities, use of mosquito nets or repellents). Additionally, these two mosquito species have distinct circadian biting activities (*e.g.,* clearly diurnal and nocturnal biting activities for *Ae. caspius* and *Cx. pipiens* mosquitoes, respectively) [31], which could further increase this inter-individual heterogeneity.

Finally, intra-genus specificity was estimated using SGA from two *Aedes* species that were not endemic at the three sites during the time of the study. The very low IgG levels against *AealSGA* and *AeaeSGA*, independent of the site and timing, indicated that the IgG responses against *AecSGA* were specific at the species level. Nevertheless, the important and significant coefficient correlations observed between the levels of IgG against mosquitoes from the *Aedes* genus were in favor of cross-reactivity. These correlations could not be attributable to dual exposure (because these mosquitoes were not present in the study area), but rather to antigen cross-reactivity within *Aedes* genus.

At the end, previous studies based on animal models suggested that antibody responses to mosquito saliva, when evaluated by ELISA, are genus-specific (*i.e., Anopheles, Culex or Aedes*), indicating little cross-reactivity between saliva from different arthropods [50]. However, to comfort the species specificity of IgG antibody response, western blotting experiments were performed in the present study. A singularity of IgG immune profiles, from a pool sera of

individuals living in Camargue sampled in September 2007, was observed both at the qualitative and quantitative levels. These results were in accordance with those reported by others [14,15,23], indicating that IgG immune profiles seems mosquito species-specific according to differences in antigenicity of saliva proteins revealed by western blots. Additionally, studies performed on phlebotomine sand flies confirmed this host singularity of antibody responses against salivary gland extracts from different sand flies species [51,52].

Collectively, these data showed that the IgG antibody response against *Aec*SGA may be related to the seasonal and geographical variations in *Ae. caspius* density. The pertinent increase and transient IgG response at the peak of exposure seems to be species specific and these results strongly suggest that human antibody responses may be used to assess the individual level exposure to mosquito bites. Nevertheless, other parameters should be considered, including historical individual exposure, which could influence the baseline IgG level. Further studies are needed to characterize the specific *Aec*SG antigens using, for instance, an immunoproteomic approach, as described previously [53,54]. Specific salivary gland antigens may be identified and recombinant protein production in the development of a more sensitive and specific immunological test to accurately assess individual exposure levels might be profitable. Thus, specific immune responses against mosquito saliva antigens could be used by control and surveillance programs to assess the efficiency of anti-mosquito strategies, to estimate exposure level and also, to identify new infestation areas. This strategy could be extended to other mosquito species that are involved in the transmission of infectious diseases and could be a tool to estimate the risk of vector-borne disease transmission. Collectively, these data confirmed that human antibody responses may be used to assess individual exposure to species-specific mosquito bites and estimate the pest level.

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Figure legends

Figure 1: Location of the study sites. Marseille city city and Fos-sur-mer town town are indicated by circles, and Camargue area is cross-hatched.

Figure 2: IgG serum levels anti-*Ae. caspius* salivary gland protein extracts. Scatter plot graphs of IgG antibody responses against *Ae. caspius* salivary gland antigens of individuals from Marseille (squares), Fos-sur-mer (circles) and Camargue (triangles) at three times February 07 (T1), September 07 (T2) and January 08 (T3) are represented in white, grey and black symbols, respectively. Antibody responses are represented by aOD: mean OD value of wells with antigen minus mean OD value of wells without antigen. Each point shows the aOD value for a single individual. Horizontal bars show medians. Differences between the two time points at a single site were tested using Wilcoxon signed-rank test. *p*-values are indicated.

Figure 3: IgG serum levels anti-*Cx. pipiens* salivary gland protein extracts. Scatter plot graphs of IgG antibody responses against *Cx. pipiens* salivary gland antigens of individuals from Marseille (squares), Fos-sur-mer (circles) and Camargue (triangles) at three times February 07 (T1), September 07 (T2) and January 08 (T3) are represented in white, grey and black symbols, respectively. Antibody responses are represented by aOD: mean OD value of wells with antigen minus the mean OD value of wells without antigen. Each point shows the aOD value for a single individual. Horizontal bars show medians. Differences between the two times in a single site were tested using the Wilcoxon signed-rank test. *p*-values are indicated.

Figure 4: IgG serum levels anti-*Ae. aegypti* and -*Ae. albopictus* salivary gland protein extracts. Scatter plot graphs of IgG antibody responses against the salivary gland antigens of *Ae. aegypti* (A) and *Ae. albopictus* (B) of individuals from Marseille (squares), Fos-sur-mer (circles) and Camargue

(triangles) at three time points February 07 (T1), September 07 (T2) and January 08 (T3) are represented in white, grey and black symbols, respectively. Antibody responses are represented by aOD: mean OD value of wells with antigen minus OD value of wells without antigen. Each point shows the aOD value for a single individual. Horizontal bars show medians. Differences between two time points at a single site were tested using the Wilcoxon signed-rank test. *p*-values are indicated.

Figure 5: Specificity of the IgG response between mosquito species using western blotting. Fifteen microgram of each salivary gland extracts of *Ae. caspius* (1), *Cx. pipiens* (2), *Ae. aegypti* (3) and *Ae. albopictus* (4) were loaded per well, separated by 12% SDS-PAGE and blotted onto a nitrocellulose membrane. IgG immune profiles from a pool sera of 5 individuals living in Camargue sampled in September 2007 were tested by immunoblotting experiments (A). Schematic representation of normalized densitometric antigenic profiles of salivary gland extracts from the four mosquito species is presented (B). Antigenic profiles against each species are indicated by the same colors at the top of each western blot profile (A) and lines on densitometric profiles (B). MW: molecular weight, kDa: kiloDalton, Rf: relative front of migration. A.U.: Arbitrary Unit.

Table 1: Correlation of IgG responses between mosquito species.

Table S1: Statistical analysis of spatial and temporal variations in IgG responses.

Table S2: Adult mosquitoes captured in each site in July 2007 using carbon dioxide dry ice traps.

Table 1: Correlation of IgG responses between mosquito species.

	Ae. caspius	*Ae. albopictus*	*Ae. aegypti*	*Cx. pipiens*
Camargue site				
Ae. caspius	1			
Ae. albopictus	0.4245 (***p*=0.0046**)	1		
Ae. aegypti	0.4526 (***p*=0.0023**)	0.6187 (***p*< 0.0001**)	1	
Cx. pipiens	0.3168 (*p*=0.0385)	0.3015 (*p*=0.0494)	0.2585 (*p*=0.0942)	1
Fos-sur-mer site				
Ae. caspius	1			
Ae. albopictus	0.5212 (***p*=0.0004**)	1		
Ae. aegypti	0.5529 (***p*=0.0004**)	0.6529 (***p*<0.0001**)	1	
Cx. pipiens	0.4300 (***p*=0.0079**)	0.0842 (*p*=0.6204)	0.2162 (*p*=0.1987)	1
Marseille site				
Ae. caspius	1			
Ae. albopictus	0.4648 (***p*=0.0029**)	1		
Ae. aegypti	0.4642 (***p*=0.0029**)	0.4796 (***p*=0.0020**)	1	
Cx. pipiens	0.1978 (*p*=0.2274)	0.2513 (*p*=0.1227)	0.0799 (*p*=0.6289)	1

Spearman's rank correlation coefficient (rho) and *p*-value between IgG responses against the salivary glands of each pair of mosquito species in September 2007 (T2) (*i.e.,* at the peak of *Ae. caspius* density) are listed. Significant correlation (0.05/6=0.0083, *i.e.,* according to the Bonferroni correction for multiple testing) are indicated in bold in parentheses.

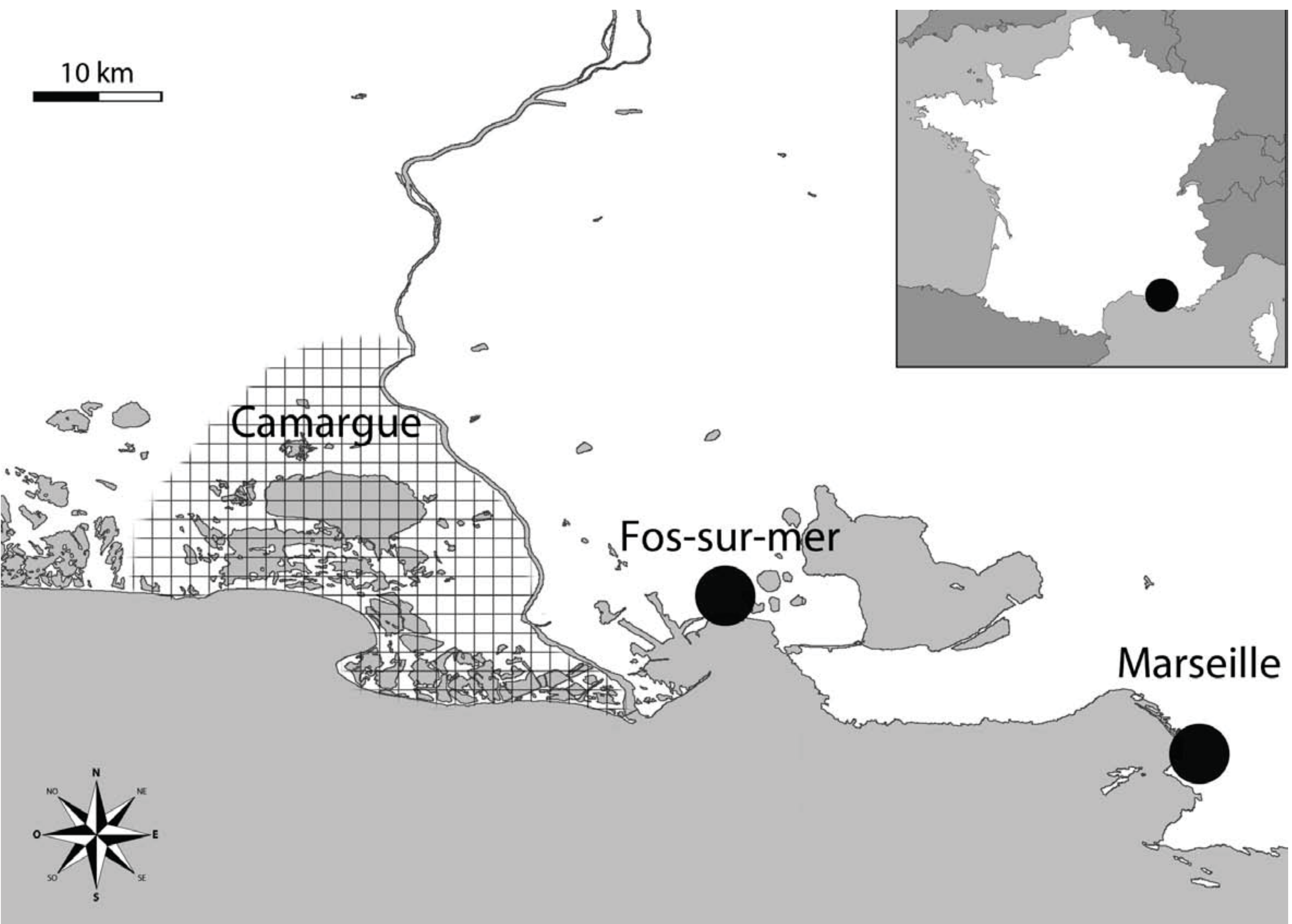


Figure 1

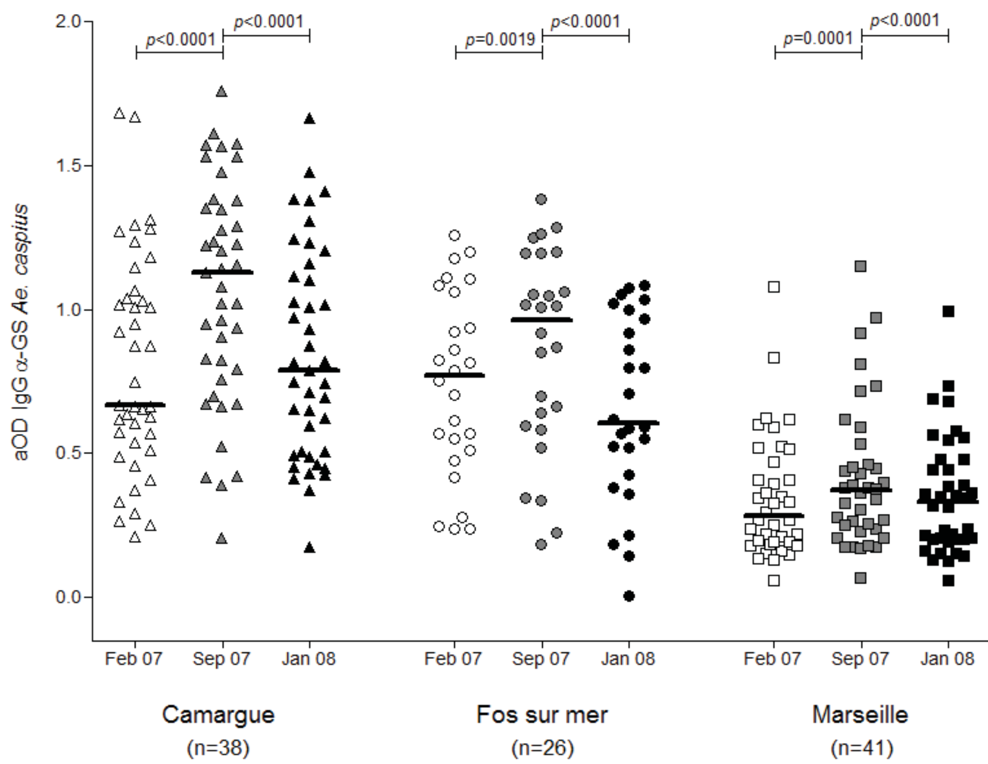


Figure 2

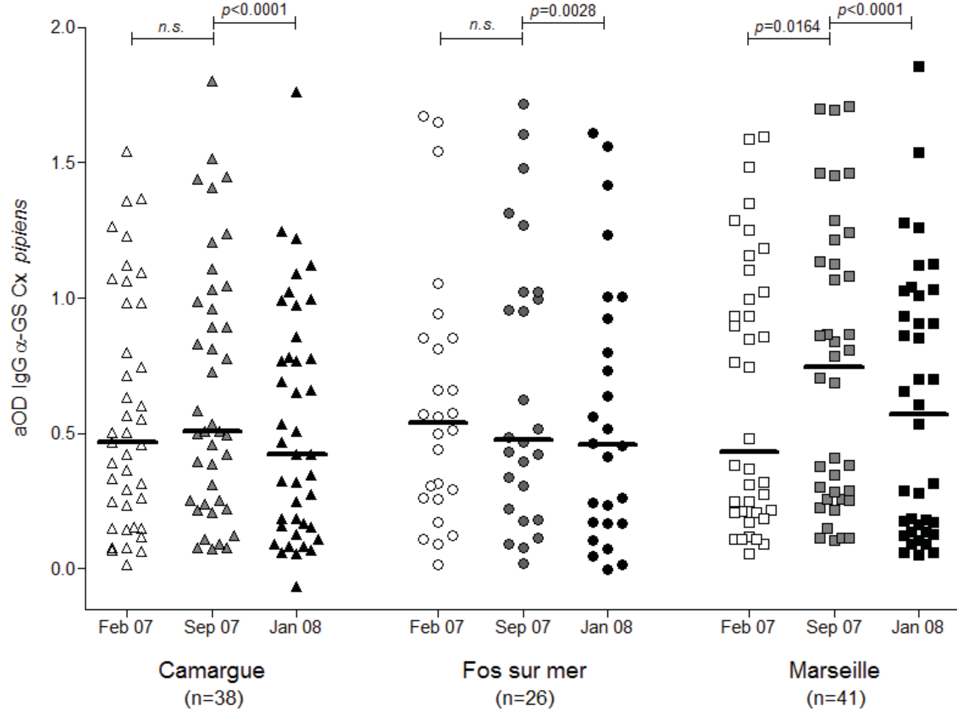
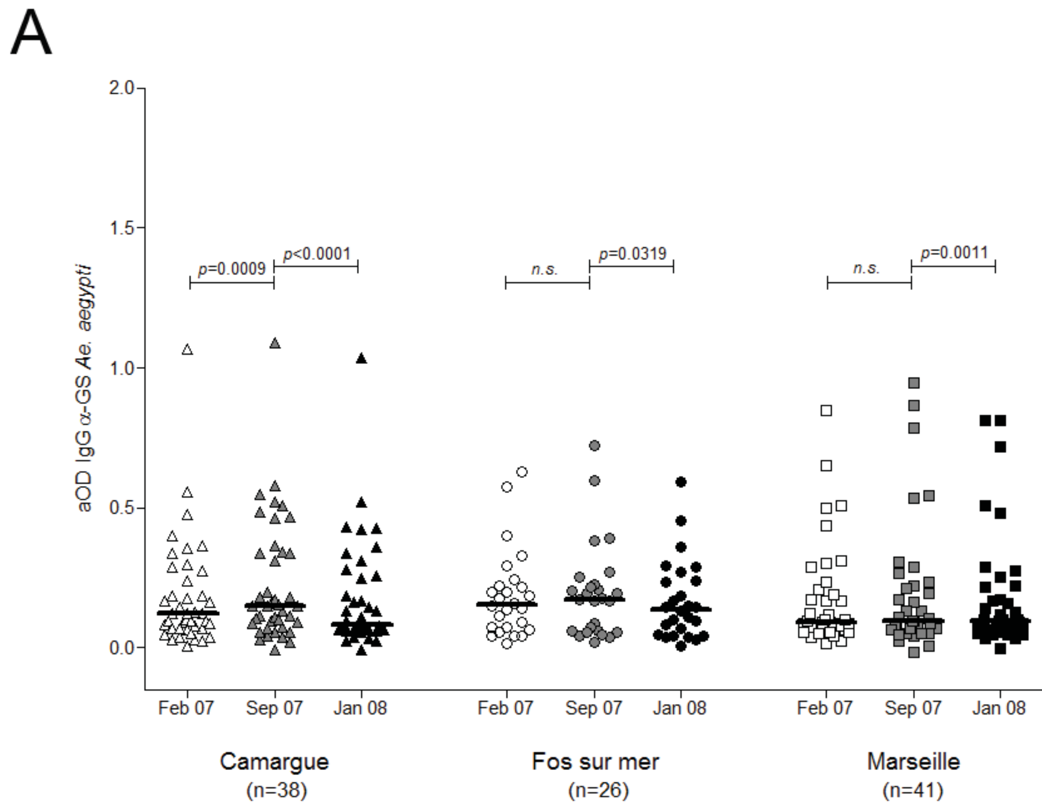


Figure 3



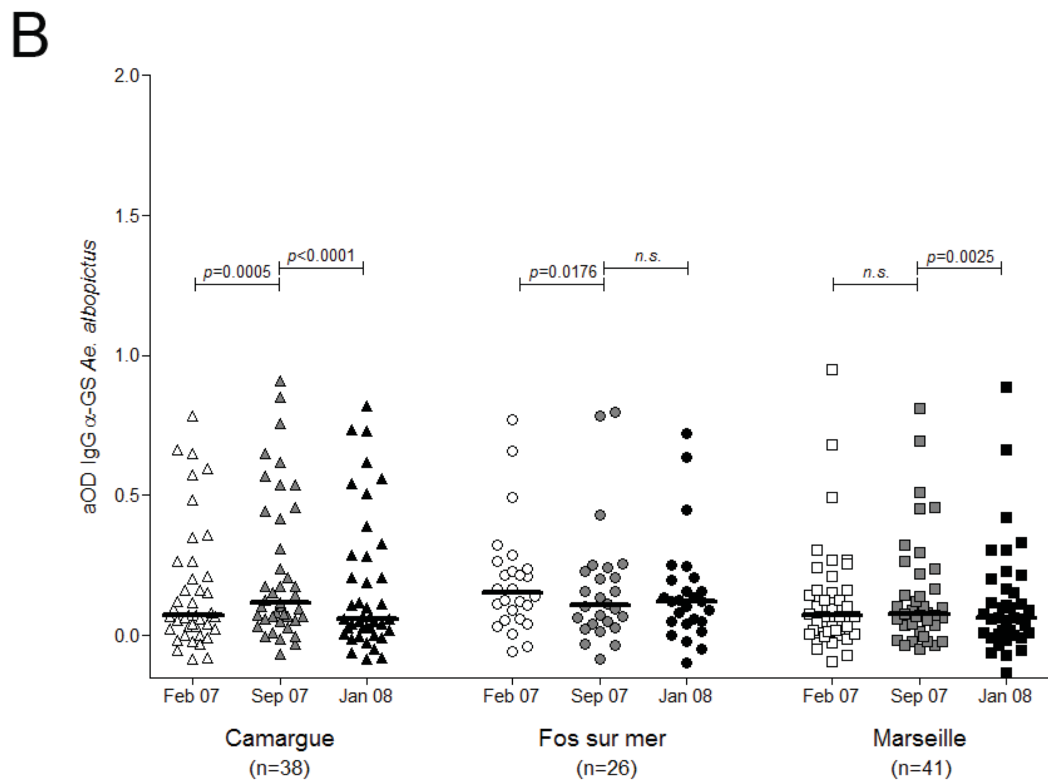


Figure 4

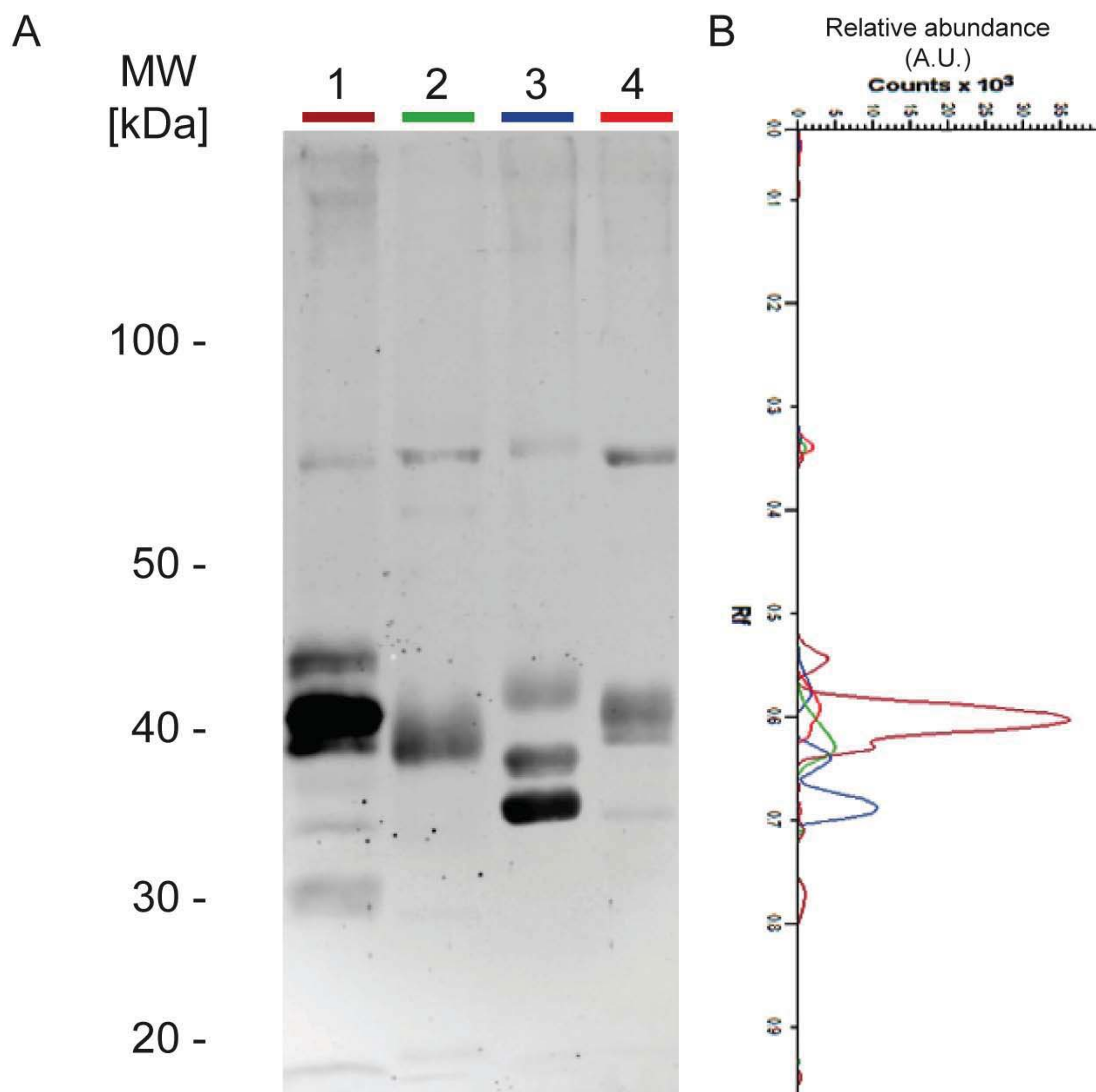


Figure 5

Table S1: Statistical analysis of spatial and temporal variations of IgG response

		Camargue (*n=41*)				**Fos-sur-mer (*n=26*)**				**Marseille (*n=38*)**				*p-value* (Kruskal-Wallis)
				95% CI of mean				95% CI of mean				95% CI of mean		
	Time	Mean	SD	Lower	Upper	Mean	SD	Lower	Upper	Mean	SD	Lower	Upper	
Ae. caspius **(aOD)**														
	T1	0.81	0.38	0.69	0.92	0.74	0.32	0.61	0.87	0.35	0.21	0.28	0.42	***< 0.0001***
	T2	1.07	0.39	0.94	1.19	0.86	0.35	0.72	1.00	0.41	0.25	0.33	0.49	***< 0.0001***
	T3	0.84	0.37	0.73	0.96	0.65	0.32	0.52	0.78	0.35	0.20	0.29	0.42	***< 0.0001***
p-value	T1 vs. T2	***< 0.0001***				***0.0018***				***< 0.0001***				
(Wilcox. Sign rank test)	T2 vs. T3	***< 0.0001***				***< 0.0001***				***< 0.0001***				
	T1 vs. T3	*0.3537*				***0.0007***				*0.9422*				
Cx. pipiens **(aOD)**														
	T1	0.57	0.43	0.44	0.71	0.61	0.47	0.42	0.80	0.64	0.49	0.48	0.81	*0.9158*
	T2	0.66	0.47	0.51	0.81	0.66	0.51	0.46	0.87	0.74	0.52	0.57	0.91	*0.7851*
	T3	0.53	0.43	0.40	0.66	0.57	0.49	0.37	0.77	0.60	0.49	0.44	0.76	*0.8662*
p-value	T1 vs. T2	*0.063*				*0.509*				***0.0174***				
(Wilcox. Sign rank test)	T2 vs. T3	***< 0.0001***				***0.0028***				***< 0.0001***				
	T1 vs. T3	*0.0519*				*0.2326*				***0.0255***				
Ae. albopictus **(aOD)**														
	T1	0.16	0.22	0.09	0.23	0.19	0.20	0.12	0.27	0.13	0.20	0.06	0.20	*0.153*
	T2	0.23	0.26	0.15	0.31	0.17	0.22	0.08	0.26	0.15	0.20	0.08	0.22	*0.3403*
	T3	0.17	0.25	0.10	0.25	0.15	0.19	0.08	0.23	0.12	0.20	0.06	0.19	*0.5195*
p-value	T1 vs. T2	***0.0005***				***0.0182***				*0.149*				
(Wilcox. Sign rank test)	T2 vs. T3	***< 0.0001***				*0.2087*				***0.0028***				
	T1 vs. T3	*0.5004*				***0.0003***				*0.2875*				
Ae. aegypti **(aOD)**														
	T1	0.18	0.19	0.12	0.24	0.19	0.15	0.13	0.25	0.18	0.19	0.12	0.24	*0.6958*
	T2	0.23	0.22	0.16	0.29	0.20	0.17	0.13	0.26	0.20	0.23	0.12	0.27	*0.5416*
	T3	0.17	0.19	0.11	0.23	0.17	0.14	0.11	0.23	0.18	0.21	0.11	0.25	*0.9002*
p-value	T1 vs. T2	***0.001***				*0.6115*				*0.5401*				
(Wilcox. Sign rank test)	T2 vs. T3	***< 0.0001***				***0.0324***				***0.0011***				
	T1 vs. T3	*0.123*				***0.0289***				*0.1845*				

Kruskal-Wallis test were used to compare antibody level between more than two independent groups (geographical comparisons). Wilcoxon matched-pairs signed-ranks test were used to compare paired sera between two time points. All significant differences ($p<0.05$) are indicated in bold. SD: standard deviation, aOD: adjusted optical density, CI: confident interval, T1: February 07, T2: September 07, T3: January 08.

Table S2: Adult mosquitoes captured in each study site in July 2007 using carbon dioxide dry ice traps. Carbon dioxide traps were hung in 5 locations in each study site during 24 hrs. Concerning the Camargue site, carbon dioxide traps were located as follow: Saliers 1: 4°29'3.08"E; 43°40'9.99"N and Saliers 2: 4°29'15.76"E; 43°39'39.24"N, Valat 1: 4°40'8.92"E; 43°30'33.00"N and Valat 2: 4°40'8.92"E; 43°30'33.00"N, and one trap was placed in Le sambuc: 4°40'14.29"E; 43°33'10.99"N. "Saliers" and "Le Sambuc" sites are about 20 km apart. The latter is 5 km from the "Valat" site. Mosquitoes were identified using morphological characteristics and identification keys. The mean number of mosquitoes sampled in each site was calculated using the results of the five traps. The proportion of each mosquito genus/species per site is indicated into brackets.

Mosquito spp.	**Camargue (%)**	**Fos-sur-mer (%)**	**Marseille (%)**
Aedes	1512 (54.2)	37 (22.4)	0
Ae. caspius	798 (28.6)	34 (20.6)	0
Ae. aegypti	0	0	0
Ae. albopictus	0	0	0
Other Aedes	714 (25.6)	3 (1.8)	0
Culex	937 (33.6)	120 (72.7)	25 (78.1)
Cx. pipiens	858 (30.8)	120 (72.7)	25 (78.1)
Other Culex	79 (2.8)	0	0
Anopheles	339 (12.2)	1 (0.6)	0
Culiseta	0	7 (4.2)	7 (21.9)

Dans ce travail, nous avons tout d'abord observé une variation de la réponse anticorps vis-à-vis d'antigènes d'extraits de glandes salivaires d'*Ae. caspius* en fonction des sites (variations spatiales) et en fonction de la saison (variations temporelles) en accord avec la densité de ce moustique. Une faible réponse anticorps anti-salive a été observée concernant les deux espèces du genre *Aedes* qui n'étaient pas présentes sur le site de l'étude suggérant une spécificité de réponse de genre, mais aussi d'espèce. Malgré ces réponses contrastées en intensité, des analyses de corrélations ont montré qu'il pouvait exister une réaction anticorps croisée, notamment entre les espèces du genre *Aedes*. L'analyse westernblot a confirmée l'existence de ces réactions croisées, mais à aussi révélée des profils antigéniques bien distincts entre ces quatre espèces. Ces résultats suggèrent fortement que des protéines salivaires spécifiques d'espèces puissent être utilisées comme marqueur immunologiques d'exposition.

De nombreuses études se sont intéressées aux propriétés immunogéniques et antigéniques de la salive de moustiques ces dernières décennies. Ce fut d'abord dans le but de caractériser des allergènes responsables de réactions cutanées inconfortables chez des individus allergiques afin de développer des outils de dépistage et des méthodes de désensibilisations efficaces (Peng et al., 1998, Peng et al., 2006, Reunala et al., 1994). Même si des candidats salivaires existent déjà pour certaines espèces de Culicidae, des progrés restent à faire pour augmenter leurs sensibilités et spécificités en vue de développer des tests de dépistage (Peng *et al.*, 2001, Peng *et al.*, 2006). De plus, la combinaison de plusieurs allergènes salivaires pourrait améliorer la sensibilité de ces tests, comme cela à déjà été mis en évidence lors de l'utilisation d'antigènes salivaires pour évaluer le contact hôte/vecteurs (Souza *et al.*, 2010). L'analyse par Western blot, des réponses anticorps d'individus allergiques exposés aux moustiques a montré que les allergènes et les antigènes étaient confondus (Peng *et al.*, 1997, Shen *et al.*, 1989, Brummer-Korvenkontio *et al.*, 1994, Brummer-Korvenkontio *et al.*, 1997). Ainsi, l'identification de nouvelles protéines salivaires antigéniques pourrait contribuer au développement de tests de dépistage d'hypersensibilités à différentes espèces de Culicidae qui ne sont pas commercialement disponibles actuellement. Par ailleurs, le traitement des allergies cutanées par désensibilisation, qui consiste à réhabituer progressivement l'organisme à l'allergène en administrant des doses croissantes de ce dernier, demeure controversé et peu utilisé concernant l'allergie aux piqûres de moustiques (Feuillet-Dassonval *et al.*, 2006). En effet, les préparations d'allergènes disponibles actuellement sont en majorité fabriquées à base de broyats de corps entiers de moustiques (principalement *Ae. communis*) qui contiennent peu de protéines salivaires et ne sont pas standardisées (Peng *et al.*, 1996). Cependant, plusieurs travaux ont rapportés que la majorité des

allergènes impliqués dans les réactions allergiques cutanées était concentrée dans la salive de moustiques (Wongkamchai *et al.*, 2010, Peng *et al.*, 1996). Ainsi, la caractérisation de nouvelles protéines salivaires antigéniques spécifiques d'espèces et leur production de façon synthétique, pourraient permettre de mettre au point de nouveaux outils pour diagnostiquer et traiter les allergies, tout en augmentant le seuil de sensibilité et en conservant un niveau élevé de spécificité (Wongkamchai *et al.*, 2010).

L'utilisation de protéines salivaires d'arthropodes vecteurs en tant que candidats vaccins semble également une piste très prometteuse pour protéger l'hôte contre certaines maladies transmissibles, notamment la leishmaniose transmise par les phlébotomes (Kamhawi et al., 2000). Il n'est pas exclu que les protéines salivaires puissent être utilisées dans la protection de certaines maladies transmises par les moustiques (Schneider *et al.*, 2008). A l'inverse, plusieurs études rapportent l'effet potentiateur de la salive lors d'infections dans plusieurs couples de vecteurs/pathogènes, tels que des moustiques du genre *Aedes* avec le virus West Nile (Schneider *et al.*, 2006) ou le virus La Crosse (Osorio *et al.*, 1996). Des études précédentes ont mis en évidence que quelques protéines salivaires ont un rôle majeur pour potentialiser certaines infections, telles que la leishmaniose transmise par les phlébotomes (Morris *et al.*, 2001) ou la borréliose de Lyme transmise par les tiques (Dai *et al.*, 2009). La caractérisation de telles protéines salivaires chez les Culicidae pourrait permettre de limiter l'infection. Cependant, un tel effet potentiateur ou protecteur de la salive de moustique n'a jamais été décrit concernant les maladies majeures transmises par les moustiques, comme la dengue ou la fièvre jaune. Concernant le paludisme, une pré-exposition à la salive d'*Anopheles* non-infectés aurait un effet protecteur sur une infection parasitaire ultérieure (Donovan *et al.*, 2007). Cependant ces résultats restent très contreversés (Kebaier *et al.*, 2010). Des études complémentaires sont donc nécessaires pour définir l'éventuel rôle

protecteur de certaines protéines salivaires de Culicidae contre les pathogènes qu'ils transmettent.
D'autres stratégies vaccinales agissant directement sur la réduction de la durée de vie et la fécondité du vecteur sont des axes de recherches très prometteurs (Billingsley *et al.*, 2008). Cependant, même si des protéines salivaires ont montré une efficacité chez d'autres arthropodes vecteurs dans ce type d'approche, notamment chez les tiques (Prevot *et al.*, 2007, Garcia-Varas *et al.*, 2009), les protéines de l'estomac ont été largement privilégiées chez le moustique (Gakhar *et al.*, 2005, Hatfield, 1988, Lal *et al.*, 2001, Srikrishnaraj *et al.*, 1995). Contrairement aux tiques, le contact hôte/vecteur est court et les protéines de l'estomac sont donc les plus exposées au système immunitaire de l'hôte.
Les protéines salivaires peuvent cependant avoir un rôle dans un autre type de vaccin bloquant la transmission. Il a été montré que les anticorps de l'hôte pouvaient traverser la barrière de l'estomac pour se retrouver dans l'hémoplymphe du moustique (Vaughan *et al.*, 1988) et dans les glandes salivaires où ils pourraient intéragir avec le pathogène (Ghosh *et al.*, 2009). De plus, des anticorps spécifiquements dirigés contre des protéines salivaires se sont montrés efficaces pour inhiber l'invasion des glandes salivaires par des sporozoïtes de paludisme murin (Brennan *et al.*, 2000, Barreau *et al.*, 1995). Ainsi, la vaccination contre des protéines des glandes salivaires impliquées dans l'interaction ou la maturation des pathogènes pourrait permettre de diminuer le taux de transmission de certaines maladies vectorielles.

Depuis les travaux de Schwartz sur l'exposition de travailleurs aux tiques dans le New Jersey (Schwartz et al., 1990), l'utilisation de la salive d'arthropodes vecteurs en tant que marqueurs immunologiques d'exposition a fait son chemin, notamment chez les Culicidae où plusieurs études montrent qu'il est possible d'utiliser de tels marqueurs pour mesurer l'exposition au niveau individuel à

certains moustiques d'importance médicale majeure (Orlandi-Pradines et al., 2007, Poinsignon et al., 2010, Remoue et al., 2007, Remoue et al., 2006, Waitayakul et al., 2006).
Dans un premier temps, nous avons montré une absence de différences majeures en comparant le protéome salivaire de différentes colonies d'une même espèce de moustique en utilisant ces méthodes, ce qui indique que le répertoire de protéines salivaires est peu hétérogène au sein de l'espèce. Ainsi, nous avons évalué la possibilité de détecter des protéines salivaires antigéniques spécifiques de genre ou d'espèce en utilisant des méthodes immunologiques (ELISA) et immuno-protéomiques (Western blots).
Par conséquent, nous avons choisi d'utiliser des stratégies complémentaires pour identifier des protéines salivaires spécifiques de genres ou d'espèces d'*Anopheles* pouvant être utilisées comme marqueurs d'exposition : (i) analyse de la diversité du répertoire protéique des glandes salivaires de plusieurs espèces de moustiques d'un même genre, et (ii) étude de la spécificité de la réponse anticorps dirigée contre des protéines salivaires.

Notre étude a permis de montrer la présence de protéines sécrétées détectées uniquement dans certaines espèces. Ce résultat est très prometteur pour définir des candidats marqueurs d'une exposition spécifique d'espèce. Cette diversité protéique se traduit par une spécificité de réponse anticorps chez des individus exposés uniquement aux moustiques du complexe *An. gambiae s.l.* En effet, les profils de réactivité obtenus sont différents aussi bien quantitativement que qualitativement. Cependant, entre des espèces apparentées (complexe *An. gambiae s.l.*), une homogénéité des protéomes salivaires provenant de moustiques d'élevages (*An. gambiae s.s*) ou du terrain (*An. arabiensis*) a été mise en évidence. La distinction d'espèces de Culicidae phylogénétiquement proches telles que ces deux espèces du complexe *An. gambiae s.l.* semble donc difficile. Toutefois, l'analyse des profils immunologiques d'individus vivant en

zone d'endémie palustre, indique qu'*An. arabiensis* présente des protéines antigéniques singulières qui pourraient etre utilisées comme marqueurs différentiels d'exposition. Ces résultats laissent envisager la possibilité de détecter des marqueurs immunologiques susceptibles de mesurer l'exposition à des vecteurs de paludisme spécifiques.

Néanmoins, les différentes espèces du complexe *An. gambiae s.l.* (actuellement au nombre de neuf avec la découverte récente d'*An. goundry* (Riehle *et al.*, 2011)) peuvent présenter des différences biologiques et comportementales non négligeables, tels que l'occupation de niches écologiques distinctes, le degré d'endophilie/exophilie ou le degré d'anthropohilie, qui peuvent avoir une conséquence sur la capacité vectorielle de ces moustiques (Robert *et al.*, 1984). Le même constat peut être fait concernant des sous-populations qui existent au sein d'une même espèce, comme les formes M et S de l'espèce *An. gambiae sensus stricto* (Simard et al., 2009, Costantini et al., 2009). Le fait de ne pas pouvoir distinguer spécifiquement l'exposition à ces espèces dans des zones d'étude ou elles sont sympatriques[2], peut engendrer des biais dans l'évaluation du risque de transmission du paludisme (ces espèces non pas la même importance médicale) ou dans l'évaluation de l'efficacité des mesures anti-vectorielles (ces espèces peuvent avoir des susceptibilités différentes face à ces mesures). Cependant, la présence de trois bandes antigéniques à 26, 23 et 10 kDa spécifiquement détectées sur les extraits de glandes salivaires d'*An. arabiensis* suggèrent l'existence de différences immunologiques au sein du complexe *An. gambiae s.l.* malgrè la forte homogénéité observée au niveau protéique et pourraient permettre de caractériser de futurs marqueurs discriminant l'exposition à des espèces phylogénétiquement très proches.

