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**EXTRACTION OF RETICULOCYTES FROM CORD BLOOD FOR USE IN THE CULTURING OF *PLASMODIUM VIVAX***

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Current techniques rely on one of three methods to culture *Plasmodium vivax* (PV). The most common method of culturing the parasite is to remove PBMCs from infected blood and allow the parasites to grow in the autologous blood. This method appears to allow only continued growth and development with limited reinvasion of uninfected red blood cells. Successful culturing of PV for more than this relies upon a steady re-supply of reticulocytes into the culture media. Studies are currently underway to supply reticulocytes from a stem cell preparation which yields a very high reticulocytemia. This method however is very costly and labor intensive. Another method of supplying reticulocytes into *vivax* cultures relies on obtaining frequent blood samples from patients with hemochromatosis, where reticulocyte counts may be 3 to 10 fold higher (up to 5% reticulocytemia) than in normal blood. This blood is then spun down using a high speed centrifuge to further enrich the reticulocytemia (up to 20%). Here we describe a simple method which takes advantage of a readily available source of reticulocytes, umbilical cord blood (2-8% reticulocytes). Irrespective of endemic or non-endemic settings, expectant mothers can be found who are willing to donate this resource which would otherwise be discarded. We further enrich reticulocytes from cord blood samples by first removing PBMCs on a CF11 cellulose column, followed by a 3 min exposure to a FACS Lysing Solution available from Becton-Dickinson. Based on osmotic pressure differences, this solution lyses all cells that do not contain RNA or DNA. By this method we observed consistent enrichment of reticulocytes from an average of 5% reticulocytemia from cord blood to a solution of 25-70% reticulocytes. Addition of 20  $\mu$ L of this solution to 2 mL *P. vivax* cultures provides a substantial number of targets for the parasites to invade while avoiding significant dilution of the hematocrit away from the standard 5%. This treatment for enriching reticulocytes improves potential for *P. vivax in vitro* culture to be grown under a variety of conditions that may lead to higher parasitemia and prolonged growth and development.

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**ERYTHROCYTE GHOSTS AS A MODEL SYSTEM FOR STUDYING MALARIA INFECTION**

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The intraerythrocytic infection caused by human malaria parasites is responsible for all of the clinical symptoms and pathologies associated with this disease. However, the identification of host and parasite proteins critical for these events is hampered by the mature erythrocyte's inability to synthesize additional proteins such as those traditionally encoded on transfected plasmids. To overcome this dilemma, we developed a method of lysing and resealing mature erythrocytes to introduce purified peptidic and proteinaceous cargoes. These ghosts retain morphological and signaling properties of normal mature erythrocytes, can be infected efficiently by malaria merozoites and support robust parasite growth and reinvasion. We validated the utility of this erythrocyte ghost-based approach by testing the role of the erythrocyte guanine nucleotide regulatory protein G<sub>3</sub> in malarial infection. Studies are now underway to assess additional host and parasite proteins implicated in malarial invasion and intraerythrocytic growth. The use of ghosts for studying interactions between the host erythrocyte and the malaria parasite may help identify potential therapeutic targets that are critical for infection.

(ACMCIP Abstract)

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**THE CLEARANCE OF LIVE *PLASMODIUM FALCIPARUM*-PARASITIZED RED BLOOD CELLS BY THE HUMAN SPLEEN**

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Red blood cells which harbour *Plasmodium falciparum* (PfrBC) become progressively less deformable as the intra-erythrocytic parasite matures, leading to PfrBC retention and clearance in the spleen. On their way to the red pulp sinus lumen, the less deformable PfrBCs can no longer squeeze through small intercellular gaps between the sinusoidal endothelial cells. Although clearance of PfrBC by the human spleen plays a major role in the outcome of *P. falciparum* infections, this process has never been confirmed experimentally. Using a previously validated isolated-perfused human spleen model we studied the clearance of PfrBC harbouring early (ring stages < 16 hours) and more mature parasite stages (late trophozoites and schizonts). The kinetics of PfrBC clearance was monitored using Giemsa-stained thin blood smears and/or flow cytometry. Live mature PfrBC were retained by the human spleen faster and to a greater extent than young ring PfrBC. Histological analysis indicated that retention of PfrBC (mature and young stages) was threefold higher in the red pulp than in the perfollicular zone. Exo-erythrocytic parasite stages were detected 10 times more frequently in the red pulp than within the perfollicular zone. As opposed to what we previously observed with artesunate-treated PfrBC, RBCs harbouring live *P. falciparum* seldom underwent pitting, suggesting that the pitting process is probably not a major mechanism of live parasite clearance by the spleen. Characteristics of the perfusate (temperature, composition) that may influence PfrBC deformability and consequently their red pulp clearance are under investigation. This new approach, based on the study of isolated-perfused human spleens, should extend our comprehension of mechanisms involved in human spleen processing and clearance of PfrBC and could lead to the identification of therapeutic or vaccine targets.

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**SINGLE NUCLEOTIDE POLYMORPHISMS IN TRAP ASSOCIATE WITH SEVERE MALARIAL DISEASE: A NOVEL PARASITE VIRULENCE GENE**

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Innate parasite properties such as increased parasite multiplication rates and red cell invasion phenotypes have been associated with severe malarial disease. The hepatocyte is the first major site of parasite-host interaction, so parasite invasion properties and host immune responses to parasite surface proteins involved in hepatocyte invasion could determine the future course of the infection and thus its virulence. This study examined the hypothesis that genes crucial in hepatocyte invasion are potential virulence loci. SNPs within hepatocyte invasion genes were analysed by sequencing for differences between asymptotically infected individuals and severe malaria patients. Polymorphisms within the gene encoding the sporozoite molecule Thrombospondin Related Adhesion Protein (TRAP) revealed strong and highly significant associations with severe malarial disease in The Gambia and Kenya. Fine mapping of these associations will be presented. We conclude that being infected with parasites carrying

different TRAP mutations affect risk of developing severe disease and we have identified TRAP as a novel virulence gene.

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### SUPPRESSION OF IFN $\gamma$ T-CELL RESPONSE TO VACCINES BY MALARIA INFECTION

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There have been several reports that patent malaria infections can suppress cell-mediated immune response to vaccines. This problem needs to be critically assessed for two reasons. First, poor immunogenicity in vaccinated parasitemic volunteers in malaria endemic area could lead to incorrectly rejecting a vaccine that could potentially benefit those without parasitemia. Second, if reduced immunogenicity of vaccine in parasitemic volunteers is accompanied by reduced efficacy, vaccinating parasitemic volunteers in malaria endemic areas could lessen the vaccine effectiveness. To address the first concern, we measured the effect of malaria blood stage infection on the induction of malaria specific CD8+T cell responses after vaccination with subunit or irradiated sporozoite vaccines in mice. We also determined the effect of malaria blood stage infection on the IFN $\gamma$  T cell response induced by a dengue DNA vaccine. For all three vaccines tested, antigen specific IFN $\gamma$  responses were reduced compared to malaria-free control mice. We conclude that during immunogenicity evaluation of pre-erythrocytic stage vaccine in malaria endemic areas, malaria blood stage infection at the time of vaccine application could potentially reduce the antigen specific IFN $\gamma$  T cell responses.

(ACMCIP Abstract)

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### CLONING AND CHARACTERIZATION OF *PLASMODIUM YOELII* MACROPHAGE MIGRATION INHIBITORY FACTOR

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During severe malaria, caused by *Plasmodium falciparum*, excessive production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) contributes to fever, hypoglycemia, cerebral malaria, bone marrow depression and severe anemia. TNF- $\alpha$  production can be enhanced by Macrophage Migration Inhibitory Factor (MIF) that prevents the glucocorticoid mediated down-regulation of the proinflammatory response. Recent studies in rodent malaria models have shown a role for host MIF in development of malaria associated anemia. MIF was shown to inhibit erythroid differentiation and hemoglobin production, as reported previously. Sequencing of the *Plasmodium* genomes predicted a gene product with significant similarity to mammalian MIF (30%). This malarial *mif*-related gene encodes a predicted protein of 116 amino acids in *P. falciparum* and *P. yoelii*. We have initiated studies to evaluate the potential role of parasite-encoded MIF in the pathogenesis of malaria, using the *P. yoelii* murine model. DNA microarray analysis indicated that *pymif*-related gene is expressed during blood stage malaria. To initiate functional studies, we generated recombinant PyMIF in *Escherichia coli* BL21(DE3)pLysS. PyMIF was purified from soluble bacterial lysates by ammonium sulfate fractionation, preparative isoelectric focusing and hydrophobic interaction chromatography. Serologic studies showed that PyMIF induced a modest antibody response during a *P. yoelii* 17X infection, indicating that the protein induces an immune response during an active infection. Assays to evaluate the functional activity of *P. yoelii* MIF are in progress. These include measuring D-dopachrome tautomerase activity and the ability to induce macrophage chemotaxis.

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### THE CSF CYTOKINE PROFILE OF CHILDREN WITH CEREBRAL MALARIA IS UNIQUE AND UNRELATED TO SERUM CYTOKINE LEVELS

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Serum cytokine levels have been measured in children with cerebral malaria (CM), but few studies have assessed cytokine and chemokine levels in the CSF. Central nervous system (CNS) levels of cytokines may differ from serum levels, and are likely of greater significance in CNS complications of CM than serum levels. For this reason, we tested CSF cytokine levels of 12 cytokines/chemokines important in other CNS disorders in 74 children with CM and 8 control children with metabolic disorders and no neurologic symptoms. CSF levels of the pro-inflammatory cytokines IL-8 and G-CSF, IL-6 (which can be pro- or anti-inflammatory), and the anti-inflammatory cytokine IL-1ra were elevated. CSF levels of MIP-1a and TNF- $\alpha$  were also elevated, though here the differences were largely due to elevation in a few samples. Median CSF IL-6, TNF- $\alpha$ , IL-8 and G-CSF levels in CM, though higher than in controls, were lower than those usually reported in viral encephalitis or meningitis, and much lower than those reported in bacterial meningitis. However, individual CSF IL-6 and IL-8 levels in several children were as high as those seen in enteroviral and tuberculous meningitis, and in a few, were as high as those seen in bacterial meningitis. Similarly, CSF G-CSF levels in some children were as high as those reported in bacterial meningitis. However, CSF IFN- $\gamma$  and IL-1 $\beta$  (pro-inflammatory) and IL-10 (anti-inflammatory) levels, which are often elevated in bacterial meningitis, and which are elevated in the serum of children with CM, were not elevated. There was no relationship between serum and CSF cytokine levels, which suggests that cytokine production occurred in the CNS. The data demonstrate, for the first time, the unique CSF cytokine profile of CM and provide new insight into the pathogenesis of CM.

(ACMCIP Abstract)

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### PROTEOMIC INSIGHTS INTO THE MAKE-UP OF *P. VIVAX* AND *P. KNOWLESI* BLOOD STAGE PARASITES

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The completion of several *Plasmodium* genome sequencing projects provides a great opportunity to more understand the make-up of several of the malaria parasites. Here we take a proteomics approach to investigate the blood stage proteomes of two species, *P. vivax* and *P. knowlesi*. Much is not known about *P. vivax* due to the difficulty in cultivation. The simian *P. knowlesi* on the other hand is readily cultured, and has recently been associated with human malarial infections. The disease associated stage of the malaria parasite is restricted to the erythrocytic stage. We analyzed protein fractions from *P. vivax* and *P. knowlesi* purified trophozoites and schizonts, purified schizont infected red blood cell membranes, and immunoprecipitated detergent-soluble fractions. In order to establish protein similarities between the two species, a panel of *P. vivax* monoclonal antibodies (mAbs) were initially used to screen *P. knowlesi* schizonts by IFA and cross-reactivity mAbs were used to immunoprecipitate proteins from *P. knowlesi* extracts. All protein fractions and immunoprecipitates were then separated on 4-20% polyacrylamide gradient gels and protein bands excised and subjected to

tryptic digests. Resulting peptides were analyzed on a MALDI TOF/TOF mass spectrometer. The resulting peptide masses were searched against all available Plasmodium proteomes. Initial data has yielded some useful insights into the make-up of both proteomes. For example a *P. vivax* mAb immunoprecipitated a complex of three *P. knowlesi* proteins, called MacP (Merozoite apical complex protein) which is present in both *P. vivax* and *P. knowlesi*. Mass spectrometry analysis revealed that this complex corresponds to the high molecular mass rhoptry protein complex (RhopH) proteins that have been described in *P. falciparum* and *P. yoelii*. Such studies enhanced by the now available proteomic tools could have major implications for vaccine and or drug development.