[2] Espèces sympatriques : Se dit des espèces qui ne s'hybrident pas, et dont les aires respectives peuvent avoir une zone commune.

A partir de ces résultats préliminaires, des protéines salivaires d'*An. gambiae* ont été sélectionnées pour être synthétisées sous forme de protéines recombinantes. Le système baculovirus/cellules d'insectes a été choisi pour produire ces protéines recombinantes en conservant les modifications post-traductionelles qui peuvent avoir un rôle dans l'antigénicité des protéines (Cloos *et al.*, 2004). De plus, des peptides issus de ces candidats protéiques ont été sélectionnés sur la base de leur antigénicité prédite par des logiciels bio-informatiques et sont en cours de synthèse. Cette stratégie, déjà utilisée avec succès sur la protéine salivaire gSG6 d'*An. gambiae* (Poinsignon *et al.*, 2008), pourrait augmenter la sensibilité et la spécificité des marqueurs d'exposition. La poursuite de l'étude comparative du répertoire salivaire des quatre espèces d'*Anopheles* permettra de définir, si possible, de nouveaux candidats. La sensibilité et la spécificité de ces potentiels marqueurs d'exposition, utilisés seuls ou en combinaison, pourront ensuite être évalués sur de larges cohortes d'individus exposés à différentes espèces de moustiques par ELISA ou Luminex™. La technologie Luminex™ est un système multi-analytique permettant avec une faible quantité de sérum et en une seule expérience, d'évaluer la réponse anticorps de plusieurs individus envers un grand nombre d'antigènes salivaires différents (jusqu'à une centaine) (Moalic *et al.*, 2004). Comme généralement, plusieurs espèces de Culicidae anthropohiles sont présentes simultanément sur un territoire donné, cet outil semble bien adapté pour definir qualitativement et quantitativement le contact homme/vecteurs.

Enfin, une étude effectuée dans le Sud de la France a confirmé l'existence d'un lien entre le niveau d'exposition aux moustiques et le niveau de la réponse anticorps dirigée contre la salive. L'étude de la cinétique de cette réponse anticorps indique qu'elle est transitoire. En outre, une diversité de la réponse anticorps a pu être mise en évidence entre genres, mais également entre espèces du même genre (*Aedes*). L'identification des protéines salivaires antigéniques

des différentes espèces de moustiques utilisées (*Ae. caspius*, *Cx. pipiens*, *Ae. aegypti* et *Ae. albopictus*) est actuellement en cours. Les candidats marqueurs d'exposition seront alors validés en utilisant une stratégie similaire à celle énoncée précédemment pour les marqueurs protéiques d'*Anopheles* (production de protéines recombinantes ou de peptides suivi de leur validation par Lumninex™).

L'ensemble de ces résultats nous permet d'être optimiste sur la mise au point de futurs marqueurs immunologiques d'exposition à la piqûre de différentes espèces de moustiques vecteurs. De tels outils immunologiques permettront d'évaluer l'exposition aux moustiques au niveau individuel grâce au suivi sérologique d'individus à risque, tels que les voyageurs, les militaires ou les travailleurs expatriés (mission de prospection pétrolière, missions humanitaires…) partant pour des durées plus ou moins longues en zone d'endémie.

En 2008, 4 440 cas de paludisme d'importation ont été déclarés en France par le Centre National de Référence du paludisme. La même année, 433 cas de paludisme ont été déclarés dans les armées sur plus de 10 000 militaires français impliqués dans des opérations extérieures (OPEX) (Haus-Cheymol et al., 2009). Il est possible de diminuer l'incidence de cette maladie vectorielle en utilisant des mesures antivectorielles adaptées aux conditions de terrain. Ainsi, le développement de marqueurs immunologiques d'exposition basé sur les propriétés antigéniques des protéines salivaires de moustiques pourrait permettre d'évaluer l'efficacité des mesures antivectorielles en mesurant le niveau d'exposition de chaque individu par une simple méthode sérologique, beaucoup plus simple à mettre en œuvre que les méthodes entomologiques actuelles. De plus, ces outils immunologiques peuvent avoir une utilité

épidémiologique en permettant d’évaluer les zones de répartitions et l’expansion géographique de certains vecteurs.

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AVANT PROPOS

J'ai été amené lors de ma thèse à côtoyer deux thématiques différentes. L'une d'elle fait suite à mes travaux débutés lors du Master 2 sur la reconnaissance d'antigènes exposés à la surface des hématies parasitées par *Plasmodium falciparum* par des voyageurs brièvement exposés au paludisme. Ce travail a donné lieu à une publication qui a été acceptée dans une revue internationale. Une introduction est exposée en première partie des annexes pour faire l'état de l'art des connaissances existantes sur ce sujet.

Le paludisme en quelques chiffres…

Le paludisme demeure la maladie parasitaire à transmission vectorielle la plus répandue à travers le monde et la plus meurtrière. Selon le rapport 2008 de l'Organisation Mondiale de la Santé (OMS), près de la moitié de la population mondiale vit en zone d'endémie palustre (3,3 milliards de personnes). Les pays tropicaux et sub-tropicaux, notamment l'Afrique, sont les plus touchés par cette maladie. En 2006, le paludisme a été responsable de près de 247 millions ($5^{ème}$ - $95^{ème}$ percentiles : 189 - 327 millions) de cas cliniques dont 86% (212 millions) sont survenus sur le continent Africain. La même année, le nombre de décès causés par la maladie a été estimé à 881 000 ($5^{ème}$ - $95^{ème}$ percentiles : 610 000 – 1 212 000) dont 91% sont survenus en Afrique et 85% chez les enfants de moins de 5 ans (WHO, 2008).

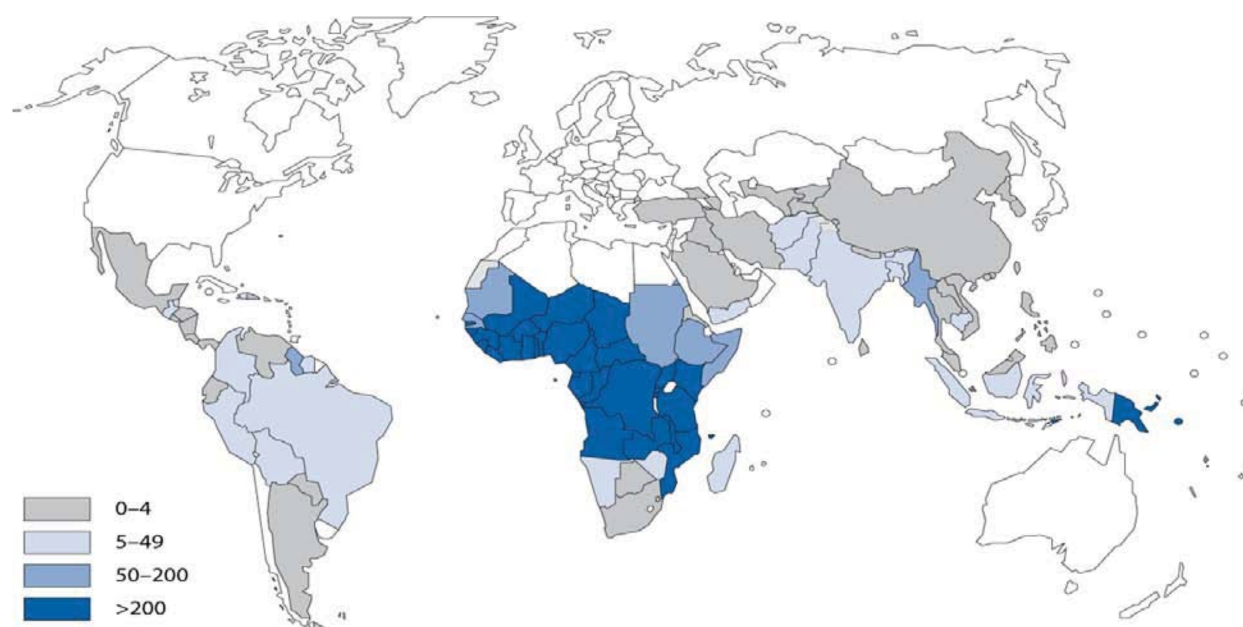

Figure 1 : Incidence estimée du paludisme en 2006 (nombre de cas pour 1000 habitants). Source : WHO, Global health Observatory map gallery (31/12/2007).

L’agent étiologique de la maladie

Le paludisme est une infection parasitaire due à un protiste de l’embranchement *Apicomplexa* et du genre *Plasmodium*. C’est un parasite très ubiquitaire dans le règne animal, il existe plus de 200 espèces de *Plasmodium* pouvant infecter diverses espèces animales (mammifères, reptiles, oiseaux) mais il existe chez ce parasite une haute spécificité d’hôte. Ainsi seulement 5 espèces de *Plasmodium* peuvent infecter l’Homme : *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* et *P. knowlesi* (Rich *et al.*, 2003). IL existe parfois des passages entre des hôtes différents, c’est le cas de *P. knowlesi* qui est à l’origine un parasite simien ayant pour hôtes naturels les macaques *Macaca fascicularis* et *Macaca nemestrina* en Asie du Sud Est. Des infections naturelles à *P. knowlesi* ont été démontrées chez l’Homme en Malaisie, Thaïlande, Philippines et Singapour, ajoutant *P. knowlesi* à la liste des *Plasmodium* pouvant infecter l’Homme (Sabbatani et al., 2010, Jongwutiwes et al., 2004, Luchavez et al., 2008, Ng et al., 2008). Souvent sous-estimées, les infections à *P. vivax* induisent néanmoins entre 70 et 80 millions de cas chaque année et comptent pour plus de 50% des infections palustres en dehors du continent Africain (Mendis et al., 2001). La plus virulente et la plus étudiée des espèces plasmodiales pouvant infecter l’homme reste *P. falciparum* qui est à l’origine de l’essentiel de la mortalité associée au paludisme, notamment en Afrique sub-saharienne (Guerra et al., 2008).

Le cycle de développement de *P. falciparum*

Le cycle de développement de *P. falciparum*, nécessite un hôte intermédiaire humain chez qui se déroule le cycle asexué ou schizogonie, et un hôte définitif invertébré (un moustique femelle du genre *Anopheles*) chez qui se déroule la multiplication sexuée ou sporogonie (Figure 2).

Le moustique infecté libère la forme infectante du parasite (les sporozoïtes) dans le sang de l'hôte humain lors du repas sanguin. Les sporozoïtes sont transportés par le sang jusqu'au foie où ils vont envahir les cellules hépatiques et entamer un processus de multiplication asexué. Ce cycle hépatique asymptomatique va libérer après quelques jours (6 à 7 jours) plusieurs milliers de mérozoïtes dans la circulation sanguine qui vont envahir les globules rouges (Todryk *et al.*, 2005). A l'intérieur du globule rouge, chaque mérozoïte mature en trophozoïte qui se divise par simple mitose pour former un schizonte pouvant contenir plus de 30 mérozoïtes (Figure 4-A). Ces mérozoïtes vont ensuite infecter d'autres globules rouges sains, formant ainsi un cycle érythrocytaire de 48 heures. La libération synchrone des mérozoïtes est à l'origine des symptômes de l'accès palustre. A un moment du cycle, une partie des mérozoïtes internalisés dans le globule rouge vont se différentier en gamétocytes mâles et femelles. Ces gamètes vont être ingérés lors d'un nouveau repas sanguin par l'anophèle femelle. Chez le moustique se déroulera alors le cycle sexué du parasite.

Les gamétocytes, stades sanguins sexués des plasmodiums, sont ingérés par un anophèle femelle au cours d'un repas sanguin. À la suite d'un processus d'exflagellation, un gamétocyte mâle se divise en 8 gamètes mâles haploïdes qui peuvent alors fusionner avec un gamète femelle haploïde issu d'un unique gamétocyte femelle. Cette fécondation aboutit à la formation d'un zygote diploïde puis d'un ookinète haploïde qui traverse activement la paroi stomacale du moustique et forme, à la surface externe de cette paroi, un oocyste, moins de 24 heures après le repas sanguin. À l'intérieur de cet oocyste, les cellules parasitaires se multiplient pour libérer, au bout de quelques jours, près d'un millier de sporozoïtes haploïdes. Les sporozoïtes gagnent ensuite les glandes salivaires et s'y accumulent. Au début d'un repas sanguin ultérieur, l'anophèle

régurgite de la salive et inocule quelques dizaines de sporozoïtes assurant ainsi la diffusion de proche en proche la maladie (Stevenson *et al.*, 2004).

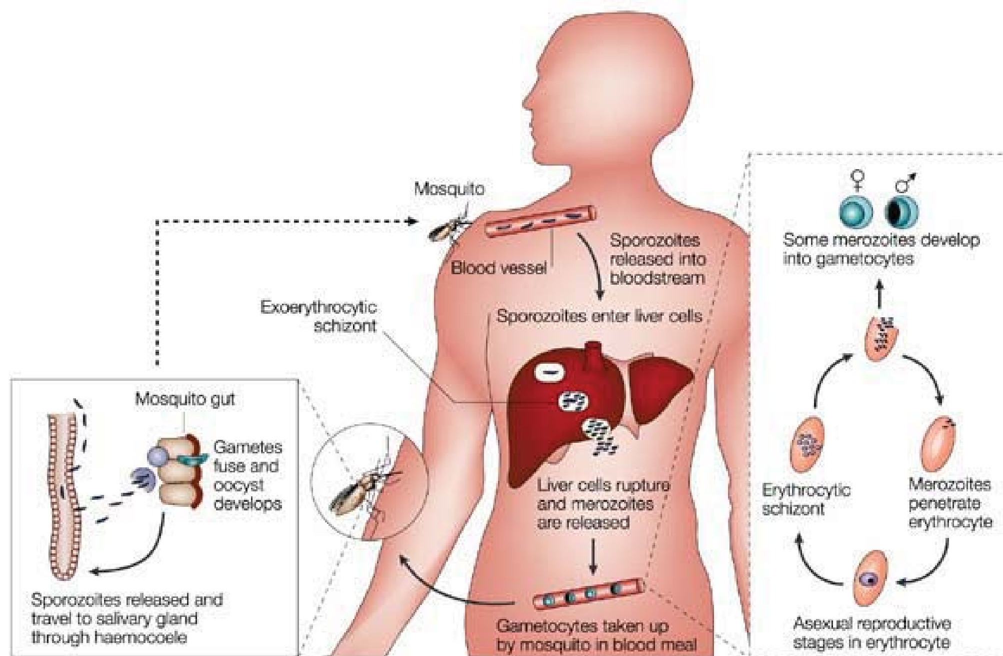

Figure 2 : Cycle de développement de *P. falciparum* chez l'hôte humain et le moustique vecteur. Source : (Stevenson *et al.*, 2004)

***Les manifestations cliniques du paludisme à* P. falciparum.**

Chez l'homme, les manifestations cliniques du paludisme sont dues aux formes asexuées intra-érythrocytaires du parasite. La symptomatologie du paludisme est très variable et peut aller de l'infection asymptomatique à l'accès pernicieux, souvent mortel. L'accès pernicieux ou neuro-paludisme est la complication la plus redoutable du paludisme à *P. falciparum* car il conduit dans la plupart des cas au décès du patient (Murphy *et al.*, 2001). Les manifestations cliniques du neuro-paludisme sont les conséquences à la fois de phénomènes mécaniques et immunitaires. Les phénomènes mécaniques sont en relation avec l'obstruction totale ou partielle des micro-vaisseaux cérébraux par les globules rouges parasités par les formes âgées de *P. falciparum*. Ce phénomène conduit à une anoxie et/ou une hypoxie dans le territoire vasculaire concerné en aval de la lésion. La pathogénèse du neuro-paludisme est un phénomène complexe qui fait

aussi intervenir d’autres facteurs tels que la réponse immunitaire et inflammatoire inappropriée et excessive de l’hôte qui est en faveur du parasite. Celui-ci trouve dans cette configuration tous les éléments nécessaires à sa survie (récepteur endothéliaux surexprimés comme ICAM-1 ou l’accumulation de plaquettes) (Wassmer et al., 2003, Faille et al., 2009). Les autres complications les plus communes du paludisme à *P. falciparum* temoignent toutes d’une détresse viscérale (insuffisance respiratoire, rénale, hépatique etc.) (English, 2000) Le paludisme gestationnel peut entrainer une défaillance placentaire partielle et ainsi perturber les échanges foeto-maternels qui peuvent être à la base de complications observées chez le fœtus et le nouveau né, telles que l’hypoxie fœtale, les retards de développement ou le faible poids à la naissance. La forme grave la plus fréquente du paludisme reste l’anémie qui est multifactorielle incluant l’éclatement cyclique des globules rouges infectés par *P. falciparum*, leur destruction par les cellules phagocytaires ainsi qu’une réduction de l’hématopoïèse (Haldar *et al.*, 2009). Les différentes formes de la maladie sont influencées par de multiples facteurs de l’hôte, du parasite lié en particulier à son polymorphisme génétique et à des facteurs sociaux et géographiques (Figure 3).

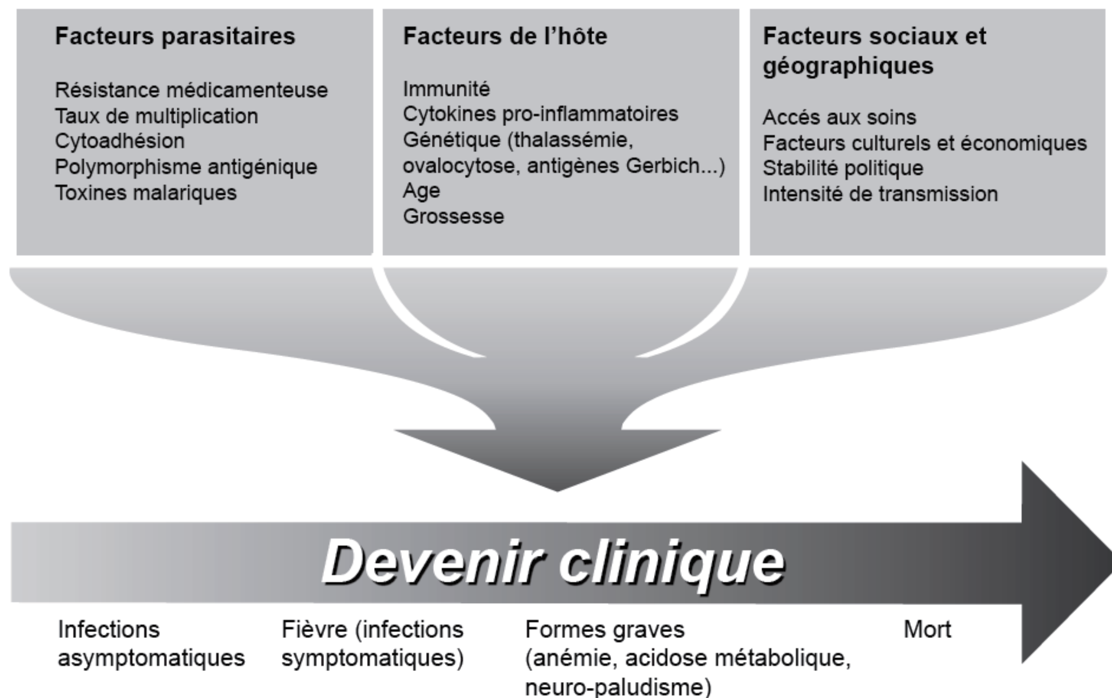

Figure 3 : Le devenir clinique des infections palustres chez un individu vivant en zone d'endémie dépend de plusieurs facteurs (parasitaires, de l'hôte, géographiques et sociaux). Source : (Weatherall et al., 2002).

Le développement de l'immunité anti-palustre

L'acquisition de l'immunité anti-palustre est lente, pouvant prendre plusieurs années même en zones à forte transmission palustre. Elle se fait aux prix d'une exposition répétée et permanente aux parasites. En zone d'endémie, cette immunité anti-palustre augmente avec l'âge des individus avec pour conséquence une baisse de l'impact clinique de la maladie. Les jeunes enfants en dessous de 5 ans vivant en zone d'endémie ont une immunité insuffisante contre les formes simples et graves de la maladie (Pierce *et al.*, 2009). Une exception peut être faite pour les enfants de moins de 6 mois qui peuvent être protégés de la maladie grâce aux anticorps maternels transmis lors de l'allaitement et à l'expression de l'hémoglobine fœtale (Larru et al., 2009). Les enfants âgés entre 5 et 10 ans développent une immunité pouvant les protéger contre les formes graves mais souffrent toujours des formes simples du paludisme. Ce n'est qu'à l'adolescence que l'immunité contre l'ensemble des

formes de la maladie est établie. Cependant, l'immunité anti-palustre n'est jamais totale et les adultes vivant en zone d'endémie palustre peuvent toujours être porteurs asymptomatiques de parasites (Doolan et al., 2009). Cette immunité dite « de prémunition » est éphémère et peut disparaitre après 1 à 2 ans chez des individus immuns quittant leurs zones d'endémie. Cette immunité acquise peut aussi disparaitre temporairement chez la femme pendant la grossesse. Ce « paludisme gestationnel » est associé à la capacité des globules rouges parasités de se fixer à un récepteur, la chondroitine sulfate A (CSA) présent dans le placenta. La soudaine susceptibilité des femmes enceintes primigestes au paludisme est due à l'apparition du placenta qui offre un environnement unique pour le développement de sous population parasitaires possédant une affinité pour les récepteurs placentaires. Les femmes multigestes sont moins touchées par cette forme de paludisme en acquérant lors des grossesses successives des anticorps protecteurs bloquant cette adhésion (Scherf *et al.*, 2001, Duffy *et al.*, 2003, Fusai *et al.*, 2000).

La physiopathologie du paludisme

Les phénomènes mécaniques contribuent à la pathogénisité de paludisme à *P. falciparum*. En ce qui concerne le neuro-paludisme, les lésions anatomopathologiques se caractérisent par plusieurs facteurs comme la présence d'hématies parasitées séquestrées au niveau des microcapillaires cérébraux (Boonpucknavig et al., 1990, Pongponratn et al., 1991, MacPherson et al., 1985), la présence d'hémorragies en anneau au niveau de la substance blanche (Boonpucknavig et al., 1990) et d'œdème cérébral. Ce dernier est présent dans 30 à 40% des cas et pourrait être expliqué par l'hypoxie cérébrale consécutive à l'obstruction vasculaire (Aikawa, 1988). Du point de vue topographique, aucune région de l'axe céphalo-rachidien (cerveau, cervelet, moelle épinière) n'est épargnée par ce phénomène de séquestration (Sein et al., 1993). Ce phénomène de séquestration permet aux parasites d'échapper aux mécanismes de défenses

de l'hôte en retirant les hématies parasitées du flux circulatoire pour éviter le filtre splénique (Engwerda et al., 2005). Cette séquestration, qui a lieu pendant la deuxième moitié du cycle érythrocytaire (à partir du stade trophozoïte âgé), met en relation des ligands parasitaires présents à la surface du globule rouge parasité et des récepteurs endothéliaux. Ces modifications induites par le parasite sur sa cellule hôte sont en relation avec l'organisation structurelle d'un globule rouge sain.

Organisation structurelle d'un globule rouge sain

Le globule rouge humain est une cellule spécialisée dans le transport de l'oxygène (O_2) et du gaz carbonique (CO_2) des poumons aux tissus et cellules du corps. Ce transport se fait grâce à l'hémoglobine contenue dans le cytoplasme de l'hématie et à l'anhydrase carbonique, une enzyme présente à la surface des hématies qui transforme le CO_2 en bicarbonates ou l'inverse, selon le besoin. Durant le processus d'érythropoïèse, le globule rouge va perdre son noyau, ses organites intra-cellulaires ainsi que sa capacité à synthétiser des protéines en quittant la moelle osseuse pour passer dans le sang. Cependant, ce globule rouge mature va entreprendre un voyage de plusieurs centaines de kilomètres à travers la circulation tout au long de ces $\approx$120 jours de vie, en se déformant à plusieurs reprises sans se fragmenter lors de ses passages répétés à travers les capillaires de la micro-vasculature qui peuvent avoir un diamètre 3 fois plus étroit que le sien (Mohandas *et al.*, 2008). Le globule rouge doit sa résistance et sa capacité à se déformer à sa membrane plasmique qui est stabilisée par un cytosquelette constitué de spectrine et de courts filaments d'actine ainsi que de la tropomyosine, tropomoduline (deux protéines empêchant l'actine de dépolymériser) et d'adducine. Ce cytosquelette est ancré sur la membrane plasmique par des interactions spectrine-ankyrine-bande 3. Les nombreuses associations de la bande 4.1 avec les fragments cytosoliques des

glycophorines et de la bande 3 stabilisent aussi le cytosquelette à la membrane érythrocytaire (Figure 6-A).

Modifications du globule rouge par le parasite

Lors du processus d'invasion du globule rouge, les mérozoïtes se retrouvent donc confrontés à une cellule hôte qui ne phagocyte pas. L'invasion du globule rouge est donc un processus actif qui fait intervenir des interactions moléculaires entre des protéines de surface du mérozoïte et des récepteurs membranaires du globule rouge. L'interaction entre les protéines parasitaires de surface EBA-175 et les glycophorines A du globule rouge a été la première à être décrite (Camus *et al.*, 1985, Duraisingh *et al.*, 2003) mais d'autres ligands parasitaires (EBA-140, MSP1, *Pf*Rh1, *Pf*Rh2a ou *Pf*Rh2b) ou érythrocytaires (glycophorines B et C) ont été identifiés (Baum et al., 2005). A la suite de ce contact initial, le mérozoïte va se réorienté de façon à présenter son pôle apical en direction de la membrane plasmique du globule rouge (Figure 4-A). Il s'établit ensuite une jonction serrée entre le mérozoïte et l'érythrocyte suivi de la sécrétion par exocytose dans le cytosol du globule rouge du contenu lipidique et protéique des rhoptries et des micronèmes situés au pôle apical du mérozoïte (Figure 4-B). Ce processus va conduire, à une désorganisation du cytosquelette du globule rouge qui va devenir flaccide et favoriser l'internalisation du parasite dans son cytosol grâce à un système de motilité basée sur le complexe actine-myosine parasitaire. L'internalisation du parasite va entraîner une invagination d'une partie de la membrane plasmique du globule rouge qui va entourer le parasite pour former la membrane parasitophore. La composition de cette membrane parasitophore est distincte de celle de l'érythrocyte. Elle contient des protéines localisées dans des fractions lipidiques « en radeaux » de la membrane plasmique du globule rouge résistantes aux détergents (DRM, detergent-resistant membrane), alors que d'autres protéines de la membrane plasmique érythrocytaire qui ne sont pas associées à ces radeaux lipidiques y sont exclues

(Murphy et al., 2006, Haldar et al., 2002). Un point de contact entre la membrane plasmique du parasite et la membrane de la vacuole parasitophore va former le cytostome, sorte de vésicule d'endocytose du cytoplasme de l'érythrocyte, qui est dirigé vers la vacuole digestive du parasite (Klemba et al., 2004). Le parasite va dégrader l'hémoglobine importée du cytoplasme érythrocytaire dans cette vacuole digestive pour avoir un apport en acides aminés nécessaire à sa croissance. Cette dégradation de l'hémoglobine produit de l'hème qui est toxique pour le parasite. Pour inhiber la toxicité de cette molécule, le parasite va transformer l'hème en cristaux d'hémozoïne qui va s'accumuler dans la vacuole digestive.

Au cours de son développement dans le globule rouge, le parasite va mettre en place un réseau de structures membranaires dans le cytosol de l'érythrocyte assurant les connections entre la membrane parasitophore et la membrane plasmique de l'érythrocyte. Un des éléments de ce réseau, connu sous le nom de structures de Maurer, est situé sous la membrane plasmique du globule rouge du côté cytoplasme (Figure 4-C). Ces structures de Maurer présentent des caractéristiques de Golgi en assurant le transfert de protéines parasitaires à la membrane érythrocytaire au niveau de petites protubérances appelées « knobs ». Il a été suggérer que le transport de protéines parasitaires de la membrane parasitophore jusqu'au structure de Maurer pouvait se faire par l'intermédiaire de vésicules de 25 à 80 nm de diamètre (Figure 5-C) ou grâce à un réseau membranaire tubo-vésiculaire (TVN, tubo-vesicular network) qui joint les structures de Maurer à la membrane parasitophore en s'étendant à travers le cytosol du globule rouge (Figure 4-D).

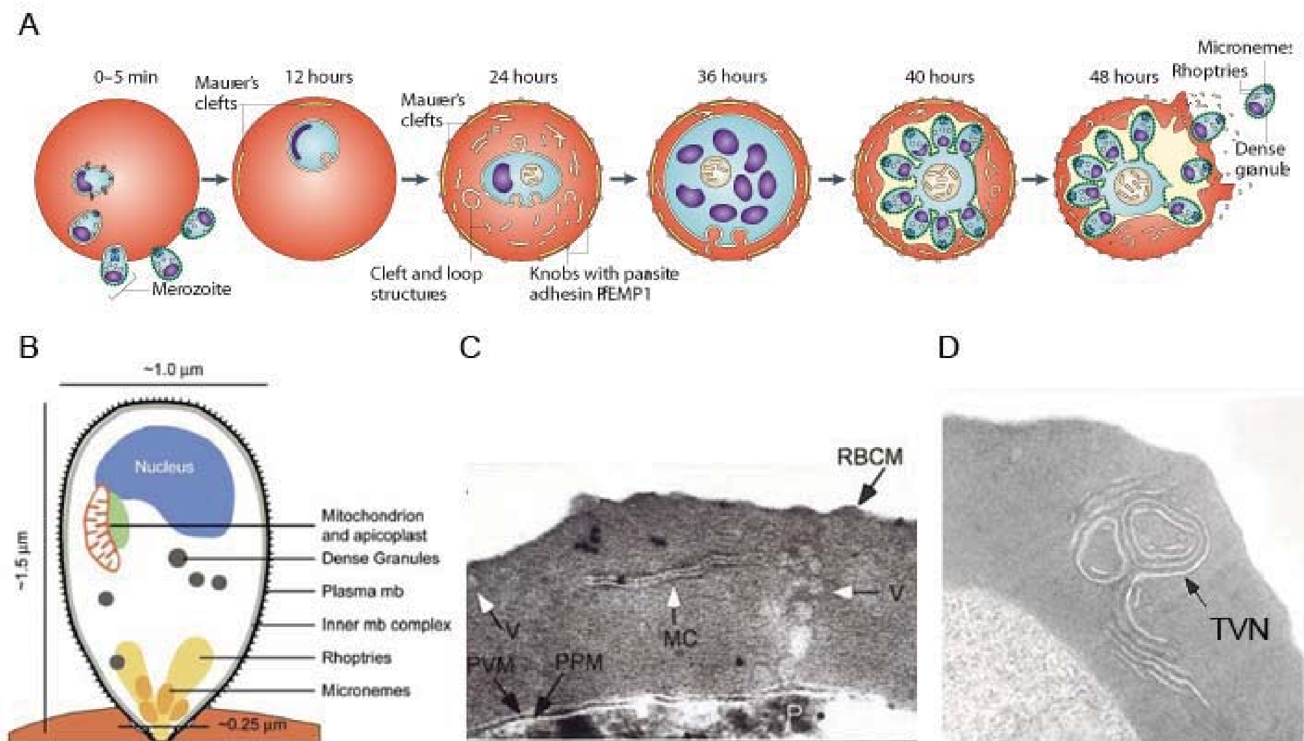

Figure 4 : Invasion érythrocytaire et développement de *P. falciparum* dans le globule rouge. RBCM : membrane du globule rouge, V : vésicules de transport, MC : structure de Maurer, PVM : membrane de la vacuole parasitophore, PPM : membrane plasmique parasitaire, TVN : réseau tubo-vésiculaire. Source : A (Maier et al., 2009), B (Baum et al., 2005), C (Taraschi et al., 2003), D (Wickert et al., 2004).

<u>Export de protéines parasitaires à la surface du globule rouge</u>

Le parasite internalisé remodèle donc sa cellule hôte en mettant en place un réseau membranaire destiné à transporter les protéines parasitaires virulentes au niveau de la membrane plasmique du globule rouge. Il a été montré que le transport de protéines au-delà de la membrane parasitophore dépendait de la présence d'un court motif d'acides aminés situé à l'extrémité N-terminale des protéines à environ 35 acides aminés en aval du peptide signal, la séquence peptidique hydrophobique qui sert à adresser les protéines vers le milieu extracellulaire. Ce motif à été identifié quasiment simultanément (avec quelques variations mineures dans le motif) par deux équipes différentes et est appelé PEXEL (Protein Export Element, motif pentamérique RxLxE/Q/D) ou VTS (Vacuolar Transport Signal, motif de 11 acides aminés RxSRILAExxx) selon que l'on fasse référence aux travaux de Marti et al. ou de Hiller et al.,

respectivement (Hiller et al., 2004, Marti et al., 2004). Ce motif d'export, appelé aussi HT (host targeting sequence) de façon générale, est retrouvé sur les protéines membranaires et solubles indiquant que le parasite est capable d'exporter des protéines à des destinations cellulaires différentes. Des travaux ultérieurs ont montré que l'extrémité C-terminale du motif HT est coupée et acétylée au niveau du réticulum endoplasmique (Figure 5-A) par une protéase aspartyle appelée plasmepsin V (Boddey et al., 2010). Cette nouvelle extrémité N-terminale pourrait présenter un motif de séquence d'acides aminés qui serait spécifiquement reconnu par des transporteurs au niveau de la membrane parasitophore, et permettrait aux protéines parasitaires préalablement dépliées (Gehde et al., 2009) de passer dans le cytoplasme du globule rouge (Figure 5-B) (Chang et al., 2008). Une étude récente à proposée l'implication d'un complexe nommé *Plasmodium* translocon of exported proteins (PTEX) dans l'export sélectif des protéines parasitaires disposant d'un motif HT (de Koning-Ward et al., 2009).

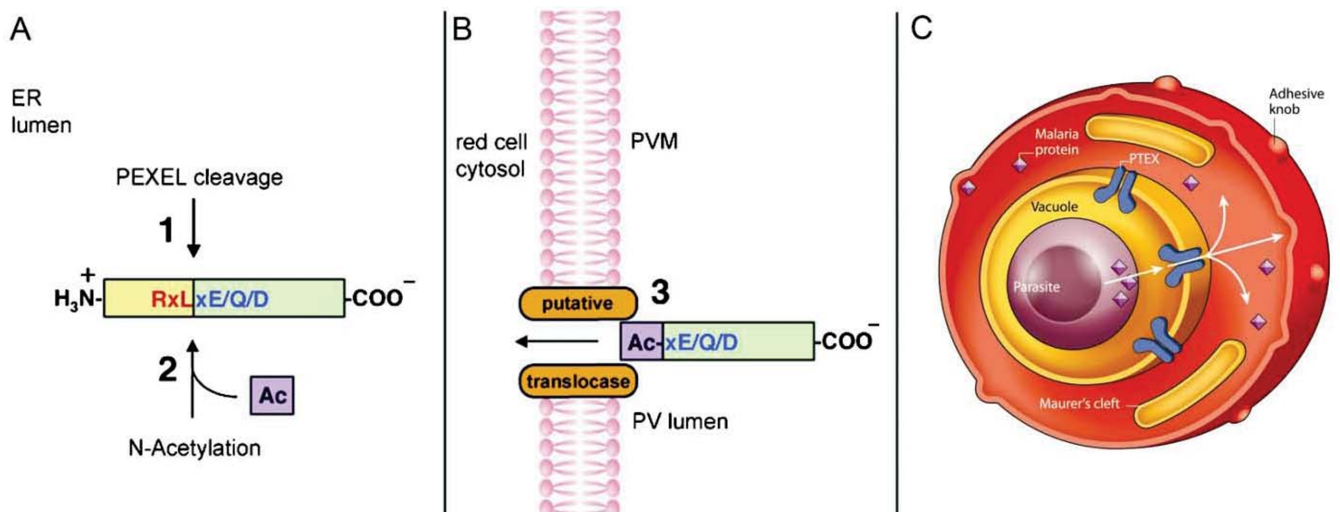

Figure 5 : Schéma modélisant le transport de protéines de *P. falciparum* médié par la coupure du motif PEXEL (A-1), l'acétylation de sa nouvelle extrémité N-terminale (A-2) dans la lumière du réticulum endoplasmique et la reconnaissance du nouveau motif acétylé par des transporteurs au niveau de la membrane parasitophore (B-3). Une représentation schématique du mécanisme d'export des protéines parasitaires grâce au

complexe PTEX est indiquée en C. Source : **A et B** (Chang et al., 2008), **C** (Reiff *et al.*, 2009).

Environ 400 protéines de *P. falciparum* ont été prédites comme pouvant être exportées basé sur la présence du motif HT (Sargeant et al., 2006). Quelques protéines, telle que REX 1 (ring exported protein 1) ou la *P. falciparum* skeleton binding protein 1 (*Pf*SBP1) ne contiennent pas ce motif mais sont pourtant bien exportées au delà de la membrane parasitophore suggérant l'existence de mécanismes d'export alternatifs (Haase et al., 2009, Dixon et al., 2008, Saridaki et al., 2009). Il a été suggéré que ces protéines peuvent être directement transférées dans les structures de Maurer pendant leur formation au niveau de la membrane parasitophore ce qui éviterait leur transport ultérieur à travers le cytoplasme du globule rouge pour rejoindre ces structures (Maier et al., 2007).

Fonctions des protéines parasitaires exportées

Les protéines parasitaires exportées peuvent être destinées à différents compartiments situés dans le cytoplasme du globule rouge. Certaines protéines parasitaires, telles que *P. falciparum* N-Ethylmaleimide-sensitive factor (*Pf*NSF), *Pf*Sar1p ou *Pf*Sec31p, sont associées à des structures vésiculaires impliquées dans le transport des protéines à travers le cytoplasme du globule rouge (Adisa et al., 2001, Hayashi et al., 2001). D'autres protéines s'associent avec les protéines du cytosquelette pour stabiliser la membrane du globule rouge, comme la ring-infected erythrocyte surface antigen (RESA) (Pei et al., 2007), la *P. falciparum* erythrocyte membrane protein 3 (*Pf*EMP3) (Knuepfer et al., 2005), la *Pf*332 (Waller et al., 2010) ou *Pf*SBP1 (Blisnick et al., 2000). Cette modification du cytosquelette du globule rouge s'accompagne d'un gain de résistance aux stress mécanique et peut empêcher l'invasion des hématies déjà parasitées. *P. falciparum* est aussi capable d'exporter des kinases (kinases de la famille FIKK) qui seraient capable de phosphoryler de façon sélective

différentes protéines du cytosquelette, comme la band 4.1 (Chishti et al., 1994), la spectrine, l'ankyrin ou la bande 3 (Murray *et al.*, 1989). Cette phosphorylation sélective peut aussi conduire à une modification de la flexibilité de la membrane du globule rouge (Eder et al., 1986, Manno et al., 1995). D'autres protéines, comme la membrane-associated histidine-rich protein-1 (MARHP-1) (Spycher et al., 2003) ou la ring-exported protein-1 (REX-1) (Hanssen et al., 2008) sont partie constituante des structures de Maurer, et peuvent avoir un rôle dans le maintien de la structure ou dans la gestion du trafic des protéines exportées au niveau des knobs (Figure 6-B).

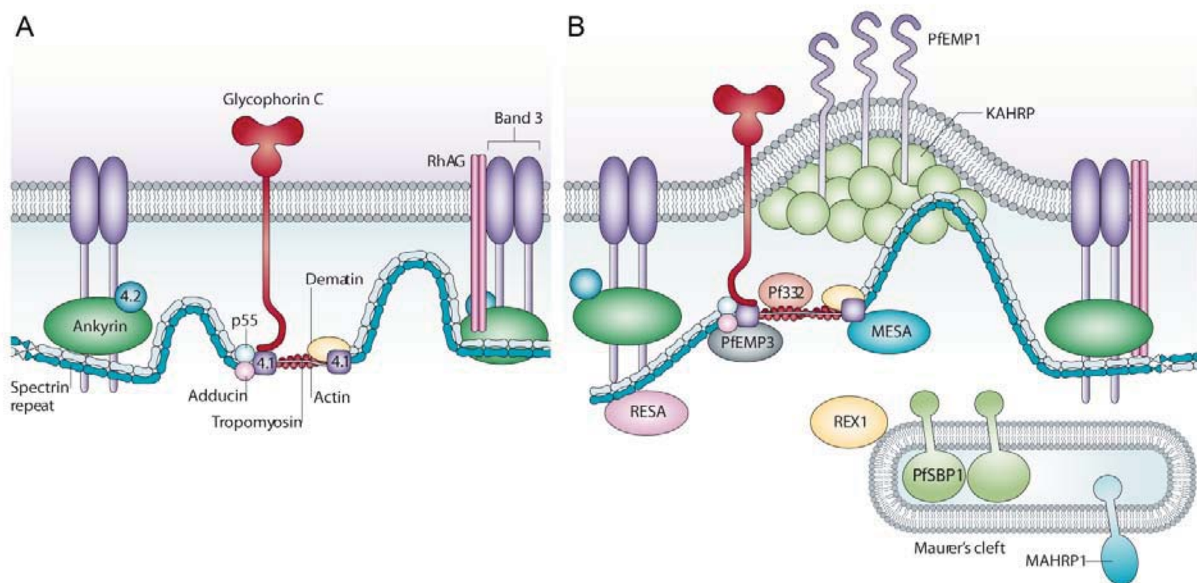

Figure 6 : Organisation du cytosquelette membranaire d'un globule rouge sain (A) et parasité par *P. falciparum* (B). Source : (Maier et al., 2009)

Il a été montré que les knobs sont le lieu d'expression de ligands parasitaires impliqués dans la cytoadhérence bien que ces structures ne conditionnent pas le phénomène car des lignées parasitaires dépourvues de knobs peuvent tout de même présenter des capacités d'adhérences *in vitro* aux cellules endotheliales (Biggs et al., 1989, Udomsangpetch et al., 1989). Les protéines parasitaires KAHRP (Knob-associated Histidine-Rich Protein) et PfEMP-3 participent à la formation de ces knobs (Figure 6-B), qui constituent le site d'ancrage de ligands

parasitaires exposés à la surface du globule rouge. Plusieurs ligands parasitaires peuvent intervenir dans la cytoadhérence, comme les membres de la famille des repetitive interspersed family (RIFINs) (Ansari et al., 2008), des cytoadherence linked asexual gene (CLAG) (Craig, 2000) ou les sequestrins (Ockenhouse et al., 1991). Cependant, il a été montré que la *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP1) jouait un rôle majeur dans la physiopathologie du paludisme grave et a fait à ce titre l'objet de nombreuses études (pour revue : (Kraemer *et al.*, 2006)).

Cette protéine est codée par une famille de gènes appelés « gènes *var* » composée d'environ 60 copies par génome haploïde. Ces gènes sont principalement localisés dans les régions subtélomériques des chromosomes et sont sujets aux phénomènes de recombinaisons qui affectent fréquemment leur structure (Scherf *et al.*, 2008, Kraemer *et al.*, 2006). Les protéines *Pf*EMP1 sont constituées de la succession de domaines de type DBL (duffy binding like domaine) et de domaines riches en cystèines. Les différentes combinaisons entre ces domaines sont spécifiques d'une liaison avec un récepteur endothélial. Il existe dans la famille des *Pf*EMP1 une grande variabilité au niveau de ces domaines qui est en partie à l'origine de l'évasion du système immunitaire de l'hôte et des propriétés de cytoadhérence du globule rouge (Figure 7). Ces propriétés donnent lieu à des phénomènes divers, tels que l'agglutination des hématies parasitées entre elles (auto-agglutination ou « clumping ») (Pain et al., 2001) ou avec des globules rouges sains (formation de rosettes ou « rosetting ») (Carlson, 1993, Handunnetti et al., 1992, David et al., 1988). L'adhérence du globule rouge aux cellules endothéliales est aussi étroitement associée à la séquestration des hématies parasitées dans les organes profonds qui est impliquée dans les formes graves du paludisme (Figure 7).

Plusieurs molécules exprimées à la surface des cellules endothéliales ont été impliquées dans l'interaction avec les hématies parasitées à travers la protéine P*f*EMP1. Dans l'ordre chronologique de leurs découvertes ces molécules sont :

la thrombospondine (TSP) (Roberts et al., 1985), CD36 (Barnwell et al., 1989), l'inter-cellular adhesion molecule 1 (ICAM-1) (Berendt et al., 1989), la vascular cell adhesion molecule 1 (VCAM-1) et l'endothelial-leukocyte adhesion molecule 1 (ELAM-1) (Ockenhouse et al., 1992), la chondroitine sulfate A (CSA) (Robert et al., 1995), la platelet endothelial cell adhesion molecule-1 (PECAM-1) (Treutiger et al., 1997), la P-selectine (Udomsangpetch et al., 1997) et plus récemment la neural cell adhesion molecule (NCAM) (Pouvelle et al., 2007).

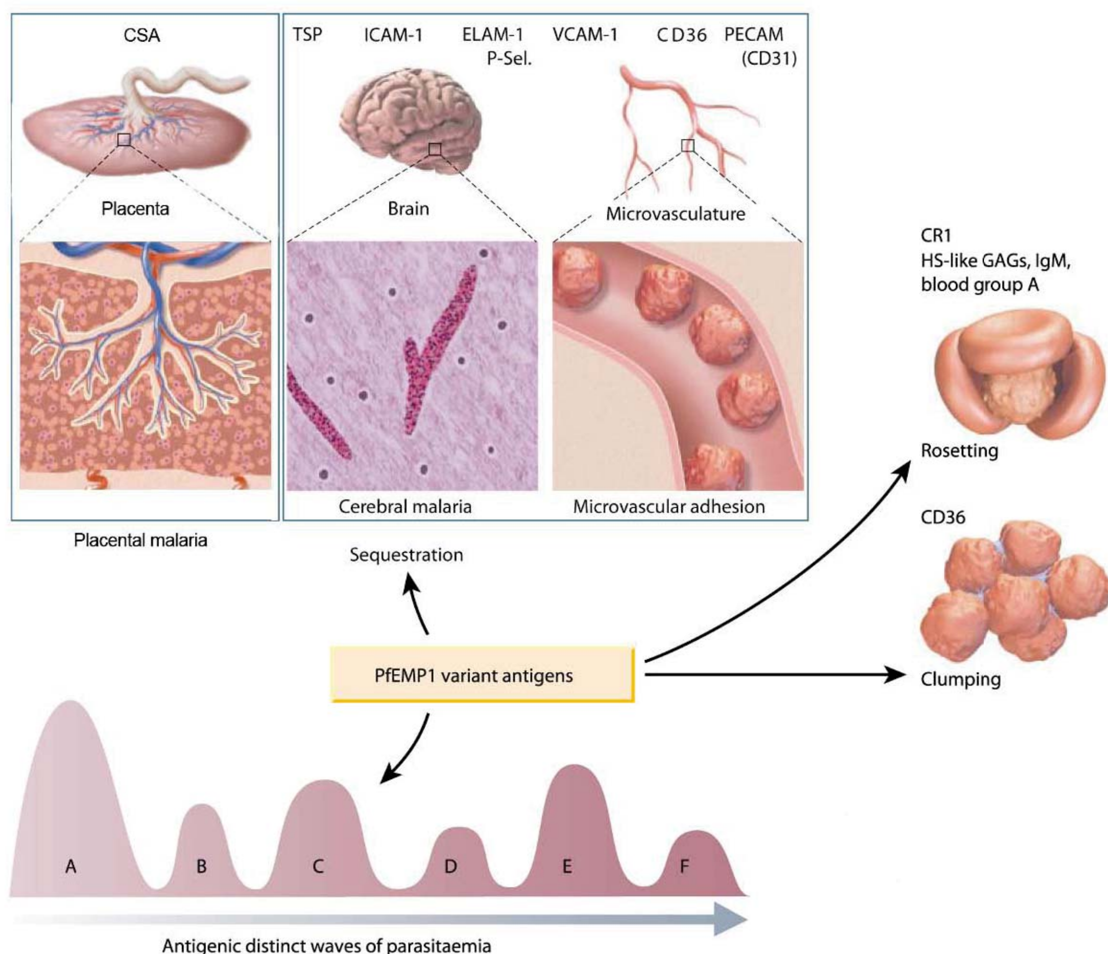

Figure 7 : Schéma montrant le rôle des variants antigéniques de PfEMP1 dans l'interaction hôte/parasite et la physiopathologie du paludisme. Source : (Miller et al., 2002)

OBJECTIFS

L'importation du paludisme dans les pays où la maladie n'est pas endémique est une cause majeure de morbidité liée aux voyages (Wichmann et al., 2004, Ansart et al., 2005). L'Europe connaît de nombreux cas de paludisme d'importation. En 2008, sur 6957 voyageurs internationaux malades, 383 (5,5%) ont été touchés par le paludisme et 12 ont développé une forme grave (Field et al., 2010). Le paludisme représente aussi une préoccupation majeure pour l'armée française. En 2008, 432 (≈3.4%) cas de paludisme ont été rapportés chez des militaires français en mission pour de courtes durées (4 mois) en zone d'endémie (Queyriaux et al., 2008). Comme l'illustrent les données sur les militaires séjournant en Côte d'Ivoire (Figure 8), le paludisme d'importation est devenu plus fréquent que le paludisme déclaré en mission depuis quelques années (Haus-Cheymol et al., 2009, Migliani et al., 2008).

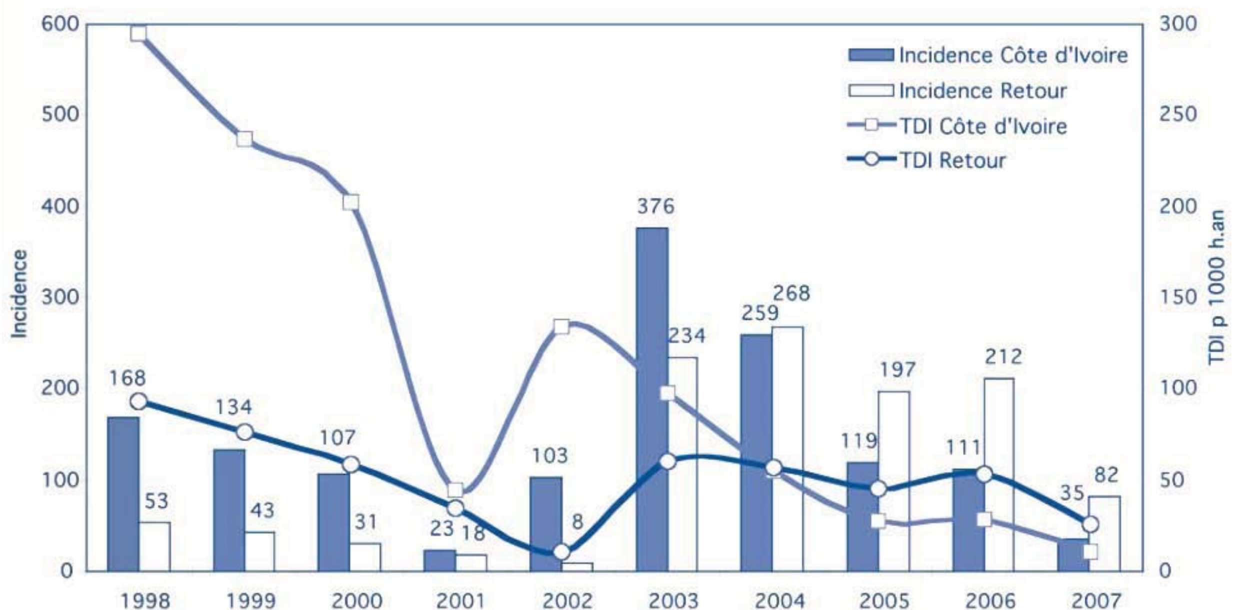

Figure 8 : Evolution de l'incidence des cas de paludisme déclarés en mission en république de Côtes d'Ivoire et au retour de mission de janvier 1998 à décembre 2007. TDI : taux d'incidence (pour 1000 hommes par an). Source : (Haus-Cheymol et al., 2009) d'après les données de (Migliani et al., 2008).

L'objectif de notre étude était de déterminer parmi les militaires ayant séjourné brièvement en zone d'endémie, la prévalence d'individus présentant une réponse IgG vis-à-vis d'antigènes présents à la surface des érythrocytes infectées par *Plasmodium falciparum* et d'identifier les protéines correspondantes. L'identification de ces protéines parasitaires antigéniques présente un intérêt majeur pour évaluer la proportion de militaires exposés au parasite en absence de signes cliniques, mais également pour le développement de candidats vaccins potentiels dans la prévention de l'apparition des formes grave de paludisme.

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ARTICLE N.6

Specific antibody responses against membrane proteins of erythrocytes infected by *Plasmodium falciparum* of individuals briefly exposed to malaria

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Specific antibody responses against membrane proteins of erythrocytes infected by *Plasmodium falciparum* of individuals briefly exposed to malaria

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Abstract

Background: *Plasmodium falciparum* infections could lead to severe malaria, principally in non-immune individuals as children and travellers from countries exempted of malaria. Severe malaria is often associated with the sequestration of *P. falciparum*-infected erythrocytes in deep micro-vascular beds via interactions between host endothelial receptors and parasite ligands expressed on the surface of the infected erythrocyte. Although, serological responses from individuals living in endemic areas against proteins expressed at surface of the infected erythrocyte have been largely studied, seldom data are available about the specific targets of antibody response from travellers.

Methods: In order to characterize antigens recognized by traveller sera, a comparison of IgG immune response against membrane protein extracts from uninfected and *P. falciparum*-infected red blood cells (iRBC), using immunoblots, was performed between non exposed individuals (*n = 31*) and briefly exposed individuals (BEI) *(n = 38)* to malaria transmission.

Results: Immune profile analysis indicated that eight protein bands from iRBC were significantly detected more frequently in the BEI group. Some of these antigenic proteins were identified by an original immuno-proteomic approach.

Conclusion: Collectively, these data may be useful to characterize the singular serological immune response against a primary malaria infection in individuals briefly exposed to transmission.

Background

The protozoan parasite *Plasmodium falciparum* is the causative agent of the most virulent form of human malaria, affecting about 500 million of persons yearly and leading to nearly two million deaths, mainly in Africa [1]. Individuals residing in endemic areas of parasite transmission acquired gradually a protective immunity to *P. falciparum* malaria after numerous disease episodes during childhood. This immunity is not sterilizing but is protective against clinical disease and especially severe malaria [2,3]. Children from endemic areas and travellers from non-endemic countries, considered as non-immune individuals, are particularly at risk of dying from severe malaria [4].

Blood stages of *P. falciparum* are responsible for all the clinical symptoms of malaria including severe cases such as severe anaemia or visceral disorders [5]. These visceral disorders are associated with sequestration of infected red blood cells (iRBC) [6,7]. This sequestration phenomenon is due to interactions between endothelial receptors and parasite proteins expressed at the surface of iRBC. The abundance and the long time display of the blood-stage malaria parasites in the human host

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render the proteins from the erythrocytic parasite stages an important target for the immune system. The absence of antigen processing in erythrocytes prevents the iRBC destruction by specific MHC-restricted T-cell response. Immunity to blood stage malaria parasites is thus primarily conferred by humoral immune responses [8]. Additionally, the passive transfer of IgG from highly exposed individuals (HEI) to non-immune patients could confer a protective effect to clinical symptoms of malaria [8-10]. Among proteins supposed to induce a protective immunity, the surface-expressed *P. falciparum* erythrocyte protein 1 (PfEMP-1) have been largely studied [11-13]. However, others erythrocytic parasite stage proteins were reported to elicit an immune response [14-17]. Collectively, these data suggest that analysis of the serological immune response from exposed individuals to malaria (briefly or continuously) could be informative for the understanding of the protective immune response development.

Serological responses from individuals living in hyperendemic areas for malaria have been largely studied. Several blood stage antigens have been characterized, and some of them are candidates for vaccine trials (for review [18,19]). Although some studies have assessed the antibody response to pre-erythrocytic antigens in travellers [20-22], seldom data are available concerning the antibody response against blood stage antigens from non-immune healthy adults briefly exposed to malaria transmission, such as travellers or individuals living in area where malaria is under elimination.

The aim of this study was to identify iRBC membrane antigenic protein repertoire recognized specifically by briefly exposed individuals (BEI). An immunoblot approach allowed us to define a singular BEI IgG response against membrane protein extract from iRBC compared to non-exposed individuals (NEI). This specific serological immune response could be useful to estimate individual exposure to malaria transmission, and to understand the first stages of the immune responses to primary malaria infection.

Methods

Population studied

Five French soldier companies (*n = 751*, mean age ± SD: 25.3 ± 4.8 years), who travelled during a five-month period in tropical Africa (Gabon or Ivory Coast, from 2002 to 2007), were included in this study. Individuals used mandatory anti-malaria prophylaxis including anti-vectorial equipment, such as permethrin-impregnated bed nets, repellents and long-sleeve battle dress at night and chemoprophylaxis (100 mg of doxycycline per day). Blood samples were collected one week after the return to France and sera were stored at -80°C. Sera from individuals living in France (*n = 31*, mean age ± SD: 31.2 ± 6.5 years, Caucasians), who have never been exposed to malaria vectors and parasite were used as negative controls. Sera from highly exposed individuals (HEI) to malaria from Congo (*n = 9*), gratefully provided by Pr. Hovette (Centre médical de secours Total, Pointe Noire, Republic of the Congo) and from Senegal (*n = 5*), were used as positive controls. All participants gave their written informed consent to take part in the study and Marseille-2 University ethical committee has approved the protocol (Ethics Statement).

Parasite culture

The RP8 *P. falciparum* cytoadherent strain was selected from the Palo Alto (FUP)1 strain by panning on Human Umbilical Vein Endothelial Cells (HUVEC, ATCC number: PCS-100-010), as described by Fried and Duffy [23]. This cell line express CD36 and ICAM-1 which are well known host receptors involved in cytoadherence of iRBC. Parasites were maintained in continuous culture in candle jar as previously described [24,25]. Briefly, parasites were cultured at 5% haematocrit of type O^+ human RBC suspended in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 25 mM HEPES (Invitrogen), 6 mM D-glucose (Sigma-Aldrich, St Louis, USA), 23 mM $NaHCO_3$ (Invitrogen), 32 μg/ml neomycine (Sigma-Aldrich), 0.2 mM L-glutamine (Invitrogen), 0.25 μg/ml orotic acid (Sigma-Aldrich), 0.5 μg/ml hypoxanthine (Sigma-Aldrich) and 10% of heat-inactivated type O^+ human serum, at 37°C in a gas mixture of 3% CO_2, 17% O_2. Parasitaemia and erythrocytic cycle stages proportion were monitored daily microscopically by examination of blood smear stained with Giemsa-stained (RAL® 555, France).

Liquid indirect immunofluorescence assay (L-IFA)

RBC and iRBC from *in vitro* cultures with about 10% parasitaemia were used to perform L-IFA as previously described [26,27]. Briefly, 50 μl of cell suspension were washed two times in RPMI 1640 at room temperature and incubated in DAPI (40 μg/ml) (Invitrogen, USA) for 30 min at 37°C. After 3 washing in PBS (Invitrogen), immuno-staining was started by incubating cells with 50 μl of diluted sera for 30 min on ice. Each sera was individually analysed using dilutions of 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1,280 in PBS pH 7.4. Positive and negative control sera were used at 1:40 dilution. After two washes with RPMI 1640, alexa-fluor 488 goat anti-human immunoglobulin G (H+L) (Molecular Probes, USA) was added to the cell suspension and incubated for 30 min on ice. A droplet of cell suspension was laid on a microscopy slide, and antibody binding and DNA staining were assessed by fluorescence microscopy on a Nikon eclipse E800 fluorescence microscope (100 × magnification). Sample titres were determined by the

maximum dilution where the fluorescence could be detected.

Sample preparation

Plasmodium falciparum adherent mature stages (trophozoite and schizonte stages) were enriched by Plasmion flotation (Fresenius France Pharma, Louvier, France) as described elsewhere [25,28]. Infected erythrocytes were washed two times in PBS medium and lysed in cold hypotonic medium (H_2O-saponin 0.1%, Sigma) for 2 min. Free parasites were discarded by centrifugation (9 300 × g for 4 min). Erythrocyte membranes were recovered from the supernatant, and this cell fraction was submitted to repeat steps of ultra centrifugations (100 000 × g for 1 h at 4°C) followed by washing until iRBC ghost were colourless, and stored at -80°C. The same protocol was used to collect membrane proteins from uninfected erythrocytes. Membrane protein extracts were then suspended in 4% (w/v) CHAPS (Sigma). Membrane aggregates were then disrupted by ultrasonication (Vibracell 72412, Bioblock Scientific, Illkirch, France) for 5 min on ice at maximum amplitude and precipitated in 100% acetone (Sigma) to discard lipids. Protein concentration was estimated using Lowry-based DC assay (Biorad, Hercules, CA, USA) according to the manufacturer's instruction. Membrane protein extracts were suspended in a buffer containing 8 M urea (Sigma), 2 M thiourea (Sigma), 4% (w/v) CHAPS (Sigma) and 30 mM Tris (Sigma), adjusted to pH 8.5 in order to obtain a protein concentration adjusted to 2.5 μg/μL.

Immunoblots and analysis procedures

The RBC and iRBC membrane samples were reduced in a Tris buffer containing dithiothreitol (1% w/v, Sigma), and 15 μg of each sample were loaded per well onto a 10% polyacrylamide gel before to be separated by SDS-PAGE in a Mini PROTEAN II (BioRad, Hercules, CA, USA). After SDS-PAGE, gels were transferred onto a nitrocellulose membrane (0.45-μm, GE Healthcare) by semidry blotting [29]. Each membrane was cut into 18-22 strips (3-4 mm wide), before incubation with a saturation buffer (5% non-fat dried milk in PBS containing tween-20 (0.1% v/v, Sigma)). Immunoblots were carried out with human sera, which were diluted at 1/100 in saturation buffer. After overnight incubation, blots were incubated with mouse anti-human Fcg/IgG horse radish peroxidase (HRP) conjugated antibody (1/5 000, Beckman Coulter, San Jose, CA, USA), and revealed using an ECL Plus western blotting (WB) detection system (GE Healthcare). Densitometric analysis of autoradiographs was performed using Diversity database™2.2 software (Biorad) to align and compare the IgG immune patterns. Two sera, one from a NEI and another one from a HEI which were used as controls, were tested on each blot in order to assess the quality of each immunoblot and to improve the accuracy of the alignment of antibody reactivities intra- and inter-gels [30,31]. Molecular weights were estimated by comparison with standard molecular weight (Biorad).

CyDye labelling

Membrane proteins from iRBC were minimally labelled with CyDye according to the manufacturer's recommended protocols (GE Healthcare). Briefly, membrane protein extracts (50 μg) were labelled with 400 pmol of Cy5, freshly dissolved in anhydrous dimethyl formamide (Sigma), and incubated on ice for 30 min in the dark. The reaction was quenched with 1 μL of free lysine (10 nM, Sigma) by incubation 10 min on ice. An equal volume of 2× sample buffer (8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM DTT, 1% (v/v) IPG Buffer 3-10 (GE Healthcare) was added to the Cy5 labelled sample before submission to two-dimensional electrophoresis (2-DE).

Two-dimensional electrophoresis (2-DE)

Reagents that were not purchased from GE Healthcare, are indicated. Destreak buffer containing 1% (v/v) IPG buffer 3-10 was used for IPG strips (18-cm) overnight rehydratation. The samples were laid at the acidic end of the IPG strip using a cup-loading technique. IEF was carried out on an Ettan IPGphor II electrophoresis unit at 20°C, for a total of 45 kVh. Prior to separation in the second dimension, IPG strips were reduced and alkylated in a equilibration buffer containing 50 mM Tris-HCl, pH 8.6 buffer, 6 M urea, 2% SDS and 30% glycerol supplemented with 1% (w/v) DTT or 2.5% (w/v) iodoacetamide instead of DTT for 10 min. IPG strips were then placed on the top of 10% uniform polyacrylamide gels. Strips were overlaid with 0.5% agarose in 1× running buffer containing bromophenol blue and the proteins were further separated by SDS-PAGE (15 W per gel) at 20°C, in Ettan DALT Six electrophoresis system. Two-dimensional gels were either stained with Imperial™Protein Stain (Thermo Scientific, Rockford, IL, USA) or transferred onto nitrocellulose membranes and incubated with human sera as described above (see Materials and Methods section: immunoblots and analysis procedures). For nitrocellulose membranes with Cy5-labelled proteins, antibody response was revealed using goat-anti-human IgG FITC-conjugate (diluted at 1:2000) (Invitrogen). Immunoblots were directly digitalized using a Typhoon™Trio Image scanner. Images were analysed with Decyder v6.5 software, allowing an accurate spot matching between 2-D protein patterns and 2-D antigenic patterns from gels and immunoblots respectively. Matched spots were selected for excision and further identification by mass spectrometry (MS).

Spots excision and in-gel digestion

Based on Decyder analysis, spots of interest were excized using Shimadzu Biotech Xcise System (Champs sur Marne, France). Protein spots were digested overnight at 37°C with sequencing-grade trypsin (12.5 µg/mL; Promega Madison, WI, USA) in 50 mM NH_4HCO_3 (Sigma). The resulting peptides were extracted with 25 mM NH_4HCO_3 for 15 min, dehydrated with acetonitrile (ACN) (Sigma), incubated with 5% acid formic (Sigma) for 15 min under agitation, then dehydrated with ACN, and finally completely dried using a SpeedVac. Samples were then stored at -20°C before analysis by mass spectrometry (MS).

Mass spectrometry analysis

The samples were analysed on a LCQ DecaXPplus (ThermoFinnigan, San Jose, CA) ion trap. Nano-liquid separation of peptides was carried out using an Ettan MDLC chromatographic system (GE Healthcare) in high throughput configuration. Ten microliters of the digest were first trapped on a zorbax 300SB-C18 5 × 0.3 mm column and eluted at a flow rate of approximately 200 nL/min on a zorbax 300SB-C18, 3.5 μm, 150 × 0.075 mm by a linear gradient of eluant B (0.1% Formic acid, 84% ACN) in eluant A (1% Formic acid). Chromatographic system was piloted by Unicorn 5.01 software (GE Healthcare). MS measurements were performed using a LCQTM Deca XP Plus ion trap mass spectrometer (ThermoFinnigan) equipped with a LCQTM nanospray ionization source. A spray voltage of 1.8 kV was applied to the liquid junction via an in-union high voltage contact coupled to a silicaTip emitter (New Objective). Operation of the mass spectrometer was fully automated during the entire procedure using Excalibur 1.3 data system (ThermoFinnigan). Continuous cycles of one full scan (m/z 500 to 1700) followed by three data-dependent MS/MS measurements at 35% normalized collision energy were performed. MS/MS measurements were allowed for the three most intense precursor ions with a maximum rejection time limit of 1 min. All MS/MS spectra were sequence database searched using Mascot Daemon v2.2.2 software (Matrix Science, London, UK).

MS data analysis

The data were searched using Mascot software, against *Homo sapiens* and *P. falciparum* National Center for Biotechnology Information non-redundant (NCBInr, NIH, Bethesda, MD) protein database (June 10th, 2009). Search parameters are set in order to allowed for one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine; precursor and product ion mass error tolerance was <0.2 Da. All identified proteins have a Mascot score greater than 41 (Mixed: *H. sapiens* + *P. falciparum*, 237,583 sequences), corresponding to a statistically significant ($p < 0.05$) confident identification. Moreover, among the positive matches, only protein identifications based on at least two different non-overlapping peptide sequences with a mass tolerance <0.05 Da were accepted. For single peptide-based identification, in addition to Mascot score significance, were considered only peptide sequence with at least six consecutive amino acids detected on MS spectra (Additional file 1). These validation criteria were added in order to limit the number of false positive matches without missing real proteins of interest.

Statistical analysis

For IgG antibody patterns analysis, the data were expressed in binary mode (0, absence of antigenic band; 1, presence of an antigenic band). The Mann-Whitney and the Fisher exact test were used as appropriate. All statistical analyses were done with the SPSS 12.0 software.

Results

BEI sera selection according to their IgG reactivity against iRBC surface proteins

IgG antibody reactivity from 751 BEI sera sampled after a five months journey in endemic area was assessed by liquid-indirect immunofluoresence assay (L-IFA). Considering reactions with titres over 1:80 as positive, 38 BEI sera (5.0%) shown an IgG reactivity directed against the surface of iRBC (Figure 1). Among the 38 selected BEI, 37 were males with a mean age ± SD of 25 ± 3.5 years. Eight BEI had a *P. falciparum* clinical episode during the journey. These positive BEI sera were selected for 1 D immunoblotting analysis. Sera from individuals never exposed to malaria (NEI, $n = 31$) were also tested by L-IFA, and none of them presented IgG reactivity against iRBC at 1:40 dilution (Figure 1). Sera from highly exposed individuals (HEI, $n = 14$) presented a high IgG reactivity against the surface of iRBC until dilution to 1:320.

Comparative IgG responses against RBC and iRBC membrane protein extracts between NEI, BEI and HEI

The IgG antibody response of selected sera from NEI ($n = 31$), BEI ($n = 38$) and HEI ($n = 14$), was tested successively against RBC and iRBC membrane protein extracts using immunoblots (Figure 2A &2B). The densitometric analysis of the different patterns of proteins recognized by IgG obtained with regard to the molecular mass, were performed for quantitative comparisons. Against RBC membrane protein extracts, 3.4 ± 2.6 (means ± SD), 3.5 ± 2.1 and 4.9 ± 1.5 antigenic bands were detected per strip for NEI, BEI and HEI sera, respectively (Figure 2C). The number of antigenic bands

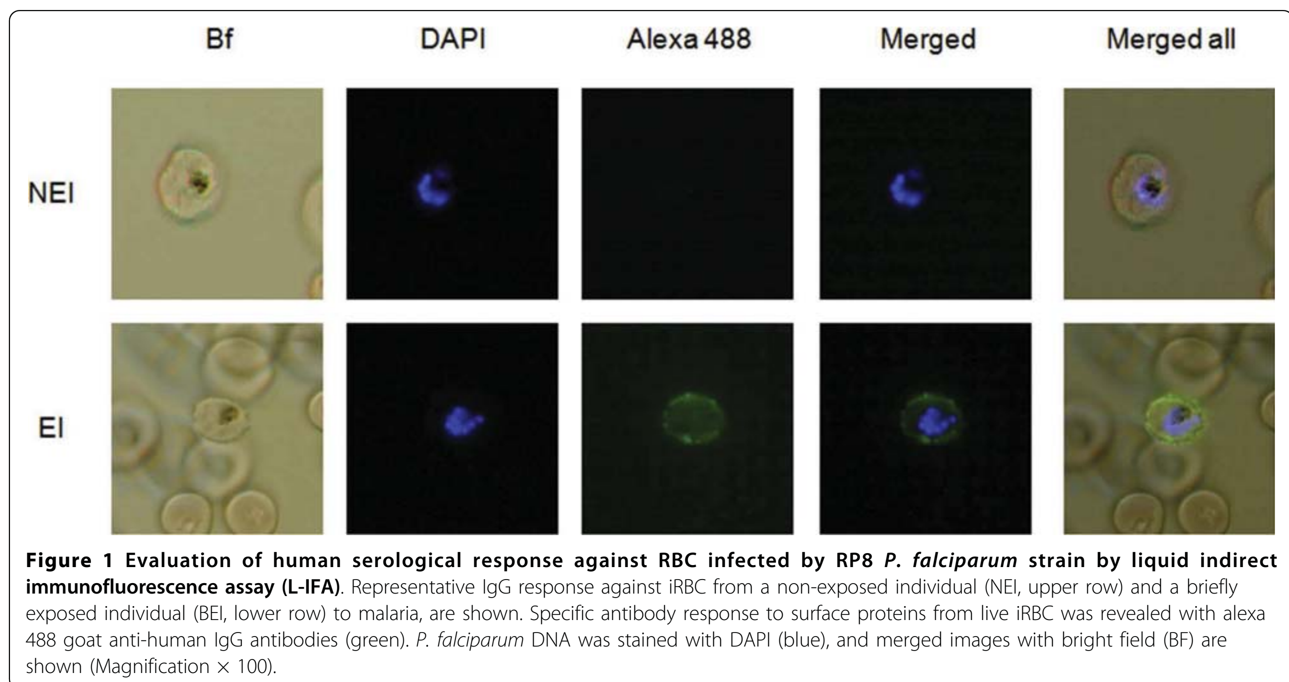

Figure 1 Evaluation of human serological response against RBC infected by RP8 *P. falciparum* strain by liquid indirect immunofluorescence assay (L-IFA). Representative IgG response against iRBC from a non-exposed individual (NEI, upper row) and a briefly exposed individual (BEI, lower row) to malaria, are shown. Specific antibody response to surface proteins from live iRBC was revealed with alexa 488 goat anti-human IgG antibodies (green). *P. falciparum* DNA was stained with DAPI (blue), and merged images with bright field (BF) are shown (Magnification × 100).

recognized by each sera group, against RBC membrane protein extracts, was low and no significant difference was observed between these groups (Mann-Whitney test, $p > 0.05$). Against iRBC membrane protein extracts, 3.4 ± 2.0 (means ± SD), 8.1 ± 4.4, 19.9 ± 4.0 antigenic bands were detected per strip for NEI, BEI and HEI sera, respectively (Figure 2C). In this case, the number of antigenic bands recognized by BEI and HEI groups were significantly more important compared to NEI group (Mann-Whitney test, $p = 1.6 \times 10^{-7}$ and $p = 4.5 \times 10^{-7}$ respectively). Moreover, the number of antigenic bands detected between RBC and iRBC membrane protein extracts is significantly increased for BEI (Mann-Whitney test, $p = 8.2 \times 10^{-8}$) and HEI (Mann-Whitney test, $p = 4.9 \times 10^{-8}$). For NEI group, between RBC and iRBC membrane protein extracts, the number of antigenic bands detected was not significantly changed (Mann-Whitney test, $p > 0.05$). Collectively, these results suggest that individuals exposed to *P. falciparum* parasites (BEI and HEI) present an IgG immune response specifically directed against iRBC membrane protein extracts. It is interesting to note that no antigenic bands could be detected above 200 kDa.

Determination of antigenic bands recognized by BEI sera

In order to define band identity of antigens recognized by BEI, a qualitative analysis of the IgG immune response was performed between NEI and BEI groups. A mapping and alignment of the IgG patterns allowed us to detect a total of 23 and 36 distinct antigenic bands against RBC and iRBC membrane protein extracts, respectively. Against RBC membrane protein extracts, no antigenic band was significantly detected more frequently in one group than to the other one (Fisher exact test, $p > 0.05$). Conversely, against iRBC membrane protein extracts, eight antigenic bands were significantly detected more frequently in BEI group compared to NEI group (Fisher exact test, $p < 0.05$; Figure 2D). Each of these antigenic bands was detected by at least 20% of BEI (Figure 2D). Additionally, each BEI sera recognized at least two of the eight selected antigenic bands. The molecular weights of these antigenic bands ranged from 18 kDa to 130 kDa, and a schematic representation of these antigenic bands was depicted on an immunoreactivity diagram (Figure 2E). Among the BEI group, five sera were selected with regard to their IgG response on 1-D immunoblots for subsequent antigens identification. Each selected BEI sera recognized at least three of the eight discriminatory antigenic bands and the pool covered the whole spectrum of these antigenic bands against iRBC membrane protein extracts. The pool was composed of these five selected BEI sera.

Characterization of iRBC membrane antigens recognized by BEI

To further characterize these discriminatory antigens, a 2-D immunoproteomic approach was adopted. A major difficulty of this approach is to perform a perfect match between antigenic spots detected on 2-D immunoblot and their counterparts on the preparative gel, on which matched spots will be excized and further analysed by mass spectrometry (MS). As the results of these protein

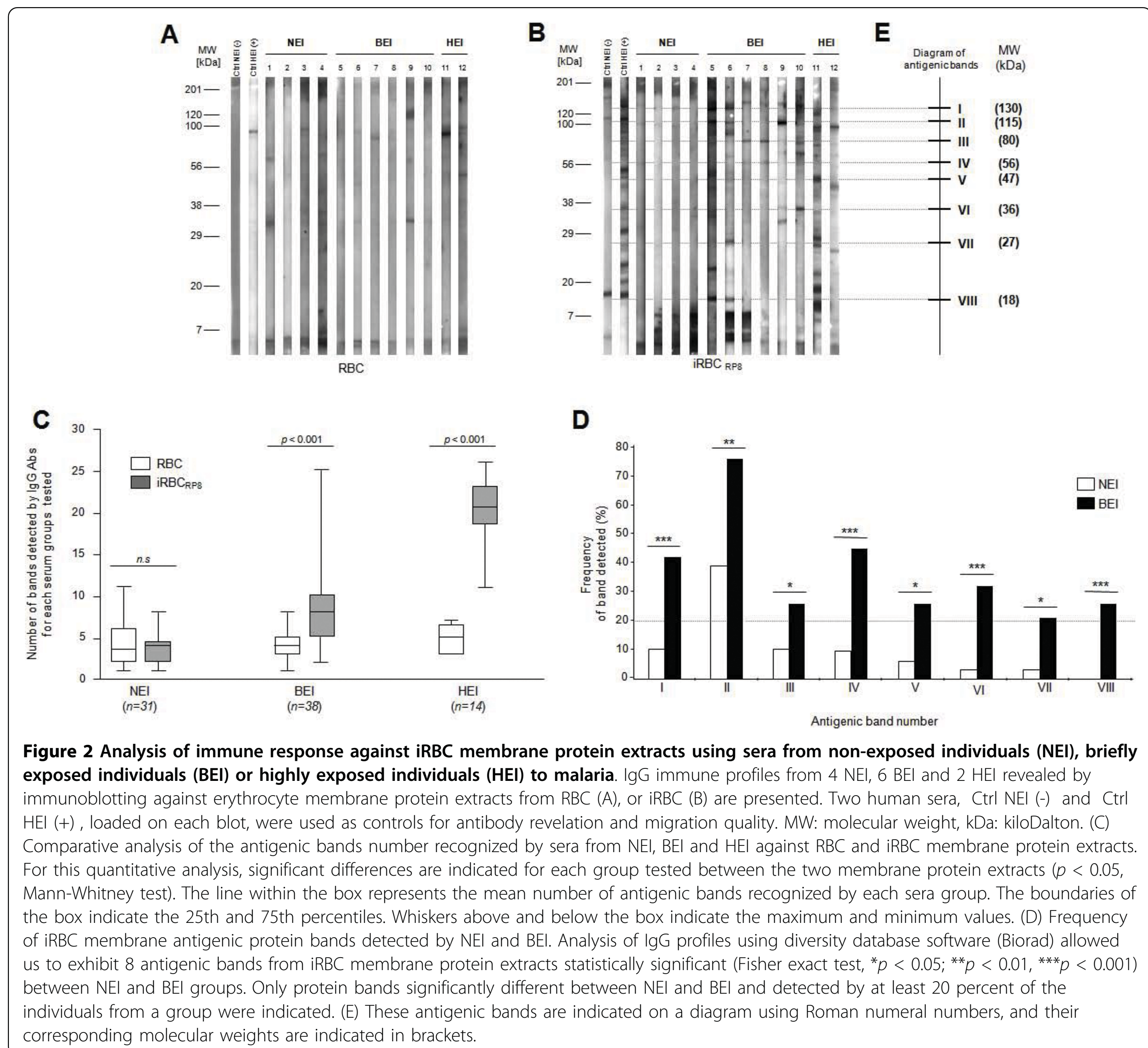

Figure 2 Analysis of immune response against iRBC membrane protein extracts using sera from non-exposed individuals (NEI), briefly exposed individuals (BEI) or highly exposed individuals (HEI) to malaria. IgG immune profiles from 4 NEI, 6 BEI and 2 HEI revealed by immunoblotting against erythrocyte membrane protein extracts from RBC (A), or iRBC (B) are presented. Two human sera, Ctrl NEI (-) and Ctrl HEI (+) , loaded on each blot, were used as controls for antibody revelation and migration quality. MW: molecular weight, kDa: kiloDalton. (C) Comparative analysis of the antigenic bands number recognized by sera from NEI, BEI and HEI against RBC and iRBC membrane protein extracts. For this quantitative analysis, significant differences are indicated for each group tested between the two membrane protein extracts ($p < 0.05$, Mann-Whitney test). The line within the box represents the mean number of antigenic bands recognized by each sera group. The boundaries of the box indicate the 25th and 75th percentiles. Whiskers above and below the box indicate the maximum and minimum values. (D) Frequency of iRBC membrane antigenic protein bands detected by NEI and BEI. Analysis of IgG profiles using diversity database software (Biorad) allowed us to exhibit 8 antigenic bands from iRBC membrane protein extracts statistically significant (Fisher exact test, *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$) between NEI and BEI groups. Only protein bands significantly different between NEI and BEI and detected by at least 20 percent of the individuals from a group were indicated. (E) These antigenic bands are indicated on a diagram using Roman numeral numbers, and their corresponding molecular weights are indicated in brackets.

identifications are the starting point of further studies, it is necessary to limit miss matching. Thus, 2-D immunoblot using a fluorescence-based method allows to increase considerably the confidence in spot matching between preparative gel and immunoblots (Figure 3).

The pool of BEI sera allowed to detect 42 antigenic spots onto the 2 D immunoblot (Figure 4). To facilitate the alignment of the antigenic bands with the corresponding spots between 1 D and 2 D immunoblot, iRBC membrane protein extracts were loaded onto 2 D gel beside the IPG strip, prior electrophoresis, as described previously [30]. For seven out of eight antigenic bands selected by 1 D analysis, their corresponding spots were detected onto 2 D immunoblot (Figure 4). No antigenic spot corresponding to the molecular weight (MW) of band “V” was detected onto 2 D immunoblot. This could be attributable to the extremes pI or low solubility of this antigenic protein in the buffer for 2DE.