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### COMPARATIVE STUDIES OF CYTOKINE PROFILES IN MURINE ANEMIA CAUSED BY PLASMODIUM YOELII INFECTION, DRUG INDUCED HEMOLYSIS OR BY HEMORRHAGE

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Malaria represents a major threat to populations in many tropical countries around the world. Two major causes of malarial death are severe anemia and cerebral malaria. Malarial anemia is characterized by the destruction of both parasitized and nonparasitized red blood cells and by the suppression of erythropoiesis (dyserythropoiesis) even in the presence of a robust host erythropoietin response. Hematopoiesis, the dynamic and complex developmental process of the formation of new blood cells, regulates the proliferation, differentiation and maturation of erythropoietic progenitor cells into erythrocytes and is under the influence of several growth factors and cytokines. To determine whether murine malaria infection affects erythropoiesis by altering the production of growth factors and cytokines, we measured the profiles of multiple cytokines during the development of murine malarial anemia with *Plasmodium yoelii*. Results indicated that murine malarial anemia was caused by significantly decreased levels of hematopoietic growth factors and cytokines; and at the same time by increased levels of factors, which negatively affect hematopoiesis. Interestingly, erythropoietin level in peripheral blood was significantly elevated during anemia in the malaria-infected mice as well as in the anemic mice caused by phenyl hydrazine hydrochloride induced hemolysis or by hemorrhage, indicating that lack of erythropoietin is not the major cause of malarial dyserythropoiesis. In addition, we determined the cytokine levels in murine anemia caused by *P. yoelii* infection, hemolysis with drug or by hemorrhage. Results indicated that the cytokine profiles in anemia caused by these methods differ significantly from each other. We observed that at the blood stage of parasite infection, several merozoite-released soluble proteins (MRSPs) bound to both parasite infected and uninfected red blood cells, peripheral leukocytes as well as nucleated bone marrow cells. We postulate that MRSPs might play a role in dyserythropoiesis by altering hematopoietic cytokine production during malaria.

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### ACTIVATION OF HUMAN BLOOD BRAIN BARRIER ENDOTHELIUM IN CEREBRAL MALARIA

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Central to the pathology of human cerebral malaria (CM) is the sequestration of *P. falciparum* infected red blood cells (Pf-IRBC) to blood brain barrier (BBB) endothelium. Although several Plasmodium proteins have been identified that interact with BBB endothelium, the effects of these molecular interactions of Pf-IRBC with the BBB endothelium are unclear. Therefore, we investigated the effect of Pf-IRBC on the activation of human brain microvascular endothelial cells (HBMEC) monolayers. The state of endothelial activation was determined by 1) ICAM-1 expression, 2) cytokine release and 3) monolayer integrity as measured by electrical

resistance. We found that Pf-IRBC but not uninfected red blood cells (RBC) activate HBMEC and increases ICAM-1 expression, cytokine release and decrease barrier integrity. Removing extracellular proteins on Pf-IRBC by trypsin treatment significantly reduced Pf-IRBC binding to HBMEC but not its ability to activate HBMEC. Selection of populations of Pf-IRBC that display a tenfold increase in binding had same effect as low-binding Pf-IRBC. Inactivation of the intracellular parasite by artemisinin, partially reduced the ability to activate HBMEC. Sub-fractionation showed that both membrane associated and soluble Pf-IRBC factors mediate the activation of BBB endothelium. Moreover, culture supernatants of Pf-IRBC activated HBMEC. HBMEC activation could also be blocked by preincubation of HBMEC with inhibitors of NFkB, reactive oxygen species (blocking increase in ICAM-1 expression) and by pretreatment of Pf-IRBC with Brefeldin A (partially prevents a decrease in electrical resistance of BBB endothelium).

Taken together, our data shows that in CM, sequestration of Pf-IRBC in brain endothelial venules leads to activation and impairment of the BBB endothelium. This is a multi-step and multifactorial process involving non-trypsin sensitive membrane components and soluble- Pf-IRBC factors. This may lead to transmittance of soluble parasite and host factors into the brain causing activation of neuroglia and neuronal dysfunction as observed in CM

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### DETERMINATION OF THE NECESSITY AND ROLE OF PLASMODIUM VIVAX RELATED PROTEINS IN MEROZOITE INVASION

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*Plasmodium knowlesi* is a simian malaria model that is related to the human malaria *P. vivax*. Two recently identified *P. knowlesi* proteins, PkNBPXa and PkNBPXb, are expressed in the erythrocytic stage of the parasite life cycle and have been localized to the microneme organelles at the apical end of the merozoite. These proteins belong to the reticulocyte-binding like (RBL) superfamily of proteins. RBL proteins identified in other Plasmodium species have been determined to be adhesive proteins that bind to unknown ligands on the surface of erythrocytes. Because PkNBPs are defined as RBL proteins, it is proposed that PkNBPs mediate the same binding function of adherence to erythrocytes. It is important to understand the role of NBPs in invasion to determine their function and develop supporting data for these proteins as malaria vaccine candidates. Through the use of transfection technologies in Plasmodium, it has become possible to determine the function of proteins by specifically targeting and disrupting the corresponding genes. Transfection technologies have been employed both *in vivo* and *in vitro* to determine if PkNBPXa and PkNBPXb are essential for merozoite adhesion and invasion of RBCs

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### TRENDS IN THE COMPLIANCE LEVELS TO ARTEMETHER-LUMEFANTRINE FOR THE TREATMENT OF UNCOMPLICATED PLASMODIUM FALCIPARUM INFECTION IN ZAMBIA AFTER FULL-SCALE DEPLOYMENT

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Due to high anti malarial drug resistance rates, Zambia has adopted Artemether-lumefantrine (AL), as its first line treatment for uncomplicated malaria. Although, AL is known to be highly efficacious, concerns have arisen regarding patient compliance. This has prompted the National

Malaria Control Centre (NMCC) to monitor treatment compliance levels annually. After a two years full deployment of AL with intensive patient counselling, compliance levels were studied and compared with the year before full-scale implementation. A descriptive study was conducted in January 2004 and 2005 in 5 districts of different social-economic and geographical localities, to ascertain levels of compliance to the 6-dose regimen in malaria patients and whether AL was being correctly administered. In the absence of national guidelines on compliance measurement, a classification of total, non-, undetermined or probable was adopted for the study, using WHO guidelines. The compliance levels for the 2 consecutive years were compared statistically. All patients suspected of having uncomplicated falciparum malaria visiting the health centers in the month of January in the five districts, were eligible. Compliance was found to be lower than the expected 90 % in both years. There was however, an improvement in the year 2005 when an aggregate of total and probable compliance for the respective years was considered (82.2% in 2005 vs 75.9% in 2004). If probable compliance is taken as non-compliance, there is still a higher compliance overall. The factors that contributed to compliance were gender, educational level and distance to health centre. Compliance has to be increased to acceptable levels due to the fact that non-adherence may lead to treatment failure and contribute to parasite resistance. There is need for the implementation of best practices to improve adherence and maintain the efficacy of AL.

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### CURING MALARIA-INFECTED RODENTS USING ARTEMISININ-DERIVED TRIOXANE DIMERS

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In only four or five chemical steps from naturally occurring artemisinin, several 3-carbon atom-linked trioxane dimers were prepared in very good overall yields. *Plasmodium berghei*-infected female mice were treated subcutaneously on the day after infection with a trioxane dimer as a single dose of 30 mg/kg murine body weight. After 30 days post-infection, the mice showed neither overt toxicity nor any behavioral modification. No malaria parasites were observed in the blood of the surviving mice on day 30 post-infection. Similarly, oral administration of some of these C-10 non-acetal trioxane dimers at a dose of 30 mg/kg mouse body weight on days 1, 2, and 3 post-infection cured the malaria-infected mice; no malaria parasites were detected in the blood of the surviving mice on day 30 post-infection. The chemical structures of the curative trioxane dimers and details of the mouse survival will be discussed, with comparison especially to malaria chemotherapy using sodium artesunate and trioxolane OZ277.

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### COSTS, EFFECTS AND COST-EFFECTIVENESS OF ARTEMETHER LUMEFANTRINE IN ZAMBIA

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In Zambia, Malaria incidence estimates indicate a threefold increase over the past 30 years to more than 3 million clinical cases annually. The decline in efficacy of common antimalarials is a contributor to this, leading to adoption of more costly drugs as first line treatment in the form of Artemisinin-based combination therapies (ACTs). In Zambia, the first line treatment for uncomplicated malaria is Artemether-Lumefantrine (AL). One of the barriers to effective deployment of ACTs in Africa has been

the cost. We evaluated the operational cost-effectiveness of malaria case management, using RDTs or microscopy or clinical assessment prior to treatment with AL from a providers' perspective. Data collection was retrospective, before the use of ACTs and prospective, over a period of 9 months, using facility based morbidity and cost data. The use of AL as first line treatment has increased the total and average cost of treatment per episode of malaria (Table 1). The average cost of AL per case treated is almost 20% higher. The average cost per case cured was less for AL (\$8.57) than SP (\$10.65) (Table 2). The cost of an additional case successfully treated was \$4.16. The study performed a cost and cost effectiveness analysis to provide guidance on issues arising from changing malaria treatment policy in the face of drug resistance to other antimalarials. On this account, AL has been found to be cost-effective.

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### DETECTION OF *PLASMODIUM*-INFECTED ERYTHROCYTES AND EVALUATION OF ANTIMALARIAL ACTIVITY IN MURINE MODELS OF MALARIA BY FLOW CYTOMETRY USING AUTOFLUORESCENCE AND YOYO-1

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Murine models are a cornerstone of the drug discovery process in malaria. In experiments of drug efficacy testing, parasitemias in peripheral blood are measured by microscopic analysis of blood smears. However, this method is subjective and labor intensive. As plasmodia invade erythrocytes suspended in blood, flow cytometry would seem the alternative technique of choice. To avoid these problems, the lack of cell nucleus in murine erythrocytes provides a simple way to detect parasitized red cells. However, due to the presence in murine blood of a significant proportion of erythrocytes containing detectable amounts of nucleic acids, only a limited specificity has been achieved. As a result, flow cytometry has not been widely used in murine models of malaria. Here we describe the application of a new flow cytometry method useful to distinguish infected erythrocytes for measuring the activity of antimalarial drugs *in vivo*. This new technique is based on the differences of autofluorescence and DNA content between infected and non-infected erythrocytes measured after staining with YOYO-1. Infected erythrocytes showed a characteristic pattern of staining that markedly increased the specificity of detection. Moreover, infected reticulocytes could be differentiated from infected normocytes. The limit of sensitivity was 0.01 % and measurements were linear at parasitemias above 0.1 % in routine conditions. When testing antimalarial compounds *in vivo*, the patterns of staining obtained from samples of blood of infected mice treated with chloroquine, artesunate, pyrimethamine or atovaquone showed a characteristic profile for each compound. The patterns correlated with the results expected according to their respective mechanisms of action and parasite stage sensitivity. This suggests that this method might be useful to assess *in vivo* the susceptibility of the parasitic stages to new antimalarials.

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### *IN VIVO* ANTIMALARIAL EFFICACY AGAINST *P. YOELII* AND *P. FALCIPARUM* OF A 4(1H)-PYRIDONE CANDIDATE FOR CLINICAL DEVELOPMENT

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4(1H)-pyridones is a novel class of selective inhibitors of the bc1 complex found in the mitochondrial respiratory chain of *Plasmodium spp.* A compound has been selected as candidate for clinical development on the basis of its *in vivo* therapeutic index. The therapeutic efficacy of the candidate compound was evaluated in a "4-day test" against *Plasmodium*

*yoelii* 17XNL or the strain *P. falciparum* PfC1N9 developed for *in vivo* assays in mice. The parameters measured were the effective doses at 50 or 90 % (ED<sub>50</sub>, ED<sub>90</sub>, respectively) on parasitemia 24 h after the last dose administered. Chloroquine was used as internal control of the assay in both models. In the standard *yoelii* model, groups of n=5 mice were inoculated intravenously with 6.4×10<sup>6</sup> infected erythrocytes and, 1 hour after infection, treated p.o. once a day for 4 days. In this assay the candidate pyridone showed potent antimalarial activity with ED<sub>50</sub> = 0.26 and ED<sub>90</sub> = 0.54 mg/Kg. In the murine model of *falciparum* malaria, groups of mice n=3 were infected with 20×10<sup>6</sup> infected erythrocytes *via* intravenous route (day 0). The treatment started at day 3 after infection as described for the previous model. The candidate pyridone showed potent antimalarial activity against *P. falciparum* with ED<sub>50</sub> = 0.65 mg/Kg and ED<sub>90</sub> = 1.47 mg/Kg. The results obtained indicate that the candidate pyridone has excellent efficacy against *P. falciparum* *in vivo*. Moreover, analysis of samples of peripheral blood by microscopy and flow cytometry suggested that the trophozoite stage is the most sensitive to the pyridone *in vivo*. A detailed pharmacodynamic study of the antimalarial activity of this candidate in our *falciparum* murine model is in progress aiming to provide efficacy data for the studies required to estimate target doses for Phase I studies in humans.