As some bands split into several protein species, 30 spots were assigned to the seven antigenic bands (Figure 4, Additional file 2). A typical example is presented by the antigenic band number “I” which can be resolved into nine different spots on the 2-D immunoblot. The 42 antigenic spots detected onto the immunoblot, were excized and submitted to MS for identification (Figure 4). The resulting fragment ion spectra were searched against *H. sapiens* and *P. falciparum*-protein databases (NCBInr). Nineteen protein spots (45%) were successfully identified and correspond to 13 distinct proteins according to their NCBI accession number (Additional

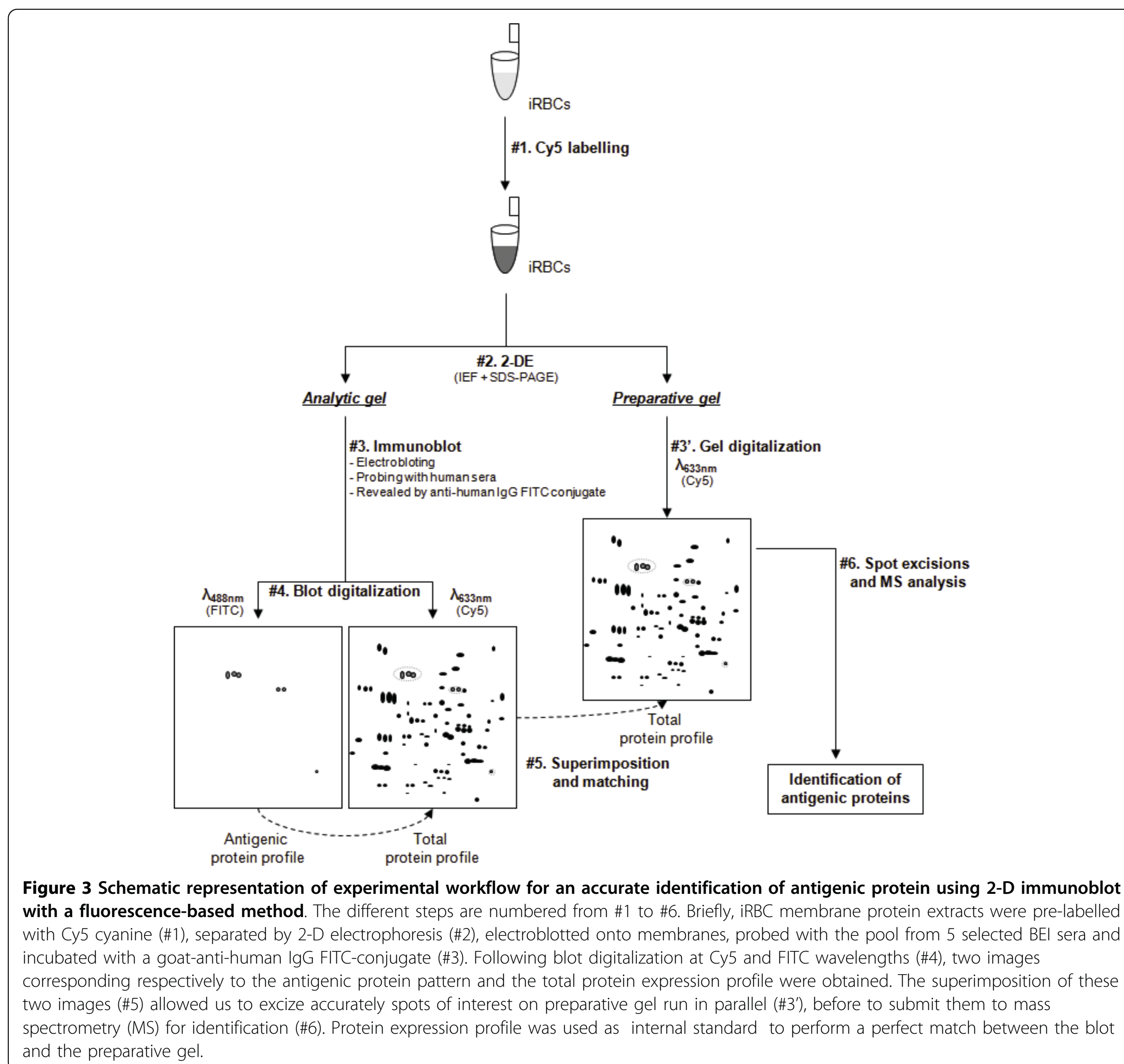

Figure 3 Schematic representation of experimental workflow for an accurate identification of antigenic protein using 2-D immunoblot with a fluorescence-based method. The different steps are numbered from #1 to #6. Briefly, iRBC membrane protein extracts were pre-labelled with Cy5 cyanine (#1), separated by 2-D electrophoresis (#2), electroblotted onto membranes, probed with the pool from 5 selected BEI sera and incubated with a goat-anti-human IgG FITC-conjugate (#3). Following blot digitalization at Cy5 and FITC wavelengths (#4), two images corresponding respectively to the antigenic protein pattern and the total protein expression profile were obtained. The superimposition of these two images (#5) allowed us to excize accurately spots of interest on preparative gel run in parallel (#3'), before to submit them to mass spectrometry (MS) for identification (#6). Protein expression profile was used as internal standard to perform a perfect match between the blot and the preparative gel.

file 2). Among them, four were identified as *P. falciparum* proteins and nine as *H. sapiens* proteins. Selected bands "I", "II" and "VII" were identified as *H. sapiens* antigens and selected bands "III", "IV" and "VI" as *P. falciparum* antigens. However, MS did not identify protein spots corresponding to the selected band "VIII", despite their apparent high abundances. This could be attributable to the low MW of these spots giving spectra with low complexity. Thus, spots corresponding to six out of eight antigenic protein bands were identified.

Discussion

A recent study performed in the laboratory shown that 35% of soldiers who travelled for a few month period in tropical Africa, presented a serological response against pre-erythrocytic antigens. Among them, only 2% had experienced a clinical malaria attack [22]. Others have reported that travellers from malaria-free countries can develop antibody response against pre-erythrocytic [20,21] and blood stages [32]*P. falciparum* antigens without progressing to a symptomatic illness. Thus, the characterization of erythrocytic stage antigens specifically detected by healthy adults shortly exposed to malaria and under chemoprophylaxis could be informative to estimate individual exposure to malaria transmission, and to clarify the mechanisms involved in the development of immune responses after malaria infection. In this aim, a comparison and an analysis of

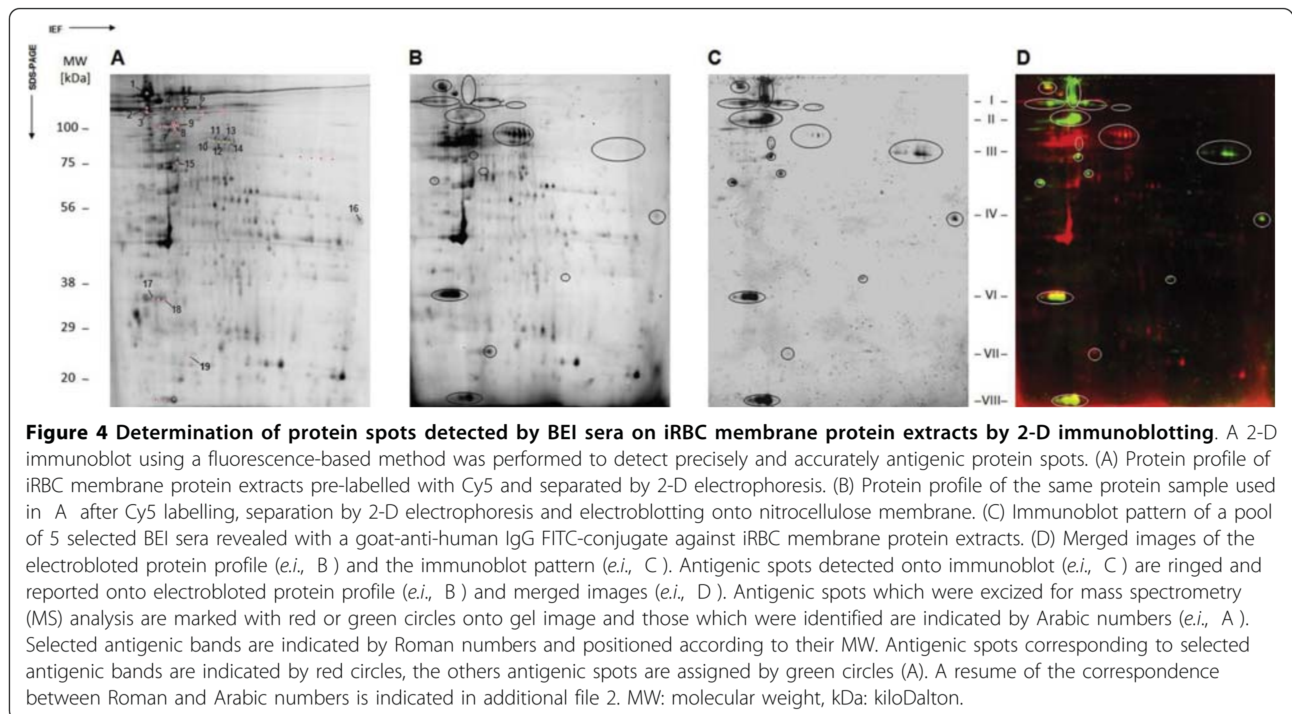

Figure 4 Determination of protein spots detected by BEI sera on iRBC membrane protein extracts by 2-D immunoblotting. A 2-D immunoblot using a fluorescence-based method was performed to detect precisely and accurately antigenic protein spots. (A) Protein profile of iRBC membrane protein extracts pre-labelled with Cy5 and separated by 2-D electrophoresis. (B) Protein profile of the same protein sample used in A after Cy5 labelling, separation by 2-D electrophoresis and electroblotting onto nitrocellulose membrane. (C) Immunoblot pattern of a pool of 5 selected BEI sera revealed with a goat-anti-human IgG FITC-conjugate against iRBC membrane protein extracts. (D) Merged images of the electrobloted protein profile (*e.i.*, B) and the immunoblot pattern (*e.i.*, C). Antigenic spots detected onto immunoblot (*e.i.*, C) are ringed and reported onto electrobloted protein profile (*e.i.*, B) and merged images (*e.i.*, D). Antigenic spots which were excized for mass spectrometry (MS) analysis are marked with red or green circles onto gel image and those which were identified are indicated by Arabic numbers (*e.i.*, A). Selected antigenic bands are indicated by Roman numbers and positioned according to their MW. Antigenic spots corresponding to selected antigenic bands are indicated by red circles, the others antigenic spots are assigned by green circles (A). A resume of the correspondence between Roman and Arabic numbers is indicated in additional file 2. MW: molecular weight, kDa: kiloDalton.

serological responses between NEI, BEI and HEI against membrane protein extracts from uninfected and *P. falciparum* infected RBC were performed. Although the use of a prophylactic treatment limiting the abundance of the blood malaria parasite could affect the antibody response development, it was observed that 5% of soldiers shown an IgG reactivity directed against the surface of iRBC. Among them, only a minor part (21%) presented a history of clinical malaria. Thus, although exposure to malaria transmission was brief and probably low because of the use of chemoprophylaxis and anti-vector devices, some BEI could develop an antibody response against blood stage antigens without history of clinical malaria attacks.

Analysis of IgG immune response indicated an association between the exposition level and the number of bands revealed by 1-D immunoblotting against iRBC membrane protein extracts. A thorough qualitative analysis of the immune profiles shown that eight protein bands from iRBC membrane protein extracts were significantly more frequently recognized by BEI sera than NEI sera. To identify unambiguously these antigenic bands, an original 2-D immunoproteomic approach using a fluorescence-based method was performed. Almeras *et al* previously demonstrated that it was possible to align, with a good confidence, antigenic bands detected by 1 D immunoblot with the corresponding spots onto 2 D immunoblot [30]. Here, this study shown that a perfect match is now conceivable between antigenic spots from the blot and their corresponding spots from the preparative gel, as recently described by Donoghue *et al* [33]. This method allowed us to identify 13 protein spots corresponding to six out of the eight antigenic bands.

Surprisingly, spots corresponding to bands "I", "II" and "VII" were identified as *H. sapiens* proteins. Auto-antibodies (aabs) against RBC membrane proteins have been already described in malaria patient's sera [34,35]. Little is known about the mechanisms that underlie the aabs production during parasite infection. Aabs production could arise from cross reactivity between host and parasite antigens. This molecular mimicry phenomenon could reduce the tolerance to self antigens and develop an auto-immunity during parasite infection [36]. However, here, all discriminatory antigenic proteins were exclusively recognized on iRBC membrane protein extracts (Additional file 3). The aabs emergence against host proteins on iRBC membrane protein extracts could be attributed to others phenomenon such as protein post-translational modifications. Effectively, it was demonstrated that protein phosphorylations of host and parasite occur during *P. falciparum* infections [37,38]. Moreover, ankyrin (gi|178646; band "I"), which was identified as a host discriminatory antigenic protein, was reported to be targeted by falcipain-2, a *P. falciparum* protease [39]. This protein cleavage could produce neo-antigens which are then recognized only in infected conditions. Nevertheless, aabs directed against these structural membrane proteins were also observed in other *Plasmodium* species infections [35] and could occur in

other diseases involving RBC abnormalities like thalassaemia or autoimmune haemolitic anaemia [40,41]. Despite a significant recognition of these host proteins by BEI sera, these discriminatory proteins did not seem to be relevant markers of malaria exposure. Another explanation of the identification host proteins could be attributed to that the spotted area could contain a mixture of both parasitic and human proteins, and the amount of parasite proteins is under-represented compared to human proteins, and so parasitic antigenic proteins may be missed by MS.

Four *P. falciparum* proteins (exported protein 2 (Exp-2) PF14_0678, band "VI"; early transcribed membrane protein (Etramp5) PFE1590w, band "VI"; Heat shock 70 kDa protein (Pf-Hsp70-1) PF08_0054, band "III"; elongation factor 1α (EF1 α) PF13_0304, band "IV") corresponding to three discriminatory antigenic bands were also identified. Exp-2 and Etramp5 which are iRBC membrane associated proteins, have been described to participate to protein transport between parasite and iRBC [42-44]. An immune response against these proteins was previously observed using sera of travellers and individuals from endemic areas [17,44]. The absence of signal detection by sera from never-exposed individuals suggests that Etramp5 could be an interesting marker of *P. falciparum* exposure.

The *Pf*-Hsp70 was detected at several parasite stages into the human host, in sporozoites [45], in liver stage [46,47], in all parasite blood stages [48,49] or in association with the iRBC plasma membrane [42], and is also possibly present on the merozoite surface [50]. Here, a specific antibody response against *Pf*-Hsp70 was revealed by BEI sera. This protein has been described as a major target in the acquisition of immunity in naturally infected humans living in areas of endemic malaria [17,49,51]. Moreover, *Pf*-Hsp70 is able to elicit protection of *Saimiri sciureus* monkey against the asexual blood stage of *P. falciparum* [52]. Collectively, these data suggest that *Pf*-Hsp70 seems to be an immunodominant antigens following parasite infection, and could be considered as a biomarker of malaria exposure.

The EF1 α is an abundant protein constituting 1-2% of the total protein in eukaryotic cells [53], and is an essential component of the translational machinery. This protein is involved in other processes as protein degradation, signal transduction and in the regulation of cytoskeletal rearrangements [54,55]. EF1 α protein was unambiguously recognized by BEI sera reflecting an exposition to the immune system. It is the first time that EF1 α protein was described as antigenic in individuals exposed to malaria.

The present study failed to identify several well described *P. falciparum* antigenic proteins from the iBRCs plasma membrane. The major part of these *P. falciparum* antigens are large hydrophobic proteins (> 150 kDa) which are generally under represented and can be difficultly detected on 2-D electrophoresis [56], such as PfEMP1 [57], Pf332 [58,59], the cytoadherence linked asexual protein 9 (Clag 9) [60,61] or the erythrocyte-binding antigen 175 (EBA-175) [62]. Furthermore, it was reported that serological immune responses against several *P. falciparum* antigens is conformation dependent [63,64]. Thus, the reducing and denaturing conditions used for the immunoblot analysis in the present study, did not allow to take into consideration conformational epitopes. Conversely, antibody reactivities against linear epitopes derived from PfEMP1 or other *P. falciparum* proteins have been already observed under reducing conditions [65,66]. The non-detection of these large proteins on 1-D and 2-D immunoblotting could be then attributed to incomplete protein transfer onto nitrocellulose membrane as previously described [67]. The use of only one parasite strain with limiting variant antigen repertoire, for BEI sera selection, could also induce a bias for identifying more antigenic membrane parasite proteins. Moreover, Fried *et al.* reported that continuous *in vitro* culture of laboratory isolates could conduct to the loss or truncation of some parasite proteins [68], which could participate to this underestimation of antigenic parasite protein detection. Additionally, a lot of known antigens from the asexual blood stage are proteins from the merozoite surface (eg. MSP1, MSP2 or RESA) [69]. In the sample condition preparations used here, iRBC membrane extracts were exempted of merozoites. Moreover, although some merozoite proteins are described to associate to the iRBC membrane during merozoite invasion (eg RSP2) [70,71], these proteins are largely under-representated on the iRBC membrane fraction. Other iRBC membrane proteins such as RIFIN (30-45 kDa) [72] and STEVOR (30-40 kDa) [73], were reported to be antigenic in individuals which have experienced several malaria infections [74,75]. A short exposure to malaria might not be sufficient to induce an antibody response against these proteins in BEI. However, complementary methods, such as the screening of *P. falciparum* expression library with BEI sera, could be envisaged for the characterization of others antigenic parasite proteins, as described previously [76].

The detection of a specific immune response against iRBC membrane extract could be unexpected using sera from individuals which have a mandatory chemoprophylaxis during their journey. Doxycycline was reported to be partially efficient on liver stages of *P. falciparum* parasites [77], and to alter asexual parasite blood stage at the end of the second erythrocytic cycle [78,79]. Thus, a possible discontinuity in chemotherapy observance would have increased the risk to develop a specific IgG response against blood stage antigens.

Conclusion

This study provides evidence that some BEI could develop a singular antibody response against blood stage antigens after a short exposure to malaria in endemic area. An original immunoproteomic approach allowed the identification of some discriminatory antigenic bands, which corresponded to *P. falciparum* and *H. sapiens* proteins. These antigens may represent promising erythrocytic biomarkers to estimate individual exposure to malaria transmission, and might help to understand the first stages of the immune responses to primary malaria infection. These antigens could be also useful in the analysis of the host-parasite relationships among travellers, or individuals living in areas where malaria is under elimination.

List of abbreviations

Aabs: Auto-antibodies; BEI: Briefly exposed individuals; CD36: Cluster of differentiation 36; EF1 α: Elongation factor 1α; Etramp: Early transcribed membrane proteins; Exp-2: Exported protein 2; HEI: Highly exposed individuals; Hsp: Heat shock protein; HUVEC: Human Umbilical Vein Endothelial Cells; ICAM-1: Intercellular adhesion molecule-1; iRBC: Infected red blood cells; MHC: Major histocompatibility complex; NEI: Non exposed individuals; PEXEL: *Plasmodium* export element; PTEX: *Plasmodium falciparum* translocon of exported proteins; TVN: Tubovesicular network; PVM: Parasitophorous vacuole membrane; RBC: Red blood cells.

Additional material

Additional file 1: Single-Peptide-Based Protein Identifications. Single peptide-based identifications of the proteasome subunit HsN3, gi565651 including the spot number, the monoisotopic mass of neutral peptide, the peptide sequence, the ion score, the expect value and the Mascot score are indicated in the table. The observed masses as well as fragment assignments are graphically presented at the bottom.

Additional file 2: Antigenic iRBC membrane proteins detected by BEI sera. The proteins were identified by LCQ DecaXPplus mass spectrometer. Band and spot numbers corresponds to numbers indicated in Figure 2 and Figure 4, respectively. The identities of protein spots, their NCBI accession numbers, the theoretical and observed MW values, the *pI* values, as well as the corresponding percentage sequence coverage, the number of peptide sequences, and the Mascot score are listed for MS/MS analysis (Protein scores greater than 41 were considered as significant ($p < 0.05$)). *As a single-peptide was used for this protein identification, the corresponding MS/MS spectrum was included in the additional file 1.

Additional file 3: Specific antigenic protein profiles recognized by pooled sera from briefly exposed individuals (BEI). Two-dimensional immunoblots were performed as described previously [31]. Briefly, RBC or iRBC membrane protein extracts were resolved by IEF on pH range 3-10 linear IPG strips (7 cm, GE Healthcare). Before SDS-PAGE (10%), 10 μg of samples were loaded at the left side of the IPG strip, and gels were electrobloted onto nitrocellulose membrane (GE Healthcare). Five sera from each group (BEI, non-exposed individuals (NEI) and highly exposed individuals (HEI)) were selected according to their representative immune profile on 1 D immunoblot, and were pooled. Each pooled sera were probed onto 2 D immunoblot, and antigenic protein spots were revealed using ECL kit on autoradiography X-ray film (GE Healthcare). Representative 1 D (#) and 2 D antigenic profiles obtained with BEI (A and B), NEI (C), and HEI (D) pooled sera against RBC (A) or iRBC (B, C and D) membrane protein extracts are illustrated. Black circles correspond to antigenic protein spots detected on 2 D immunoblots. Antigenic spots detected by BEI and NEI or BEI and HEI pooled sera on iRBC membrane protein extracts are encircled in dashed line (C and D). Roman numbers correspond to antigenic bands indicated in Figure 2 and additional file 2.

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Authors' contributions

Conceived and designed the experiments: AL, FT, RC, FA. Performed the experiments: FA, AL, BS, DC. Analysed the data: FA, AL, PM, RC, BM, FT, LD. Contributed reagents/materials/analysis tools: PM, VC, BM, FP. Wrote the paper: FA, AL, RC. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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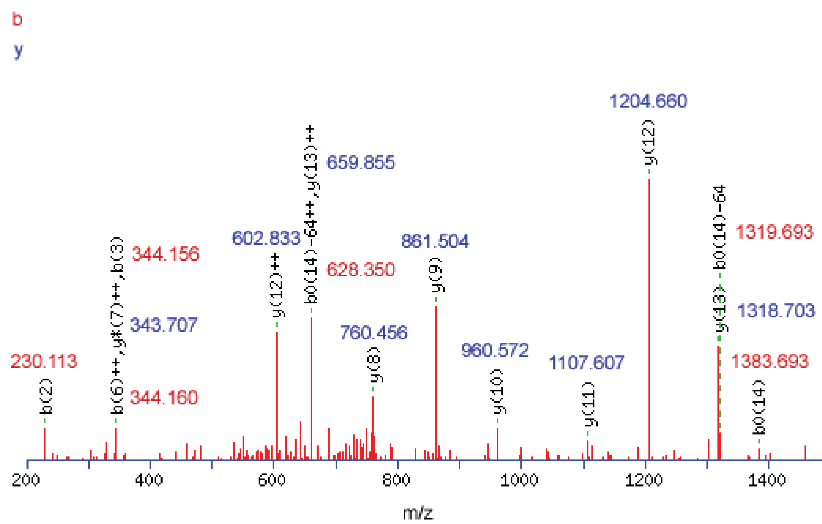
Additional file 1: Single-Peptide-Based Protein Identifications

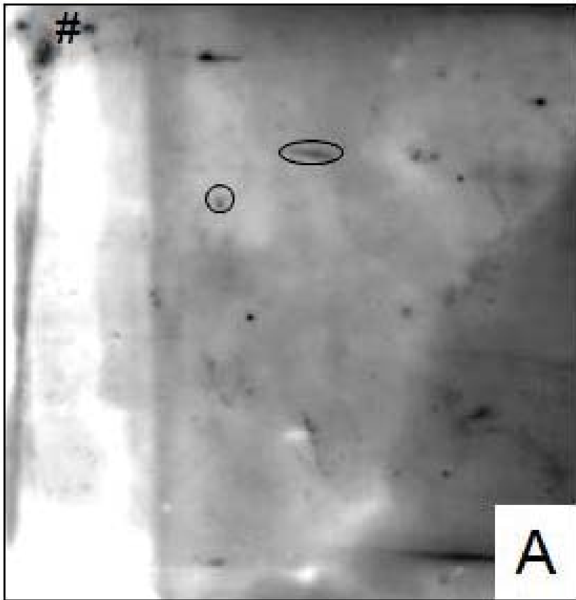
Spot number	Protein name	Accession number	m/z	Peptide	Ion score	Expect	Significance (Mascot score)
19	proteasome subunit HsN3 [*Homo sapiens*]	gi|565651	1547.5023	TQNPMVTGTSVLGVK	48	0.012	63

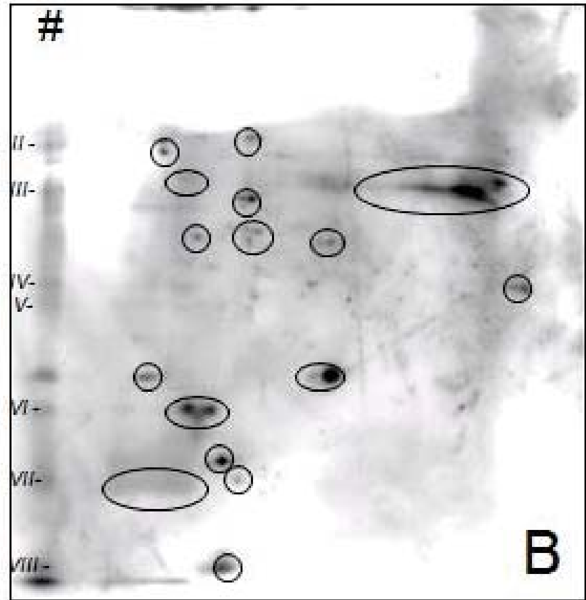


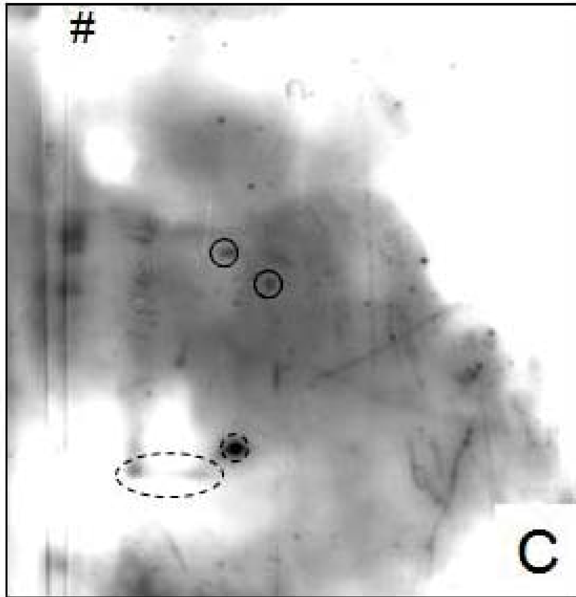
Additional file 2: Antigenic iRBC membrane proteins detected by BEI sera.

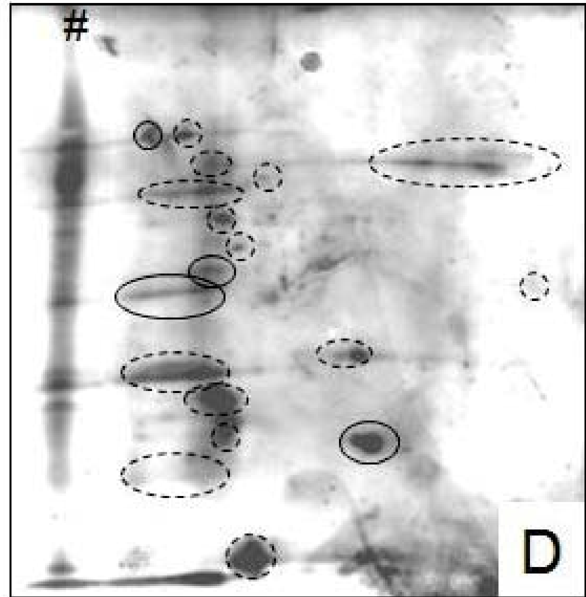
Band number	Spot number	Protein name	Accession no. (NCBI)	Accession no. (Pf-locus)	*MW* (kDa)		*p*I value (Theor.)	Coverage (%)	Number of MS/MS peptide sequences	Significance (Mascot score)
					Theor.	Observ.				
		Selected antigenic spots								
	2	Ankyrin [Homo sapiens]	gi|178646	-	207.14	131	5.8	5	7	211
	3	beta-spectrin [Homo sapiens]	gi|338441	-	247.03	131	5.1	2	4	52
I	4	erythrocyte ankyrin[Homo sapiens]	gi|226788	-	207.33	120	5.6	11	16	454
	5	Ankyrin [Homo sapiens]	gi|178646	-	207.14	120	5.8	15	24	489
	6	Ankyrin [Homo sapiens]	gi|178646	-	207.14	121	5.8	20	33	778
	7	adducin 2 isoform a [Homo sapiens]	gi|9257180	-	81.26	98	5.3	13	8	218
II	8	adducin 2 isoform a [Homo sapiens]	gi|9257192	-	81.26	95	5.7	14	9	245
	9	adducin 2 isoform a [Homo sapiens]	gi|9257192	-	81.26	94	5.7	15	10	230
III	15	Heat shock 70 kDa protein [Plasmodium falciparum]	gi|123598	PF08_0054	74.75	76	5.5	18	12	188

IV	16	elongation factor 1 alpha [Plasmodium falciparum]	gi|124513850	PF13_0304	49.16	55	9.1	12	5	115
VI	17	unnamed protein product/etramp5 [Plasmodium falciparum]	gi|829215	PFE1590w	10.80	33	5.0	19	3	96
	18	Exp-2 [Plasmodium falciparum]	gi|3021540	PF14_0678	33.16	33	5.3	7	4	44
VII	19	proteasome subunit HsN3 [Homo sapiens]*	gi|565651	-	29.23	24	5.7	5	1	63
Antigenic spots										
	1	Spectrin alpha erythrocytic 1[Homo sapiens]	gi|115298659	-	281.04	145	4.9	45	106	2249
	10	erythroid protein 4.1 isoform A [Homo sapiens]	gi|182073	-	87.06	84	5.3	17	10	130
	11	erythroid protein 4.1 isoform A [Homo sapiens]	gi|182073	-	87.06	84	5.3	18	13	101
	12	erythroid protein 4.1 isoform A [Homo sapiens]	gi|182073	-	87.06	82	5.3	15	9	176
	13	erythrocyte membrane protein band 4.1, isoform 3 [Homo sapiens]	gi|4758274	-	66.76	83	6.8	24	12	146
	14	erythrocyte membrane protein band 4.1, isoform 3 [Homo sapiens]	gi|4758274	-	66.76	82	6.8	26	13	179









Additional file 3

AUTRES TRAVAUX

ARTICLE N.7

Plasmodium falciparum proteome changes in response to doxycycline treatment

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MALARIA JOURNAL

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Plasmodium falciparum proteome changes in response to doxycycline treatment

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Abstract

Background: The emergence of *Plasmodium falciparum* resistance to most anti-malarial compounds has highlighted the urgency to develop new drugs and to clarify the mechanisms of anti-malarial drugs currently used. Among them, doxycycline is used alone for malaria chemoprophylaxis or in combination with quinine or artemisinin derivatives for malaria treatment. The molecular mechanisms of doxycycline action in *P. falciparum* have not yet been clearly defined, particularly at the protein level.

Methods: A proteomic approach was used to analyse protein expression changes in the schizont stage of the malarial parasite *P. falciparum* following doxycycline treatment. A comparison of protein expression between treated and untreated protein samples was performed using two complementary proteomic approaches: two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and isobaric tagging reagents for relative and absolute quantification (iTRAQ).

Results: After doxycycline treatment, 32 and 40 *P. falciparum* proteins were found to have significantly deregulated expression levels by 2D-DIGE and iTRAQ methods, respectively. Although some of these proteins have been already described as being deregulated by other drug treatments, numerous changes in protein levels seem to be specific to doxycycline treatment, which could perturb apicoplast metabolism. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed to confirm this hypothesis.

Conclusions: In this study, a specific response to doxycycline treatment was distinguished and seems to involve mitochondrion and apicoplast organelles. These data provide a starting point for the elucidation of drug targets and the discovery of mechanisms of resistance to anti-malarial compounds.

Background

The parasitic protozoon *Plasmodium falciparum* is responsible for approximately 247 million cases of malaria and one million deaths each year, particularly in sub-Saharan Africa [1]. Anti-mosquito measures and new artemisinin-containing treatments have been recently adopted in hopes of achieving the global eradication of malaria. Novel drugs, vaccines and insecticides, as well as deeper insights into parasite biology, human immunity, and vector behaviour, are essential to support these efforts [2].

Over the past 30 years, experimental observations obtained *in vitro* and in clinical studies have demonstrated the anti-malarial activity of tetracycline and its derivatives [3]. Daily doxycycline (DOX) has been shown to be an effective chemoprophylactic in Thailand [4], Indonesia [5], and Kenya [6]. DOX is currently one of the recommended chemoprophylactic regimens for travellers visiting malaria endemic areas in Southeast Asia, Africa and South America [7]. DOX is now recommended by the French Consensus Conference for chemoprophylaxis in countries with a high prevalence of *P. falciparum* resistance to chloroquine or multiple drugs [8]. However, while no instances of *P. falciparum* malaria clinical failure with DOX have been reported yet, three different phenotypes (low, medium and high DOX susceptibility groups) have been identified among *P. falciparum* clinical isolates

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[9]. These different phenotypes have been associated with *pfmdt* and *pftetQ* copy number variations and *pftetQ* sequence polymorphisms [10].

DOX has long been known to inhibit protein synthesis in bacteria [11] by binding the S4, S7, S9 and S17 proteins of the small 30S ribosomal subunit and various ribonucleic acids of the 16S rRNA, which prevents the binding of aminoacyl transfer RNA to site A of the ribosome [12]. In *P. falciparum*, tetracyclines have been reported to directly inhibit mitochondrial protein synthesis [13] and also to decrease dihydroorotate dehydrogenase activity, which is involved in *de novo* pyrimidine synthesis [14]. DOX inhibits *P. falciparum* synthesis of nucleotides and deoxynucleotides [15]. Minocycline, another tetracycline derivative, also decreases the transcription of mitochondrial genes and plastid genes, indicating that it may target these two organelles [16]. More recently, two research groups [17,18] reported specific action by cyclines on the apicoplast of *P. falciparum* via cell biology and transcriptome approaches. Collectively, these published data indicate that organelles from *P. falciparum* seem to be primary targets for cyclines; however, the molecular mechanisms involved in this plastid regulation are not yet clearly defined, particularly at the protein level.

Proteome studies have contributed substantially to our understanding of parasite biology and host-parasite interactions [19]. Mass spectrometry (MS) methods have been used to enable large-scale identification of proteins at different stages of the malarial parasite life cycle [20,21]. However, few proteomic analyses have been undertaken to better understand the mechanisms of drug action or resistance in *P. falciparum*. The effects of chloroquine and artemisinin derivatives on *P. falciparum* have been studied using different proteomic techniques, such as a gel-based approach [22], SELDI (Surface Enhanced Laser Desorption Ionization) TOF (Time of Flight) MS analysis [23] and, more recently, isoleucine-based SIL (Stable Isotope Labelling) [24]. Until now, *P. falciparum* proteome response following doxycycline treatment has not been studied.

The present study aimed to highlight the metabolic pathways that are affected in *P. falciparum* following DOX treatment. To accomplish this objective, two complementary proteomics approaches were used: two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and isobaric tagging reagents for relative and absolute quantification (iTRAQ). The combination of these two technologies allowed us to identify proteins that are deregulated in response to doxycycline and were involved in various cellular functions such as redox homeostasis, stress response, protein synthesis, lipid synthesis and energy metabolism. These results indicated that *P. falciparum* organelles seem perturbed by DOX treatment, suggesting that these are the drug's primary targets.

Methods

Plasmodium falciparum growth conditions and protein extraction

Parasites (chloroquine-resistant W2 clone) were maintained in continuous culture as described elsewhere [25], at 10% haematocrit of type A^+ human RBCs suspended in supplemented RPMI 1640 (Invitrogen) and 10% heat-inactivated type A^+ human serum at 37°C in a gas mixture of 5% CO_2, 10% O_2 and 85% N_2. The medium was changed twice daily. Parasitaemia was monitored daily via microscope by examination of blood smears stained with a RAL® 555 kit (Réactifs RAL). Parasite synchronization was performed by sorbitol treatment (D-sorbitol, ICN Biomedicals) as described elsewhere [26]. At the ring stage, parasites were or were not exposed to DOX (Sigma) at 10 µM (the IC_{50} as previously determined [27]) for a period of 24 h. Parasites at the schizont stages during the second cycle after DOX exposure were extracted from the RBCs. Control and treated groups consisted of four biological replicates for the DIGE experiment and three biological replicates for the iTRAQ experiment. IRBCs were washed 3 times in PBS (Invitrogen) and lysed by 0.1% saponin (Sigma) for 5 min. Free parasites were sedimented by centrifugation (9,300 g for 5 min) and washed with PBS 3 times and stored at -80°C. Parasites were resuspended in 10 mM Tris-HCl buffer (pH 8) and disrupted by ultrasonication (Vibracell 72412, Bioblock Scientific) for 5 min on ice at maximum amplitude. After ultracentrifugation (100,000 g for 1 h at 4°C), soluble protein fractions were recovered from the supernatant and the pellet containing membrane protein fractions was then suspended in 4% (w/v) 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate (CHAPS) (Sigma). All of the fractions were precipitated in 100% acetone (Sigma) to remove lipids, and the protein concentration of each sample was estimated using the Lowry-based DC assay (Biorad) according to the manufacturer's instructions. All of the samples were suspended in standard cell lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris base, pH 8.5 (Sigma)) to obtain a protein concentration adjusted to 2.5 µg/µL.

2D-DIGE

Protein samples were minimally labelled with CyDye according to the manufacturer's recommended protocols (GE Healthcare). Briefly, soluble protein samples from the control parasites (50 µg) and the DOX-treated parasites (50 µg) were labelled with 400 pmol of either Cy3 or Cy5 (in four biological quadruplicates, with a dye swap) and an internal standard (50 µg) was labelled with 400 pmol of Cy2, freshly dissolved in N, N-dimethylforma-

mide (DMF) (Sigma), and incubated on ice for 30 min in the dark. The reaction was quenched with 1 μL of free lysine (10 nM) by incubation for 10 min on ice. Cy3-, Cy5- and Cy2-labeled samples were then pooled, and an equal volume of 2 × sample buffer was added (8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM dithiothreitol (DTT), and 1% (v/v) immobilized pH gradient (IPG) Buffer 3-10 (GE Healthcare). The membrane protein samples were treated as described above with either IPG Buffer 4-7 or IPG Buffer 6-11 (GE Healthcare). The mixture of labelled proteins was then separated by two-dimensional gel electrophoresis (2-DE) (See additional file 1 for more details).

2-D Image analysis

Gel images were acquired with a Typhoon® Trio Image scanner (GE Healthcare) at different excitation wavelengths (Cy3, 580 BP 30/green (532 nm); Cy5, 670 BP 30/red (633 nm); Cy2, 520 BP 40/blue (488 nm)). Images were cropped with ImageQuant® software (GE Healthcare) and further analysed using DeCyder v6.5 (GE Healthcare). The software was used to perform gel alignment, spot averaging and normalization and Student's *t*-test to determine which protein spots changed in abundance in response to DOX-treatment. The number of detected spots showing a difference with a *p*-value of < 0.05 was then determined.

In-gel trypsin digestion

After imaging, the gels were stained either with Sypro Ruby (Bio-Rad) according to the manufacturer's protocol and then scanned using the typhoon scanner or with Coomassie Brilliant Blue (CBB) G-250 as previously described [28]. Spots of interest were manually excised. Protein spots were digested overnight at 37°C with sequencing-grade trypsin (12.5 μg/mL; Promega Madison) in 50 mM NH_4HCO_3 (Sigma). The resulting peptides were extracted with 25 mM NH_4HCO_3 for 15 min, dehydrated with acetonitrile (ACN) (Sigma), incubated with 5% formic acid (Sigma) for 15 min under agitation, dehydrated with ACN, and finally completely dried using a SpeedVac. Samples were then stored at -20°C before analysis by MS.

iTRAQ labelling and strong cation exchange

After protein precipitation in acetone, the samples were dissolved in 20 μL of dissolution buffer, reduced, alkylated, trypsin-digested and labelled using the iTRAQ reagents four-plex kit according to the manufacturer's instructions (Applied Biosystems). The resulting peptide solutions from control and DOX-treated soluble protein samples were labelled with iTRAQ114 and iTRAQ117, respectively, and incubated at room temperature for 1 h. Labelled peptides were then pooled and acidified by mixing with the cation buffer load iTRAQ reagent for a total volume of 1 ml. The peptide mixture was subsequently fractionated by strong cation exchange (SCX) chromatography (See additional file 1 for more details). The elution was monitored by absorbance at 214 and 280 nm (Additional file 2), and 40 fractions were collected. These experiments were conducted in three different biological replicates. The same protocol was applied to the membrane proteins samples, but labelling was done with iTRAQ115 (control) and iTRAQ116 (DOX-treatment). Each fraction of iTRAQ-labelled sample was dried using a Speedvac, reconstituted in 12 μL of buffer (1% v/v formic acid in H_2O) and analysed by nano-liquid chromatography tandem mass spectrometry (nano-LC-MS/MS).