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#### COMPARATIVE MULTI-DOSE PHARMACOKINETIC MODELLING AND DETERMINATION OF NON-RECRUDESCENCE LEVEL FOR *PLASMODIUM YOELII* INFECTION IN MOUSE OF ANTIMALARIAL 4(1H)-PYRIDONES

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4(1H)-Pyridones are a novel class of antimalarials acting as selective inhibitors of *Plasmodium* mitochondrial function with demonstrated activity both *in vitro* and in animal models of infection. Compounds GW308678 and GW844520 were selected as lead compounds for pre-clinical evaluation. In terms of therapeutic efficacy *in vivo* (efficacy against *Plasmodium yoelii* in mice, standard '4-day Test'), GW308678 appeared to have similar potency than GW844520 with ED50 and ED90 values of 0.3 and 0.5 mg/Kg compared with ED50 and ED90 of 0.2 and 0.4 mg/Kg for GW844520. However, whereas GW844520 was able to completely eradicate *P. yoelii* infection in mouse after four 4 mg/Kg doses, animals treated with GW308678 showed recrudescence at any dose deployed (up to 32 mg/Kg). The two compounds displayed important differences in terms of Clearance (Cl) (0.6 ml/min/Kg for GW844520 compared with 3 ml/min/Kg for GW308678) and terminal half-life (t<sub>1/2</sub>) (24.1 and 3.6 hours for GW844520 and GW308678 respectively) in mice. According to these data, current hypothesis to explain the differences observed was to consider that these differences were related to the longer exposure over time obtained for GW844520 compared with GW308678. In order to test this hypothesis, we proceed to model the concentration-time data of both drugs from available experimental PK data (single 10 mg/Kg oral dose, compound levels determined in total blood). Once the best-fitted PK model for each compound was achieved, simulated PK profiles corresponding to the different doses administered in therapeutic efficacy *in vivo* assays were obtained and simulated GW308678 PK profiles were extended to match the expected terminal exposure of GW844520 after four doses. Applying the predicted extended dosage model, GW308678 was able to fully eradicate *Plasmodium yoelii* infection at the same dose (4 mg/Kg) than GW844520.

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#### COUNTERFEIT ANTI MALARIA DRUGS: THE DRAMA OF DIHYDROARTEMISININ (DHA)

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In recent literature attention is drawn to the distribution of counterfeit antimalarial drugs, in particular in Southeast Asia. It is mainly done by adding insufficient active ingredient to the pills or even by selling pills without active ingredient. The impression is given that a large proportion of artesunate based malarial medications are fake. This could also happen in Africa and therefore the content of a series of artesunate tablet preparations, randomly purchased on the street markets in Kinshasa (RDC), was investigated. The content of all packs (n=15) gave results of at least 90 % of artesunate in the tablets. For other variables of tablet quality several of the packs were far below GMP requirements but, fortunately for the patients, they received an acceptable quantity of artesunate in line with the 90-110% recommendation of International Pharmacopoea (Int.Ph). Also a series of Dihydroartemisinin containing tablets (n=6) was obtained and subjected to a similar analysis. None of the tested packages complied with the specifications of the Int. Ph. The amount of impurities and related substances found varied between 3% and 50% (max 2%). In general one major breakdown product is found. It does not contain the peroxide bridge and it is inactive against malaria. Breakdown of DHA is not surprising. This molecule, being a peroxide, is an unstable chemical substance with high potential energy level. The hemi-acetal hydroxyl group can switch from alpha to beta position and this involves ring opening bringing the molecule in an unstable transition state. Under appropriate circumstances (rising temperatures and humidity) the peroxide moiety is decomposed when singlet oxygen is expelled. This wasting of energy has an explosive character akin to that of trinitrotoluene (TNT). Chemical handling of artemisinin and derivatives must be done carefully in order not to cause an explosion of the peroxide bridge. It is clear that therapy with DHA in tropical countries can cause some serious problem where not the manufacturer as such, but climatic conditions are responsible for the damage. This breakdown of DHA also holds for preparations containing the DHA combinations like e.g. DHA/Piperazine. Prescribers are warned for the pitfalls with DHA and its likely breakdown and, hence, underdosing in the tablets.

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#### DETECTION OF FOUR *PLASMODIUM* SPECIES BY GENUS- AND SPECIES-SPECIFIC LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR CLINICAL MALARIA PATIENTS

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Loop-mediated isothermal amplification (LAMP), a novel nucleic acid amplification method, was developed for the rapid detection of four species of human malaria parasites, *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. This LAMP method amplified 18S small subunit ribosomal RNA (SSU rRNA) gene of malaria parasites with high specificity, efficiency and rapidity under isothermal conditions using a set of *Plasmodium* genus- and species-specific primer sets and a DNA polymerase with strand displacement activity. In this study, we evaluated the specificity and sensitivity of these LAMP assay in comparison with conventional microscopic examination and established nested-PCR assay. The LAMP assay was evaluated by using blood specimens collected on filter paper from 103 individuals (50 of the individuals diagnosed with malaria infection under microscopic observation and nested-PCR assay,

and 53 samples of negatively diagnosed) from malaria clinics in Mae Sod district, Tak province, northwestern Thailand. Results were interpreted by measuring turbidity in real time or visualization at the end of the assay. Out of 50 positive cases, 36 from 37 vivax cases, all of 17 falciparum cases, one case of malariae malaria were species specifically diagnosed by the LAMP method. Forty eight out of fifty three negative cases, diagnosed by both microscopy and nested-PCR assay, were confirmed by LAMP, and five cases were diagnosed with malaria parasites. LAMP can yield results within about 30 min for detecting of *Plasmodium* genus, 40 min for *P. falciparum*, and 45 min for *P. vivax*. This method does not require post-reaction processing, reduces sample handling, and minimizes the risks of contamination. These results indicate that the LAMP assay is an extremely rapid, highly sensitive, specific, cost effective and suitable for routine screening of *Plasmodium* sp. infection in clinical laboratories. Further studies are warranted to validate this technique for routine diagnosis of malaria and surveillance of malaria prevalence in malaria endemic areas where there are asymptomatic populations with low parasite density.

(ACMCI Abstract)

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#### COMPARISON OF PERFORMANCE CHARACTERISTICS OF THE BINAX NOW® MALARIA TEST USING VENOUS AND FINGERSTICK SAMPLES

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The impact of the choice of clinical sample, venous blood obtained via phlebotomy or fingerstick-derived blood, on the performance of malaria rapid diagnostic tests (RDTs) is not known. 800 subjects with symptoms consistent with malaria were enrolled at two malaria clinics near the Thai-Burmese border during July/August 2003. Subjects underwent both fingerstick and phlebotomy within 5 minutes. Both blood samples were blindly evaluated by reference microscopy and by the same lot of the NOW® Malaria test (Binax, Inc., Scarborough, Maine, USA). Microscopic analysis of the Giemsa-stained venous blood smears revealed 81 cases of *Plasmodium falciparum*, 126 cases of *P. vivax* and 588 negatives. 5 additional malaria cases (< 1%) were detected from the fingerstick samples at very low densities. When compared against microscopic examination of venous blood, sensitivity of the Binax NOW® Malaria RDT was 100% (95%CI 96-100%) for falciparum, and 82% (95%CI 74-88%) for vivax malaria. Specificity was 95%. Similar results were obtained from the fingerstick samples, although more faint 'false positive' T1 lines (HRP-2 antigen) were detected from the fingerstick samples. The percent agreement between the venous and fingerstick samples was 94% (752 of 799) with most disagreements being the absence versus presence of weakly positive HRP-2 lines. This 6% discrepancy rate is similar that to seen in our lab among our trained microscopists interpreting the same thick/thin blood smear. The Binax NOW® Malaria RDT can effectively be used with venous or fingerstick blood samples

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#### DEVELOPMENT OF AN ALDOLASE CAPTURE ELISA FOR USE IN QUALITY CONTROL OF MALARIA RAPID DIAGNOSTIC TESTS AND MEASURING PARASITE GROWTH IN-VITRO

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Several antigen-specific capture assays to detect and diagnose malaria parasites or to measure parasite developmental growth *in vitro* have been developed over the past decade or so. These colorimetric based assays have been developed to reduce or eliminate a need to use radioactive chemicals to measure growth for *in vitro* drug susceptibility studies or

the necessity of microscopy to make a specific diagnosis of malaria. In the latter case, the antigen-capture assays have taken the form of rapid diagnostic test (RDT) devices using lateral-flow immunochromatographic technology. The two principal target antigens in these assays have been Histidine-Rich Protein II (HRP II) and parasite Lactate Dehydrogenase (pLDH). However, unlike pLDH, HRP II is only specific to *Plasmodium falciparum* and RDTs designed to detect this antigen in blood will not detect the other human species of *Plasmodium*. A number of RDTs have now been designed to detect parasite aldolase in addition to HRP II to give the diagnostic assays a pan-species detection capability. We have now developed a quantitative ELISA based aldolase capture assay using two sets of monoclonal antibodies, biotinylation of the detection antibody and horseradish peroxidase conjugated Neutraavidin. The ELISA can reliably detect parasite levels from 100-200/μl to over 40,000/μl. Aldolase levels per infected erythrocyte increase 5 to 8 fold from ring-stage to late trophozoite/early schizont stage of development. The levels of aldolase expressed in a parasite are comparable to that of HRP II. The assay recognizes recombinant *P. falciparum* and *P. vivax* aldolase equally well. This aldolase capture assay now provides an additional tool to measure malaria parasite development for growth inhibitor/drug sensitivity studies in the laboratory or field as well as a means to standardize materials for the quality control of malaria RDTs, which are increasingly being used for the diagnosis of malaria.

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#### STAGE-SPECIFIC ACTIVITY OF ANTIMALARIAL COMPOUNDS MEASURED BY OPTICAL MICROSCOPY, HYPOXANTHINE UPTAKE AND A NEW METHOD OF FLOW CYTOMETRY

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Development of new antimalarial agents must consider stage-specific activity to improve drug efficacy. The evaluation of stage-specific activity of new antimalarial agents using standard methodology is laborious and time-consuming. The present study has been focused in the comparison of three different methods to assess the advantages of each one and determine their ability to evaluate stage-specific activity. We prepared a highly synchronic culture of *Plasmodium falciparum* parasites which was treated during each specific stage with different antimalarial compounds: artemisinin, pyrimethamine and a member of the pyridones family (GW844520). Their stage-specific activity was measured by optical microscopic observation, incorporation of 3H hypoxanthine and a new flow cytometry method. This technique is based on the differences of autofluorescence and DNA content between infected and non-infected erythrocytes measured after staining with YOYO-1. Optical microscopy is the standard method to assess the level of development of parasites and to differentiate morphologically each stage. Flow cytometry could distinguish each stage of the parasite although microscopy was more precise determining the morphological changing during the evolution of stages. Regarding antimalarial activity, hypoxanthine incorporation is the most sensitive methodology. Flow cytometry was also able to discriminate between healthy and damaged parasites and showed a good correlation with the data determined by microscopy. In general, artemisinin showed a huge inhibition for all the stages, measured by hypoxanthine and microscopy, flow cytometry showed the same inhibition than the others methods only for trophozoite stage; pyrimethamine was predominantly effective against late trophozoite and schizont stage by the three methods, meanwhile pyridones derivate was effective against early and late trophozoites. This new method of cytometry presents the main advantage of allowing the measurement of a large amount of samples with a good correlation with the parasitemia levels determined by optical microscopy.

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**BRINGING HEMOZOIN CRYSTALS AND SURFACES INTO THE LIGHT**

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Hemozoin, the unique material produced by an equally unique malarial biochemistry, remains one of the parasites Achilles' heel for drug development. The structure determination of hemozoin offered an important insight into its potential drug binding sites but heme's general insolubility has prevented rigorous testing of many hypothetical drug/crystal interactions. This presentation will concern our efforts to develop soluble versions of the dimer present in hemozoin. In particular the hematin derivatives of deuterio and meso porphyrin-IX form the same dimer and exhibit key solubility lacking in the parent natural material. For the first time drug/dimer interactions can be examined in solution without competing equilibria from biologically irrelevant aggregation and oxo-bridged dimer formation. New structures related to hemozoin have been determined and attempts to label the faces of the crystallites will be described.

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**THE ANTIPARASITIC DIAMIDINE DB75 TARGETS THE PLASMODIUM FALCIPARUM NUCLEUS**

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DB289 is a broad spectrum anti-parasitic compound which has been shown to be effective against both Human African Trypanosomiasis and malaria in recent clinical trials. DB75, (2,5-bis(4-amidinophenyl)furan) is the active metabolite of this drug. Previous work by our group showed that DB75 localizes in and disrupts mitochondria of yeast and of Trypanosoma brucei. The objective of this study was to determine the subcellular target of DB75 in *P. falciparum*. The inherent fluorescent properties of the drug allowed us to visualize it with UV scanning confocal microscopy and colocalize it with organelle-specific fluorescent probes including MitoTracker® Red (mitochondria), LysoTracker® Red (food vacuole) or Draq5™ (nuclear DNA). In addition, a GFP-PfPREX transfected falciparum strain was used to determine if DB75 localizes to the apicoplast. Our results show DB75 localizes only in the nucleus of *P. falciparum* and not in the mitochondrion, apicoplast or food vacuole. Previous studies show that DB75 binds to the minor groove of DNA. Taken together, these results suggest that the mechanism of action of DB75 against *P. falciparum* may be different than its mechanism of action against trypanosomes and may involve inhibition of nuclear DNA synthesis.

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**DEVELOPMENT OF ANOPHELES DIRUS SPOOROZOITE-INDUCED MOUSE MALARIA MODEL**

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*Anopheles dirus* is one of the major malaria vectors in Thailand and the most efficient vector used in experimental malaria infection to produce sporogonic stages of human and primate parasites at Armed Forces Research Institute of the Medical Sciences. A new exo-erythrocytic (EE)

ICR mice model inoculated with sporozoites harvested from *A. dirus* mosquitoes was successfully developed and optimized. We compared two mice malaria strains, *Plasmodium berghei* (ANKA strain) and *P. yoelii yoelii* (17XL strain) at different doses and routes of infection in order to select the best model with 100% consistent fatal infection. Gametocytes were first detected in the blood of donor mice 2 to 5 days after intraperitoneal (IP) inoculation of infected blood. Fifty mosquitoes were then fed on each anesthetized donor mouse on the first or second day of gametocyte detection with parasitemia exceeding 1%. Engorged mosquitoes were kept at 21°C for a minimum of 21 days. Twenty one to 30 days after feeding on the mice, infective sporozoites were harvested from the mosquitoes' salivary glands. Sporozoite inoculations via IP and intravenous (IV) routes were compared at multiple doses from 25,000 to 100,000 sporozoites per mouse for *P. berghei* and 100 to 17,500 sporozoites per mouse for *P. yoelii* in 105 and 62 mice respectively. The IV route induced the highest infection rate for both sporozoite strains. The lowest *P. berghei* sporozoite inoculation dose to induce consistent 100% infection was 75,000. Prepatent period of *P. berghei* infection was 3 to 6 days and resulted in 100% mortality within 7 to 28 days post inoculation. The lowest *P. yoelii* sporozoite inoculation dose to induce consistent 100% infection was 500 but only 13.5% of mice died with the remainder self curing. Pilot direct feeding of 5 and 25 *P. berghei* infected mosquitoes induced infection in all 5 and 8 mice respectively. Two confirmation studies showed that the 50,000 sporozoites produced 100% infection in 20 mice but produced only 41.8% infection when 55 mice were inoculated. The *P. berghei* model was selected with an optimal dose of 100,000 sporozoites which induced 100% fatal infection in 4 confirmation experiments of 20, 20, 7 and 30 mice resulting in 100% mortality within 7 to 24 days. This model is being validated for EE antimalarial drug screening.

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**REVERSED CHLOROQUINES: AN UPDATE ON MOLECULES DESIGNED TO SUBVERT CHLOROQUINE RESISTANCE IN P. FALCIPARUM**

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Drug resistant strains of malaria, including chloroquine-resistant (CQR) *Plasmodium falciparum*, are a key factor in the worldwide and increasing public health crisis of malaria. One hope for remedying this situation has been offered in the form of 'reversal agents', molecules which have little or no antimalarial activity on their own, but affect CQR malaria such that it becomes sensitive to CQ (i.e., acting as if it were CQs). In this report we present our new work in the development of 'reversed CQs' (RCQs), made by covalently linking CQ and a reversal agent. Such 'reversed CQs' (RCQs) have conceptual advantages over CQ/reversal agent cocktails. We also present evidence pertaining to the RCQ mechanism, in part by designing and testing molecules that resemble RCQs, but which were designed to fail.

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**SYNTHESES AND EVALUATIONS OF REVERSED CHLOROQUINES**

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Malaria kills more than a million people annually, mostly from the parasite *Plasmodium falciparum*, with a further 300-500 million nonfatal cases each year. Chloroquine (CQ) has been one of the most inexpensive and widely used drugs in the treatment of malaria, but resistance to CQ is becoming widespread. It has been known for nearly two decades that CQ resistance in *P. falciparum* can be reversed by co-administration with

compounds known as 'reversal agents'; however there are potential pitfalls in their implementation. We undertook a novel strategy, namely the chemical linking of a reversal agent to a CQ-like molecule to make a 'reversed chloroquine', or RCQ. This presentation highlights and updates the chemical syntheses and antimalarial activities of several such compounds, which can have very good antimalarial activity against both CQ sensitive and CQ resistant strains of *P. falciparum*.

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### INVESTIGATIONS ON EMBRYO FETAL DEVELOPMENT IN RATS AND RABBITS WITH RBX11160 AND ITS SAFETY AS COMPARED TO ARTESUNATE

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RBx11160(RBx) is a synthetic endoperoxide antimalarial. The objective of this study was to assess the embryo-fetal toxicity in rats and rabbits with RBx at 10, 30 and 90 mg/kg/day dose. More than dose proportional exposure from 10-30 mg/kg and dose proportional exposure from 30-90 mg/kg doses was seen both species. In rats, all females survived until C-section and no test item-related clinical signs, of discomfort and gross changes were noted. At 10 and 30 mg/kg/day doses, no changes in food consumption, mean body wt, reproductive parameters, and fetus wt were noticed. At 30 mg/kg/day, a VSD was noted in one fetus out of 257 fetuses examined, this defect was also seen in controls. At 90 mg/kg/day dose level, reduction in mean food consumption (-10.7% compared with the vehicle control), body wt gain (57 g vs with 74 g in control)s, post-implantation loss (10/22 females), litter size (8.2 fetuses per dam compared with 13.6 in the vehicle control) and fetal weight (4.1 g vs 4.8 g in controls) were seen. Further, abnormal findings viz., VSD (5 fetuses), aortic arch dilated (6 fetuses), ductus arteriosus and/or pulmonary trunk narrowed (5 fetuses), spleen reduced in size (4 fetuses) were seen. No test item-related effects were noted on sex ratios, at fresh external or skeletal examinations at all doses. The maternal NOEL and reproductive parameters was considered to be 30 mg/kg body wt/day (~7X the human exposure at 100 mg/day). The fetal NOEL was considered to be 10 mg/kg body wt/day (equivalent to human exposure at 100 mg/day). In rabbits, at 90 mg/kg/day dose, labored respiration was noted for four females and one female was found dead on day 19 pc, reduction in food consumption (-27.3% vs controls), mean body weight development (+0.1% vs with +2.4% controls), fetal wt (31.5 g vs with 32.7 g controls), At 10 and 30 mg/kg/day doses, no mortality/clinical signs, changes in mean body weight and post-implantation loss. At 30 mg/kg body wt/day, slight reduction in food consumption was noticed. No macroscopic findings or fetal anomalies were seen at any dose. The maternal NOEL was 10 mg/kg body weight/day (= to human exposure at 100 mg/day), and the fetal NOEL was 30 mg/kg body weight/day (3X the human exposure 100 mg/day). Under the conditions described for this study, RBx11160 did not reveal any teratogenic potential at any dosages (16X human exposure 100 mg/day). artemisinins are embryo-lethal and teratogenic in this model suggesting RBx maybe safer in pregnancy.

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### FIXED DOSE ACT: PHARMACOKINETIC STUDY OF ARTESUNATE-SULFAMETHOXYPIRAZINE-PYRIMETHAMINE (CO-ARINATE FDC) IN A BLACK POPULATION IN IVORY COAST

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Malaria kills approximately 1.5 to 2.7 million people each year. In spite of the introduction of artemisinin and its derivatives, even as combination

therapy with other antimalarials, the treatment of malaria is hampered by problems like insufficient efficacy, recrudescence, early re-infection, bad patient compliance and high cost price of the drug. For these reasons new and more acceptable medicines are developed. One of them is the combination Artesunate /Sulfamethoxy-pyrazine/ Pyrimethamine (Co-Arinate FDC) in a fixed dose tablet. Clinically, three of these tablets administered over a total of 24 h period (one day) cure malaria. The clinical part of study was done with fourteen healthy volunteers in Ivory Coast in the Urban Sanitary Formation in the Suburban Community of Abidjan. Blood samples were taken after oral administration of one unit (1.5 tablet) containing 300 mg Artesunate, 750 mg Sulfamethoxy-pyrazine and 18.75 mg Pyrimethamine. The blood plasma levels of these three active ingredients were determined by Liquid Chromatography-Mass Spectrometry. For Sulfamethoxy-pyrazine the  $C_{max}$  was achieved in 2 to 4 hours and an elimination half life of approximately 65 hours. For Pyrimethamine a similar curve is obtained.  $C_{max}$  was reached after 2 hours and an apparent elimination half life of approximately 54 to 148 hours. Peak levels are in agreement with literature data for these compounds. For Artesunate the levels of Artesunate and its active metabolite Dihydroartemisinin are determined. For none of the volunteers there was a detectable concentration (2,5 ng/ml) of Artesunate found in the plasma samples investigated. Only DHA was found. The  $T_{max}$  was achieved after 2 hours and after 12 hours the DHA fell below detection limit. The area under the curve is fully in line with what was obtained in a similar study with artesunate administered from a monotherapy tablet. In conclusion, from the fdc tablet proposed the three active ingredients are properly released and give rise to adequate plasma concentrations.  $T_{max}$ ,  $C_{max}$  and area under the curve are not different from those obtained after separate administration. Co-arinate FDC is a good galenic preparation for the treatment of malaria.

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### IS ARTESUNATE OR ITS ACTIVE METABOLITE DIHYDROARTEMISININ BEING EXCRETED IN THE MILK OF LACTATING MOTHERS?

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In Sub-Saharan Africa malaria remains a major problem. It affects a lot of people and therefore its treatment causes concern for certain groups like for young children and pregnant women in the first trimester. Although the use of artemisinin derivatives is already generally accepted and applied, there still remain some questions. Up to now, there is no published evidence that artesunate and/or its active metabolite dihydroartemisinin is excreted in mother milk. If so the next question would be whether the amount present in the total amount of milk sucked by the baby constitutes a danger? The presence of artesunate and of dihydroartemisinin (DHA) in the milk of lactating mothers, who had decided to stop breast feeding their child was studied. Artesunate was given as a single dose of a 200 mg tablet at hour zero after milk was collected using a vacuum pump. Then, at time 1, 2, 4, 6 and 10 hour milk was collected and all samples were deep frozen (-20°C) until analysis. This analysis was done by appropriate preparation of samples and concomitant measurement of artesunate (limit of detection 5 ng/ml) and its major metabolite dihydroartemisinin (limit of detection 2.5 ng/ml) using HPLC/mass spectrometry. Artesunate in mother milk remains below the detection limit at all time points. However, DHA is present from the first hour onwards and reaches a maximum after 90 minutes (around 35 ng/ml). The concentration then lowers off and it disappears from milk after the 6th hour. Total amounts of DHA present in a milk meal for a baby can be calculated to be of microgram level only for the total amount secreted over a period of time. In conclusion, treatment of a lactating mother with artesunate does not constitute a danger for the baby who will receive only very low quantities of the DHA metabolite and no quantity of artesunate during a meal.