Protein identification by nano-LC MS/MS

Protein digests extracted from excised DIGE gel spots were analysed by nano-LC-ESI-MS/MS. Purification and analysis were performed on a C18 capillary column using a CapLC system (Waters) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Ultima, Waters). Chromatographic separations were conducted on an RP capillary column (AtlantisTM dC18, 3 μm, 75 μm × 150 mm Nano EaseTM, Waters) with a 180-200 $nl.min^{-1}$ rate of flow (See additional file 1 for more details).

DIGE protein database search

The data were searched using Mascot software against the *P. falciparum* National Center for Biotechnology Information non-redundant protein database (NCBInr, NIH, Bethesda, MD, March 27th, 2008). Search parameters allowed for one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine; the precursor and product ion mass error tolerance was < 0.2 Da. All identified peptides had a Mascot score greater than 28 (*P. falciparum*, 12,220 sequences), corresponding to a statistically significant ($p < 0.05$) confident identification. Moreover, among the positive matches, only protein identifications based on at least two different non-overlapping peptide sequences of more than six amino acids and with a mass tolerance < 0.05 Da were accepted (Additional file 3). These additional validation criteria struck a balance that limited the number of false positive matches without missing real proteins of interest.

iTRAQ protein database search and quantification

Mascot distiller software (v2.1.1, Matrix Science) was used to convert MassLynx.raw MS/MS data files into mascot generic files (mgf). (See additional file 1 for more details). For protein identification, mgf data files were searched against a mixed file of *Homo sapiens* and *P. falciparum* sequences in the NCBInr (NIH, Bethesda, MD)

protein database (229,804 sequences on September 12th, 2008 for soluble proteins and 230,260 sequences on October 21st, 2008 for membrane proteins) using the MASCOT algorithm (v2.2, Matrix Science). (See additional file 1 for more details). For protein quantification, data analysis was performed with Multi-Q 1.6.1.1. as described elsewhere [29]. The MassLynx.raw data files from the Q-TOF Ultima (Waters) were previously converted into files of the mzXML format by the massWolf program [30]. (See additional file 1 for more details). Geometric means of the ratios of DOX-treated protein to control protein (iTRAQ117/iTRAQ114 for soluble proteins and iTRAQ116/iTRAQ115 for membrane proteins) and the standard deviation were calculated. Proteins with ratios ≤ 0.80 or ≥ 1.20 between DOX-treated and untreated experimental conditions were considered regulated proteins, as reported elsewhere [31].

Bioinformatics predictions of biological processes and subcellular localization of identified proteins

The NCBI GI (GeneInfo Identifier) numbers of *P. falciparum* proteins identified were converted into standard gene names for retrieval from the UniprotKB ID module [32]. Then, in UniprotKB, PlasmoDB accession numbers of proteins were retrieved for further analysis. Biological processes and subcellular localization of differentially expressed proteins were assessed using Gene Ontology annotations downloaded from PlasmoDB [33]. The transit peptides that enable proteins to target the apicoplast were identified by the PlasmoAP tool [34].

Quantitative real-time RT-PCR

The same parasite cultures used for the proteomic analysis were also used to perform quantitative RT-PCR. Total RNA was extracted with TRIZOL® reagent following the manufacturer's recommendations (Invitrogen) and treated with DNase (DNAfree®, Ambion). Total RNA was quantified with the NanoDrop ND-1000 (Labtech), followed by quality assessment with the 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's protocol. Acceptable A260/A280 ratios were in the range of 1.8-2.2. Acceptable rRNA ratios (28S/18S) needed to be > 0.9, and RIN (RNA Integrity Number) values needed to be > 8.0. Total RNA (1 μg) was reverse transcribed with the High-Capacity cDNA Archive Kit as described by the manufacturer (Applied Biosystems). The primer pairs used (Eurogentec), (Additional file 4) were designed with Primer Express software v2.0 (Applied Biosystems). Real-time transcript quantification was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems). Amplification reactions and the $2^{-\Delta\Delta Ct}$ method of relative quantification to estimate relative expression of mRNA targets were performed as previously described [35]. All data were expressed as means ± standard deviation. A two-tailed Student's *t*-test was employed to compare RT-PCR gene expression levels. Statistical significance was defined as $p < 0.05$.

Results

Phenotypic effect of DOX treatment

Ring stage parasites (> 95%) were incubated with 10 μM DOX (*i.e.* corresponding to IC_{50}) for 24 h followed by a chase period until the end of the successive cycle at 84 h. Then, parasites were collected at the schizont stage for several reasons. First, DOX exerts delayed effects against *P. falciparum* [36], *i.e.* exposing the parasites to DOX does not lead to phenotypic effects at the end of the first cycle but instead at the end of the successive cycle. A chase period was applied because continuous DOX exposure leads to almost 100% parasite lethality. Secondly, this drug has an increased potency against the schizont stage [17], and finally, protein synthesis in parasites is maximal during trophozoïte and schizont stages [37]. At the beginning of the experiments, all cultures had a parasitaemia equal to 2%. At the end of the experiments, parasitaemia obtained from untreated and DOX-treated iRBCs were 7.9% ± 0.7 and 3.7% ± 0.5, respectively. Both control and treated cultures were at the schizont stage (> 90%).

Plasmodium falciparum response to DOX treatment according to 2D-DIGE analysis

Since membrane-associated proteins are generally under-represented by two-dimensional electrophoresis, proteins extracted from the schizont stages were separated into soluble and membrane fractions to circumvent this limitation. Then, four biological replicates from untreated or DOX-treated samples were divided into soluble or membrane protein fractions and their protein expression profiles were compared using 2D-DIGE methods. After imaging, DeCyder software was used to detect spot levels that were significantly deregulated by DOX treatment (Figure 1 and additional file 5). Among the three type of gels (with 18-cm 3-10, 4-7 and 6-11 linear IPG strips), a total of 150 spots were considered to be significantly deregulated (45, 45 and 60 spots respectively in the membranaire protein gels with 4-7 linear IPG strip, in the membranaire protein gels with 6-11 linear IPG strip and in the soluble protein gels with 3-10 linear IPG strip); 95 spots (63%) were up-regulated and 55 (37%) spots down-regulated in response to DOX treatment ($p < 0.05$, Student's *t*-test, and spot ratios ≤ 0.74 or ≥ 1.35). Thirty five spots (11, 12 and 12 spots respectively) could not be excised manually (invisible after gel coloration compared to scanning images with Typhoon). Then, 115 spots were submitted to identification by nano LC MS/MS (34, 33 and 48 spots respectively). Sixty seven spots were identified by mass spectrometry (MS) (15, 20 and 32 spots respectively). Forty eight spots were not identified (19, 13

Table 1: Differentially expressed proteins in DOX-treated parasites (DIGE quantification).

Accession number	Name	Spot	MASCOT score	Peptides matched	Ratios[a]	Biological process[b]	Predicted Localization[c]	Fraction[d]
PF13_0304	Elongation factor 1 alpha	1078	236	5	2.85*	Translation	Cytoplasm	S
		1111	226	4	2.65*			S
		1143	246	5	2.42*			S
		1026	309	6	2.31*			S
		848	411	9	2.04**			M (2)
		833	306	6	2.03**			M (2)
		1168	254	6	1.65*			S
PF13_0143	Phosphoribosylpyrophosphate synthetase	964	174	3	2.36*	Carbohydrate metabolism	Apicoplast	M (2)
PFB0445c	DEAD box helicase, UAP56	2246	138	2	2.25*	Nucleic acid binding	Unknown	S
PFI1105w	Phosphoglycerate kinase	1025	515	10	2.14*	Carbohydrate metabolism	Unknown	S
		993	343	6	2.07*			S
PF14_0598	Glyceraldehyde-3-phosphate dehydrogenase	1288	114	2	2.13*	Carbohydrate metabolism	Cytoplasm	S
		1295	276	5	1.89*			S
		1325	472	8	1.81*			S
PFI1090w	**S-adenosylmethionine synthetase**	**1236**	**88**	**2**	**1.99***	**One carbon compound metabolism**	**Cytoplasm**	**M (1)**
PFF1300w	Putative pyruvate kinase	800	217	4	1.95*	Carbohydrate metabolism	Apicoplast	S
		788	120	3	1.86*			S
		1672	110	2	1.70*			M (1)
		553	149	3	1.61**			M (2)
PF14_0368	2-Cys peroxiredoxin	1821	116	2	1.94*	Anti oxidative stress	Unknown	M (2)
PFE0690c	Rab1 protein	2221	171	4	1.89**	Intracellular protein transport	Cytoplasm	S
PF11_0396	Protein phosphatase 2C	2309	144	2	1.84*	Protein amino acid dephosphorylation	Cytoplasm	S
PFF1335c	**4-methyl-5(B-hydroxyethyl)-thiazol Monophosphate biosynthesis enzyme**	**2198**	**282**	**5**	**1.69***	**Thiamin biosynthesis**	**Cytoplasm**	**S**
MAL13P1.283	TCP-1/cpn60 chaperonin family	958	828	13	1.64*	Protein folding	Cytoplasm	M (1)
		936	315	6	1.38*			M (1)

Table 1: Differentially expressed proteins in DOX-treated parasites (DIGE quantification). (Continued)

PFF1155w	**Hexokinase**	**795**	**123**	**3**	**1.61***	**Carbohydrate metabolism**	**Unknown**	**S**
PF14_0425	Fructose-bisphosphate aldolase	1180	552	10	1.60***	Carbohydrate metabolism	Unknown	S
		917	561	10	1.43*			M (2)
PF14_0076	Plasmepsin 1 precursor	1406	215	4	1.58***	Haemoglobin Catabolism	Membrane	S
MAL8P1.69	**14-3-3 protein**	**1647**	**71**	**2**	**1.54***	**Protein folding**	**Unknown**	**S**
PF08_0131	**1-Cys peroxiredoxin**	**2251**	**83**	**2**	**1.52***	**Anti oxidative stress**	**Unknown**	**M (1)**
PF08_0074	**DNA/RNA-binding Protein Alba**	**1237**	**113**	**3**	**1.51***	**Nucleic acid binding**	**Nucleus**	**M (2)**
PF14_0486	**Elongation factor 2**	**1264**	**164**	**4**	**1.48***	**Translation**	**Cytoplasm**	**S**
PF13_0214	Elongation factor 1-gamma	1984	77	2	1.47*	Translation	Apicoplast	S
PF10_0155	Enolase	1066	439	8	1.43**	Carbohydrate metabolism	Cytoplasm	M (1)
		1089	584	10	1.35*			M (1)
PFL0960w	D-ribulose-5-phosphate 3-epimerase	2248	94	2	1.35**	Carbohydrate metabolism	Unknown	M (1)
PF11_0117	Replication factor C subunit 5	1020	106	2	0.70*	DNA replication	Nucleus	M (2)
PF08_0109	Proteasome subunit alpha type 5	2057	79	2	0.63*	Ubiquitin dependent protein catabolism	Unknown	S
PFL0185c	Nucleosome assembly protein 1	967	170	4	0.61**	Nucleosome assembly	Nucleus	S
PF11_0313	60S ribosomal protein P0	2081	100	2	0.60*	Translation	Mitochondrion	M (1)
PF11_0282	Deoxyuridine 5'-triphosphate nucleotidohydrolase	2332	306	5	0.60*	DNA replication	Nucleus	S
MAL8P1.95	**Conserved *Plasmodium* protein**	**1532**	**241**	**3**	**0.58***	**Unknown**	**Unknown**	**S**
PF14_0678	Exp-2	1421	153	3	0.57**	Unknown	Membrane	M (1)
PF11_0183	GTP-binding nuclear protein ran/tc4	1451	98	2	0.49*	Nucleus transport	Nucleus	M (2)
PF13_0033	26S proteasome regulatory subunit	854	341	8	0.47*	Ubiquitin dependent protein catabolism	Nucleus	M (2)
PF14_0359	HSP40, Subfamily A	648	204	4	0.35*	Protein folding	Unknown	M (2)

In bold: differentially expressed proteins commonly identified in DIGE and iTRAQ. [a]Significant modification in protein ratios between DOX and control cells with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. [b]Data depicted from PlasmoDB (gene ontology biological process annotation). [c]Data depicted from PlasmoDB (gene ontology location annotation or PlasmoAP to predict apicoplast addressing). [d]S = soluble, pI 3-10; M = membrane (1) corresponds to pI 4-7 and (2) to pI 6-11.

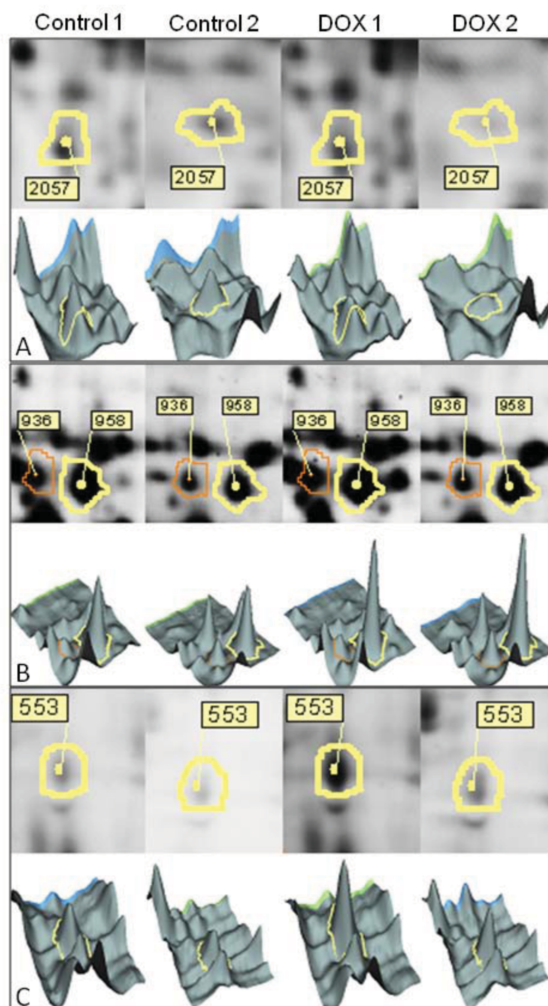

Figure 1 Examples of 2D and 3D representations of fluorescence intensity of spots that were up and down regulated in response to DOX treatment, as visualized with Decyder software in 2D-DIGE analysis. (A) In the soluble proteins gel with pI 3-10, spot 2057 was down regulated under DOX treatment and was identified as a putative protein: proteasome subunit alpha type 5 (PF08_0109). (B) In the membrane proteomic map with pI 4-7, spots 936 and 958 were up regulated under DOX treatment and identified as isoforms of the same putative protein: TCP-1/cpn60 chaperon family (MAL13P1.283). (C) In the membrane proteomic map with pI 6-11, spot 553 was up regulated and identified as a putative pyruvate kinase (PFF1300w).

and 16 spots respectively) and 20 spots (5, 9 and 6 spots respectively) were not retained, because comigration of proteins confounded the ability to identify individual proteins. Finally, 47 protein spots were identified (Table 1). However, some proteins were detected in more than one spot, indicating different isoforms. A total of 32 distinct proteins, according to their accession numbers, were finally identified (Figure 1) (Table 1, peptide details in Additional file 2). Among these proteins, 22 were up-regulated, and 10 were down-regulated. They were classified according to their biological functions in Table 1. Some proteins were identified as being involved in primary metabolism (*e.g.*, carbohydrate, protein and amino-acid metabolism, and DNA replication). Proteins involved in anti-oxidative stress (PF08_0131, 1-Cys peroxiredoxin in spot 2251 and PF14_0368, 2-Cys peroxiredoxin in spot 1821) and two proteins with unknown functions were also identified. Several isoforms of the same protein (PF10_0155, enolase in spots 1066 and 1089; PF14_0598, glyceraldehyde-3-phosphate dehydrogenase in spots 1288, 1295 and 1325 in Figure 1A and Table 1) were identified in adjacent spots.

Plasmodium falciparum response to DOX treatment according to iTRAQ analysis

The same soluble and membrane protein extracts used for 2D-DIGE analysis were subjected to iTRAQ analysis. For this analysis, three biological replicates from untreated or DOX-treated parasites were digested, and the peptides were labelled with different isobaric tags. Of the soluble protein samples, 422 proteins were confidently identified including 246 plasmodial proteins (58.3%) and 176 human proteins (41.7%) in the three biological replicates. Among them, 169 were quantified (*i.e.*, with at least 4 labelled, non-degenerated peptides), including 14 human proteins (8.3%) and 155 plasmodial proteins (91.7%). Twenty-two proteins displayed significant differences in expression levels (proteins with fold change $\leq$ 0.80 or $\geq$ 1.20 were considered as differentially expressed proteins); 18 of these proteins were up-regulated, and four were down-regulated following DOX exposure (Table 2). In membrane protein samples, 308 proteins were confidently identified, including 204 plasmodial proteins (66.2%) and 104 human proteins (33.8%) in the three biological replicates. Among them, 156 were quantified, including 9 human proteins (5.8%) and 147 plasmodial proteins (94.2%). Eighteen proteins displayed significant differences in expression; 14 of these proteins were up-regulated and four were down-regulated following DOX exposure. In total, 40 proteins were differentially expressed in response to DOX treatment (Table 2); 80% were up-regulated (32 out of 40), and 20% were down-regulated (8 out of 40). The proteins up-regulated by DOX treatment were associated with haemoglobin catabolism, protein synthesis, protein processing, anti-oxidative stress and phospholipid metabolism. Down-regulated proteins were mostly associated with protein synthesis/processing or nuclear transport. A substantial proportion of the proteins (20%, 8 out of 40) have not been assigned to a biological function yet. Two up-regulated proteins were identified as human proteins: biliverdine reductase and S100-calcium binding protein A4. Their function and location in human cells were precised in Table 2.

Table 2: Differentially expressed proteins in DOX-treated parasites (iTRAQ quantification).

Accession number[a]	Name	Peptides quantified	Ratios ± SD	Biological process[b]	Predicted Localization[c]
PF13_0130	Vacuolar ATP synthase subunit g	3	2.47 ± 1.07	Vacuolar acidification	Membrane
PFC0735w	40S ribosomal protein S15A putative	3	1.93 ± 0.71	Translation	Cytoplasm
PF08_0074	**DNA/RNA-binding Protein Alba, putative**	**6**	**1.82 ± 0.08**	**Nucleic acid binding**	**Nucleus**
MAL13P1.214	Phosphoethanolamine N-methyltransferase	6	1.79 ± 0.47	Phosphatidylcholine biosynthesis	Unknown
PF10_0068	RNA binding protein putative	3	1.76 ± 0.16	Nucleic acid binding	Apicoplast
PFB0340c	Serine repeat antigen 5 (SERA-5)	8	1.76 ± 0.37	Proteolysis	Unknown
PFC0920w	Histone H2A variant putative	3	1.59 ± 0.25	Nucleosome assembly	Apicoplast
PFI1090w	**S-adenosylmethionine synthetase**	**5**	**1.57 ± 0.25**	**One carbon compound metabolism**	**Cytoplasm**
PFB0915w	Liver stage antigen-3	4	1.54 ± 0.04	Unknown	Membrane
PFL1545c	Chaperonin cpn60	3	1.52 ± 0.40	Protein folding	Apicoplast
PFF0835w	Conserved *Plasmodium* protein	5	1.50 ± 0.01	Unknown	Unknown
PF08_0110	Rab18 GTPase	3	1.49 ± 0.13	Intracellular protein transport	Cytoplasm
PF14_0078	Plasmepsin III|HAP protein	6	1.48 ± 0.04	Haemoglobin Catabolism	Membrane
PF14_0324	Hsp70/Hsp90 organizing protein putative	17	1.46 ± 0.21	Protein folding	Unknown
PF10_0115	QF122 antigen	9	1.45 ± 0.04	Nucleic acid binding	Apicoplast
PF14_0655	RNA helicase-1 putative	5	1.44 ± 0.01	Translation	Cytoplasm
PF14_0201	Surface protein putative Pf113	6	1.44 ± 0.07	Unknown	Membrane
PF14_0486	**Elongation factor 2**	**16**	**1.41 ± 0.30**	**Translation**	**Cytoplasm**
PF10_0323	Early transcribed membrane protein 10.2	4	1.40 ± 0.08	Unknown	Membrane
PFL1170w	Polyadenylate-binding protein putative	12	1.35 ± 0.05	Transcription	Unknown
gi|544759	Biliverdin reductase B	2	1.35 ± 0.16	Porphyrin metabolism	Cytoplasm
PFE0870w	Transcriptional regulator putative	4	1.34 ± 0.10	Transcription	Nucleus
gi|4506765	S100 calcium-binding protein A4	2	1.34 ± 0.06	Cell growth	Cytoplasm
PF11_0062	Histone H2B	7	1.34 ± 0.12	Nucleosome assembly	Apicoplast
PF14_0391	60S ribosomal protein L1 putative	2	1.32 ± 0.13	Translation	Cytoplasm
MAL8P1.69	**14-3-3 protein**	**6**	**1.30 ± 0.19**	**Protein folding**	**Unknown**
MAL13P1.56	M1-family aminopeptidase	5	1.28 ± 0.06	Haemoglobin Catabolism	Apicoplast
PF14_0439	Leucine aminopeptidase putative	4	1.27 ± 0.18	Haemoglobin Catabolism	Apicoplast
PF08_0096	RNA helicase putative	3	1.24 ± 0.04	Transcription	Unknown

Table 2: Differentially expressed proteins in DOX-treated parasites (iTRAQ quantification). (Continued)

PF08_0131	**1-Cys peroxiredoxin**	**9**	**1.22 ± 0.15**	**Anti oxidative stress**	**Unknown**
PFE0585c	Myo-inositol 1-phosphate synthase putative	10	1.22 ± 0.03	Phospholipid biosynthetic process	Unknown
PFL1720w	Serine hydroxymethyltransferase	3	1.21 ± 0.01	One carbon compound metabolism	Unknown
PF14_0167	Prefoldin subunit 2 putative	4	0.80 ± 0.05	Protein folding	Cytoplasm
PFI1780w	*Plasmodium* exported protein (PHISTc)	2	0.80 ± 0.19	Unknown	Apicoplast
MAL8P1.95	**Conserved *Plasmodium* protein**	**5**	**0.76 ± 0.09**	**Unknown**	**Unknown**
PFE0290c	Conserved *Plasmodium* protein	3	0.75 ± 0.06	Unknown	Unknown
PFA0110w	DnaJ protein putative	5	0.74 ± 0.24	Protein folding	Membrane
PF08_0087	Importin alpha putative	5	0.73 ± 0.12	Nucleus transport	Nucleus
PFD0090c	*Plasmodium* exported protein (PHISTa)	4	0.61 ± 0.14	Unknown	Apicoplast
PF11_0351	Heat shock protein hsp70 homologue	6	0.54 ± 0.04	Protein folding	Mitochondrion

In bold: differentially expressed proteins commonly identified in DIGE and iTRAQ. [a]PlasmoDB accession number, except for two human proteins (NCBI accession number). [b]Data depicted from PlasmoDB (gene ontology biological process annotation). [c]Data depicted from PlasmoDB (gene ontology location annotation or PlasmoAP to predict apicoplast addressing).

Comparison of differentially expressed proteins identified by 2D-DIGE and iTRAQ

Of the deregulated proteins identified by DIGE, 19% (six out of 32) were also identified by iTRAQ. The proteins identified by both approaches (DIGE and iTRAQ) were similarly deregulated; for example, S-adenosylmethionine synthetase (DIGE fold change of 1.99 in Table 1 and 1.57 with iTRAQ in Table 2) and 1-Cys peroxiredoxin (fold change of 1.52 with DIGE and 1.22 with iTRAQ). The metabolic processes in which the proteins are involved are similar and, for the most part, changes in protein expression levels were similar between the two approaches (Figure 2). The main metabolic systems identified by both proteomics approaches were protein metabolism, the anti-oxidant response mechanism, nucleic acid binding and transport mechanisms. Both approaches revealed a down-regulation of proteins involved in protein synthesis metabolism and transport mechanism and an up-regulation of proteins involved in protein metabolism and anti-oxidant response mechanisms (Figure 2). However, proteins involved in nucleic acid binding were characterized differently by the two methods; these proteins were up-regulated according to the iTRAQ data and down-regulated according to the DIGE data. Proteins involved in carbohydrate metabolism were all up-regulated and only identified by the DIGE method (Table 1). In addition, proteins involved in specific metabolic pathways were only identified by iTRAQ (Figure 2 and Table 2). Those proteins identified by iTRAQ alone were either membrane proteins involved in vacuolar acidification or enzymes involved in phospholipid metabolism (Table 2). Fifteen soluble proteins were identified in the soluble fractions but not in the membrane fractions (Table 1). Fourteen proteins were identified only in membrane fractions, but only two are actually thought to belong to the membrane, calling into question the effectiveness of the 2D-PAGE approach for characterising membrane proteins. The iTRAQ method, in contrast, allowed identification and quantification of a large number of high molecular weight proteins and membrane proteins (Table 2).

Subcellular localization of regulated proteins

As predicted by gene ontology in PlasmoDB and PlasmoAP (for apicoplast addressing), the cellular localization (Table 1 and Table 2) of the 64 differentially regulated plasmodial proteins identified by the two proteomic approaches was as follows: 21% in the cytoplasm, 19% in the apicoplast, 13% in the membrane, 13% in the nucleus, 5% in the mitochondria and 29% unknown. Cytoplasmic, apicoplastic and membrane proteins were generally up-regulated, while nuclear and mitochondrial proteins were more often down-regulated (Figure 3). All of the identified apicoplastic proteins were encoded by the nuclear genome of *P. falciparum* and not by its plastid genome.

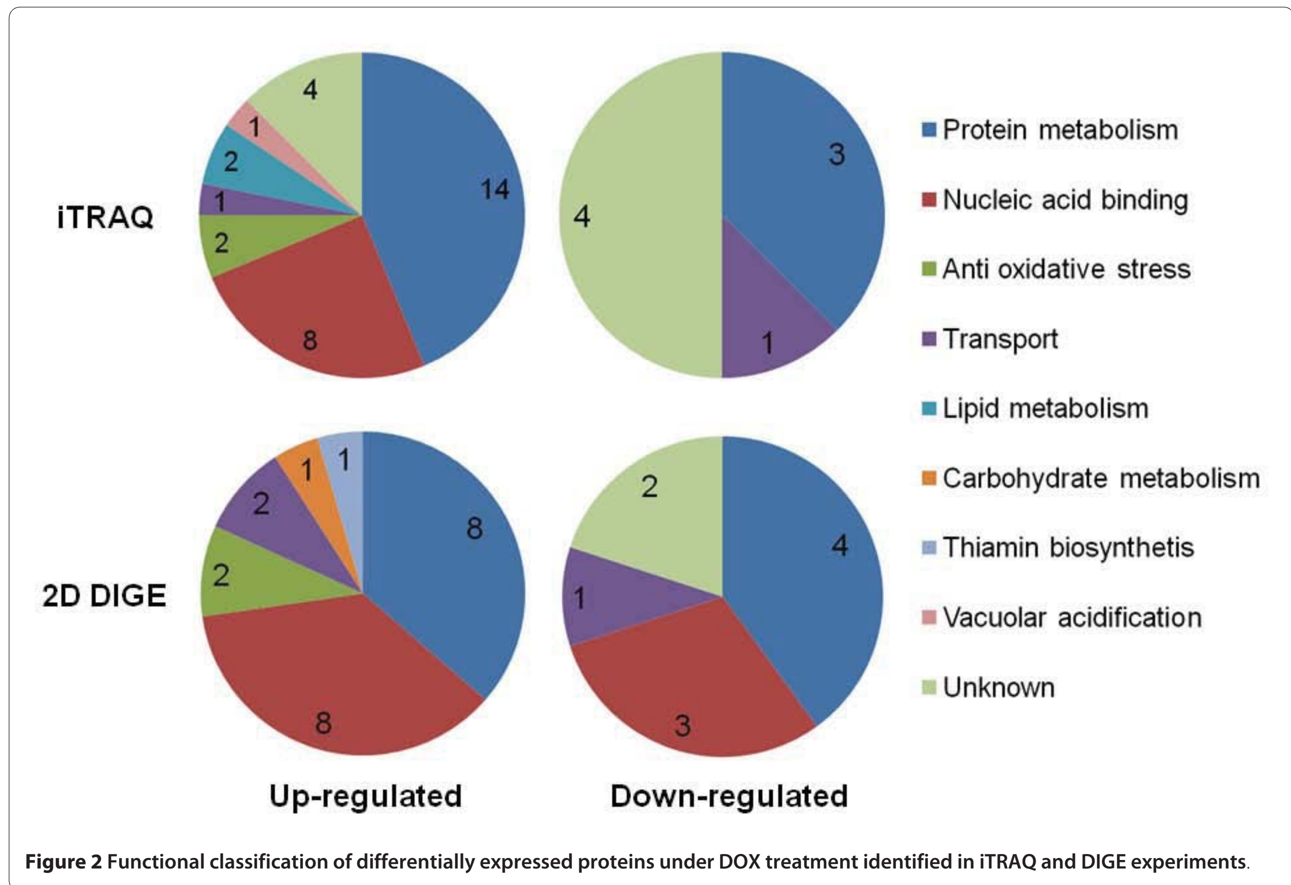

Figure 2 Functional classification of differentially expressed proteins under DOX treatment identified in iTRAQ and DIGE experiments.

RT-PCR quantifications

To validate the proteomics results, quantitative RT-PCR was performed because antibodies against *P. falciparum* are not commercially available, and the proteomics data suggest that apicoplast function was particularly perturbed by DOX-treatment. Thus, three apicoplast transcripts were chosen to evaluate the modifications observed in this organelle following DOX-treatment. PFI1090w (the S-adenosylmethionine synthetase) and MAL8P1.95 (a conserved *Plasmodium* protein), which had similar expression profiles (up and down regulation respectively) according to both DIGE and iTRAQ, also had similar expression profiles at the transcript level (Table 3). PF14_0439 (leucine aminopeptidase) was shown to be upregulated by iTRAQ analysis and was also up-regulated at the transcript level. Moreover, mRNA for the *PftufA*, *PfsufB* and *PfclpC* proteins encoded by the plastid genome, which were not quantified at the protein level by DIGE or iTRAQ, were down-regulated at the transcript level. Two transcripts of PF07_0033 (CG4) and PFE1195w (Karyopherin beta) proteins were used as positive controls in RT-PCR experiments (Their qRT-PCR ratios were 1.05 ± 0.09 and 0.98 ± 0.12 respectively) because they were not deregulated in iTRAQ proteomic approach (iTRAQ ratios were 1.01 ± 0.03 and 1.07 ± 0.05 respectively), (Table 3).

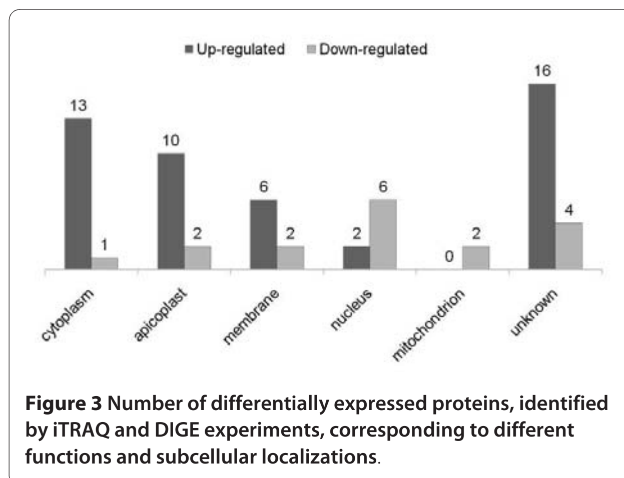

Figure 3 Number of differentially expressed proteins, identified by iTRAQ and DIGE experiments, corresponding to different functions and subcellular localizations.

Discussion

In the present study, proteome changes was researched in *Plasmodium falciparum* following DOX treatment in order to clarify the action mechanisms of this drug using two proteomic approaches, 2D-DIGE and iTRAQ. These techniques have been shown to be complementary in studying protein changes as they have distinct physico-

Table 3: Quantitative RT-PCR results.

Accession nr	Description	DIGE ratios	iTRAQ ratios	qRT-PCR ratios[a]
PFCOMPIRB-TufA	TufA	NA	NA	0.30 ± 0.04
PFCOMPIRB-SufB	SufB	NA	NA	0.32 ± 0.12
PFCOMPIRB-ClpC	ClpC	NA	NA	0.26 ± 0.08
PFI1090w	S-adenosylmethionine synthetase	1.99	1.57 ± 0.25	1.72 ± 0.16
MAL8P1.95	Conserved *Plasmodium* protein	0.57	0.76 ± 0.09	0.44 ± 0.03
PF14_0439	Leucine aminopeptidase putative	NA	1.27 ± 0.18	1.48 ± 0.23
PF07_0033	Cg4 protein	NA	1.01 ± 0.03	1.05 ± 0.09
PFE1195w	Karyopherin beta	NA	1.07 ± 0.05	0.98 ± 0.12

[a]qRT-PCR ratios correspond to the relative expression of target mRNA between the DOX treated and the control (mean of three biological replicates). NA: not available.

chemical properties that favour identification of different proteins [38,39]; that is the reason why a 19% overlap was observed between deregulated proteins identified by the both methods. Analysis of the DIGE results detected differentially expressed proteins with PTMs (post translational modifications) after DOX treatment (Table 1 and Figure 1). In particular, enolase and aldolase have previously been reported to possess several differentially expressed isoforms during the schizont stages of *P. falciparum* [40]; these two proteins are localized in Maurer's cleft [41] and in the food vacuole [42]. However, little is known about the role of these PTMs in the plasmodial regulation of protein expression under either physiological conditions or anti-malarial treatment.

Few proteomic studies have been undertaken to elucidate the mechanisms of drug action in *P. falciparum* but all share some common features with the present work [22,24,43]. After exposure to anti-malarial treatment, proteome analysis has generally revealed a low number of differentially expressed proteins with an upregulation of proteins involved in glycolysis, chaperoning or redox metabolism. In a stable isotope labelling experiment after artemisinin and chloroquine treatment in schizont stages of *P. falciparum*, among more than 800 quantified proteins, only 41 and 38 were up-regulated, respectively [24]. However, none of these proteins were associated with heat shock response or glycolysis functions, probably because the design of the study did not allow it, *e.g.* the SILAC method, which explores only newly synthesized proteins, was used. In a gel-based study, arthemeter and lumefantrine treatment were respectively associated with an up-regulation of 22 and 41 proteins [43]. In another study, chloroquine treatment increased the number of oxidized proteins in the schizont stage of parasites [22]. In these two last studies, four glycolysis enzymes (enolase, aldolase, phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase) and one heat shock protein (HSP 70 homolog) were commonly identified as up-regulated proteins under lumefantrine or chloroquine treatment, which was similar to the results seen under DOX treatment. The increased expression of redox metabolism proteins (1-Cys peroxiredoxin, 2-Cys peroxiredoxin) and 11 other "associated proteins" (Table 1 and Table 2), which have been recently shown to be potential targets of thioredoxin, glutaredoxin and plasmoredoxin [44], might represent another non-specific common feature of parasite responses to drug treatment.

Alternately, some metabolic pathways (Figure 4) might represent a specific response of parasites to DOX. Several studies have shown that tetracyclines, which are members of the DOX family, directly inhibit mitochondrial protein synthesis [13,45,46]. This inhibition would lead to a decrease in the mitochondrial respiratory chain activity [16] because the plasmodial mitochondrial genome encodes cytochrome *c* oxidase subunits I and III and apocytochrome *b*. The mitochondrial respiratory chain is coupled to dihydroorotate dehydrogenase activity, which has been shown to be depressed under tetracycline treatment [14]. This enzyme is involved in *de novo* pyrimidine biosynthesis, and its inhibition is associated with decreased levels of nucleotides and deoxynucleotides in *P. falciparum* in response to tetracycline treatment [15]. DOX inhibition of mitochondrial protein synthesis could be responsible for DNA replication impairment as suggested in the present study, *i.e.* replication factor C subunit 5 and deoxyuridine 5'-3P nucleotidohydrolase were down-regulated (Figure 4).

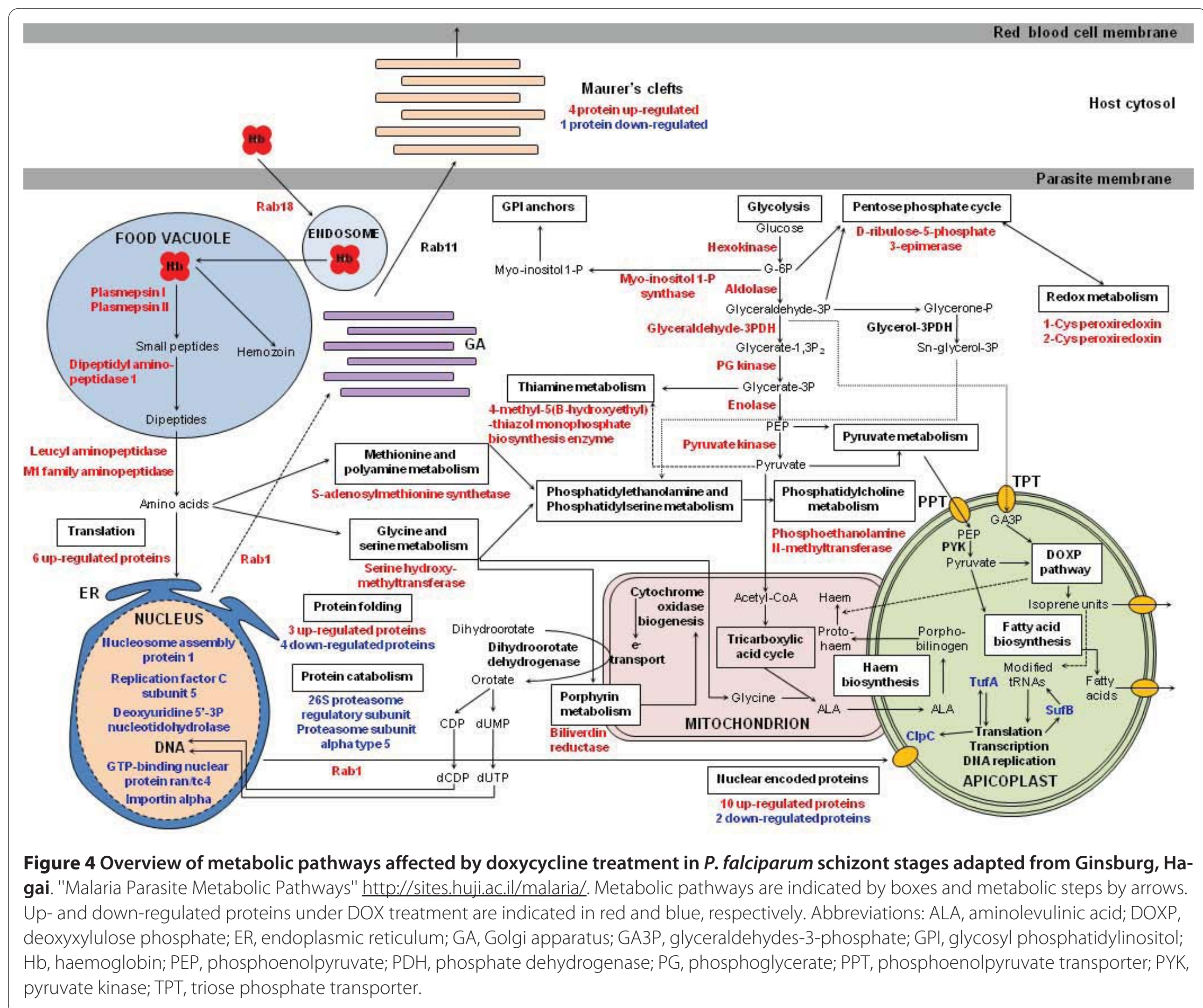

Figure 4 Overview of metabolic pathways affected by doxycycline treatment in *P. falciparum* schizont stages adapted from Ginsburg, Hagai. "Malaria Parasite Metabolic Pathways" http://sites.huji.ac.il/malaria/. Metabolic pathways are indicated by boxes and metabolic steps by arrows. Up- and down-regulated proteins under DOX treatment are indicated in red and blue, respectively. Abbreviations: ALA, aminolevulinic acid; DOXP, deoxyxylulose phosphate; ER, endoplasmic reticulum; GA, Golgi apparatus; GA3P, glyceraldehydes-3-phosphate; GPI, glycosyl phosphatidylinositol; Hb, haemoglobin; PEP, phosphoenolpyruvate; PDH, phosphate dehydrogenase; PG, phosphoglycerate; PPT, phosphoenolpyruvate transporter; PYK, pyruvate kinase; TPT, triose phosphate transporter.

Two recent works have confirmed specific action by the cyclines on the *P. falciparum* apicoplast [17,18]; replication and transcription of the plastid genome as well as the import of nuclear encoded proteins into the plastid matrices' were inhibited in response to cycline treatment. Cyclines are assumed to inhibit plastid protein synthesis, but the precise mechanism of action has not yet been identified. The apicoplast genome encodes different tRNAs, rRNA, TufA (a translational elongation factor), SufB (involved in iron metabolism and modification of tRNAs [47]), ClpC (a protease required for nuclear-encoded protein import into the apicoplast) and a DNA dependent RNA polymerase (involved in transcription). This organelle is implicated in fatty acid and isoprenoid precursor synthesis and in heme biosynthesis in tight association with mitochondria [48]. In the present study, ClpC, TufA and SufB were found to be down-regulated under DOX treatment, but only at the transcriptional level. The inhibition of ClpC could explain the defect in protein import into the apicoplast and consequently the overexpression of 12 encoded nuclear proteins that are localized to the apicoplast (Figure 4).

Conclusions

The present study has given the first insights into changes in protein regulation in *P. falciparum* upon DOX treatment, suggesting that *P. falciparum* apicoplasts and mitochondria are the targets of DOX. It has also confirmed that 2D-DIGE and iTRAQ are powerful and complementary techniques in studying protein changes in response to drug treatment. However, more experiments will be needed to characterize the specific molecular mechanisms of DOX treatment. In order to prove that DOX inhibits plastid or mitochondrial translational further biochemical approaches would probably be required.

Additional material

Additional file 1 Methods. Supplementary Methods

Additional file 2 SCX separation profiles of iTRAQ labelled peptides from the three biological replicates of soluble proteins at 214 nm. Supplementary Figure

Additional file 3 Differentially expressed proteins in DOX-treated parasites identified from differential 2D-DIGE (pH 3-10, 4-7 and 6-11) analysis. Supplementary Table

Additional file 4 Primer pairs used in quantitative RT-PCR. Supplementary Table

Additional file 5 The 2D gel proteomic map of schizont stages of *P. falciparum*. Spots with a significant intensity change between doxycycline treatment and untreated are indicated by a circle in the soluble proteomic map with pI 3-10 (A), and in the membrane proteomic map with pI 4-7 (B) and pI 6-11 (C). (D) Enlargement of panel (A) to focus on differentially expressed protein spots following DOX treatment.

Abbreviations
DOX: doxycycline; RBCs: red blood cells; iRBCs: infected red blood cells.

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

Authors' contributions
SB, LA, TF, CR and BP conceived and designed the experiments, SB, LA, MB, NW and AF performed the experiments, SB, LA, MB, EB, NW, AF and CR analyzed the data, MB, EB and SG contributed reagents/materials/analysis tools, and SB, LA, CR and BP wrote the paper.

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ARTICLE N.8

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Platelet microparticles: a new player in malaria parasite cytoadherence to human brain endothelium

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ABSTRACT **Cerebral malaria (CM) is characterized by accumulation of circulating cells within brain microvessels, among which platelets play an important role. *In vitro*, platelets modulate the cytoadherence of *Plasmodium falciparum*-parasitized red blood cells (PRBCs) to brain endothelial cells. Here we show for the first time that platelet microparticles (PMPs) are able to bind to PRBCs, thereby transferring platelet antigens to the PRBC surface. This binding is largely specific to PRBCs, because PMPs show little adherence to normal red blood cells. PMP adherence is also dependent on the *P. falciparum* erythrocyte membrane protein 1 variant expressed by PRBCs. PMP binding to PRBCs decreases after neutralization of PRBC surface proteins by trypsin or after treatment of PMPs with a mAb to platelet-endothelial cell adhesion molecule-1 (CD31) and glycoprotein IV (CD36). Furthermore, PMP uptake is a dynamic process that can be achieved by human brain endothelial cells (HBECs), inducing changes in the endothelial phenotype. Lastly, PMPs dramatically increase PRBC cytoadherence to HBECs. In conclusion, our study identifies several mechanisms by which PMPs may participate in CM pathogenesis while interacting with both PRBCs and HBECs. PMPs thereby provide a novel target for antagonizing interactions between vascular cells that promote microvascular sludging and blood brain barrier alteration during CM.—Faille, D., Combes, V., Mitchell, A. J., Fontaine, A., Juhan-Vague, I., Alessi, M.-C., Chimini, G., Fusaï, T., Grau, G. E. Platelet microparticles: a new player in malaria parasite cytoadherence to human brain endothelium. *FASEB J.* 23, 3449–3458 (2009). www.fasebj.org**

Key Words: vesiculation internalization antigen transfer microvessels pathogenesis neuroinflammation

CEREBRAL MALARIA (CM) is the most severe neurological complication that occurs upon infection with *Plasmodium falciparum.* It is most common in young children living in sub-Saharan Africa, where it affects >500,000 individuals each year and has a mortality rate of 20%. Furthermore, neurocognitive sequelae have been increasingly recognized in >10% of survivors (1). Clinical symptoms of this acute encephalopathy consist of febrile headaches, seizures, and deep coma and are associated with typical pathological features such as brain swelling and ring microhemorrages due to blood-brain barrier disruption (2). Interactions between parasitized red blood cells (PRBCs) and the other cell types within host cerebral blood vessels appear to be extremely important for the development of pathological changes. Several histopathological studies have demonstrated excessive accumulation of not only PRBCs (3) but also leukocytes (4, 5) and platelets (6) in brain postcapillary venules of patients who have died of CM. PRBC cytoadherence to endothelium is a consequence of the interactions *of P. falciparum*-derived surface proteins, such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (7), with the host ligands chondroitin sulfate A (CSA) (8), thrombospondin, platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31) (9), glycoprotein (GP) IV (CD36), E-selectin (CD62-E), intercellular cell adhesion molecule 1 (ICAM-1; CD54), or vascular cell adhesion molecule 1 (VCAM-1; CD106) (10). Sequestration is increased when adherent PRBCs bind to other PRBCs (autoagglutination or clumping) or to noninfected red blood cells (rosetting) (11–14). In addition to mechanical obstruction caused by PRBCs, a pronounced proinflammatory context with high plasma levels of cytokines, notably interferon- , TNF, and lymphotoxin, causes up-regulation of surface molecules on vascular cells, which then leads to enhanced interactions between various cell types within the vascular lumen (15). *In vitro*, platelets have been shown to bind to PRBCs (16, 17) and to bridge PRBCs and brain endothelial cells, thus enhancing the binding of the former (18). Furthermore, platelets can have a direct cytotoxic effect on the monolayer of human brain endothelial cells (HBECs), an effect that is amplified by the presence of PRBCs (19). *In vivo*, injection of TNF increases platelet binding to brain microvasculature (20).

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Injection of anti-lymphocyte function-associated antigen 1 (LFA-1) mAb (21), anti-GPIIb mAb (22), anti-GPIb mAb, aspirin, or clopidogrel (20) and experimentally induced thrombocytopenia (23, 24) have confirmed the involvement of platelets in the pathogenesis of experimental murine CM caused by *Plasmodium berghei* ANKA (PbA).

The proinflammatory environment occurring during CM is associated with the shedding of submicrometer elements from plasma membranes known as microparticles (MPs). These MPs are characterized by the increased exposure on the external membrane leaflet of anionic phospholipids, such as phosphatidylserine (PS), and the presence of proteins, including functional adhesive receptors, derived from the membrane of the cell of origin (25). *In vitro* studies have shown that platelet microparticles (PMPs) are able to activate macrovascular endothelial cells, such as human umbilical vein endothelial cells (HUVECs), enhancing surface expression of ICAM-1, production of IL-8, IL-1β, and IL-6 (26), and adherence of monocytes (27). Moreover, PMPs are also able to bind to circulating neutrophils, transferring functional GPIIb/IIIa (CD41/CD61) receptor and thus participating in inflammatory signaling (28). Elevated plasma levels of MPs of endothelial origin have been found in malaria-infected children, specifically during the acute phase of CM (29), and these levels positively correlated with corresponding plasma TNF levels (unpublished results). These MPs reflect the damage at the endothelial level, but they may also have a central role in the pathogenesis of CM. This observation is supported by studies in ATP-binding cassette A1 (ABCA1)-deficient mice, which have a blunted plasma MP response during PbA infection. In these mice, MP numbers failed to rise in response to infection, in contrast to the response in wild-type mice, and this absence of response was associated with full protection against CM (30). Another study correlated PMP number with syndrome severity in a murine model (31). Because platelets constitute a major source of circulating MPs (30, 32), we studied the potential role of PMPs in the sequestration phenomenon. To this end, an *in vitro* model of human CM was used to study the interactions of PMPs with two major cell types involved in CM pathogenesis, PRBCs and HBECs, and to investigate the ability of PMPs to influence adherence of PRBCs to HBECs.

MATERIALS AND METHODS

Reagents and monoclonal antibodies

Human recombinant TNF was purchased from PeproTech EC Ltd. (London, UK). The following mAbs were purchased from Beckman Coulter Immunotech (Marseille, France): mAb to PECAM-1 (CD31, clone1 F11, IgG1), mAb to GPIV (CD36, clone FA6-152, IgG1), mAb to GPIb (CD42b, clone SZ2, IgG1), mAb to GPIIIa (CD61, clone SZ21, IgG1), irrelevant isotype-matched mAb IgG1 (clone 679.1Mc7), and phycoerythrin (PE)-conjugated mAb to GPIIb/IIIa (CD41, clone P2, IgG1) and the corresponding PE-labeled isotype mAb (clone H143.225.8, IgG1). Alexa Fluor 546-goat anti-mouse IgG and Alexa Fluor 555-conjugated wheat germ agglutinin (WGA) were purchased from Invitrogen (Karlsruhe, Germany). FITC-conjugated annexin V was purchased from AbCys SA (Paris, France). PKH26 and PKH67 fluorescent cell linker kits were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

PRBCs

IPPAM ($PRBC^{GPIV}$) and RP8 ($PRBC^{CSA}$) strains of *P. falciparum* (gift from Dr. C. Behr, Institut Pasteur, Paris, France) were obtained by regular panning on endothelial cells expressing either GPIV (CD36) or CSA using the method described by Fried and Duffy (33). Parasites were maintained in continuous culture at 2% hematocrit using type O+ human red blood cells as described elsewhere (34). Uninfected normal red blood cells (NRBCs) used as controls were cultured the same way for at least 2 wk before experiments. Late trophozoite-stage PRBC preparations were enriched to 80–85% by gelatin flotation using Plasmion (Fresenius Kabi France, Couvier, France) as described elsewhere (35). Adherent stages were resuspended in the cytoadherence medium composed of RPMI 1640 adjusted to pH 6.8.

Microvascular endothelial cells

HBECs-5i were derived by Dorovini-Zis *et al.* (36) from small fragments of human cerebral cortex obtained from patients who had died of various causes. These immortalized cells were then characterized in our laboratory, as described elsewhere (19). HBECs were seeded on 0.1% gelatin-coated culture flasks (BD Falcon; BD Biosciences, Le Pont De Claix, France) and grown to confluence in DMEM/Ham's F12 containing 15 mM HEPES and L-glutamine, pH 7.4 (Life Technologies, Inc., Carlsbad, CA, USA), supplemented with 10% heat-inactivated FCS (Life Technologies, Inc.). Conservation of the phenotype of this cell line was checked over passages, and the cell line tested negative for mycoplasma contamination every 5 passages.

Blood collection and preparation of washed platelets

Venous blood was obtained from informed healthy volunteers and anticoagulated with 0.32% sodium citrate (final concentration) in BD Vacutainer tubes (BD Biosciences). The volunteers had not taken any drugs for ≥14 d. Platelet-rich plasma (PRP) was prepared by centrifugation at 200 *g* for 15 min at room temperature, and then platelets were pelleted by centrifugation of the PRP for 6 min at 2000 *g* and washed in HEPES buffer warmed to 37°C (0.137 M NaCl, 2.68 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, and 0.1% glucose, pH 6.8). Platelets were adjusted to 1×10^9/ml in the same buffer.

Platelet activation and PMP purification

Platelets were incubated without stirring for 45 min at 37°C in the presence of calcium ionophore A23187 (10 μM). Platelets and cell debris were removed by double centrifugation for 6 min at 2000 *g* and then for 2 min at 13,000 *g*. PMPs were pelleted by centrifugation for 60 min at 20,800 *g*, washed once in PBS, resuspended in cytoadherence medium, and kept at 4°C before use. Supernatants resulting from the final wash were used as a control. Supernatants were free of PMPs, as confirmed by flow cytometry analysis (data not shown).

Flow cytometry analysis of PMPs

To determine PMP numbers, 10 μl of PMP suspension were labeled by PS probing using 2 μl of FITC-annexin V and 4 μl

of binding buffer, containing 10 mM $CaCl_2$, for 20 min in the dark at room temperature. Incubation was stopped by the addition of binding buffer containing 2.5 mM $CaCl_2$. Samples were analyzed using an EPICS XL flow cytometer operated at a high flow rate setting for 60 s (System2 software; Beckman Coulter Immunotech). MPs were identified on their forward scatter and side scatter and binding of annexin V as described elsewhere (37). The number of MPs per microliter was calculated as $n/\mu l = (N \;\; 500)/(60 \;\; 10)$, where 500 is the total volume in the tube before analysis, 60 is the sample volume analyzed, and 10 is the original volume of PMP suspension.

Membrane labeling of PMPs

A green fluorescent amphiphilic cell linker dye kit (PKH67) was used. PMPs were labeled as recommended by the manufacturer with minor modifications. In brief, PMPs were mixed volume to volume with the dye by gentle pipetting for 1 min at room temperature. PMPs were then washed extensively with DMEM without phenol red (Life Technologies, Inc.) by centrifugation.

PMP binding to PRBCs

PRBCs ($4 \;\; 10^6$) were incubated with PMPs (2000/μl) in cytoadherence medium under mild shaking and were then washed to remove unbound PMPs. PMP binding to PRBCs was measured by investigating surface expression of platelet GPIIb/IIIa within the PRBC population by flow cytometry after labeling of each sample with 2 μl of PE-conjugated mAb to GPIIb/IIIa. The corresponding PE-labeled isotype mAb was used as control.

In some experiments, PMPs were pretreated with 1 U/ml chondroitinase ABC for 1 h at 37°C. To analyze the role of extracellular PRBC proteins in PMP binding to PRBCs, PRBCs were treated by trypsin using the method of Leech *et al.* (38) with minor modifications. PRBCs were washed in PBS and treated with 10 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma-Aldrich Corp.) for 10 min at room temperature and then were washed in cold cytoadherence medium. To evaluate the role of PS in PMP binding to PRBCs, both PRBCs and PMPs were preincubated with 40 μg/ml annexin V in binding buffer for 30 min at 4°C. To investigate the role of platelet antigens present on the surface of PMPs on their binding to PRBCs, PMPs were preincubated with 10 μg/ml blocking mAb against PECAM-1, GPIV, GPIb, or GPIIIa or with a mixture of the four mAbs for 30 min.

To analyze PMP binding to PRBCs by confocal microscopy, PKH26-labeled PRBCs were incubated with PKH67-labeled PMPs as described above. After washing, PRBCs were fixed in 2% paraformaldehyde and left to adhere to a poly-L-lysine-coated glass slide (Menzel Gläser, Braunschweig, Germany).

PMP binding to HBECs

To quantify PMP binding to HBECs, cell monolayers were treated with PKH67-labeled PMPs (2000/μl) in 96-well plates (BD Falcon) for 30 min to 5 h. After incubation, HBECs were washed, detached, and analyzed by flow cytometry. External fluorescence was quenched by adding trypan blue at a final concentration of 0.05% for 10 min before analysis as described by Bjerknes and Bassoe (39).

For microscopy analysis, HBECs were grown to confluence on gelatin-coated 8-well Labtek slides (Nunc, Wiesbaden, Germany) incubated with PKH67-labeled PMPs, washed to remove unbound PMPs, and fixed with 4% paraformaldehyde. For confocal microscopy, HBEC membranes were labeled with 10 μg/ml Alexa Fluor 555-coupled WGA. For surface antigen expression analysis by fluorescence microscopy, cells were indirectly labeled using a primary mAb against PECAM-1 or GPIV revealed with a secondary Alexa Fluor 546-conjugated goat anti-mouse IgG. A nonspecific isotype-matched mouse immunoglobulin was used as a negative control.

Cytoadherence assays

HBECs were cultured to confluence on gelatin-coated 48-well plates. Cells were either left unstimulated or incubated overnight with 10 ng/ml TNF. HBECs were then washed in PBS and incubated with PMPs and PRBCs following two sequence schemes. *1*) PMPs were first incubated with HBECs, HBECs were washed to remove unbound PMPs, and PRBCs were then added for the second incubation. *2*) PMPs were first incubated with PRBCs under mild shaking, PRBCs were then washed, and added to HBECs for the second incubation. The first and second incubation steps were performed at 37°C for 5 h and 90 min, respectively. Wells were washed to remove nonadherent PRBCs, and the remaining cells were fixed by incubation in 4% paraformaldehyde for 10 min.

Confocal laser scanning microscopy and fluorescence microscopy

PMP interactions with PRBCs and HBECs were analyzed by confocal microscopy using an Axiovert 200 inverted microscope, equipped with a Zeiss LSM 510 scanning module and Zeiss LSM510 software (Zeiss, Oberkochen, Germany). Surface antigen expression on HBECs was analyzed by standard fluorescence microscopy using a DMRB microscope (Leica GmbH, Wetzlar, Germany). Images were captured with a ProgRes CF camera and visualized with ProgRes Capture Pro 2.6 software (Jenoptik, Jena, Germany).

Statistical analyses

Continuous variables were tested for normality distribution with the Kolmogorov-Smirnov test and are presented as continuous variables (mean ± SD or mean ± SE). Means between two groups were compared using a 2-tailed unpaired Student's *t* test or, for skewed variables, a Mann-Whitney test. Values of $P < 0.05$ were accepted as significant. All statistical analyses were performed using GraphPad Prism 4.0 for Windows (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

PMPs adhere preferentially to PRBCs

The presence of adhesion molecules such as PECAM-1, GPIV, or GPIIb/IIIa on PMPs (unpublished results) suggested that they could interact with other cells of the vascular compartment, such as PRBCs. To address the possible interactions of PMPs with PRBCs, PRBCs were incubated with increasing numbers of PMPs, and the presence of platelet GPIIb/IIIa on PRBC surface was then quantified by flow cytometry. After enrichment of PRBC suspension in mature stages, it still contained a significant proportion of noninfected red blood cells (NIRBCs). To differentiate these two forms, parasite nuclei within PRBCs were stained with

acridine orange and identified by flow cytometry. After 90 min of coincubation, 14.5 ± 7.9% of PRBCs were positive for GPIIb/IIIa staining (**Fig. 1*Aiv***, top right quadrant), whereas only 0.21 ± 0.21% of NIRBCs and 0.18 ± 0.16% of control NRBCs presented the platelet antigen on their surface (Fig. 1*Aiv*, *ii*, respectively, bottom right quadrant). Moreover, no GPIIbIIIa-positive signal was detected on red blood cells (RBCs) in the absence of PMPs (Fig. 1*Ai*, *iii*). In addition, PMP binding to PRBCs was dependent on PMP number added, as the percentage of GPIIb/IIIa-positive PRBCs increased with PMP number (Fig. 1*B*).

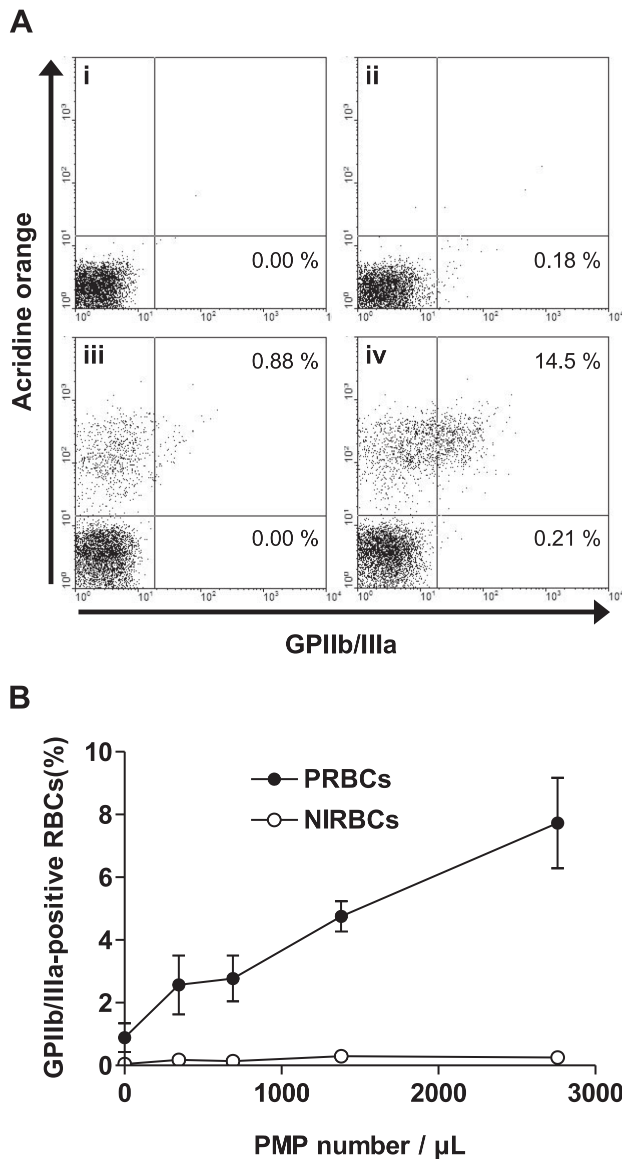

Figure 1. *A*) PMP binding to RBCs and transfer of GPIIb/IIIa from PMPs to RBCs. NRBC (*i*, *ii*) or $PRBC^{GPIV}$ (*iii*, *iv*) suspensions were incubated for 90 min at 4°C with PMPs (*ii*, *iv*) or with supernatant from the final PMP washing step as a negative control (*i*, *iii*) and were analyzed for the presence of GPIIb/IIIa by flow cytometry. *x*-Axis cutoffs were fixed according to staining with an isotype control mAb. PRBCs were differentiated from NIRBCs by staining with acridine orange 0.01% before coincubations. Numbers in top right quadrants represent fraction of GPIIb/IIIa-positive PRBCs. Numbers in bottom right quadrants represent fraction of GPIIb/IIIa-positive NRBCs or NIRBCs. *B*) Dose-response curve of PMP binding to RBCs. PRBCs were incubated with increasing PMP numbers and were analyzed for presence of GPIIb/IIIa by flow cytometry. PMP numbers were determined by flow cytometry using annexin V staining. Graph shows percentage of GPIIb/IIIa-positive PRBCs (●) or NIRBCs (○). Data are means ± SD.

To visualize PMP binding to PRBCs, PKH26-labeled PRBCs (red fluorescence) were coincubated with PKH67-labeled PMPs (green fluorescence) and analyzed by confocal microscopy. Fluorescent PMPs were identified either on the cell surface or integrated within the plasma membrane of PRBCs (**Fig. 2**, bottom), but never inside the cell. No interactions were observed with NRBCs or NIRBCs (data not shown).

To investigate the possible mechanisms involved in PMP binding to PRBCs, we first used two laboratory *P. falciparum* lines expressing PfEMP1 variants with different receptor-binding specificities. We compared a GPIV (CD36)-binding line ($PRBCs^{GPIV}$) and a CSA-binding line ($PRBCs^{CSA}$) for their ability to bind to PMPs. Both $PRBCs^{GPIV}$ and $PRBCs^{CSA}$ were able to bind to PMPs, but PMP binding was significantly lower for $PRBCs^{CSA}$ ($n=6$, $P=0.0002$) (**Fig. 3*A***) and was further decreased by PMP pretreatment with chondroitinase ABC ($P=0.04$) (Fig. 3*A*). These results indicate that interactions of PfEMP1 with its specific ligands, here GPIV or CSA/glycoaminoglycans on the surface of PMPs, may be directly involved in PMP binding. As $PRBCs^{GPIV}$ showed the greatest PMP binding, we used this strain for the next experiments, and we inhibited potential adhesion molecules expressed on either PRBCs or PMPs. First, the degradation of extracellular proteins on PRBCs by trypsin decreased PMP binding to 26% compared with the positive control ($n=6$, $P<0.0001$) (Fig. 3*B*), thus confirming that parasite proteins are directly implicated in this binding. Second, and unexpectedly, PS blockade by annexin V preincubation on both PMPs and PRBCs not only failed to inhibit PMP binding but also significantly increased the adherence ($n=6$, $P=0.005$) (Fig. 3*B*). This result suggests that PS is not directly involved in PMP binding to PRBCs. Finally, blockade of platelet antigens by mAb directed against PECAM-1 or GPIV significantly decreased PMP binding to 34 and 53%, respectively ($n=9$, $P<0.0001$ and $P=0.0006$, respectively, compared with the isotype control) (Fig. 3*C*). In contrast, mAbs directed against GPIba or GPIIIa alone did not significantly reduce PMP binding. However, the inhibitory effect of blocking mAbs was stronger when added together ($P<0.0001$), suggesting interactions *via* several receptors simultaneously.

PMPs adhere to and penetrate into HBECs

After investigating the interaction of PMPs with PRBCs, we tested PMP adherence to HBECs. HBECs were coincubated with PKH67-labeled PMPs, and nonadherent PMPs were removed by washing. Fluorescence microscopy images obtained before and after washing still showed a significant proportion of PMP binding (**Fig. 4*Ai*, *ii***, respectively).

To better visualize PMP interactions with HBECs,

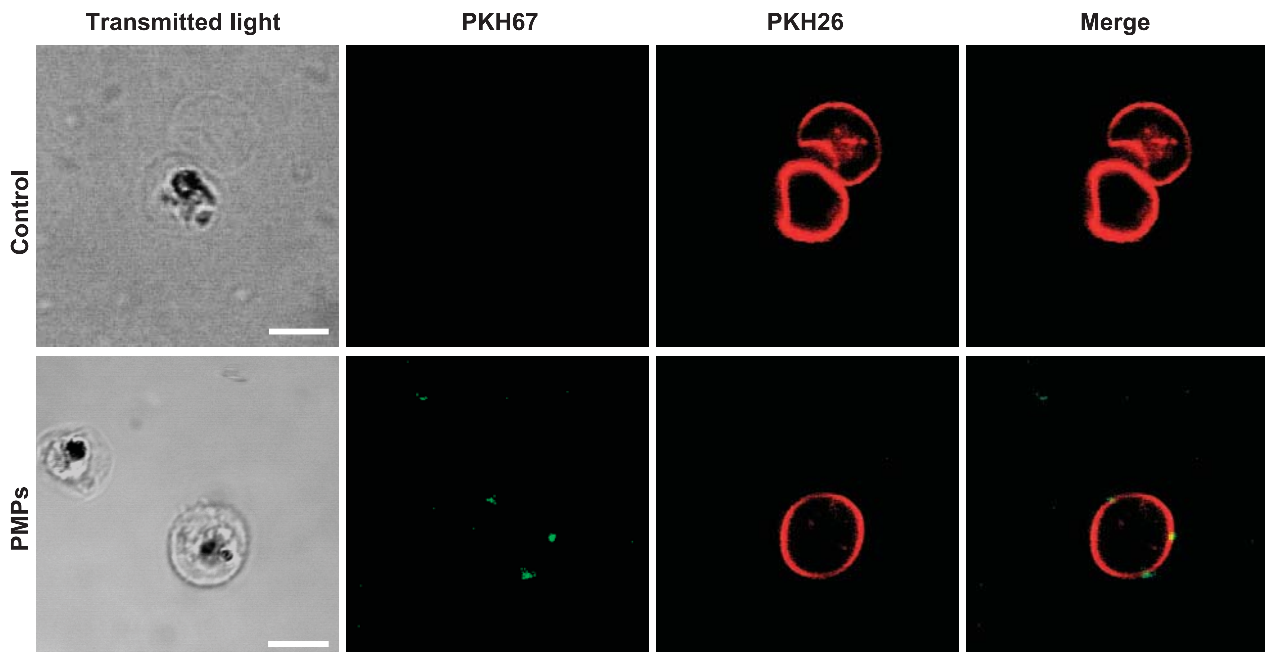

Figure 2. Visualization of PMP binding to PRBCs. Red PKH26-labeled PRBCs^{GPIV} were incubated with green PKH67-labeled PMPs (bottom) or with supernatant as control (top) for 90 min at 37°C and analyzed by confocal microscopy. PRBCs were differentiated from NIRBCs by identification of their nuclei under transmitted light. For each selected sample, ≥20 random RBCs were observed individually, and 20 optical sections separated by 0.5-nm steps were recorded. Lens used was a Plan-Apochromat 100/1.40 oil-immersion objective (Zeiss). Observations are representative of 3 independent experiments. Scale bars = 10 μm.

HBEC membranes were labeled with Alexa 555-WGA (red fluorescence), and samples were examined by confocal microscopy. Two localizations of PMPs could be observed. PMPs were either adherent to the cell surface (Fig. 4*Bi*, *Ci*) or internalized within HBEC cytoplasm (Fig. 4*Bii*). The majority of internalized PMPs appeared to be surrounded by intracellular membranes, which were also stained by WGA (Fig. 4*Cii*, insert).

PMP binding and internalization were then quantified using flow cytometry. PMP interactions with HBECs at 4°C were limited as shown by the 13 ± 2% of HBECs becoming fluorescent after incubation with PKH67-labeled PMPs (**Fig. 5*Aii***), whereas they were dramatically augmented at 37°C as 82 ± 4% of HBECs were becoming fluorescent (Fig. 5*Aiii*). These interactions increased with the time of incubation (Fig. 5*B*) and with the number of PMPs (data not shown). Quenching of external fluorescent surface-bound PMPs with trypan blue allowed us to differentiate binding from internalization. Although a significant decrease in HBEC fluorescence after quenching was observed at 4°C, no such difference was retrieved at 37°C (Fig. 5*C*). This result indicates that a significant part of PMPs were bound to the HBEC surface when incubated at 4°C, whereas the major part of PMPs were internalized by an active process when incubated at 37°C, confirming the confocal microscopy observation of a large majority of PMPs internalized within vesicular structures. Interestingly, PMP internalization was not modulated after overnight treatment of HBECs with TNF (data not shown).

HBECs acquire PECAM-1 and GPIV surface antigens *via* PMPs

We then evaluated the consequences of these interactions between PMPs and HBECs on HBEC phenotype. When analyzed for expression of surface antigen expression by fluorescence microscopy, HBECs did not express either PECAM-1 or GPIV at a basal level (**Fig. 6*A***, left and middle panels). After 5 h of incubation with PMPs, both antigens could be detected on their surface (Fig. 6*B, C*). Surprisingly, the expression of these antigens did not always colocalize with the membranous material from PMPs traced with PKH67 (Fig. 6*B, C*; inserts).

PMPs increase PRBC cytoadherence

Because PMPs do bind and bring new adhesion molecules to both PRBCs and HBECs, we investigated whether PMP interactions have a functional consequence on the cytoadherence of PRBCs to HBECs. To this end, two different sequences of tripartite coculture were carried out, as described in Materials and Methods. Although, as expected, basal PRBC cytoadherence to HBECs was significantly higher than that of NRBCs (14.9±10.4 *vs.* 2.3±2.0 RBCs/field, respectively, mean±SD, $P<0.0001$), adherence of both NRBCs and PRBCs was significantly increased by HBEC prestimulation with TNF (**Fig. 7**, no PMP, *vs.* ■). Adherence of both NRBCs and PRBCs increased in the presence of PMPs when PMPs were first added to HBECs or to RBCs [Fig. 7, PMP on endothelial cell (ECs) or PMP on RBCs *vs.* no PMP,]. This increase was not significantly differ-

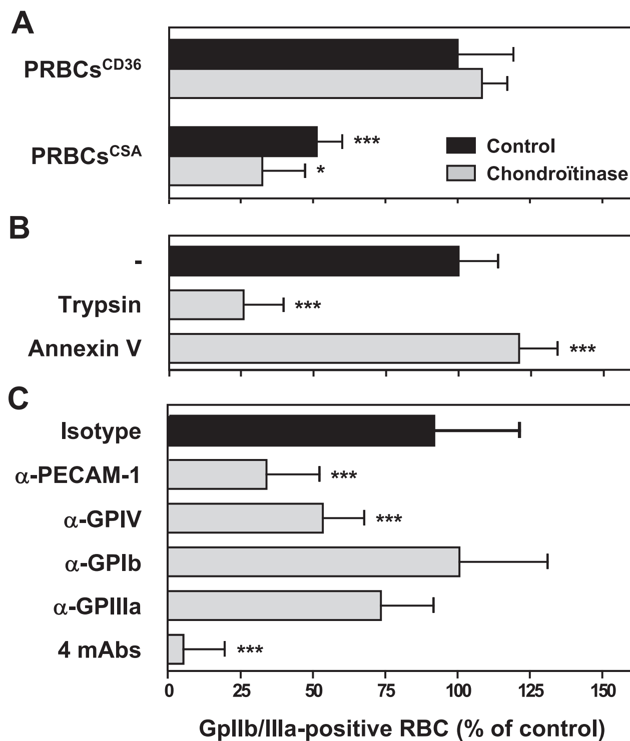

Figure 3. Role of adhesion molecules in PMP binding to PRBCs. *A*) PRBCs^{GPIV} or PRBCs^{CSA} were incubated with PMPs that were pretreated or not with chondroitinase ABC. *B*) PRBCs were pretreated with trypsin or both PRBCs and PMPs were pretreated with annexin V. *C*) PMPs were incubated in the presence of the isotype control or blocking mAbs directed against platelet adhesion molecules before addition of PRBCs. PMP binding was determined by measuring GPIIb/IIIa exposure on acridine orange-positive RBC subpopulation. Results are mean ± SD percentage of PMP binding *vs.* positive control. $^{*}P < 0.05$, $^{***}P < 0.001$; 2-tailed, unpaired Student's *t* test.

ent between the two sequence schemes of PMP addition. In addition, HBEC prestimulation with TNF failed to promote any further increase in PRBC adherence in the presence of PMPs whatever the addition scheme, whereas it slightly but significantly enhanced PMP-increased adherence of NRBCs (Fig. 7, PMP on ECs or PMP on RBCs, *vs.* ■).

DISCUSSION

During the final stages of CM, a sequence of events occurs that ultimately leads to the sequestration of PRBCs, platelets, and leukocytes within the brain microvasculature and to the alteration of the endothelial blood-brain barrier. Recently, we proposed that MPs are likely to be a further critical component of this cascade (29). Although these studies have shown a correlation between plasma MP levels and the development of CM, a direct mechanism by which MPs could affect CM pathology had not been explicitly demonstrated. Based on our previous studies in which platelets were able to enhance PRBC binding to brain endothelial cells and subsequently alter their phenotype, it seemed plausible that PMPs could act in a similar manner. Because PRBCs and HBECs are two key players in the pathogenesis of CM, we therefore investigated the interactions of PMPs with both cell types.

Although several animal models have been established to investigate CM pathogenesis, they cannot reproduce all the features of human disease. This is particularly true for PRBC cytoadherence that occurs in a less prominent fashion in murine CM because, among other reasons, of the absence of membrane knobs on mouse PRBCs (40). We thus chose to investigate the potential role for PMPs in the modulation of PRBC adherence to brain endothelium *in vitro* using human RBCs infected with *P. falciparum* and the HBEC-5i cell line displaying distinctive characteristics of human brain microvascular endothelium (19).

In this *in vitro* model of human CM, we show for the first time that MPs, shed after platelet activation, have the capacity to interact with both PRBCs and HBECs. One of the functional consequences of these combined phenomena is a dramatically increased cytoadherence of PRBCs to HBECs, suggesting that PMPs could be involved in the phenomenon of erythrocyte sequestration observed in cerebral postcapillary venules during the neurological syndrome of severe malaria.

By two independent methodologies we demonstrated that PMPs bind to PRBCs. Using flow cytometry we quantified PMP binding to RBCs *in vitro*. PMPs adhered preferentially to RBCs that were parasitized and only marginally adhered to NRBCs or to NIRBCs. This adhesion appears to occur *via* both proteins of parasite origin expressed on PRBCs and platelet antigens because digestion of extracellular proteins present on PRBC surface by trypsin or blockade of PECAM-1 or GPIV on PMPs decreased this binding. Interactions between PfEMP-1 on the PRBC surface and platelet GPIV have already been described and are known to confer to platelets their capacity to adhere to PRBCs. Thus, platelets enhance clumping (16), which is associated with severe malaria and more specifically with CM (41). The possible involvement of PfEMP-1 interactions with GPIV can be supported by the fact that PMPs adhere preferentially to a *P. falciparum* line selected to bind to GPIV rather than to a CSA-binding line. Platelet glycosaminoglycans such as CSA (42) are partially but significantly involved in PMP adherence to the CSA-binding line as PMP treatment with chondroitinase ABC removed a substantial portion of PMP adherence to those PRBCs. In the same way, the partial inhibition of PMP adherence to the GPIV-binding line with a mAb against GPVI indicates that other mechanisms must be involved.

MPs released by various cells have been implicated in transferring of antigens between cells and providing them with specific biological properties. For example, MPs containing CCR5 can transfer this receptor for HIV-1 to CCR5-deficient peripheral blood mononuclear cells and thus enable infection of these cells with HIV-1 (43). In the same way, monocyte-derived MPs have been shown to bind activated platelets, transferring proteins such as tissue factor and P-selectin glycoprotein ligand 1 to their surface (44). We show here that PMPs are able to transfer platelet antigens such as GPIIb/IIIa to PRBCs and can thus bring additional

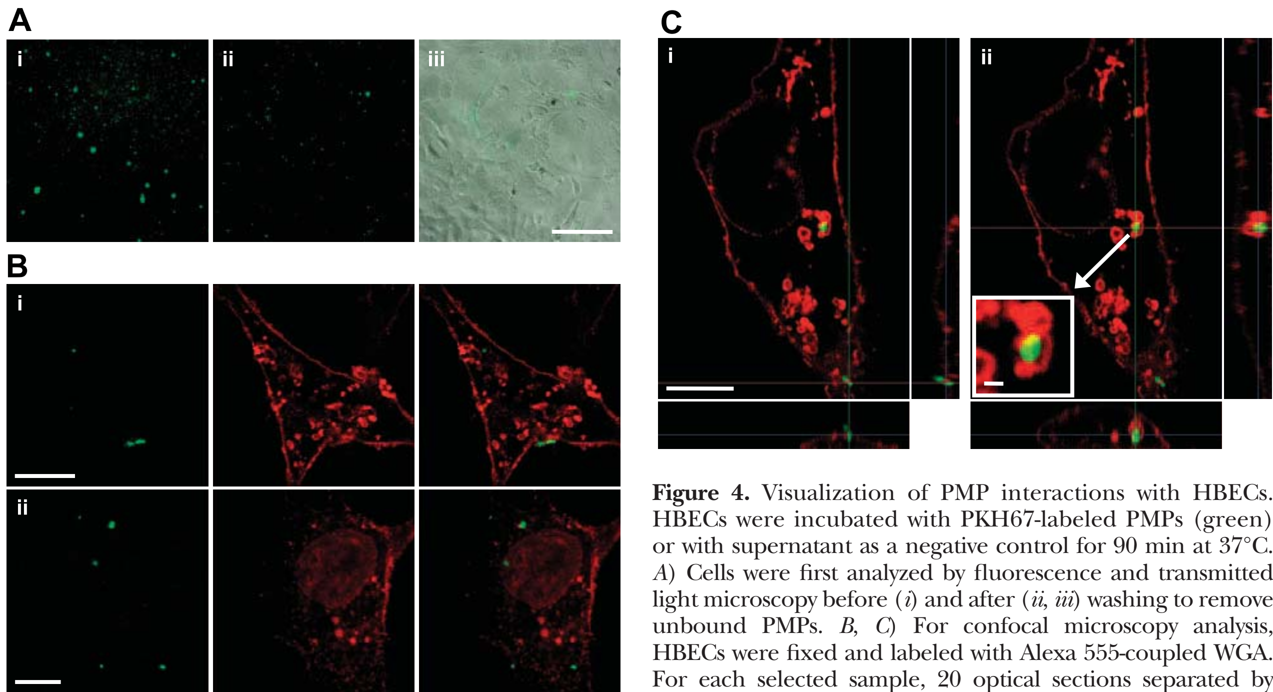

Figure 4. Visualization of PMP interactions with HBECs. HBECs were incubated with PKH67-labeled PMPs (green) or with supernatant as a negative control for 90 min at 37°C. *A*) Cells were first analyzed by fluorescence and transmitted light microscopy before (*i*) and after (*ii, iii*) washing to remove unbound PMPs. *B, C*) For confocal microscopy analysis, HBECs were fixed and labeled with Alexa 555-coupled WGA. For each selected sample, 20 optical sections separated by 0.4-nm steps were analyzed. The lens used was a Zeiss Plan-Apochromat 63/1.40 oil immersion objective lens. *B*) Merged *xy* images of adherent and internalized PMPs (*B,* i, ii, respectively) and WGA-labeled HBEC membranes (red). *C*) *xy* image with *z* projection in ortho-cuts. Observations are representative of four experiments. Scale bars = 100 μm (*A*); 10 μm (*B, Ci*); 1 μm (*Cii*).

adhesion molecules to their surface. Whether acquired GPIIbIIIa is functional and participates in any signal transduction within RBCs remains unknown.