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### ASSESSING THE SPREAD OF DIHYDROFOLATE REDUCTASE AND DIHYDROPTEROATE SYNTHASE MUTANT ALLELES IN *PLASMODIUM VIVAX* POPULATIONS

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*Plasmodium vivax* is a serious health concern in many regions; inadvertent treatment with sulfadoxine/pyrimethamine (S/P) due to misdiagnosis or co-infection with *P. falciparum* is common. Mutations in dihydrofolate reductase (*dhfr*) confer resistance to pyrimethamine and diminish clinical responsiveness to S/P; the effect of *dhps* mutations on clinical responsiveness to S/P is less clear. Point mutations in *dhps* are more common in regions with high versus low S/P drug pressure, and specific point mutations are believed to account for the presumed innate resistance of *P. vivax* to sulfadoxine. Studies in *P. falciparum* have suggested that resistance-conferring mutations in *dhfr* are the result of selective sweep. However, mutations in *P. vivax dhfr* and *dhps* may have arisen and spread in a different manner, as *P. vivax dhfr* and *dhps* are highly polymorphic, with a variety of point mutations, variable repeats and insertions/deletions (indels) observed. Single nucleotide polymorphisms (SNPs) flanking *dhfr* and *dhps* and indels/repeats within the genes may provide information about the manner in which resistance-conferring alleles have moved through natural populations. We amplified *dhfr-ts* and 792 bp upstream/ 683 bp downstream from 28 global samples (Sri Lanka, Vietnam, Thailand, Nicaragua, Panama and Colombia). We found a range of polymorphisms both within the *dhfr* coding region and in the flanking intergenic regions. At present, informative indels within *dhfr* have been identified, as well as six informative SNPs upstream and two downstream. The repeat region between bp 604 and 644 in *dhps* was assessed in 21 field isolates from Indonesia and Papua New Guinea, and a bi-allelic point mutation at bp 382 was observed. A variety of repeat structures were found. These polymorphism data may be used to understand the way in which resistance-conferring mutations arise and spread through natural *P. vivax* populations.

(ACMCIP Abstract)

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### COMPREHENSIVE AND SYSTEMATIC APPROACH TO MONITORING ANTIMALARIAL DRUG RESISTANCE IN THE PERUVIAN AMAZON

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Antimalarial drug resistance is on a steady increase worldwide. In Peru, much of the circulating *Plasmodium falciparum* is resistant to chloroquine and areas with a very high level of resistance towards sulfadoxine-pyrimethamine (SP) have been documented and well studied. There have been reports of chloroquine (CQ) resistant isolates of *P. vivax* but to date, the resistance of only a couple of isolates has been confirmed by HPLC. Currently, Peru has two treatment policies for uncomplicated *P. falciparum* malaria. In the Amazon basin, centered near the jungle city of Iquitos, treatment is with MQ-ART while in the north coast the treatment policy is SP-ART. The current treatment policy for *P. vivax* in the whole of Peru is CQ and primaquine (PQ). There is little information as to the extent of the spread of SP resistance in *P. falciparum* and CQ resistance in *P. vivax* in the Peruvian Amazon, so a comprehensive effort has been made to monitor the spread of resistance using various methods. Currently, active surveillance sites have been set up to monitor *in vivo* drug resistance in *P. vivax* in the Iquitos city region while passive surveillance has been

instituted for both *P. falciparum* and *P. vivax* malaria in more than 20 sites extending west from Iquitos. The passive surveillance includes obtaining samples from patients with confirmed case of malaria followed by a detailed questionnaire and a rigorous follow up program to monitor compliance with treatment and to identify treatment failures. When possible, fresh clinical isolates of both *P. vivax* and *P. falciparum* are used for *in vitro* drug susceptibility testing using in-house pre-dosed plates and SYBR Green incorporation to determine IC<sub>50</sub> values. Isolates collected during the active and passive surveillance are also subjected to analysis for the currently known molecular markers that have been shown to confer drug resistance in both *P. falciparum* and *P. vivax*. More recently, microsatellite technology has been used to monitor the spread of SP resistance through the Amazon basin. By combining all the various means to assess drug resistance, it is the long-term goal of the group to better understand the spread and to monitor where antimalarial treatments will eventually fail so treatment policies can be amended prior to treatment failures in the local clinics.

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### SIGNIFICANT ASSOCIATION BETWEEN THE PRESENCE OF THE BOLIVIA REPEAT INSERTION IN THE *DHFR* GENE OF *PLASMODIUM FALCIPARUM* AND *IN VIVO* RESISTANCE TO SULFADOXINE-PYRIMETHAMINE (SP) IN ISOLATES COLLECTED IN THE AMAZON REGION OF PERU

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Treatment of *Plasmodium falciparum* malaria with the antifolate drug sulfadoxine-pyrimethamine (SP) became first-line therapy in the Amazon region of Peru in 1997. Efficacy of this combination therapy is compromised by the development of resistance in the parasite caused by point mutations in dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) genes that are associated with pyrimethamine and sulfadoxine resistance respectively. Previous reports from South America have documented the presence of an insertion after codon 30 in *Pfdhf* named the Bolivia repeat (BR), which was found in combination with mutations at codons 50, 51, 59, 108 and 164 in isolates with high levels of resistance to pyrimethamine and cycloguanil, however no association was studied in samples collected as part of an efficacy trial to evaluate *in vivo* SP resistance. Therefore, we sought to determine the presence of the BR in isolates previously shown to contain mutations at codons 51, 108 and 164 but were wild type at positions 50 and 59. Following PCR and DNA sequencing of *Pfdhfr* gene, the BR was found in 100% association with the 51I and 164L mutant alleles while isolates that did not have the BR were wild type at these locations. All isolates had the 108N mutation. Interestingly, 82% of the patients that failed treatment (42/51) harbored isolates with the BR and had in total, 6 or 7 mutations in the *Pfdhfr* and *Pfdhps* genes. Interestingly, of the 9/51 patients that did not have BR but failed treatment with SP, they only harbored the 108N mutant allele. This could signify a series of unknown mutation in other genes involved in folic acid synthesis. Of the 35 patients that were sensitive to SP treatment, 6/35 (17%) of them harbored the BR but had wild type alleles at N51 and I164. It is still not known what role the BR has in resistance to SP but according to our results its presence is associated with *in vivo* drug resistance as verified by logistic regression analysis and could therefore serve as a marker for monitoring resistance to this combinational therapy in the Peruvian Amazon.

## CAMBODIAN'S RESPONSE TO HIGH RESISTANCE OF P.F TO ANTI-MALARIA DRUG

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In Cambodia, malaria is a rural disease occurring especially among people who work or live in the forest and forest fringes and it is ranked among the leading causes of mortality and morbidity. Multiple drug resistance of *Plasmodium falciparum* has been a big problem for malaria treatment in Cambodia, especially in the western provinces. The threats to this issue were few patients with malaria received corrected and appropriated malaria diagnosis and treatment and the widespread availability use of fake anti-malarial drugs by risk group who sought first line treatment in the private sector, as fake artesunate and mefloquine is 70-80% in drug outlets (NMCP,2004). This raises the important policy issue of the mechanism of drug delivery (where people get their drugs from), and its impact on drug intake and treatment outcome. In order to facilitate correct dosing, to protect people from fake drugs and to help improve the compliance of patients and health care providers, since early 2002 the sub-com of malaria decided that malaria treatment should be pre-packaged in blister packs for different age and weight groups and distribute by three channels delivery, public and private sector and Village Malaria Workers. On the other hand, more control of the source of drug supplies had been considered thought strengthening the management of pharmaceuticals and other medical supplies and appropriate inventory system. Although in Cambodia, the application of legislation to control the drugs in the market and knowledge of people about correct malaria diagnosis and treatment were still weak, but some action taken have improved the situation. For example, public announcements about fake drugs, the distribution of warning posters and their obligatory display in pharmacies and increase community awareness through mass media such as radio, TV and newspapers, and conducted anti malaria drug sensitivity monitoring at selected sentinel sites may all help to prevent the development and widespread of anti-malaria drug resistance in Cambodia.

## GENOME-WIDE GENE EXPRESSION AND MECHANISM OF CHLOROQUINE RESISTANCE IN THE HUMAN MALARIA PARASITE *PLASMODIUM FALCIPARUM*

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Mutations in the *pfcr* (*P. falciparum* chloroquine-resistant transporter) play a key role in conferring chloroquine (CQ) resistance (CQR) to *P. falciparum* (*Pf*). Mutations in *pfmdr1* (*Pf* multiple drug resistance gene 1) also affect the levels of CQR. Additionally, parasites with the same *pfcr* and *pfmdr1* mutant alleles differ dramatically in their response to CQ, suggesting the involvement of other genes in determining drug susceptibility levels. In areas where CQ use has ceased, the reemergence of CQ sensitive (CQS) parasites suggests that at least some mutations in *pfcr* are deleterious to parasite survival. To understand the effects of *pfcr* mutations on parasite survival and their responses to drugs, we investigated gene expression profiles of two *pfcr* mutant parasite clones derived from a CQ sensitive parasite (106/1) under lethal doses of either CQ or quinine (QN). A total of 96 genes were significantly differentially expressed in the two mutants, including 14 putative transporters. Of the 63 genes that are significantly up-regulated in association with the PfcRT (K76I) mutation, many are involved in parasite invasion or growth, signal transduction, and posttranslational regulation, suggesting compensation for the altered effects of mutant *pfcr*. On the other hand, a total of 33 genes were significantly down-regulated, ~30% of which encode

transporters and membrane proteins (including *pfmdr1*). As a member of a drug/metabolite transporter superfamily, mutations in PfcRT not only affect the transport of CQ and QN, but may also influence other transport processes, leading to changes in expression of relevant transporters. An additional mutation at amino acid position 352 reverses the CQR parasite back to a CQS parasite. Only two genes, however, showed significantly reversed expression patterns in response to this additional change. Further characterization of genes that are differentially expressed between CQS and CQR parasites will lead to a better understanding of drug resistance and normal biological PfcRT functions in malaria parasites.

## EVALUATION OF RAPID DIAGNOSTIC TESTS FOR DIAGNOSIS OF MALARIA AND MONITORING THE EFFICACY OF ANTI-MALARIA THERAPY IN SUDAN

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To improve the diagnosis of malaria and treatment strategies, two RDTs, Paracheck test (HRPII) and optimal test (pLDH) were evaluated to monitor the therapeutic efficacy of CQ. The above mentioned diagnostic tests were performed on blood samples collected during the follow-up days 1, 2, 3, 7, 14, 21 and 28. The total number of blood samples investigated was 570 (470 from the enrolled *P. falciparum* malaria cases and 100 from the blood bank donors as a control). Microscopy showed that 218 cases were positive and 352 were negative. The Paracheck test (HRPII) showed that 249 individuals were positive and 321 negative, whereas the optiMAL (pLDH) showing that 214 were positive and 356 negative. The findings indicated that there were 31 false positives demonstrated by the Paracheck test and no false negative with this test. In contrast, the optiMAL test resulted in only 4 false negative samples and no false positive cases. The results of Paracheck test showed that its sensitivity was 88.9%, its specificity was 91.2%, and its reliability was 80.1%. The optiMAL had in this study a sensitivity of 98.2%, a specificity of 98.9% and reliability of 97%. The study also indicated that CQ resistance frequencies were 43.7%, 47.9% and 41.7% (**P-value = 0.0001**) by using microscopy, Paracheck test and optiMAL tests respectively.

It was concluded from this study that the OptiMal test is the most sensitive and specific and reliable RDT for the diagnosis of malaria, and monitoring antimalarial drugs efficacy, but microscopy remains superior.

## PFMDR1 AND FALCIPARUM MALARIA RESISTANCE TO ARTEMISININ COMBINATION THERAPY IN CAMBODIA

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The combination of artesunate and mefloquine was introduced as the national first line treatment for *Plasmodium falciparum* malaria in Cambodia in 2000. However, recent clinical trials performed at the Thai-Cambodian border have already pointed to declining efficacy of this

combination and to artemether-lumefantrine as well. The aim of this study was to assess the link between *pfmdr1* copy number and artesunate-mefloquine or artemether-lumefantrine treatment failure. Blood samples were collected from *P. falciparum*-infected patients enrolled in two *in vivo* efficacy studies in north-western Cambodia: 113 patients were treated with artemether-lumefantrine in Sampovloun in 2002 and 2003, and 119 patients with artesunate-mefloquine in Sampovloun and Veal Veng in 2003 and 2004. For all these samples, the *in vitro* IC50 was measured and the strains were genotyped for *pfmdr1* copy number by Real Time PCR.

An increase in *Pfmdr1* copy number was positively correlated with mefloquine IC50 ( $r=0.325$ ,  $p=0.001$ ) and lumefantrine IC50 ( $r=0.325$ ,  $p=0.021$ ). Strains with three or more copies of *pfmdr1* were associated with recrudescence in artesunate-mefloquine treated patients (hazard ratio (HR) = 7.16 [95%CI: 1.92 - 26.71],  $p=0.003$ ) but not with recrudescence in artemether-lumefantrine treated patients (HR= 1.14 [95%CI: 0.26 - 4.91],  $p=0.861$ ). In conclusion, *Pfmdr1* copy number was linked to artesunate-mefloquine treatment failure. No association between artemether-lumefantrine treatment failure and increased *pfmdr1* copy numbers could be established. Isolates with decreased *in vitro* susceptibility to mefloquine were common along the border with Thailand. Our results demonstrate that *pfmdr1* copy number is a valid molecular marker of artesunate-mefloquine treatment failure in *falciparum* malaria on the Thai-Cambodian border. The implication for its use to monitor the prevalence and spreading of ACT resistance in this area is discussed.