Not only do PMPs bind to PRBCs but PMPs also interact with HBECs. PMPs have been shown to activate macrovascular endothelium and to support interac-

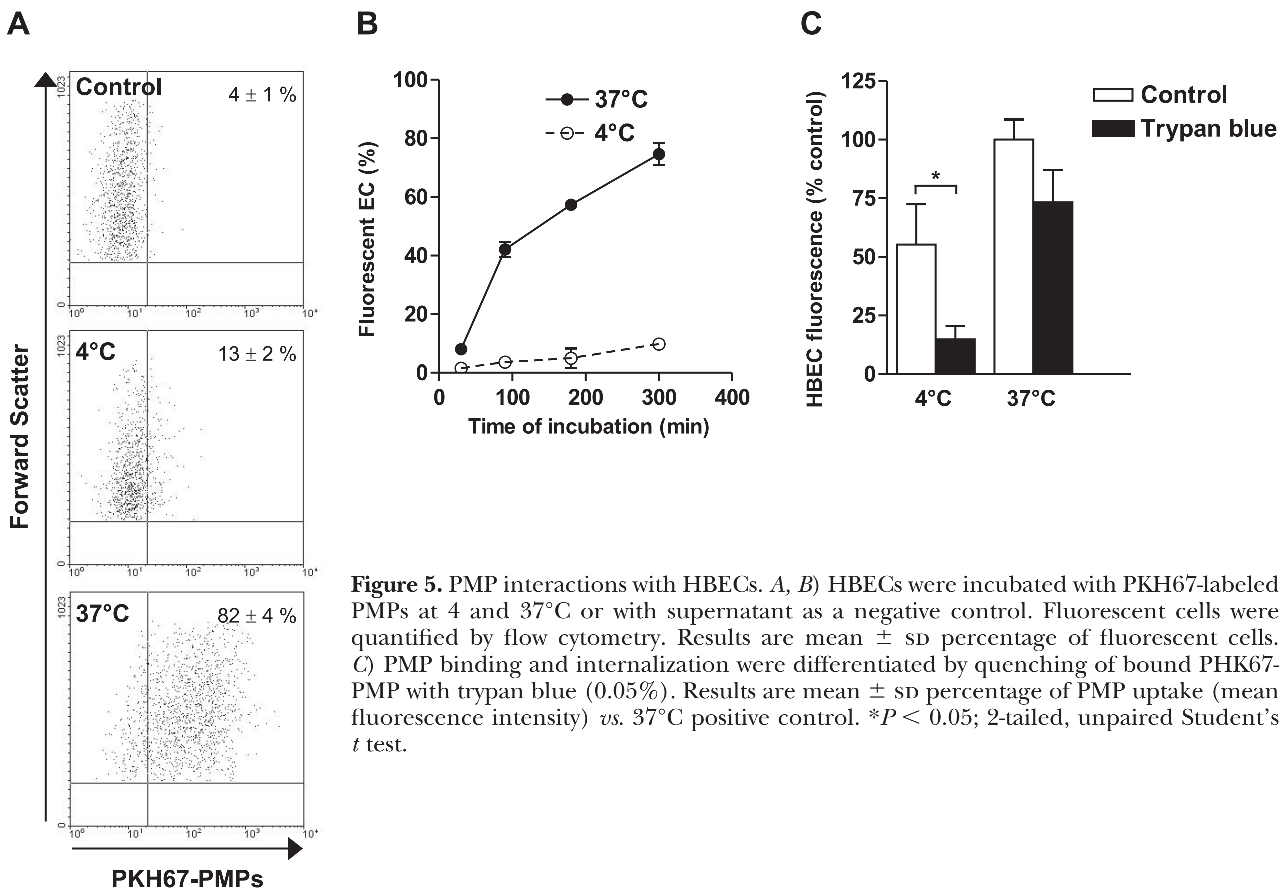

Figure 5. PMP interactions with HBECs. *A, B*) HBECs were incubated with PKH67-labeled PMPs at 4 and 37°C or with supernatant as a negative control. Fluorescent cells were quantified by flow cytometry. Results are mean ± SD percentage of fluorescent cells. *C*) PMP binding and internalization were differentiated by quenching of bound PHK67-PMP with trypan blue (0.05%). Results are mean ± SD percentage of PMP uptake (mean fluorescence intensity) *vs.* 37°C positive control. *$P < 0.05$; 2-tailed, unpaired Student's *t* test.

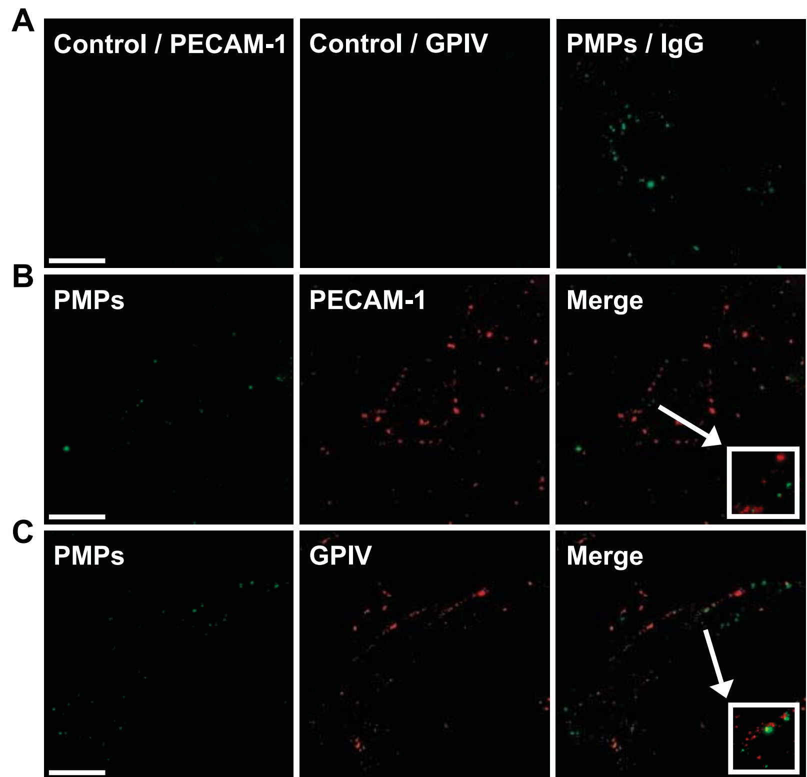

Figure 6. Detection of PECAM-1 and GPIV staining on HBECs after incubation with PMPs. HBECs were incubated with green PKH67-labeled PMPs or with supernatant as a negative control for 5 h at 37°C. Cells were washed, fixed, and indirectly stained with a primary antibody against PECAM-1 or GPIV or isotype control revealed with a secondary Alexa Fluor 555-conjugated goat anti-mouse IgG (red). Cells were then analyzed by fluorescence microscopy. *A*) Merged images of controls. *B, C*) Green channel, red channel, and merged image of samples. Scale bars = 10 μm.

tions with other intravascular cell types (27), but the present study is the first to show that this occurs with microvascular cells of brain origin. A consequence of this interaction is a change in the endothelial surface phenotype as illustrated by the acquisition of PECAM-1 and GPIV *via* PMPs, molecules that are not present on this cell line (19). The further observation that PMPs adhere to and subsequently penetrate into HBECs is striking. The majority of internalized PMPs were present in an as-yet unidentified subcellular compartment. Receptor-mediated engulfment of MPs by "scavenger endothelium" has been evoked to be relevant in the clearance of MPs from the circulation (45, 46). Indeed, as PS is one of the better explored "eat me" signals (47), the notion that endothelial cells might help scavenge PS-positive MPs warrants further investigation. Also, Del Conde *et al.* (44) described the transfer of fluorescent phospholipids from monocyte-derived MPs to the membrane of activated platelets, thus distinguishing a passive attachment of MPs to platelets from the fusion of their membranes. Here we show that PMP membranous material traced with PKH67 fluorescent dye can be processed inside endothelial cells.

Moreover, antigens derived from PMPs could be detected on the HBEC surface with a distinct pattern from PMP membranous material. This finding suggests that once PMPs become internalized, their different compounds follow separated degrading or recycling pathways inside their target cell. The clear endocytic/recycling pathways involved in PMP internalization and the mechanisms according to how PMP antigens are processed to HBEC surface membranes are under study (unpublished results). Given that PMPs were able to interact with both PRBCs and HBECs, we investigated whether these interactions had a functional consequence on PRBC cytoadherence to HBECs.

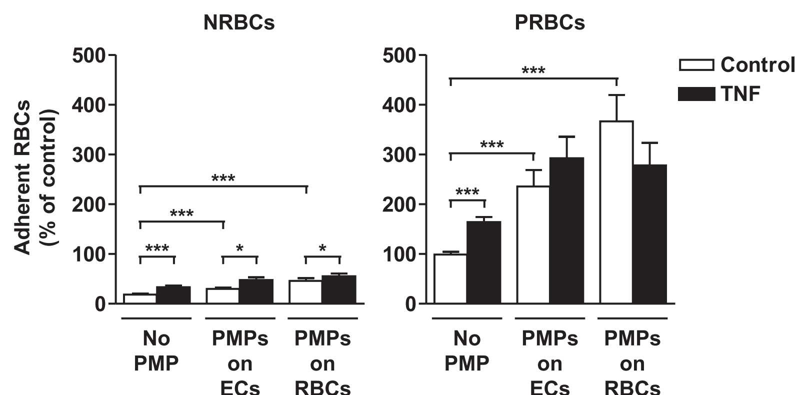

Figure 7. Effect of PMPs on PRBC cytoadherence to HBECs. Confluent HBEC monolayers were either left unstimulated ( ) or incubated with TNF (■). Cells were then incubated with PMPs and RBCs after two sequence schemes in which PMPs were either first incubated with HBECs or with RBCs. Adherent RBCs to HBEC monolayers were then counted in 20 random fields for each sample under Hoffman modulation contrast phase, with an inverted microscope (CKX41; Olympus, Tokyo, Japan) using the 40 objective. Means ± SE of ≥5 experiments are plotted as percentage of control adherence of PRBCs^{GPIV} on resting HBECs in the absence of PMPs, which is set to 100%. *$P < 0.05$, ***$P < 0.001$; 2-tailed Mann-Whitney test.

In the absence of PMPs, PRBC cytoadherence to HBECs increased significantly by endothelial prestimulation with TNF. Indeed, the IPPAM strain (PRBCs^{GPIV}) is known to adhere to endothelial GPIV (absent on these cells) and also to ICAM-1, the latter being strongly up-regulated by TNF (19). The presence of PMPs, irrespective of the sequence of incubation, induced a significant increase in PRBC adherence to HBECs. Once PMPs have adhered to either HBECs or to RBCs, depending on the sequence of incubation, they bring additional adhesion molecules between both cell types. Interestingly, TNF stimulation of HBECs failed to increase PRBC cytoadherence in the presence of PMPs, confirming that neither PMP interactions with HBECs nor PMP-increased PRBC adherence is modulated by TNF. This result suggests that PMP internalization is not mediated by TNF-up-regulated receptors such as VCAM-1 or ICAM-1.

Recently, Setty and Betal (48) identified a novel functional receptor at the surface of human lung microvascular endothelial cells that recognizes cell surface-expressed PS (PSR) and that is able to support PS-positive RBC adherence in the absence of plasma ligands. In our *in vitro* model, we demonstrate that PMPs bind PRBCs and are present on the PRBC surface, rendering RBCs positive for platelet antigens. In the same way, we can postulate that PMPs, when incubated first with PRBCs, can also transfer PS on their surface and allow the PS-positive RBCs to bind to the endothelial PSR or to the immobilized subendothelial matrix thrombospondin (49).

Thus, in this study, we show for the first time *in vitro* that PMPs are more than a simple marker of enhanced platelet activity, in that they act as a potential player in pathogenesis of CM by interacting with other cell types of the vascular compartment. Interestingly, in a recent clinical study, we found that high plasma MP levels from various cellular origins were correlated with neurological complications during malaria infection (unpublished results). Among these, PMPs were the most abundant MP type in the different patient groups and more particularly in the CM group. The roles of platelets in CM pathogenesis have to be clarified as they can be either deleterious (23) or protective (50, 51). In addition, because the PMP surface has been described as being enriched in phospholipids and integrins compared with the platelet surface, they could have roles different from those of platelets (52). PMPs also are more mobile than platelets and thus are more susceptible to interaction with other cell types (53). Taken together, the phenomena described here converge toward a conclusion that, beyond platelets, PMPs strongly increase the adherence of RBCs to the microvascular endothelium. In addition, *in vitro*, platelets could have an effect that is in fact partially due to PMPs that are likely to be released during the experiments. With CM severity being partly related to both PRBC sequestration within cerebral microvessels and to the ensuing mechanical phenomenon of vascular obstruction, these data provide novel insight into the understanding of a potential pathogenic role for MPs in the development of CM. If confirmed *in vivo*, this observations could provide new potential targets for therapeutic amelioration of CM. FJ

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In Vitro Activity of Ferroquine Is Independent of Polymorphisms in Transport Protein Genes Implicated in Quinoline Resistance in *Plasmodium falciparum*

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In Vitro Activity of Ferroquine Is Independent of Polymorphisms in Transport Protein Genes Implicated in Quinoline Resistance in *Plasmodium falciparum*▽

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The in vitro activity of ferroquine (FQ) (SR97193), a 4-aminoquinoline antimalarial compound that contains a ferrocenic nucleus, against 15 *Plasmodium falciparum* strains was assessed and compared with those of chloroquine (CQ), quinine (QN), monodesethylamodiaquine (MDAQ), and mefloquine (MQ). These 15 strains were genotyped for polymorphisms in quinoline resistance-associated genes such as Pf*crt*, Pf*mdr1*, Pf*mrp*, and Pf*nhe-1*. FQ was highly active against CQ-resistant parasites or in parasites with reduced susceptibility to QN, MDAQ, or MQ. Encouragingly, we did not find a correlation between responses to FQ and those to other quinoline drugs. These results suggest that no cross-resistance exits between FQ and CQ or quinoline antimalarial drugs. Mutations in codons 74, 75, 76, 220, 271, 326, 356, and 371 of the Pf*crt* gene; codons 86, 184, 1034, 1042, and 1246 of the Pf*mdr1* gene; and codons 191 and 437 of the Pf*mrp* gene were not significantly associated with *P. falciparum* susceptibility to FQ. Neither the number of ms4760 DNNND or DDNHNDNHNN repeats in Pf*nhe-1* nor the profile of ms4760 was significantly associated with the FQ in vitro response. These data suggest the FQ may not interact with transport proteins in quinoline-resistant parasites. The present results justify further clinical trials of FQ in multidrug resistance areas.

Two of the current options to reduce the morbidity and mortality of malaria are chemoprophylaxis and chemotherapy. During the past 20 years, many strains of *Plasmodium falciparum* have become resistant to chloroquine and other antimalarial drugs (24). This has prompted a search for an effective alternative antimalarial drug with minimal side effects. The emergence and spread of parasites that are resistant to antimalarial drugs has caused an urgent need for novel compounds to be discovered and developed.

An approach to remove aminoquinoline resistance in parasites is to modify the position and the chemical nature of the substituents or the length of the side chain on the quinoline nucleus of the aminoquinoline (12, 34). Recently, many different metals have been incorporated into antimalarial agents (29). Indeed, several organometallic compounds based on chloroquine with a ferrocene nucleus localized at different sites have been synthesized (5–8). This approach is currently being developed by J. Brocard and colleagues (URA-CNRS 402, Lille, France), who have synthesized ferroquine (FQ) {i.e., 7-chloro-4-[(2-*N*,*N*′-dimethylaminomethyl)ferrocenylmethylamino]quinoline} (Fig. 1). FQ is currently under phase II clinical trial investigations.

Only six previous studies investigated the activity of ferroquine against *P. falciparum* strains isolated from infected patients (1, 2, 10, 21, 28, 30). The drug susceptibilities of *P. falciparum* strains vary among different locations, where isolates have different antimalarial resistance backgrounds. It seems that ferroquine activity is independent of chloroquine resistance in *P. falciparum* (21), and ferroquine antimalarial activity is not influenced by polymorphisms in the Pf*crt* gene (*Plasmodium falciparum* chloroquine resistance transporter), which encodes a protein located in the parasite digestive vacuole and is involved in drug transport and chloroquine resistance (10, 11).

The objective of this study was to determine whether genetic polymorphisms in genes associated with quinoline resistance modulate in vitro responses to ferroquine. We assessed polymorphisms in genes that are potentially associated with quinoline resistance: Pf*crt*, Pf*mdr1* (*P. falciparum* multidrug resistance gene 1), Pf*nhe-1* (*P. falciparum* sodium/hydrogen exchanger), and Pf*mrp* (*P. falciparum* multidrug resistance protein). There is strong evidence that Pf*crt* is associated with chloroquine resistance (18, 32). Pf*mdr1* is involved in mefloquine resistance (15, 31). The evidence of the involvement of Pf*nhe-1* in resistance is compelling but weaker than those for Pf*crt* or Pf*mdr1*. Pf*nhe-1*, which encodes a proton transporter localized to the plasma membrane, may alter quinine activity (4, 17). The evidence for Pf*mrp* being involved in resistance is still debated. However, it seems that Pf*mrp* is associated with decreases in chloroquine and quinine susceptibility (20, 26, 33).

MATERIALS AND METHODS

***Plasmodium falciparum* cultures.** Fifteen monoclonal strains isolated from patients from a wide panel of countries (Brazil, Cambodia, Cameroon, Comoros,

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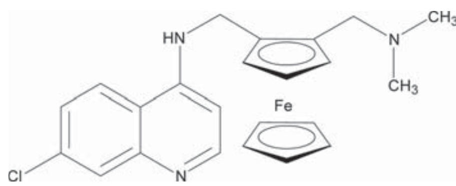

FIG. 1. Chemical structure of FQ {7-chloro-4-[2-*N*,*N*-dimethylaminomethyl)ferrocenylmethylamino]quinoline}.

Djibouti, The Gambia, Honduras, Indochina, Niger, Senegal, Sierra Leone, and Sudan) were maintained in culture in RPMI 1640 medium (Invitrogen, Paisley, United Kingdom) supplemented with 10% human serum (Abcys S.A., Paris, France) and buffered with 25 mM HEPES and 25 mM $NaHCO_3$ (Invitrogen). Parasites were grown in A-positive human blood under controlled atmospheric conditions, which consist of 10% O_2, 5% CO_2, and 85% N_2 at 37°C in 95% humidity.

The strains were synchronized with sorbitol twice before use (23). The susceptibility of each strain to antimalarial drugs was assessed in five independent experiments.

Drugs. FQ base (SR97193) was obtained from Sanofi-Aventis (France). Chloroquine diphosphate (CQ) and quinine hydrochloride (QN) were purchased from Sigma (St. Louis, MO). Monodesethylamodiaquine (MDAQ) was obtained from the World Health Organization (Geneva, Switzerland), and mefloquine (MQ) was obtained from Hoffman-LaRoche (Bale, Switzerland). FQ and CQ were resuspended in water in concentrations ranging between 0.125 and 500 nM for FQ and 5 and 3,200 nM for CQ. QN, MDAQ, and MQ, which were first dissolved in methanol and then diluted in water to obtain final concentration ranges of 5 to 3,200 nM for QN, 1.56 to 1,000 nM for MDAQ, and 3.2 to 400 nM for MQ.

In vitro assay. For in vitro isotopic microtests, 25 μl/well of antimalarial drug and 200 μl/well of the parasitized red blood cell suspension (final parasitemia, 0.5%; final hematocrit, 1.5%) were distributed into 96-well plates. Parasite growth was assessed by adding 1 μCi of tritiated hypoxanthine with a specific activity of 14.1 Ci/mmol (Perkin-Elmer, Courtaboeuf, France) to each well at time zero. The plates were then incubated for 48 h in controlled atmospheric conditions. Immediately after incubation, the plates were frozen and thawed to lyse erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter GF/B; Perkin-Elmer) and washed using a cell harvester (Filter-Mate cell harvester; Perkin-Elmer). Filter microplates were dried, and 25 μl of scintillation cocktail (Microscint O; Perkin-Elmer) was placed into each well. Radioactivity incorporated by the parasites was measured with a scintillation counter (Top Count; Perkin-Elmer).

The drug concentration able to inhibit 50% of parasite growth (IC_{50}) was assessed by identifying the drug concentration corresponding to 50% of the uptake of tritiated hypoxanthine by the parasite in the drug-free control wells. The IC_{50} value was determined by nonlinear regression analysis of log-based dose-response curves (Riasmart; Packard).

Nucleic acid extraction. Total genomic DNA of each strain was isolated by using the E.Z.N.A. blood DNA kit (Omega Bio-Tek, GA) extraction method. RNA of each strain was purified by using the QIAamp Blood Mini kit (Qiagen, Germany).

Pf*crt* single-nucleotide polymorphisms (SNPs). A 1,250-nucleotide-length fragment of the Pf*crt* gene was amplified by reverse transcription-PCR using primers F1sense (5′-TAA TTT CTT ACA TAT AAC AAA ATG AAA TTC-3′) and F1antisense (5′-TTA TTG TGT AAT AAT TGA ATC GAC-3′) and sequenced using primers F2sense (5′-TAG GTG GAG GTT CTT GTC TTG GTA-3′) and F2antisense (5′-TCG ACG TTG GTT AAT TCT CCT TC-3′) (16). Amplifications were performed according to the manufacturer's instructions (Access reverse transcription-PCR system kit; Promega, WI). Sequencing was conducted using ABI Prism Big Dye Terminator v1.1 (Applied Biosystems, CA) cycle sequencing ready reaction kits.

Pf*mdr1* SNPs. Pf*mdr1* was amplified by PCR using primers 5′-TTA CAT TTT ATT TGA TTT TGT GTT G-3′ and 5′-CAT CTT TTC TAG TAT CAT AAT GAA-3′ to amplify codons 86 and 184 and 5′-ACG GGT TTA GTA AAT AAT ATT GTT-3′ and 5′-ATG GGT TCT TGA CTA ACT ATT G-3′ to amplify codons 1034, 1042, and 1246. Amplifications were performed with the Titanium PCR kit (Clontech Ozyme, France) according to the manufacturer's instructions. The amplified fragments were sequenced as previously described.

Pf*mrp* SNPs. PCR amplification followed by sequencing was used to detect SNPs in Pf*mrp* at positions 191 and 437. The primers used for amplification and sequencing were pfmrp-501F (5′-TTT CAA AGT ATT CAG TGG GT-3′) and pfmrp-1409R (5′-GGC ATA ATA ATT GAT GTA AA-3′).

Pf*nhe-1* microsatellite profile. A sequence containing the ms4760 microsatellite described previously (17) was amplified using primers pfnhe-3802F (5′-TTATTAAATGAATATAAAGA-3′) and pfnhe-4322R (5′-TTTTTTATCATTACTAAAGA-3′). The amplified fragments were sequenced as previously described.

Statistical analysis. Assessment of standard antimalarial drug cross-resistance with FQ was estimated by determining the coefficient of correlation (r) and coefficient of determination (r^2). The Kruskal-Wallis test or the Mann-Whitney U test was used, when appropriate, to compare equalities of populations for each mutation.

RESULTS

Fifteen *P. falciparum* strains were tested for their in vitro susceptibilities to FQ, CQ, QN, MQ, and MDAQ. FQ had a considerably higher level of activity than did all quinolines tested. The IC_{50} values for FQ ranged from 1.8 to 13.4 nM, with a 5.3 nM mean (standard deviation, ±3.2 nM) (Table 1).

In vitro cross-resistance was measured by the pairwise correlation of IC_{50} values of all 15 strains. Neither FQ and CQ

TABLE 1. In vitro susceptibilities of 15 strains of *Plasmodium falciparum* to FQ, CQ, QN, MQ, and MDAQ

Strain	Origin	Mean IC_{50} (nM) (95% CI)[a]				
		FQ	CQ	QN	MQ	MDAQ
3D7	Sierra Leone	3.4 (3.1–3.8)	21 (18–25)	104 (73–148)	52 (47–58)	21 (15–28)
W2	Indochina	7.6 (6.5–8.8)	485 (377–625)	684 (576–812)	32 (27–37)	146 (113–189)
D6	Africa	5.6 (5.1–6.2)	23 (19–28)	36 (30–42)	59 (50–68)	18 (11–25)
FCM29	Cameroon	6.8 (6.3–7.4)	517 (449–594)	597 (512–696)	28 (21–36)	318 (278–369)
FCR3	The Gambia	1.8 (1.6–2.1)	477 (376–607)	665 (546–811)	30 (26–34)	110 (99–122)
HB3	Honduras	13.4 (11.0–16.2)	18 (11–27)	44 (37–53)	51 (40–65)	35 (29–45)
106/1	Sudan	3.4 (3.0–3.8)	27 (21–33)	66 (60–71)	25 (20–30)	29 (26–33)
IMT Bres	Brazil	3.3 (2.6–4.2)	398 (349–454)	501 (434–579)	33 (28–39)	98 (87–117)
IMT 8425	Senegal	1.9 (1.6–2.2)	29 (26–33)	29 (24–34)	44 (39–50)	26 (21–33)
IMT 10336	Comoros	8.6 (5.8–12.9)	18 (11–27)	220 (180–270)	21 (16–28)	23 (20–27)
IMT K14	Cambodia	7.8 (6.4–9.4)	597 (512–696)	1130 (1042–1225)	33 (28–39)	199 (163–243)
IMT K2	Cambodia	2.2 (1.9–2.6)	531 (441–639)	614 (572–827)	38 (32–45)	91 (78–107)
IMT K4	Cambodia	2.1 (1.7–2.7)	309 (260–367)	1047 (935–1172)	14 (10–18)	146 (113–189)
IMT L1	Niger	4.5 (4.0–5.2)	274 (235–319)	531 (441–639)	38 (32–45)	54 (42–68)
IMT Vol	Djibouti	7.3 (5.6–9.5)	262 (236–292)	422 (377–472)	25 (20–30)	113 (102–125)

[a] Values are mean IC_{50}s of five experiments for each strain. CI, confidence interval.

TABLE 2. Correlation of in vitro responses of 15 strains of *Plasmodium falciparum* to FQ, CQ, QN, MQ, and MDAQ

Drug	Drug partner	r	r^2	P value
FQ	CQ	−0.147	0.0216	0.600
FQ	QN	−0.066	0.0044	0.816
FQ	MQ	−0.011	0.0001	0.970
FQ	MDAQ	+0.086	0.0074	0.760
CQ	QN	+0.756	0.5715	0.001
CQ	MQ	−0.313	0.0980	0.256
CQ	MDAQ	+0.806	0.6496	0.0003
QN	MQ	−0.421	0.1697	0.118
QN	MDAQ	+0.728	0.5300	0.002
MQ	MDAQ	−0.585	0.3422	0.022

(r^2 = 0.0216) nor FQ and the other quinolines tested were correlated (Table 2). On the contrary, CQ and QN, CQ and MDAQ, QN and MQ, QN and MDAQ, and MQ and MDAQ were significantly correlated.

The following mutations were identified for at least one strain: Pf*crt* M74I, N75E, K76T, A220S, Q271(E/V), N326S, I356T, and I371R; Pf*mrp* H191Y and S437A; and Pf*mdr1* N86Y, Y184F, S1034C, N1042D, and D1246Y (Table 2). Six different ms4760 microsatellite profiles of Pf*nhe-1* were observed (Fig. 2). The numbers of DNNND and DDNHND NHNN repeats on ms4760 ranged from 1 to 4 and 1 to 2, respectively (Table 3).

Polymorphisms in the Pf*crt*, Pf*mdr1*, or Pf*mrp* gene were not associated with *P. falciparum* susceptibility to FQ ($P > 0.386$). On the contrary, in vitro resistance to CQ and reduced susceptibility to QN and MDAQ were significantly associated with mutations in codons 74, 75, 76, 220, 271, 326, 356, and 371 in the Pf*crt* gene ($0.005 < P < 0.05$) and in codons 191 and 437 in the Pf*mrp* gene ($P < 0.007$). Reduced susceptibility to MQ was significantly associated with mutations in codons 74, 75, 76, 220, 271, 326, and 371 in the Pf*crt* gene ($0.017 < P < 0.05$) and in codons 191 and 437 in the Pf*mrp* gene ($P = 0.05$). In addition, in vitro resistance to CQ and reduced susceptibility to QN were significantly associated with mutations in codons 1034 and 1042 in the Pf*mdr1* gene ($P = 0.014$).

The number of ms4760 DNNND repeats in Pf*nhe-1* was not significantly associated with the FQ response ($P = 0.923$), in opposition to those of CQ, QN, and MDAQ ($P < 0.066$).

Statistical analysis was performed for various profiles including ms4760-6 and ms4760-7, which were the most commonly observed profiles. No significant association between FQ or MQ IC_{50} and Pf*nhe-1* ms4760 profiles was established. On the contrary, a significant association was observed for the most frequent profiles (ms4760-6 and ms4760-7) for CQ, QN, and MDAQ ($0.021 < P < 0.049$). Profile 6 was significantly associated with reduced susceptibility to CQ, QN, and MDAQ. Profile 7 was significantly associated with a high level of in vitro resistance to CQ, QN, and MDAQ.

DISCUSSION

FQ, a CQ derivative, is highly active against CQ-resistant *P. falciparum* laboratory strains (13) and against *P. falciparum* strains isolated from infected patients (1, 2, 10, 21, 28, 30). FQ shows good antimalarial and toxicity profiles in rodent malaria models (8). FQ is therefore an interesting candidate for clinical development. FQ is even highly active against parasites with reduced susceptibility to QN, MDAQ, or MQ. FQ is more active than CQ, QN, MDAQ, and even MQ. Encouragingly, we did not find a correlation between FQ and the other quinoline drugs, i.e., CQ, QN, MDAQ, or MQ. These results suggest that no cross-resistance between FQ and CQ, or quinoline antimalarial drugs, exits. These data are in accordance with previous studies, which showed weak coefficients of determination, between 0.096 and 0.127, for correlation between FQ and CQ (2, 21, 28). The potency of FQ against CQ-, QN-, MDAQ-, or MQ-resistant *P. falciparum* strains and the absence of cross-resistance suggest that both drugs have different modes of action or mechanisms of resistance. CQ is believed to act by concentrating in the parasite digestive vacuole and preventing the crystallization of toxic heme in the hemozoin, leading to membrane damage and parasite death (14, 35). Like CQ, FQ forms complexes with hematin in solution and is an inhibitor of β-hematin formation (9). Nevertheless, the absence of cross-resistance between FQ and the other quinolines suggests that FQ may not work exactly as does chloroquine, react with heme and hemozoin differently than the other quinolines, or have a different molecular target.

IC_{50} values for FQ were found to be unrelated to mutations occurring in transport protein genes involved in quinoline antimalarial drug resistance, such as Pf*crt*, Pf*mdr1*, Pf*mrp*, or Pf*nhe-1*. The absence of association with FQ activity and polymorphisms in the Pf*crt* gene is consistent with previous results for Cambodian isolates (10). These data suggest that FQ may not be expelled by transport proteins in quinoline-resistant parasites, possibly as a result of the strong affinity of *P. falciparum* for the iron moiety of the molecule (25). In comparison to CQ, the presence of a ferrocene moiety with a different shape, volume, lipophilicity, effects on basicity, and electrostatic profile dramatically modifies the pharmacological behavior of the parent drug (9). Therefore, FQ appears to present reduced affinity for the transporters involved in the resistance to CQ and quinoline drugs. This may partially explain the high level of activity of FQ against multidrug-resistant *P. falciparum* parasites. This is consistent with results that indicate the ability of mutant PfCRT to confer CQ resistance is precisely

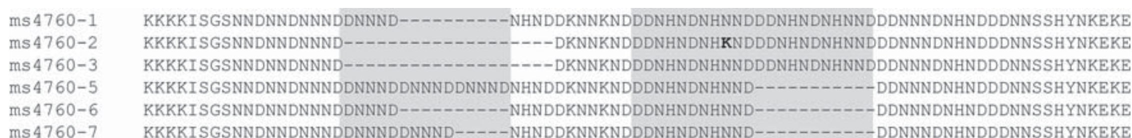

FIG. 2. Sequences of Pf*nhe-1* microsatellite ms4760 detected among the 15 studied *P. falciparum* strains. Profiles 1 to 7 were previously described (16).

TABLE 3. Pf*crt*, Pf*mdr1*, Pf*mrp*, and Pf*nhe-1* polymorphisms in *Plasmodium falciparum* strains[a]

Strain	Amino acid encoded by:																						No. of Pf*nhe-1* microsatellite ms4760 DNNND repeats	No. of Pf*nhe-1* microsatellite ms4760 DDNHNDNHNN repeats	Mutation at Pf*nhe-1* microsatellite ms4760[a]	Pf*nhe-1* microsatellite ms4760 profile
	Pf*crt* codon:															Pf*mdr1* codon:					Pf*mrp* codon:					
	72	74	75	76	97	144	148	194	220	271	326	333	356	367	371	86	184	1034	1042	1246	191	437				
3D7	C	M	N	K	H	A	L	I	A	Q	N	T	I	G	**R**	N	Y	S	N	D	H	S	1	2	**K**	2
W2	C	**I**	**E**	**T**	H	A	L	I	**S**	**E**	**S**	T	**T**	G	I	**Y**	Y	S	N	D	**Y**	**A**	2	2	N	1
D6	C	M	N	K	H	A	L	I	A	Q	N	T	I	G	**R**	N	Y	S	N	D	H	S	1	2	N	3
FCM29	C	**I**	**E**	**T**	H	A	L	I	**S**	**E**	**S**	T	I	G	I	**Y**	Y	S	N	D	**Y**	**A**	3	1	N	7
FCR3	C	**I**	**E**	**T**	H	A	L	I	**S**	**E**	**S**	T	I	G	I	**Y**	Y	S	N	D	**Y**	**A**	2	1	N	6
HB3	C	M	N	K	H	A	L	I	A	Q	N	T	I	G	**R**	N	**F**	S	N	D	H	S	4	1	N	5
106/1	C	**I**	**E**	K	H	A	L	I	**S**	**E**	**S**	T	I	G	I	**Y**	Y	S	N	D	**Y**	**A**	2	1	N	6
IMT Bres	C	**I**	**E**	**T**	H	A	L	I	**S**	**E**	**S**	T	I	G	I	**Y**	Y	S	N	D	**Y**	**A**	2	1	N	6
IMT 8425	C	M	N	K	H	A	L	I	A	Q	N	T	I	G	**R**	N	Y	S	N	D	H	S	1	2	**K**	2
IMT 10336	C	M	N	K	H	A	L	I	A	**E**	N	T	I	G	**R**	N	Y	S	N	D	H	S	1	2	N	3
IMT K14	C	**I**	**E**	**T**	H	A	L	I	**S**	**E**	**S**	T	**T**	G	I	N	**F**	**C**	**D**	**Y**	**Y**	**A**	3	1	N	7
IMT K2	C	**I**	**E**	**T**	H	A	L	I	**S**	**V**	**S**	T	**T**	G	I	N	**F**	**C**	**D**	D	**Y**	**A**	3	1	N	7
IMT K4	C	**I**	**E**	**T**	H	A	L	I	**S**	**E**	**S**	T	**T**	G	I	N	Y	**C**	**D**	D	**Y**	**A**	3	1	N	7
IMT L1	C	**I**	**E**	**T**	H	A	L	I	**S**	**E**	N	T	I	G	I	**Y**	Y	S	N	D	**Y**	**A**	2	1	N	6
IMT Vol	C	**I**	**E**	**T**	H	A	L	I	**S**	**E**	**S**	T	I	G	I	**Y**	Y	S	N	D	**Y**	**A**	2	1	N	6

[a] Polymorphism types are detailed in Fig. 2. Boldface type indicates point mutations.

configured for CQ (22). Resistance was rapidly lost following subtle structural modifications of the basic diethylamino side chain linked to the 4-aminoquinoline ring structure. Cross-resistance was clearly evident with analogs that varied by only a single CH_2 group and absent when two CH_2 groups were removed or six were added (22). In addition, *Dictyostelium discoideum* transformants expressing the CQ resistance phenotype PfCRT were not able to expel piperaquine, a bisquinoline analog of CQ (27). The absence of an interaction of FQ with PfCRT suggests that the phenotypic response to FQ would not be modified by resistance reversers such as verapamil. Nevertheless, the effects of reversers on aminoquinoline analogs are still debated. Verapamil did not affect the relative piperaquine response in *D. discoideum* transformants expressing the CQ resistance phenotype PfCRT at concentrations that completely reverse CQ resistance (27), while desipramine could reverse resistance to bisquinoline WR268,668 (3). The ability of verapamil to enhance the activity of a drug is inversely related to the log *D* of this drug (19). In addition, no resistance of *P. falciparum* to FQ has been found in vitro in either patient isolates or laboratory-adapted strains under drug pressure (11).

In conclusion, FQ is highly active against parasites with reduced susceptibility to QN, MDAQ, or MQ. No cross-resistance between FQ and CQ, or quinoline antimalarial drugs, exists. IC_{50} values for FQ were found to be unrelated to mutations occurring in transport protein genes involved in quinoline antimalarial drug resistance, such as Pf*crt*, Pf*mdr1*, Pf*mrp*, or Pf*nhe-1*. The present results justify clinical trials of FQ in multidrug resistance areas. A phase II study is now in progress in Gabon.

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REVUE N.2

Analyses protéomiques et parasitoses :
Principes et applications

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ANALYSES PROTÉOMIQUES ET PARASITOSES : PRINCIPES ET APPLICATIONS

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RÉSUMÉ • Il y a plus de 30 ans, O'Farrel décrivit une méthode permettant la séparation des protéines selon deux dimensions. Cette technique a extraordinairement évolué depuis. Les avancées technologiques réalisées en parallèle en spectrométrie de masse, ainsi que les programmes de séquençages du génome de différents organismes en ont favorisé l'expansion. L'électrophorèse bi-dimensionnelle (2-D) est devenue un outil important en protéomique, permettant la séparation de milliers de protéines et aboutissant à un vision globale de l'état du protéome. Cet article présente les différentes étapes et limites d'une analyse protéomique : la préparation du matériel biologique, l'électrophorèse 2-D, les systèmes de détections protéiques, et les outils disponibles pour l'identification protéique. Des approches protéomiques alternatives à l'électrophorèse 2-D sont aussi exposées. Enfin quelques exemples d'application notamment lors d'infections parasitaires permettent de décrire les différentes possibilités des analyses protéomiques dans l'étude des mécanismes de virulence, de résistance au traitement, ou de réponse immunologique vis-à-vis d'un agent pathogène.

MOTS-CLÉS • Paludisme - Protéomique - Electrophorèse bidimensionnelle - Spectrométrie de masse - Parasitose.

PROTEOMIC ANALYSIS AND PARASITOSIS: PRINCIPLES AND APPLICATIONS

ABSTRACT • O'Farrel described a method allowing two-dimensional (2D) protein separation more than 30 years ago. Since then the original technique has made enormous progress. This progress has been accompanied by advances in mass spectrometry technology as well as various genome-sequencing programs. Today 2D electrophoresis has become the workhorse of proteomics, allowing resolution of complex structures containing thousands of proteins and providing a global view of the state of a proteome. This article presents the different steps and limitations of proteomic analysis: preparation of biological material, 2D electrophoresis, protein detection systems, and available tools for protein identification. Alternative proteomic approaches to 2D electrophoresis are also presented. A few applications are described as examples to illustrate the utility of proteomic analysis for studying the mechanisms underlying virulence, resistance to antimalarial therapies and immune response against pathologic agents.

KEY WORDS • Malaria – Proteomics – Bidimensional electrophoresis – Mass spectrometry – Parasitosis.

Abréviations : bidimensionnelle (2-D), spectrométrie de masse (SM), point isoélectrique (pI), gradient de pH immobilisés (IPG), gamme dynamique linéaire (GDL), électrospray ionisation (ESI), masse/charge (m/z), Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF), peptide mass fingerprinting (PMF), electrospray ionization quadripole time-of-flight mass spectrometry in tandem (ESI Q-TOF MS/MS), isotope-code affinity tag method (ICAT), surface-enhanced laser desorption/ionization (SELDI), multidimensional protein identification technology (MudPIT).

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Les protéines représentent plus de la moitié du poids sec des cellules, et jouent un rôle majeur dans leur structure et la communication intercellulaire. Les protéines résultent de la transcription puis de la traduction des gènes aboutissant à la formation de chaînes polypeptidiques constituées d'un assortiment de 20 acides aminés. L'explosion de différents programmes de séquençages de génomes a permis de définir l'ensemble des chaînes polypeptidiques (ou protéines) susceptible d'être synthétisé par un organisme. Le fait de disposer de ce répertoire de gènes ne permet de définir ni l'identité, ni le niveau d'expression des gènes associés au type et/ou à l'environnement cellulaire. En outre, les chaînes polypeptidiques peuvent subir des modifications post-traductionnelles tels que la glycosylation ou le clivage protéolytique, modi-

fications non détectables par la seule analyse du génome ou des transcrits des gènes (transcriptome).

La protéomique est l'étude de l'ensemble des protéines codées par le génome (protéome) (1). Contrairement au génome, qui reste constant dans les cellules d'un même organisme et au cours de la vie d'une cellule, le protéome subit des modifications permanentes en réponse aux différentes conditions environnementales (2). Un organisme renferme donc un seul génome, mais il possède une très grande diversité de protéomes. Cette différence fondamentale entre protéome et génome nécessite des techniques pour la séparation et la quantification individuelle des protéines ainsi que leurs identifications. La protéomique offre la possibilité de caractériser le répertoire des protéines exprimées, ainsi que leurs régulations au cours de divers processus cellulaires comme la différenciation, l'infection cellulaire ou l'action d'agents pharmacologiques (3).

On distingue classiquement deux types d'analyse protéomique, d'une part l'approche systématique qui consiste à faire l'inventaire des protéines présentes à un moment donné, dans un compartiment cellulaire donné, et d'autre part l'approche pragmatique dont l'objectif est de déterminer les modifications d'expression protéique (qualitatives et quantitatives) suite à des variations environnementales (ex. stress, effets de médicaments, infection). Les deux approches sont complémentaires et permettent d'obtenir une vision globale de la physiologie de la cellule.

Cette revue décrit différentes approches techniques protéomiques classiques, en particulier l'électrophorèse bidimensionnelle (2-D), et des systèmes d'identification protéique par spectrométrie de masse (SM). Quelques exemples sont présentés pour illustrer les différents champs d'applications, notamment dans l'étude des infections parasitaires.

L'ANALYSE PROTÉOMIQUE PAR ÉLECTROPHORÈSE BI-DIMENSIONNELLE

L'analyse protéomique (Fig. 1) peut être subdivisée en 4 grandes étapes : (i) la préparation et le traitement de l'échantillon, (ii) la séparation des protéines classiquement en utilisant l'électrophorèse 2-D, (iii) la détection/coloration des protéines, et (iv) leur identification ou celle de leurs modifications post-traductionnelles par SM.

Préparation de l'échantillon

Les modalités de préparation de l'échantillon conditionnent la réussite d'une analyse protéomique. L'objectif étant l'obtention de protéines individualisées après séparation par électrophorèse 2-D pour une identification en SM, une destruction des cellules, des tissus ou organismes doit être réalisée tout en préservant l'intégrité des protéines, et leur représentativité biologique. Or les protéines cellulaires diffèrent par leur solubilité en relation avec leurs caractéristiques physico-chimiques. Les protéines cytoplasmiques sont généralement solubles dans l'eau, tandis que les protéines nucléaires et membranaires sont souvent extrêmement hydrophobes et insolubles en milieu aqueux (4, 5). Ces différences

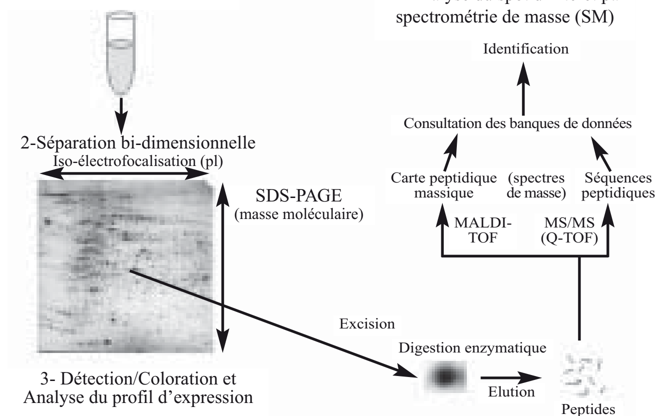

Figure 1 - Représentation schématique des différentes étapes de l'analyse protéomique par électrophorèse bi-dimensionnelle couplée à la spectrométrie de masse (SM). L'échantillon est homogénéisé avant de séparer le mélange de protéines en fonction de leur point isoélectrique (pI), puis de leur masse moléculaire. Une détection des spots est réalisée par une coloration (e.g nitrate d'argent), l'analyse du gel par un logiciel dédié permet la sélection des spots d'intérêts. Ces derniers sont excisés, digérés par une enzyme (e.g trypsine), dont l'éluât est analysé par spectrométrie de masse. La confrontation des résultats obtenus par MALDI-TOF (carte peptidique massique) ou MS/MS (séquençage peptidique) avec les banques de données permet l'identification de la protéine d'intérêt et/ou de ses modifications post-traductionnelles.

de solubilité biaisent la représentativité des protéines analysées : les protéines faiblement solubles sont sous représentées en électrophorèse 2-D (6). L'absence de méthodes universelles d'extraction des protéines implique une optimisation des conditions de préparation des échantillons selon le type d'échantillon, mais également selon la question biologique posée.

La solution de solubilisation est composée généralement d'un agent chaotrope (agent biochimique désorganisant la structure protéique), d'un détergeant non ionique ou zwitterionique, d'un agent réducteur (prévient l'oxydation des groupements SH (thiol) et rompt les ponts disulfures), et d'un mélange d'ampholytes (composé chimique pouvant jouer le rôle d'un acide ou d'une base) qui facilitent la séparation des protéines lors de l'isoélectrofocalisation (séparation des protéines en fonction de leur point isoélectrique) (Tableau I) (7, 8). Afin de minimiser les risques de dégradation protéique lors de la préparation de l'échantillon, un mélange d'inhibiteurs de protéases est couramment inclus dans la solution de solubilisation.

Aucune préparation des échantillons ne permet à l'électrophorèse 2-D de séparer et visualiser sur un seul gel l'ensemble des protéines exprimées (9). Les biais sont principalement liés à la grande hétérogénéité des propriétés physico-chimiques des protéines issues des différents compartiments cellulaires, et à la dynamique de l'expression des protéines dans la cellule. Les différences d'expression entre les protéines les moins abondantes tels que les récepteurs

Tableau I - Réactifs couramment utilisés pour la préparation des échantillons pour l'électrophorèse 2-D

	Réactifs	Propriétés
Agents chaotropes	Urée (5-9 M) (<37°C) Urée (7M)/Thiourée (2 M)	↘ Liaisons hydrogènes ↘ Interactions hydrophobes
Détergents (1 - 4% w/v)		↘ Interactions hydrophobes Solubilisation des protéines sans modifier leur pI
Non ioniques	NP-40 Triton X100 Dodecyl maltoside	
Zwitterioniques	CHAPS / CHAPSO Sulfobetaines (SB 3-10; ASB 14) Octylglucoside	
Agents réducteurs (0.5 - 1% w/v)	Dithiothreitol (DTT) Dithioerythritol (DTE) Tributylphosphine (TBP) (2mM) Tris (2-carboxyethyl)phosphine (TCEP)	Réduction des ponts disulfures (cystéines)
Carrier Ampholytes (2% v/v)	Ampholynes	↘ Précipitation ↗ Migration
Inhibiteurs de protéases	APMSF EDTA Leupeptine PMSF Bestatine	↘ Sérines protéases ↘ Métalloprotéases ↘ Sérine et thiol protéase ↘ Serine protéase ↘ aminopeptidase

↘ diminution, ↗ augmentation.

(< 100 copies par cellule) et celles exprimées à des taux importants comme les protéines de structures (10^5 à 10^6 copies par cellules) rendent illusoire la détection des protéines minoritaires dans un extrait cellulaire total. Enfin, le mélange extrêmement complexe de protéines présentes dans une cellule de mammifère (environ 100 000 protéines différentes) (9), est incompatible avec le nombre de protéines distinguables sur un gel 2-D (généralement 2 000 protéines, et pouvant atteindre dans certains cas 5000 protéines) (7).

Pour pallier ces limites, l'analyse de sous-protéomes permet de diminuer la complexité protéique d'un échantillon ou d'enrichir les formes protéiques minoritaires (9-11). Les techniques les plus couramment utilisées sont : (i) l'isolement de compartiments ou d'organelles cellulaires par centrifugations successives avec ou sans gradients de densité, (ii) le fractionnement de l'échantillon à l'aide de colonnes de chromatographie, (iii) l'isolement d'un groupe de cellules spécifiques par cytométrie de flux, (iv) la microdissection d'un tissu à l'aide d'un laser (12). Un exemple de réalisation de sous-protéomes de gels 2-D (fraction membranaire et fraction cytoplasmique) par rapport au même échantillon non fractionné d'une lignée cellulaire humaine est illustré sur la figure 2A.

L'électrophorèse bidimensionnelle (2-D)

L'électrophorèse 2-D sépare d'abord les protéines selon leur point isoélectrique (pI), puis selon leur masse moléculaire. Les protéines sont des composés amphotères, c'est à dire chargés positivement et négativement. Le pI d'une protéine correspond au pH pour lequel les charges positives compensent les charges négatives.

La première description de la séparation de protéines dans une première dimension selon leur pI, et dans une seconde dimension selon leur masse moléculaire remonte au milieu des années 70 par O'Farrell (13). Cette technique a révolutionné l'analyse des protéines puisqu'elle permet de séparer un nombre important de protéines (forte résolution) tout en bénéficiant d'une bonne sensibilité (quantification des protéines). Cependant, la séparation sur la première dimension posait des problèmes de reproductibilité rendant difficile la comparaison de résultats entre laboratoires (7).

L'introduction des gradients de pH immobilisés (IPG) au début des années 80, a permis de résoudre ces problèmes de reproductibilité (14). Ces IPGs se présentent sous la forme de bandelettes d'acrylamide contenant des immobilines déshydratées pré-coulées sur un film plastique de 3-4 mm de large et de 7 à 24 cm de long. Les gradients peuvent couvrir de larges gammes de pH (ex. pH 3 à 10) ou des gradients de pH étroits (1 unité de pH ou moins) conférant une résolution élevée (15, 16). Des protéines de masses moléculaires proches ou identiques, parfaitement indistinguables en électrophorèse SDS-PAGE (une dimension, 1D), peuvent être séparés en fonction de leur pI. Ces propriétés font de l'électrophorèse 2-D, la technique de choix pour l'étude des modifications post-traductionnelles (17, 18). Les IPGs prêtes à l'emploi ont «démocratisé» les analyses protéomiques, aboutissant à une simplification de manipulation, et à une reproductibilité accrue inter-gels permettant

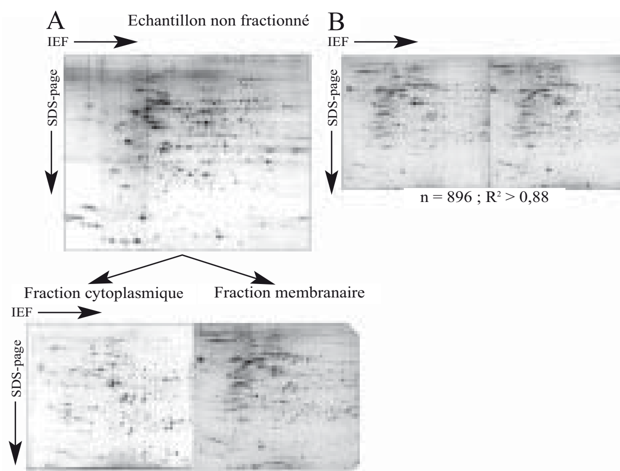

Figure 2 - Gel bidimensionnel de lignées cellulaires humaines en cultures. (A) Modification du profil protéique selon que l'on s'intéresse à l'ensemble des protéines cellulaires (en haut), ou à ses sous-protéomes membranaires ou cytoplasmiques (en bas). Détection d'un nombre plus important (non détectable sur la fraction totale) de spots protéiques grâce au fractionnement cellulaire. (B) Comparaison de deux électrophorèses 2-D réalisées à partir de culture de la même lignée cellulaire à 1 semaine d'intervalle dans les mêmes conditions. L'analyse à l'aide d'un logiciel dédié (PDQuest 7.2, Biorad), permet d'évaluer la reproductibilité entre ces deux gels (R : coefficient de corrélation ; n : nombre de spots pris en compte pour l'analyse).

une standardisation des protocoles entre laboratoires et des analyses comparatives (Fig. 2B).

Malgré ces développements techniques, les protéines de pI < 3 ou > 9, et/ou de masses moléculaires < 10 kDa ou > 150 kDa, ne sont généralement pas détectables en gels 2-D classiques (19). Or, les prédictions bioinformatiques des cartes protéomiques faites à partir de l'analyse des séquences des gènes estiment que jusqu'à 20 % des protéines exprimées dans un organisme possèderaient un pI supérieur à 10 (20). Des stratégies ont été développées par différentes équipes pour élargir la fenêtre d'analyse en pI (21-23), sans pour autant permettre la détection de l'ensemble des protéines de pI extrêmes. Inversement, quelques modifications mineures de la seconde dimension, comme l'augmentation de la réticulation du gel, et le changement d'un tampon de migration permettent de mieux séparer les protéines de faibles masses moléculaires (jusqu'à 1 kDa) (24). Pour les protéines de hauts poids moléculaires, le problème est différent car il ne provient pas de la séparation en SDS-PAGE, mais principalement des tampons de solubilisation des échantillons. Ces derniers, ayant des forces ioniques faibles et utilisant des détergents non chargés, rendent particulièrement difficiles l'extraction et le maintien en solution de ces protéines. Les protéines à plusieurs domaines transmembranaires cumulent souvent ces différents facteurs limitant leur analyse (masse moléculaire élevé, pI > 9, solubilité faible et nombre de copies par cellule peu important) (25).

Des stratégies alternatives à l'électrophorèse 2-D, s'affranchissant partiellement de certaines de ces limitations technologiques sont présentées dans la partie consacrée aux nouvelles approches d'analyse protéomique.

Détection/Coloration et systèmes d'analyses de gels 2-D

Plusieurs méthodes existent pour la détection des protéines après une électrophorèse 2-D (26, 27). Les plus couramment utilisées sont basées sur l'emploi de colorants (bleu de Coomassie R-250 ou G-250), de métaux lourds (nitrate d'argent), de radioéléments (^{3}H, ^{14}C, ^{35}S, ou ^{32}P), ou de sondes fluorescentes (SYPRO Ruby, SYPRO Orange, Deep purple, etc...). Ces méthodes de détection diffèrent en termes de sensibilité, de gamme dynamique linéaire (GDL) (ratio d'intensité sur lequel on peut faire une quantification linéaire), de coût, et surtout de compatibilité avec la SM (28). Un résumé des propriétés de ces principales méthodes de détection est présenté dans le tableau II. Ces méthodes colorent l'ensemble des protéines présentes sur un gel 2-D correspondant à des systèmes de détection universelle.

D'autres méthodes détectent uniquement une partie du protéome présent sur le gel 2-D correspondant à des systèmes de détection spécifique. Par exemple, des systèmes de détection spécifique fluorescente ont été développés pour l'analyse des modifications post-traductionnelles (29, 30). La caractérisation des modifications post-traductionnelles est un des enjeux majeurs pour la compréhension de nombreux mécanismes de régulation cellulaire. L'étude de l'ensemble des protéines phosphorylées ou glycosylées est par conséquent en plein essor, et l'on parle respectivement d'analyses du phosphoprotéome et du glycoprotéome (18, 31). Récemment, des systèmes de détection spécifique fluorescente ont été développés pour l'analyse du phosphoprotéome et du glycoprotéome par l'utilisation respectivement du Pro-Q Diamod et du Pro-Q Emerald (29, 30). L'immunoprotéomique est une autre façon de détecter spécifiquement des protéines antigéniques transférées sur membrane de nitrocellulose et révélées à l'aide d'anticorps. Ce type d'approche peut permettre la caractérisation de marqueurs physiopathologiques ou d'évolution d'une pathologie (32).

L'idéal est de disposer d'une technique de détection la plus sensible et la plus linéaire possible, permettant à la fois de consommer le moins d'échantillon biologique possible et de pouvoir réaliser une analyse quantitative aussi précise que possible. En pratique, il s'agit de choisir le meilleur compromis pour l'objectif fixé.

Ces analyses qualitatives et quantitatives sont réalisées à l'aide de logiciels dédiés à l'étude comparative des gels 2-D. Plusieurs logiciels sont disponibles et la comparaison de leurs capacités analytiques a fait l'objet de quelques publications (33-35). Brièvement, les logiciels réalisent successivement à partir des images numérisées, une détection automatique des spots protéiques, leur quantification puis une comparaison des profils protéiques des gels 2-D dans des conditions identiques (tests de reproductibilité, figure 2B), et entre différentes conditions expérimentales (protéines associées aux modifications environnementales). Ces logiciels réalisent des analyses quantitatives

Tableau II - Propriétés des méthodes courantes de détection des protéines sur gel 2-D :

Type de marquage	Sensibilité	Gamme dynamique linéaire (GLD)	Système de détection	Compatibilité avec la SM	Durée / nombre d'étapes	Coût	Références
Bleu de Coomassie R-250	10-25 ng	10^2	Scanner densitométrique	+ + + +	2,5 h/2	+	(65)
Bleu de Coomassie G-250	5-10 ng	10^2	Scanner densitométrique	+ + + +	2,5 h/5	+	(65)
Nitrate d'argent	0,5-1 ng	$< 10^2$	Scanner densitométrique	+ +*	3h/7	+ +	(36, 66, 67)
Méthionine ^{35}S	0,1 ng	$> 10^3$	Film autoradiographique, phosphor imager	+ + + +**	Variable# / 3	+ + +	(68-70)
SYPRO Ruby	0,5-1 ng	$> 10^3$	UV-transilluminateur, caméra CCD, scanner laser	+ + +	3h / 2	+ + + +	(26, 71-73)

* Uniquement en l'absence de glutaraldéhyde, son utilisation lors de la coloration rend le système complètement incompatible avec la SM.
** Rarement utilisé en SM dû à la présence de radioactivité.
# De quelques heures à plusieurs jours.
+ : faible, + + : intermédiaire, + + + : élevé, + + + + : très élevé.
SM : spectrométrie de masse.

précises (de l'ordre de ± 10%, figure 2B), ce qui permet de considérer des variations faibles de quantités de protéines (de l'ordre d'une facteur 2) comme statistiquement significatives ($p<0.05$). Les spots protéiques retenus par l'analyse statistique sont généralement sélectionnés pour être identifiés par SM.

Spectrométrie de masse (SM)

L'ultime étape de l'analyse protéomique est l'identification des spots protéiques d'intérêt et/ou de leurs modifications post-traductionnelles. Cette identification protéique a longtemps été réalisée par le microséquençage chimique

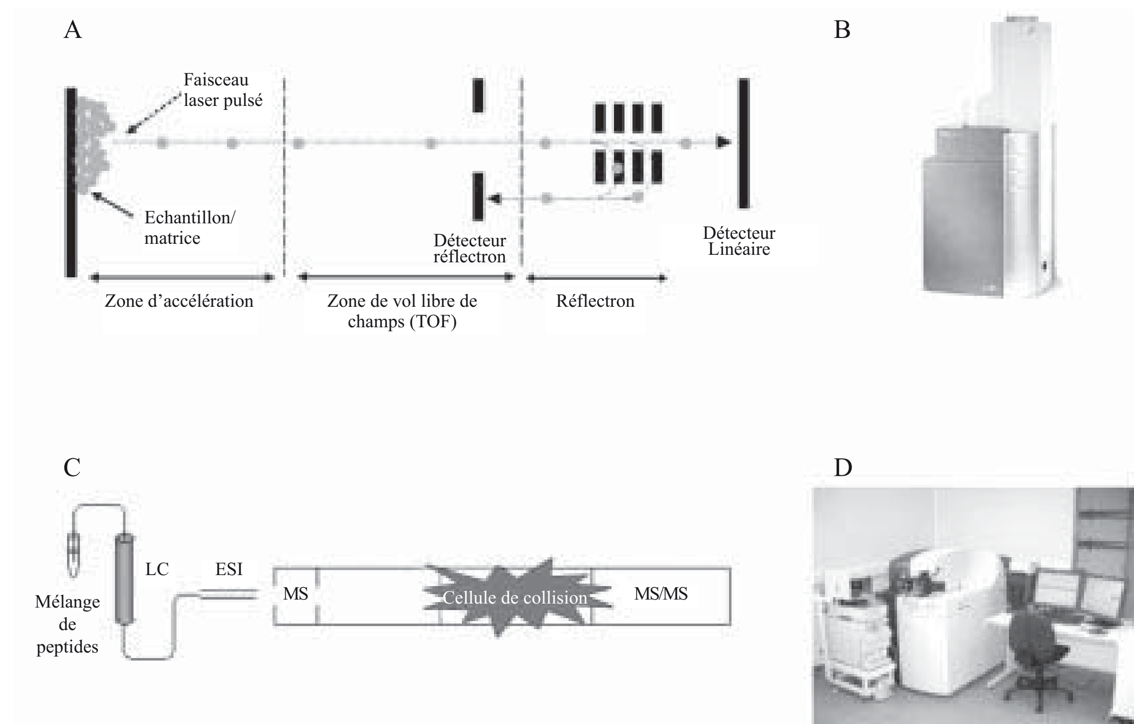

Figure 3 - Spectromètres de masse : MALDI-TOF et Q-TOF. (A) Représentation schématique d'un MALDI-TOF. L'échantillon peptidique ionisé par le laser est accéléré puis séparé dans la zone libre de champs avant d'atteindre le détecteur. Ce dernier couplé à l'enregistreur permet d'obtenir une carte peptidique massique spécifique du composé. (B) Photographie de l'Ettan pro MALDI-TOF de chez Amersham biosciences disponible sur la plate-forme protéomique de la Faculté de Pharmacie de Maseille. (C) Représentation schématique d'un Q-TOF. Les peptides sont injectés par électrospray ionisation (ESI) dans le spectromètre de masse après leur séparation par chromatographie liquide (LC). Les peptides entrent dans un premier spectre de masse (MS) sélectionnant les peptides qui seront séquencés dans le second SM (MS/MS) après avoir été fragmentés dans la cellule de collision. (D) Photographie d'un Q-TOF de chez Micromass disponible au Centre d'Analyse Protéomique de Marseille (CAPM) situé au niveau de la Faculté de Médecine Nord de Marseille.

(chimie récurrente d'Edman). Aujourd'hui, les techniques de SM, beaucoup plus sensibles et rapides, ont remplacé cette approche. Préalablement à l'analyse par SM, les spots protéiques d'intérêt, sont excisés du gel, soumis à une digestion par une protéase (généralement de la trypsine), précédant l'élution des peptides générés (Fig. 1). Selon le type de coloration utilisée et l'appareil de SM choisi pour l'identification de la protéine, la méthode de préparation de l'échantillon varie sensiblement (36).

Un SM est un appareil qui permet de mesurer le rapport masse/charge (m/z) des ions formés à partir de l'échantillon à analyser. Il est généralement constitué des parties suivantes : (i) une source d'ions dans laquelle se produit le passage en phase gazeuse (vaporisation/sublimation/désorption) et l'ionisation des molécules à analyser (source MALDI ou ESI), (ii) un analyseur qui sépare les ions selon leur rapport masse/charge (ex. TOF, quadripôle), (iii) un détecteur qui donne l'intensité des ions en leur associant leur rapport m/z, (iv) et un enregistreur pour le traitement du signal et la visualisation des spectres.

Il existe plusieurs types de spectromètres de masse utilisables pour l'identification protéique. Cette partie présente les principes généraux de deux appareils : le MALDI-TOF et l'ESI-Q-TOF MS/MS.

MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time-of-Flight)

Dans le MALDI, l'échantillon est mélangé avec une matrice (ex. α-cyano-4-hydroxycinnamic acid, (37)) favorisant la désorption et l'ionisation des peptides. Le MALDI-TOF peut fonctionner soit en mode linéaire soit en mode réflectron. Un aperçu du fonctionnement de l'appareil est illustré sur la figure 3A. Brièvement, un faisceau laser pulsé ionise l'échantillon. Ce dernier est accéléré vers l'analyseur par l'application d'un champ électrique. Dans la seconde zone se trouve un tube de vol (TOF pour Time-Of-Flight) libre de champs, où les ions se séparent en fonction de la vitesse acquise dans la zone d'accélération jusqu'à atteindre le détecteur. Ce dernier couplé à l'enregistreur permet d'obtenir un spectre (carte peptidique massique) de la distribution des masses des fragments peptidiques obtenus par la digestion trypsique. La trypsine coupe les protéines après les résidus lysine ou arginine. Les cartes peptidiques massiques sont spécifiques de chaque protéine, et constituent une véritable empreinte digitale d'où le nom de «peptide mass fingerprinting» (PMF) (38, 39). L'identification de la protéine s'effectue en comparant la carte peptidique massique obtenue expérimentalement avec celles résultant du clivage théorique de l'ensemble des protéines dont la séquence est connue dans les banques de données (ex. SwissProt, TrEmbl, MSDB). Plusieurs programmes d'analyse de carte peptidique massique sont disponibles sur internet pour cette identification (Mascot : http://www.matrixscience.com, ProFound : http://bioinformatics.genomicsolutions.com/prowl/profound/profound_E_adv.htlm) (40, 41). Si l'information requise pour l'identification de la protéine est absente de la banque de données (ex. séquençage incomplet du génome), ou si l'échantillon à analyser est composé d'un mélange de protéines (ex. une bande protéique d'un gel SDS-PAGE), les identifications ne peuvent pas être effectuées par cette méthode. On préfèrera alors utiliser un spectromètre de masse en mode tandem de type Q-TOF.

Le MALDI-TOF, généralement dédié à l'identification de protéines séparées par gel 2-D, présente de nombreux avantages : il allie la sensibilité (500 femtomoles), (42) à la rapidité d'analyse (quelques minutes par analyse), et peut être automatisé permettant des analyses à hauts débits (> 1000 échantillons par jour) (43).

ESI Q-TOF MS/MS (electrospray ionization quadripole time-of-flight mass spectrometry in tandem)

Le séquençage peptidique par SM en tandem est basé sur le clivage des liaisons entre les résidus d'acides aminés. Un aperçu du fonctionnement de l'appareil est illustré sur la figure 3C. L'electrospray ionisation nécessite la génération d'ions peptidiques à partir d'une solution aqueuse (44). Le mélange peptidique en solution est séparé sur une colonne de chromatographie avant de passer au travers d'une aiguille soumise à un très haut voltage, puis le solvant est éliminé par un gaz inerte ou chauffage. Les ions formés entrent dans le premier analyseur. Parmi ces ions, un ion précurseur est sélectionné, et seuls les ions ayant un rapport m/z sélectionné entrent dans une zone libre de champs, où est placée une cellule de collision. A ce niveau, les ions précurseurs entrent en collision avec des molécules de gaz (*e.g* argon), qui provoque leur fragmentation. Les fragments pénètrent dans un second analyseur permettant d'obtenir des informations sur la séquence ou les modifications post-traductionnelles de la protéine. L'identification est obtenue par l'utilisation de banques de données protéiques et génomiques corrélée avec les données obtenues du spectre de masse (ex. Mascot). En biologie, ce type d'expérience est notamment utilisé pour déterminer la séquence de peptides, ou pour rechercher des modifications ou des mutations. Les analyses en *electrospay* avec la configuration Q-TOF (Quadripole-time-of-flight) correspondent à la configuration MS/MS classique. Le premier analyseur est un quadripôle (Q), et le second est un temps de vol (TOF).

NOUVELLES APPROCHES D'ANALYSE PROTÉOMIQUE

L'objectif de ces nouvelles approches est de réaliser des analyses protéomiques à haut débit et haute sensibilité à partir de mélanges plus ou moins complexes de protéines, en s'exonérant de la réalisation de gels 2-D.

Dans cette optique, Washburn et ses collaborateurs (45) ont développé une technique fondée sur la séparation des protéines par chromatographie à deux dimensions (deux colonnes en séries avec des phases différentes) couplée directement à un appareil de spectrométrie de masse. Dans cette approche, un échantillon complexe est digéré par une enzyme, puis séparé par chromatographie liquide (généralement utilisation de colonnes à phase inverse puis échan-

geuses d'ions, en série) pour réduire la complexité du mélange de peptides délivrés simultanément au SM. Les peptides séparés selon leur rapport m/z sont séquencés et les informations de séquence sont utilisées pour rechercher l'identité de la protéine dans les banques de données. Cette technique présente l'avantage de pouvoir identifier un nombre important de protéines en une seule analyse, mais également des protéines de masse moléculaire et de pI extrêmes ainsi que des protéines membranaires (45, 46). Cette technique fut nommée MudPIT pour *multidimensional protein identification technology.* Malgré ses performances impressionnantes, la technique MudPIT ne détecte qu'une partie des protéines visualisées par l'approche classique d'électrophorèse 2-D couplé à la SM (47). De plus, cette technique ne permet pas d'évaluer les quantités relatives des différentes protéines.

Afin de comparer les quantités relatives de protéines, une approche basée sur l'utilisation d'isotopes stables fut développée (nommée ICAT pour *isotope-code affinity tag method*) (48). Cette technique est utilisable pour l'étude quantitative des protéines membranaires (49).

Enfin, Hutchens et Yip (50) ont développé un système composé de différentes matrices de puce, qui exploite les caractéristiques biophysiques et chromatographiques des différentes protéines pour leur sélection préférentielle. Cette technique manufacturée par Ciphergen (Fremont, Californie, USA) nommée SELDI (surface-enhanced laser desorption/ionization), ne nécessite aucune digestion enzymatique préalable de l'échantillon, et est principalement utilisée pour des comparaisons de profils d'expression protéique (51). Brièvement, le principe consiste à déposer un échantillon complexe sur un support chromatographique (puce), puis à l'éluer partiellement pour en réduire la complexité. Les protéines fixées sont alors analysées dans un SM. Sa principale limite est l'obtention uniquement de la masse moléculaire de la protéine, aucune information sur son identité n'est disponible. Cette technique est généralement utilisée pour la recherche de biomarqueurs (52).

APPLICATIONS

Pour compléter cette revue, quelques exemples d'utilisation de l'outil protéomique appliqués à l'étude des infections parasitaires sont présentés. Jusqu'à récemment, les études protéomiques parasitaires étaient limitées par l'absence du séquençage de leur génome. Les programmes de séquençage génomique de nombreux parasites, tel que le protozoaire *Leishmania major* ou *Plasmodium falciparum* rendent de telles études réalisables (53). Il est ainsi possible par une approche protéomique de s'intéresser aux effets d'agents pharmacologiques et le cas échéant aux mécanismes de résistance du parasite, mais également de définir des marqueurs immunologiques induits par l'infection parasitaire pouvant servir à terme au développement de tests diagnostiques ou de cibles thérapeutiques ou vaccinales.

La leishmaniose est une pathologie induite par un parasite protozoaire du genre *Leishmania* dont l'évolution clinique peut être fatale, notamment dans les formes viscérales (54). Bien que des traitements existent, des phénomènes de résistance sont couramment décrits (55). Une comparaison quantitative du protéome d'une souche de *L. major* sensible vs. résistante au traitement par des antifolates (méthotrexate), a permis à Drummelsmith *et Coll* (56) d'identifier une nouvelle cible de ce médicament (la ptéridine réductase PTR1). Une mutation du gène ptr1 induit une surexpression de la protéine responsable du phénomène de résistance. L'intérêt d'une telle identification permet d'envisager le dépistage des souches résistances et le développement d'agents pharmacologiques plus efficaces.

Les études protéomiques sont particulièrement intéressantes dans l'étude de corrélations entre protéome et phénotype parasitaire comme par exemple la virulence. Il a été décrit des infections par *Entamoeba histolytica* chez des sujets ne présentant aucun signe de colite amibienne ou d'amibiase hépatique (57, 58). Des analyses comparatives par gels 2-D, du protéome de deux souches *d'Entamoeba histolytica* différant par leur virulence, on été réalisées par Davis *et Coll*, (59). Cette étude associe la virulence parasitaire à la dérégulation de l'expression de six protéines, dont deux d'entres elles présentent des propriétés anti-oxydantes. Ce type d'approche permet ainsi d'identifier des marqueurs biologiques de virulence d'infections parasitaires.

Le paludisme à *Plasmodium facilparum* est responsable du décès de millions de personnes chaque année (60). En l'absence de vaccins efficaces, la chimiothérapie reste le principal moyen de contrôle de la maladie. Cependant, l'apparition de résistances vis-à-vis des traitements anti-paludiques nécessite le développement de nouvelles molécules ou combinaisons de molécules anti-paludiques (61). Récemment, une équipe anglaise (62) s'est intéressée à déterminer les cibles d'un nouveau médicament, le CoArtem (association d'un dérivé de l'artemisinine et de la luméfantrine). Dans cette optique, ils ont comparé les protéomes de *P. falciparum* traité par l'un ou l'autre des anti-paludiques contenus dans le CoArtem. Ils ont pu mettre en évidence une action antagoniste de ces molécules sur les enzymes de la glycolyse (métabolisme énergétique) du parasite, mais une action similaire sur plusieurs protéines de choc thermique. Ce travail a disséqué quelques uns des effets de ces molécules sur le protéome de *P. falciparum* permettant une meilleure compréhension de leur mécanisme d'action.

La protéomique peut également être utilisée pour l'identification de protéines d'agents infectieux pouvant induire une réponse immunitaire. *Chlamydia trachomatis* est une bactérie sexuellement transmissible dont le site d'infection privilégié est le tractus urogénital (63). Afin d'identifier des protéines antigéniques de *Chlamydia trachomatis,* Sanchez-Campillo *et Coll*, ont analysé les profils de réactivité immunologique par Western blot 2-D, de sujets infectés par cette bactérie (64). Malgré une hétérogénéité de reconnaissance antigénique entre les patients, cette approche immuno-protéomique, leur a permis d'identifier une protéine antigénique commune (outer membrane protein-2, OMP2) qui pourrait servir de marqueur biologique d'infection pour le développement de tests diagnostics. Les auteurs évoquent également que certaines des protéines antigéniques identifiées

(les protéines ribosomales L7/L12 et la protéase induite par le stress HtrA) pourraient être des cibles de choix pour des stratégies vaccinales.

CONCLUSION

Les nombreux progrès technologiques réalisés ces 20 dernières années, aussi bien dans la préparation et l'analyse de gels bidimensionnels de protéines, que dans les analyses par SM ont favorisé l'émergence de la protéomique. Malgré les limitations de l'électrophorèse 2-D évoquées précédemment, cette technique est capable de séparer simultanément un mélange complexe de protéines, permet des analyses comparatives qualitatives et quantitatives d'expressions protéiques, et permet l'étude de leurs modifications post-traductionnelles. L'ensemble de ces propriétés fait de l'électrophorèse 2-D un outil de choix toujours compétitif pour l'analyse de protéomes.

L'explosion des programmes de séquençages des génomes de nombreux organismes, notamment ceux de parasites et de leurs vecteurs, a permis d'envisager le développement d'approches protéomiques lors d'infections parasitaires. Comme cela est présenté ci-dessus, l'analyse protéomique peut être utilisée dans de très nombreux domaines, tant au niveau d'études fondamentales qu'au niveau d'études appliquées.

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ARTICLE N.10

Atorvastatin is 10-fold more active in vitro than other statins against *Plasmodium falciparum*

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Atorvastatin Is 10-Fold More Active In Vitro than Other Statins against *Plasmodium falciparum* ▽

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, influence a broad array of pathogenic microorganisms. Lovastatin reduces the intracellular growth of *Salmonella enterica* serovar Typhimurium in cultured macrophages, while atorvastatin does the same in a mouse model (2). Lovastatin additionally reduces the growth of *Candida albicans* by inhibiting the sterol pathway (11). Statins interfere severely with the growth of protozoan parasites of the *Trypanosomatidae* family such as *Trypanosoma cruzi* and various *Leishmania* species (8, 12, 13). HMG-CoA reductase has been detected in *Trypanosoma* and *Leishmania* (3, 8). The presence of an HMG-CoA homolog was not revealed by BLASTX analysis of the *Plasmodium falciparum* sequence with other protozoal HMG-CoA protein sequences. However, as reported previously, treatment in vitro of *Plasmodium falciparum* with 120 or 240 μM mevastatin inhibited parasite growth (4, 9).

The susceptibilities to simvastatin, simvastatin sodium salt, pravastatin sodium salt, lovastatin, fluvastatin sodium salt, mevastatin, mevastatin sodium salt (Calbiochem, Merck, Germany), and atorvastatin calcium salt (Molekula, United Kingdom) were assessed in vitro against chloroquine-susceptible *P. falciparum* strains 3D7 (Africa), D6 (Sierra Leone), and IMT031 (Gabon) and chloroquine-resistant strains W2 (Indochina), Bre1 (Brazil), and FCR3 (The Gambia). Lovastatin and mevastatin were converted to the active form by dissolving the lactone form in 100 μl of 100% ethanol, adding 200 μl of 0.2 M KOH, and then adding 0.2 M HCl for neutralization to pH 7.2 (5). Simvastatin, simvastatin sodium salt, pravastatin sodium salt, lovastatin, fluvastatin sodium salt, mevastatin, mevastatin sodium salt, and atorvastatin calcium salt were dissolved in dimethyl sulfoxide 1% (vol/vol) in RPMI. Twofold serial dilutions, with final concentrations ranging from 1.5 μM to 200 μM, were prepared in dimethyl sulfoxide 1% in RPMI and distributed into Falcon 96-well plates just before use. The isotopic microdrug susceptibility test used was described previously (10).

Table 1 presents the 50% inhibitory concentrations (IC_{50}) of the different statins for *P. falciparum*. Simvastatin, fluvastatin, lovastatin, and atorvastatin, in the salt active forms, are more active than simvastatin, mevastatin, and lovastatin, in the lactone form. Pravastatin and mevastatin sodium or potassium salts are inactive against *P. falciparum* (>200 μM). The results indicate that susceptibility to the salts of simvastatin, fluvastatin, lovastatin, and mevastatin is not dependent on the status of chloroquine resistance. The results observed with the simvastatin salt were similar to those reported by other authors (5). Atorvastatin salt, in the range of 5 to 12 μM, is 10-fold more active against *P. falciparum* than the other salts. Atorvastatin IC_{90}s ranged from 14.8 to 39 μM. The activity of atorvastatin is independent of the status of chloroquine resistance (4.8 to 5.8 μM against chloroquine-resistant strains versus 5.3 to 11.8 μM for the susceptible strains).

The chemical structures of simvastatin, lovastatin, mevastatin, and pravastatin are closely related. Those of fluvastatin and atorvastatin are very different from the others. The structural differences between atorvastatin and the other statins could explain differential activity. However, we cannot rule out the action of calcium in the differential activity of atorvastatin.

Multiple daily doses of 2.5 to 80 mg of atorvastatin produced steady-state maximum plasma concentrations of 1.95 to 252 μg $liter^{-1}$ (in the range of 0.2 to 0.3 μM for the maximum) (1). In L6 cells (rat skeletal muscle cell line), atorvastatin at 100 μM induced death in 27% of the cells (7). Although the atorvastatin IC_{50} for *P. falciparum* exceeds these reported plasma concentrations, it may be below toxic concentrations.

Parasites treated with mevastatin show depressed biosynthesis of dolichol and isoprenoid pyrophosphate (4). In addition, mevastatin decreases the viability of cells by inhibiting proteasome activity. Atorvastatin is an inhibitor for phosphoglyco-

TABLE 1. In vitro activities of statins against chloroquine-susceptible (3D7, D6, and IMT031) and chloroquine-resistant (W2, Bre1, and FCR3) *P. falciparum* strains

Drug	Mean IC_{50} in μM against indicated *P. falciparum* strains (95% confidence interval)[a]					
	3D7 (Africa)	D6 (Sierra Leone)	IMT031 (Gabon)	W2 (Indochina)	Bre1 (Brazil)	FCR3 (The Gambia)
Simvastatin	>200	>200	>200	>200	>200	>200
Simvastatin sodium salt	69.8 (61.5–79.2)	51.3 (42.1–62.6)	36.9 (30.3–45.0)	58.9 (52.8–65.6)	48.4 (35.3–66.4)	76.7 (71.7–82.9)
Lovastatin	>200	>200	>200	>200	>200	>200
Lovastatin potassium salt	95.3 (85.9–105.7)	44.3 (32.3–60.7)	57.8 (39.8–84.1)	103.0 (98.4–107.3)	52.6 (38.2–72.5)	43.9 (27.9–68.9)
Fluvastatin sodium salt	107.9 (94.7–123.0)	92.3 (85.4–99.6)	87.1 (76.4–99.3)	90.2 (83.5–97.4)	92.5 (77.2–110.8)	115.9 (104.0–129.1)
Mevastatin	>200	>200	>200	>200	>200	>200
Mevastatin sodium salt	>200	>200	>200	>200	102.1 (85.2–122.3)	>200
Mevastatin potassium salt	>200	>200	>200	88.5 (81.2–96.4)	>200	>200
Pravastatin sodium salt	>200	>200	>200	>200	>200	>200
Atorvastatin calcium salt	11.8 (9.9–14.0)	5.8 (5.2–6.5)	5.3 (4.1–6.8)	4.8 (4.4–5.1)	5.8 (5.1–6.6)	5.1 (4.3–6.0)
Atorvastatin calcium salt	39.0 (25.3–60.1)[b]	18.1 (15.1–21.7)[b]	15.8 (11.8–21.1)[b]	14.8 (10.4–20.5)[b]	24.0 (17.3–33.4)[b]	23.6 (16.2–34.3)[b]

[a] Values are means of three to eight independent experiments.
[b] Mean IC_{90} in μM (95% confidence interval).

protein, an efflux protein, and may be a substrate for this transporter as well (6). A phosphoglycoprotein in *P. falciparum*, Pgh1, is implicated in quinoline resistance.

In conclusion, the present observation suggests that atorvastatin is a good candidate for further studies on the use of statins in malaria treatment.

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