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### ISLAND-WIDE DIFFERENCES IN SINGLE NUCLEOTIDE POLYMORPHISMS IN THE *P. VIVAX* DIHYDROFOLATE REDUCTASE GENE (*PVDHFR*) IN SAMPLES FROM SRI LANKA

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Single nucleotide polymorphisms (SNPs) in the *Plasmodium vivax* dihydrofolate reductase gene (*Pvdhfr*) is causing resistance to the antimalarial drug, pyrimethamine and is likely to result in resistance to the drug combination sulfadoxine/pyrimethamine (SP) *in vivo*. We have developed a simple enzyme-linked immunosorbent assay (ELISA) system using sequence specific oligonucleotide probes (SSOPs) for high-throughput screening of SNPs in the *Pvdhfr* gene at codon 57, 58, 61 and 117. The sensitivity and specificity of the SSOP-ELISA method was found comparable to results obtained by restriction fragment length polymorphism (RFLP). The SSOP-ELISA was used to examine the prevalence of 10 different SNPs/haplotypes in 384 samples from individuals tested positive for *vivax* malaria mainly between 2004 and 2005 living in 9 different districts of Sri Lanka. In 32% of the samples, 5 different mutant *Pvdhfr* haplotypes were detected, of which 77% were the double mutant haplotype at 57 and 58. The distribution of the remaining mutant haplotypes differed significantly according to geographical origin of infection. Furthermore, a significant temporal distribution of the haplotypes was found with most of the mutations detected succeeding the malaria peak season (March-September 2005). The high frequency of mutations in the *Pvdhfr* was unexpected since SP is only rarely used against *P. vivax* infections in Sri Lanka. It indicates that drug resistant parasites can evolve despite a low level of SP drug pressure possibly attributed to e.g. the use of other antiparasitic drugs.

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### PREDICTORS OF MALARIA INCIDENCE IN A COHORT OF CHILDREN LIVING IN KAMPALA, UGANDA

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Malaria is often considered a homogenous disease in children living in Africa. However, malaria risk may be heterogeneous in urban areas with lower endemicity, which could have important implications for malaria control interventions. We identified predictors of malaria incidence in children living in Kampala, Uganda, using a prospective cohort design. A representative sample of 601 children aged 1-10 were recruited from a census population and followed for all of their health care needs in a designated study clinic. Malaria was diagnosed each time a child presented with a new episode of fever and a positive thick blood smear. Episodes of malaria diagnosed within 14 days of a previous episode were considered treatment failures rather than new episodes. Independent predictors of malaria incidence were identified using a negative binomial regression model with adjustment for clustering of children living in the same household. A total of 671 new episodes of malaria were diagnosed after 682 person years of follow-up (incidence = 0.98/person year). Malaria incidence was highly heterogeneous, with no malaria in 283 children, 1 episode in 146 children, and >1 episode in 172 children (range 2-11 episodes). Malaria incidence decreased with increasing age at enrollment (incidence rate ratio (IRR) = 0.95 per 1 year increase,  $p=0.03$ ). Reported use of an insecticide treated net (ITN; IRR=0.35,  $p=0.001$ , but not use of an untreated net (IRR=0.96,  $p=0.81$ ) was associated with a lower incidence of malaria compared to children with no reported bednet use. Patients with low glucose-6-phosphate dehydrogenase activity had a lower incidence of malaria than those with normal activity (IRR=0.67,  $p=0.02$ ). Sickle cell trait, gender, and low height-for-age or weight-for-height scores were not associated with malaria incidence. An additional spatial analysis is planned. Identification of predictors of malaria incidence can improve our understanding of the epidemiology of this disease and help guide targeted interventions for prevention.

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### MALARIA AND HELMINTH CO-INFECTION IN A SEMI-URBAN POPULATION OF PREGNANT WOMEN IN UGANDA

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Helminths infections currently affect approximately one billion people worldwide, and malaria mortality is estimated a 1 million deaths per year. Recent studies suggest an association between helminth infections and malaria that may be biological in nature. Pregnant women may be at particularly high risk from adverse effects of these infections, such as anaemia and adverse birth outcomes. The aim of this study was to identify risk factors for helminth and malaria infections and describe the spatial distribution of co-infections in pregnant women in Entebbe, Uganda. This cross-sectional study used base-line data from a trial of anti-helminthics based at Entebbe hospital antenatal clinic. Helminth and malaria infections were quantified in 2515 women from stool and blood

samples, a questionnaire recorded socioeconomic and demographic details, and residences were georeferenced. *Mansonella perstans* was independently associated with malaria infection (odds ratio adjusted for age, tribe, socioeconomic status, HIV and residential location: 1.65, 95% confidence interval 1.21-2.21,  $p=0.001$ ) but the probability of co-infection depended on an individual's residential location. An apparent association between malaria and hookworm infections was abrogated after adjustment for confounders. Other helminths showed no significant association with malaria. In conclusion, statistically significant association was observed between *M. perstans* and malaria parasitaemia in this population of pregnant women, which is biologically not understood and may merit further investigation. Location of residence may be important, as it may be associated with overlapping occurrences of the vectors involved.

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### MALARIA IN TRAVELERS RETURNING TO OTTAWA FROM 1995-2004: A RETROSPECTIVE DESCRIPTIVE REVIEW

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Malaria affects 1 per 1000 travelers returning from endemic areas, with a fatality of 0-1.1%. A lack of appropriate pre-travel advice is a major risk factor for severe malaria, yet other factors leading to malaria contraction and complicated disease have not been examined in our country. The aims of this study were to: 1) identify local risk factors active contraction of malaria; 2) describe risk factors based on demographics, pre-trip advice, chemoprophylaxis, and trip details; and 3) evaluate risk factors for presenting with or developing severe, cerebral, or fatal disease. A retrospective, descriptive study of malaria presenting to The Ottawa Hospital (TOH) and the Children's Hospital of Eastern Ontario (CHEO) from 1995 to 2004 was conducted. Subjects included all those with laboratory-confirmed malaria. 72 adults and 34 children had smear-confirmed malaria during the study period, most commonly *Plasmodium falciparum*. 58.3% of adults and 79.4% of children were born in endemic areas, residing in Canada for a mean of 5.2 years. 4 patients were foreign visitors, and 30 newly arrived immigrants. Excluding these, 72 patients were "returned travelers", most commonly from Sub-Saharan Africa (77.8% of patients.) The mean trip duration was 216.6 days. Visiting friends and relatives (VFR) was the most common reason for travel (56.9% of patients.) Of 68 travelers with available data, only 38.2% were on appropriate chemoprophylaxis - 25.6% of 39 VFR's, 55.2% of 29 other travelers. Among all 106 patients, 100 patients had symptom onset post-arrival from a malarious area and 6 patients had symptom onset while in a malarious area. 81.1% of patients presented with mild disease, 18.9% with severe or cerebral malaria. 45.3% of patients had 1 physician visits prior to a suspected diagnosis, leading to delays in obtaining studies (46.1 hours versus 4.2 hours [ $p=0.0019$ ]) and initiating therapy (65.6 hours versus 8.1 hours [ $p=0.0017$ ].) 5 patients had adverse outcomes (1 death, 3 relapses, and 1 lost pregnancy.) In conclusion, we assessed 106 patients with malaria over one decade. Most were travelers, over half of whom traveled as VFR's. Patients presented with malaria across the spectrum of severity, yet adverse outcomes were rare. Chemoprophylaxis is underused, particularly amongst VFR travelers, suggesting a need for targeted outreach of pre-travel programs. Delays are common in diagnosis and treatment of malaria, suggesting a need for awareness programs.

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### DECREASING MALARIA MORTALITY IN VALLE DEL CAUCA, COLOMBIA

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Malaria deaths should be exceptional in non-endemic areas and in endemic countries that have achieved relative good standards of health services. However, Valle del Cauca, one of the largest and developed states in Colombia, reports persistently high numbers of deaths attributable to malaria. The potential factors responsible for malaria deaths were studied and interventions deployed. Baseline indicators for total malaria deaths and lethality rates in the previous 5 years were generated by searching all available sources of malaria-related information. A malaria mortality response group was constituted to analyze each reported death attributable to malaria in 2004 and 2005 and to generate an intervention plan. Emphasis was given to follow-up the interventions. Death certificates were identified as the most reliable source of information. From 2000 to 2004, 115 malaria-related deaths (lethality rate of 2.65/1000 cases) were reported. In 2004, 21 deaths were reported, the majority (8) in residents of the capital of the state which is malaria-free. A program was initiated at the end of 2004 to reduce 50% malaria-related mortality in the state. Travelers from non-endemic to endemic areas were targeted with educational material (such as TV ads, and flyers at transport terminals) aimed at preventing exposure to mosquitoes and to recognized malaria symptoms and complications at an early stage. In 2005, a total of 13 malaria-related deaths (lethality rate 1.9/1000 cases) were reported (a reduction of 38% in number of deaths with respect to 2004) and only one case was in a resident of the capital of the state. The project will continue in 2006 with the objective to further reduce 50% malaria mortality in Valle del Cauca. In conclusion, Decreasing malaria mortality in Valle del Cauca requires multistage interventions: improving surveillance and access to health care, deploying interventions targeted to travellers to endemic areas, and increasing awareness of malaria in health services in both endemic and non-endemic areas.

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### USING HOUSEHOLD SURVEYS TO EVALUATE THE IMPACT OF GLOBAL FUND ACTIVITIES ON MALARIA PREVENTION PRACTICES IN VANUATU

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We propose a model for using household surveys to assess the impact of Global Fund activities on prevention and bednet use as well as malaria burden, and present the preliminary results of our baseline survey of malaria prevention practices, including bednet use and ownership, conducted on a subset of the 80+ islands which comprise Vanuatu. The results of the baseline survey will be used as a guide for health officials in identifying policy priorities regarding the roll out and distribution of the LLNs over the next 2 years, in addition to laying the foundation for a mid-term evaluation. Vanuatu is one of the few countries in the world that has made the introduction and widespread distribution of LLNs (to replace the country's existing, and rapidly aging stock of insecticide-treated nets (ITNs)), the centerpiece of their malaria control strategy. A questionnaire collecting information on malaria knowledge, attitudes, and prevention

practices, and including basic demographic and socio-economic information, was administered to 921 households in 34 villages selected using two-stage cluster sampling. Preliminary survey results indicate that 70% of the households surveyed own at least one net, and households possess 2.2 nets on average. 28% of households have no net at all. Of those households having at least one net, 50% have never retreated any of their nets with insecticide, and 51% report that the oldest net in the household still in use as a bednet is more than three years old. Household size does not predict net ownership, nor does the presence of children < 5. However, SES was found to be predictive of net ownership. 44% of respondents reported having slept under a bed net the night before, compared to 59% who reported that they had not. 52% of women reported sleeping under a net the night before compared to 39% of men). Only 36% of smokers and 37% of drinkers reported net use the night before, compared to 47% of non-smokers and 49% of non-drinkers. We review policy implications of baseline results which serve to justify the shift to long-lasting nets because retreatment rates are low, underscore the need for replacing the existing stock of aging nets in Vanuatu, and highlight the need for better targeting of poorer households and those with children under the age of 5 years.

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### PARASITE CLEARANCE CURVES AND TIMES IN BLANTYRE, MALAWI: EFFECT ON PATIENT OUTCOME AND EVOLUTION OVER A NINETEEN YEAR TIME PERIOD

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We undertook a retrospective analysis of parasitemia for patients with clinically defined cerebral malaria between 1986-2005 (n= 1368). Peripheral parasite density was measured every six hours after the start of treatment with intravenous quinine. By setting the parasitemia at time of admission (T0) at 100%, we were able to plot clearance curves. The initial parasitemia did not have an effect on outcome. In 28% of patients, the parasitemia actually increased over the T0 level; this did not have an effect on outcome either, however, of children with an increase over baseline parasitemia at six hours, those who died had a higher mean increase compared to those who survived (p=0.0065). No significant change in parasite clearance time was detected over the nineteen year observation period. Further examination of the relationship between initial parasitemia and any subsequent increase may yield a useful prognostic tool to identify patients who might benefit from more aggressive treatment. Our data suggest that quinine sensitivity has not significantly changed in the past 19 years.

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### THE BURDEN OF MALARIA IN PREGNANCY IN SOUTH AFRICA

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Malaria in pregnancy is associated with adverse effects such as anaemia, low birth weight, prematurity, stillbirth, miscarriage and maternal death. To prevent these effects, the World Health Organization recommends implementation of intermittent preventive treatment and use of insecticide-treated bednets for all pregnant women at risk of malaria. In South Africa, information is required to provide a better understanding of the burden of malaria in pregnancy, particularly in the malaria highest risk province of KwaZulu-Natal. This information will further support decisions on whether South Africa needs malaria preventive strategies specific for pregnant women. A study was undertaken in KwaZulu-Natal province during 2004 - 2005 to determine the burden of malaria in pregnancy and the effects of malaria on birth outcomes. Data was collected from pregnant women attending antenatal care at 3 health

facilities and included demographic details, current and previous malaria status, haemoglobin level, HIV status, use of insecticide-treated bednets and birth outcomes. Of the 1395 enrolled pregnant women, more than a quarter were teenagers, three quarters attended antenatal care clinic early. Both the prevalence and incidence of malaria were less than 1.5%. The prevalence of anaemia and HIV were high. The fact that the majority of the women attended antenatal care clinic early could be beneficial for implementation of interventions aimed at improving pregnancy outcomes. The low prevalence and incidence of malaria suggest that: 1) anaemia is not due to malaria, but some other factor which needs further investigation; 2) the available malaria control measures aimed at the general population are beneficial for pregnant women; and 3) the findings provide no basis to recommend prevention measures specific for malaria in pregnancy in Kwazulu-Natal province and South Africa.

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### MALARIA INFECTION AND MORTALITY IN CHILDREN IN RURAL NORTHERN GHANA

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Prevalence surveys were carried out in the Kassena-Nankana district (KND) of northern Ghana at the end of the high (November) and low (May) malaria transmission seasons of 2001 and 2002 to document the age specific malaria infection, fever, haemoglobin levels and bednet use. The Navrongo Demographic Surveillance System (NDSS) was used in the random selection of forty compounds (indexed compounds) which formed the nuclei about which clusters were formed. Individuals were radially recruited about the indexed compounds into the study until a minimum number (5) per age group was enrolled per cluster per season. The age groups were: under 1 year, 1-4 years, 5-9 years, 10-19 years, 20-39 years and 40 years and above. A total of 6,991 people aged 1 month to 89 years were sampled. Among the sampled population were 1,929 (27.6%) children under five years of age. Data collected on the under five-year-old children were linked with data on malaria specific mortality in children <5 years from the NDSS to establish the relationship between malaria infection and mortality. Malaria infections were due mainly to *Plasmodium falciparum* (94%). The prevalence of infections (<5years) were highest (p<0.001) and seasonal (dry=37.7%; wet 72.2%, p<0.001) in the rural non-irrigated areas; with no seasonality in the rural irrigated (dry=41.3%; wet 55.4%, p>0.05) and central more urbanized (dry=22.6%; wet 34.3%, p>0.05) areas. There was an association between severe anemia (Hb<6.0g/dl) and malaria infection (p=0.002). Similarly, there was a strong association between fever (axillary temperature ≥ 37.5 oC) and malaria infection (p<0.001). All cause mortality in children <5 years in the KND was 294.5/1000 live births out of which malaria specific mortality was 44.9/1000 live births. All cause mortality in rural non-irrigated areas was higher (mortality = 310.4/1000 live births [CI: 297.3-324.1]) than in rural irrigated areas (mortality = 227.4/1000 live births [CI: 195.5-263]). Malaria specific mortality was also higher in rural non-irrigated areas (mortality = 48.4/1000 live births [CI: 43.4-54.0]) than in rural irrigated areas (mortality = 26.6/1000 live births [CI: 16.3-40.0]). High malaria infections may contribute significantly to malaria mortalities in rural communities.

### INTERLEUKIN-23 AND INTERLEUKIN-12 CYTOKINE PATTERNS DURING MALARIAL ANEMIA IN YOUNG KENYAN CHILDREN NATURALLY EXPOSED TO FALCIPARUM MALARIA

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Severe malarial anemia (SMA) in African children is characterized by an imbalance in pro- and anti-inflammatory cytokine production. We have recently shown that immunoprotective responses, such as interleukin (IL)-12 production, are suppressed in children with SMA, due to parasite-induced over-production of IL-10. Although the role of IL-23, a newly discovered pro-inflammatory cytokine, in mediating the immunopathogenesis of SMA has not been reported, altered levels of IL-23 are associated with chronic inflammatory anemia. IL-23 shares similarities with IL-12, including a common p40 subunit and suppression by IL-10. IL-23 is also suppressed by IL-12. To further define the pathogenesis of SMA, we investigated interactions between IL-10, IL-12, and IL-23 in Kenyan children (n=69; aged <36 months) from a holoendemic *Plasmodium falciparum* transmission area of western Kenya. These investigations revealed that IL-23 and IL-10 levels were elevated in children with SMA (Hb less than 6.0 g/dL) relative to gender- and age-matched aparasitemic healthy controls (Hb greater than 11.0 g/dL, P=0.09 and P<0.001, respectively), while circulating IL-12 levels were suppressed in children with SMA relative to the HC group (P<0.01). Although IL-23 levels were not significantly associated with either Hb levels or parasitemia, IL-10 levels were inversely correlated with Hb levels (r=-0.418; P<0.001) and positively associated with parasitemia (r=0.437; P=0.002). IL-12 levels were positively correlated with Hb levels (r=0.278; P=0.022) and inversely associated with parasitemia (r=-0.329; P=0.024). Furthermore, although the IL-12/IL-23 ratio was positively correlated with Hb (r=0.228, P=0.08) and inversely associated with parasitemia (r=-0.330, P=0.031), IL-23 and IL-12 were inversely correlated (r=-0.233, P=0.056). Taken together, these results suggest that IL-12 and IL-23 have distinct patterns of expression in children with SMA. Additional studies examining the downstream effector functions of IL-12 and IL-23 may offer insight into the roles of these cytokines in the pathogenesis of SMA.

### EVALUATION OF HOST HUMORAL ANTIBODY MECHANISMS AGAINST *PLASMODIUM FALCIPARUM* RECOMBINANT CIRCUMSPOROZOITE ANTIGEN IN NIGERIAN CHILDREN

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Facing the challenge of controlling the malaria vector (the mosquito), and parasite resistance, a malaria vaccine suitable for young children and women of child bearing age offers an ideal solution. Efforts in this study were devoted to evaluate the reactivity of IgG and its subclasses in the test serum specific to circumsporozoite protein. Twenty-five serum samples (>10,000 parasites/ $\mu$ l of blood) were collected from malaria-infected children at the General-Outpatient clinic, University College Hospital, Ibadan during the transmission season. Samples were analyzed using two different ELISA. The assays were done in triplicates. The mean absorbance values of IgG subclasses reactive against *Plasmodium falciparum*

circumsporozoite protein appeared to be age dependent and ranged from 0.01 for IgG 4 in elder patients to 0.98 for IgG 3 in youngsters. The study also demonstrated that babies <6 month had a certain proportion of IgG subclasses with IgG 3 been the highest. The twenty-five subjects investigated in this study had significantly higher mean IgG 1 and IgG 3 than the uninfected controls. This follows the order IgG 3>IgG 1>IgG 2>IgG 4 which confirmed the prevalence of the cytophilic antibodies (IgG 1 and IgG 3) over the non-cytophilic subclasses (IgG 2 and 4). There was low production of IgG 4 and IgG 2 level in 35% of the subjects. A particular 4 month old subject was at borderline to IgG 4 with a cut-off value of 0.03. IgG was also detected in the North American serum (NAS) which served as negative control for CSP-specific IgG subclasses, although NAS titre was lower than each of the malaria subjects in Nigeria, except its IgG2 that was higher (0.16) than other subclasses. Age-dependent variations suggest that acquired immunity could play a significant role in the overall protection from malaria infections. Findings from this study revealed that IgG3 and IgG1 were the most prevalent co-expressed subclasses in 65% of the malaria infected children and could play a potential role as valuable target of the CSP vaccine development.

(ACMCI Abstract)

### DIVERSITY OF TLR SNPS IN MALARIA ENDEMIC AREAS

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Toll-Like Receptors (TLRs) are components of the innate immune system that recognize molecular patterns associated with invading microorganisms. TLR Single Nucleotide Polymorphisms (SNPs) may result in differential responsiveness to microbial TLR ligands, and thereby result in within-population variability in susceptibility to infectious diseases. Our hypothesis is that exposure to infectious pathogens introduces selective pressure on the human genome that results in genetic diversity of TLRs. TLRs 2, 4, and 9 are activated by glycosylphosphatidylinositol and hemozoin ligands produced by the malaria parasite *Plasmodium falciparum*. We examined the frequency of the four most common TLR9 SNPs in human populations from three areas with differing malaria transmission levels- high transmission holoendemic areas of Papua New Guinea and lowland Western Kenya and a low transmission and unstable transmission of highland Kenya. Preliminary data analysis indicates that there are no differences in TLR9 polymorphisms between the high and low transmission study sites in Kenya, and TLR9 polymorphism in these ethnicities is similar to that of European populations with no recent history of malaria exposure. In contrast, less polymorphism exists in the TLR9 gene of Papua New Guineans compared to Kenyans. These results indicate that malaria transmission is not associated with the distinct patterns of the TLR9 SNPs examined here. Future analyses will include characterization of TLR2 and TLR4 polymorphism, and estimating the level of TLR genetic diversity within and between these populations.

(ACMCI Abstract)

### THE ROLE OF *BIR* GENES IN RODENT IMMUNITY TO *PLASMODIUM BERGHEI* NK65

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Understanding the development of immunity to malaria in the rodent and the mechanisms employed by the parasite to evade the immune system will lead to new approaches to fight malaria in humans. One understudied family of potential variant antigens in *Plasmodium berghei* is the *bir* gene family. A multi-copy family of subtelomeric genes, the *bir* genes belong

to the larger group of homologous antigens known as *Plasmodium* interspersed repeats (*pir*). *bir* homologues have been identified in many malaria species, including *P. vivax*, *P. knowlesi*, *P. chabaudi*, and *P. yoelii*. We set out to examine the effect of immune pressure on *bir* gene repertoire. In an immune model, mice are infected i.p. with  $1 \times 10^6$  *P. berghei* NK65-infected red blood cells (iRBCs) and drug cured with a regimen of chloroquine starting when parasitemia reaches 2-3%. Mice are challenged with  $1 \times 10^6$  iRBCs at least 40 days after clearance of the initial infection. We are in the process of identifying changes in *bir* gene expression between parasites used for immunization, those used for subsequent challenge, and those able to escape immunity. Analysis of *bir* gene expression was performed by both RT-PCR using conserved primers and hybridization of cDNA to specific *bir* genes on a blot. We observe at least one *bir* gene expressed in all parasites examined, and multiple *bir* genes are expressed in each parasite population.

(ACMCIP Abstract)

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### CYTOKINE-ASSOCIATED NECROBIOSIS AMONG PLASMODIUM FALCIPARUM INFECTED CHILDREN UNDER THE AGE OF SIX

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Relating distinct erythrocyte and leukocyte disorders to circulating cytokine levels may elucidate the role the immune system plays in falciparum malaria pathology in young children. In this investigation, blood samples from 21 Nigerian children under six years old diagnosed with clinically-uncomplicated falciparum malaria were obtained before and 7 days after treatment with sulfadoxine-pyrimethamine (SP). Abnormal blood cells were found in all 21 pretreatment samples, revealing an unexpected neutrophil necrobiotic activity in a leukemoid reaction, which correlated with specific cytokine levels. This study explores the relationships of plasma cytokine levels with erythrocyte aggregation and apoptosis-like vacuolation (eryptosis), which may cause anemia directly during chronic falciparum exposure, and with leukocyte necrobiosis, which may suppress lymphocyte activity or induce production of antibodies that might cause an autoimmune response that contributes to malaria pathogenesis or that might provide immunoprotection against the parasites. When humoral and/or cell mediated immune responses are compromised by the low levels of IL-2 found, neutrophil necrobiosis and circulating fibrinoid aggregates may stimulate an autoimmune response to molecular patterns, which may over time provide an alternative mechanism of protection against the parasite. The high levels of IL-10 and CRP found with necrobiotic activity suggest a mechanism by which these cytokines modulate the immune response to necrobiotic activity to suppress its effects. On the other hand, the high levels of TNF $\alpha$  suggest that inflammation caused by necrobiotic leukocyte release of intracellular contents may stimulate adjuvant activity of released self-DNA contributing to an autoimmune leukemoid reaction similar to that found in systemic lupus erythematosus production of anti-nuclear antibodies (ANA), or the production of anti-neutrophil cytoplasmic antibodies (ANCA). Necrobiotic activity may determine maintenance of homeostasis, generation of induced pathology, or a protective mechanism as yet undiscovered.

(ACMCIP Abstract)

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### IFN- $\gamma$ IS NECESSARY FOR THE SUPPRESSION OF PLASMODIUM YOELII 17XL MALARIA IN MEROZOITE SURFACE PROTEIN-8 IMMUNIZED BALB/C MICE

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C57BL/6 and BALB/c mice have been effectively used to evaluate polarized Th1 and Th2 immune responses induced by protein immunization and/or infection with a variety of pathogens. Previously, we reported that BALB/c mice could be protected against lethal *Plasmodium yoelii* 17XL malaria by prior immunization with recombinant PyMSP-8, a 46 kDa glycosylphosphatidylinositol anchored merozoite surface protein. To better understand PyMSP-8 dependent protection, we compared the response of BALB/c and C57BL/6 mice to immunization with rPyMSP-8 and to subsequent challenge with *P. yoelii* 17XL. Analysis of prechallenge sera revealed high and comparable levels of PyMSP-8-specific IgG in these two strains of mice. The isotype profiles of vaccine-induced antibodies showed that BALB/c mice produced significantly more PyMSP-8-specific IgG1 relative to C57BL/6 mice and a somewhat greater proportion of PyMSP-8-specific antibodies that recognized conformational epitopes. Upon challenge, PyMSP-8 immunized BALB/c mice were protected against lethal *P. yoelii* 17XL malaria while parasitemia in PyMSP-8 immunized C57BL/6 mice was not significantly different than in control animals. These data suggest that PyMSP-8 induced protection correlates with a Th2 and IgG1 biased immune response. To investigate this association further, sera from PyMSP-8 immunized BALB/c and C57BL/6 mice were tested in a set of passive protection assays. In contrast to the active immunization data, passive transfer of both sets of immune sera to naive mice significantly delayed the onset of parasitemia in *P. yoelii* 17XL challenged mice. In addition, we observed that PyMSP-8 immunized IL-4-/- knock-out mice were still protected against *P. yoelii* 17XL malaria despite an impaired Th2 response and a dramatic reduction in IgG1 production. Surprisingly, PyMSP-8 immunized IFN- $\gamma$ -/- knock-out mice, which exhibited an enhanced production of PyMSP-8-specific IgG1 antibodies succumbed to *P. yoelii* 17XL malaria during the second week of infection. We then analyzed serum IFN- $\gamma$  levels and splenic IFN- $\gamma$  mRNA levels in PyMSP-8 immunized mice challenged with *P. yoelii* 17XL. Throughout the course of infection, BALB/c mice produced a greater amount of IFN- $\gamma$  than C57BL/6 mice. Combined, these data indicate that in addition to PyMSP8-specific antibodies, infection-induced IFN- $\gamma$  is necessary for the complete control of *P. yoelii* 17XL parasitemia in PyMSP-8 immunized mice.

(ACMCIP Abstract)

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### REVISITING THE INTERACTION OF DENDRITIC CELLS WITH MALARIA BLOOD STAGE PARASITES

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More than a century since Alphonse Laveran first discovered malaria parasites in the blood of an infected patient, the host immune response against malaria remains poorly understood. Epidemiological data suggest that malaria-specific immune responses develop slowly with short duration. Several studies in recent years have focused on the interaction of blood stage parasites with antigen-presenting dendritic cells (DCs). Overall, the data from these investigations are conflicting. While some investigators reported that blood stage parasites suppressed DC functions, other investigators demonstrated activation of DCs. This prompted us to reinvestigate the interaction of *Plasmodium falciparum* blood stage parasites with DCs. Our results demonstrated that both schizont lysate and purified hemozoin poorly induced human monocyte-derived DC

(Mo-DC) and peripheral blood CD11c<sup>+</sup> DC maturation as monitored by up-regulation of co-stimulatory molecule expression. In contrast to human DC responses, mouse bone marrow CD11c<sup>+</sup>B220<sup>-</sup> DCs were significantly activated by schizont lysate as indicated by up-regulation of co-stimulatory molecule expression and IL-12p40 production. Purified hemozoin had no effect on mouse bone marrow CD11c<sup>+</sup>B220<sup>-</sup> DCs. The difference in responses of DCs derived from human and mice against *P. falciparum* blood stage products are of considerable interest and may depend on the differential expression of TLRs, especially TLR9. Our results suggest that unlike mouse CD11c<sup>+</sup>B220<sup>-</sup> DCs, human myeloid DCs are poorly activated by *P. falciparum* blood stage parasites and this may explain poor anti-malaria immune responses observed in infected individuals.

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### MALARIA EXACERBATES MYCOBACTERIAL INFECTION *IN VITRO* AND *IN VIVO*

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Malaria and tuberculosis (TB) are both intracellular pathogens which cause significant morbidity and mortality on a global scale. In the geographic areas co-endemic for the two diseases, co-infection with malaria and TB is likely to be common. Host immune responses to *Plasmodium* are modulated by concurrent mycobacterial infections. The macrophage (mφ) is an effector target for both TB and malaria. We hypothesize that malaria may increase intracellular mycobacterial replication and accelerate the clinical course of infection *in vivo*. Using a murine macrophage cell line (RAW 264.7) with *Mycobacterium marinum* and *Plasmodium falciparum* as an *in vitro* model of malaria-mycobacterium interaction, we demonstrate that the growth of *M. marinum* was significantly greater ( $p < 0.05$ ) in mφs exposed to *P. falciparum*-infected erythrocytes than in mφs infected with *M. marinum* alone. Subsequently, we used a well-characterised murine malaria model to study the effects of malaria and mycobacterium co-infection *in vivo*. C57BL/6 mice were infected with *Mycobacterium bovis* BCG and *Plasmodium chabaudi* AS. The animals were sacrificed at various times and their organs were harvested for histologic evaluation and quantitative assessment of mycobacterial growth. Cytokine profiles were obtained for TGF-β, IFN-γ and TNF-α, using mouse sera in sandwich ELISA. Mice co-infected with *M. bovis* BCG and *P. chabaudi* had higher mycobacterium loads in their spleens ( $p = 0.04$ ), livers ( $p = 0.02$ ) and lungs ( $p < 0.05$ ), compared to mice infected with *M. bovis* BCG alone. Studies currently underway aim to elucidate the mechanism(s) underlying the loss of control of mycobacterial infection in the presence of malaria.

(ACMCIP Abstract)

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### NEW *PLASMODIUM VIVAX* GENOTYPES IN THE NORTH COAST OF PERU

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Piura, in the north coast of Peru, is endemic area for *Plasmodium vivax* malaria. Malaria is sporadic and prone to epidemics in Piura, in contrast to an endemic pattern of transmission in the Peruvian Amazon. Different *P. vivax* strains with different antigenic characteristics might be responsible for vivax malaria in the two regions. The objective of this study was to compare *Plasmodium vivax* genotypes from the north coast of Peru to

those in the Iquitos Amazon region of Peru. 71 febrile subjects were enrolled in two health posts with high malaria endemicity in Piura from 12/04 to 01/06. Thick smear was used for case detection. Genotyping was performed using PCR for tandem repeat polymorphism markers and PCR-RFLP of PvMSP-3α using *HhaI* and *AluI* digests. Phylogenetic multilocus sequence typing analysis was performed using Start2 to generate a neighbor-joining tree from allelic profile data. Of 71 febriles, 27 were positive for *P. vivax* by light microscopy. 41% of the smear-positives were rice farmers or merchants (7%); 22% of the smear-positive cases had had one episode of malaria in the previous year. Of 12 strains analyzed, 11 were distinguishable by a combination of Tandem Repeat Polymorphisms and PvMSP-3α. These strains clustered separately from 24 strains previously obtained from a village in the Peruvian Amazon as determined by neighbor-joining phylogenetic tree analysis. In conclusion, the *P. vivax* genotypes found in Piura differ from those found in the Amazon region of Iquitos. The difference in genotypes may indicate differences in antigenic types in the two regions. Further studies are needed to determine whether different genotypes found by PvMSP-3α and the Tandem Repeat Polymorphic markers correspond to allelic differences in malaria vaccine candidates.

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### INVESTIGATING THE FUNCTION OF MAEBL IN THE INVASION OF SALIVARY GLANDS

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*Plasmodium falciparum* is the malaria parasite responsible for the majority of fatalities, causing at least 2 million deaths each year. An essential step for the transmission of the disease is the passage through the *Anopheles* mosquito. In fact, the *Plasmodium* sporozoites have to invade the salivary glands of the mosquito in a process in which parasite ligands are involved through the recognition of specific host receptors. One of the proteins considered essential for the invasion of salivary glands is MAEBL, which is a conserved protein expressed abundantly in midgut sporozoites. The precise molecular form of the mature MAEBL protein in invasive midgut sporozoites is not known, since MAEBL is proteolytically processed and different potential isoforms are formed by alternative splicing of the 3' end. In order to understand the function of *P. falciparum* (NF54) MAEBL in the sporozoite invasion of salivary glands, we created a series of constructs by allelic replacement of the 3' end. The transfected parasites express only one of the two main molecular forms of MAEBL. The characterization of the transfected parasites will be presented. The understanding of the biological function of MAEBL in invasion of salivary glands will help validate the use of MAEBL as a target for vector-based strategies for transmission blocking.

(ACMCIP Abstract)

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### EXPRESSION OF THE 19-KDA C-TERMINAL PORTION OF *PLASMODIUM FALCIPARUM* MSP1 WITH AND WITHOUT GPI ANCHOR SIGNAL SEQUENCE IN MAMMALIAN CELLS AND EVALUATION OF THEIR IMMUNOGENICITY

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Malaria caused by *Plasmodium falciparum* is a major global public health problem and causes millions of death yearly. A safe and effective vaccine is essential for combating malaria. Among candidate antigens of *P. falciparum* that have been studied, the merozoite surface protein 1 (MSP-1), especially its metabolically processed 19-kDa portion (MSP-119), is of particular interest. In vitro studies showed that mouse antibodies against the cysteine rich EGF-domains of MSP-119 inhibit *P. falciparum* merozoite invasion of erythrocytes. In *falciparum*-*Aotus* model, protection has been achieved by immunizing with complete Freund's adjuvant,



but immunization with other adjuvant formulations gave no protection. In a recent clinical trial in humans, MSP-119 adsorbed onto alum was only moderately immunogenic and showed low antibody and T cell responses. These studies suggested antibody titer and its subtypes play an important role in the protective immunity against malaria. In this study, we constructed two expression plasmids that encode the carboxyl terminal MSP-119 from the *P. falciparum* 3D7 strain with and without the GPI anchor signal sequence. The polypeptides were expressed in HEK-293 cells. Immunofluorescence analysis demonstrated that the polypeptide produced from the plasmid containing MSP-119 without GPI anchor signal sequence was localized intracellularly, whereas, the polypeptide produced from the plasmid with GPI anchor signal was predominantly expressed on the cell surface. Metabolic labeling of cells with [<sup>3</sup>H]glucosamine and analysis of radiolabeled MSP-119 by SDS-PAGE/fluorography and Western blotting confirmed the presence of GPI anchor in the cell surface expressed MSP-119, and that lack of GPI moiety in MSP-119 expressed intracellularly. Immunization of mice with cDNA from the two plasmids elicited MSP-119-specific IgGs in both cases, but without significant difference in antibody titer. We are currently analyzing the antibody isotypes.

(ACMCIP Abstract)

## 571

### REGULATION OF GENE EXPRESSION IN THE MALARIA PARASITE *PLASMODIUM FALCIPARUM* BY THE HISTONE ACETYLTRANSFERASE PFGCN5

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Histone acetylation is an important epigenetic mark that plays a major role in transcription regulation. The dynamics of histone acetylation is regulated by the opposing actions of histone acetyltransferases and histone deacetylases. In the malaria parasite *Plasmodium falciparum*, the GNAT (GCN5-related N-acetyltransferase) family member, PFGCN5, preferentially acetylates histone H3 at K9 and K14. Here we applied chromatin immunoprecipitation and DNA microarray to map the global distribution of histone H3-K9 acetylation and PFGCN5 in the *P. falciparum* genome. We have demonstrated that H3K9 acetylation was distributed on all 14 chromosomes and it was highly correlated with the distribution of PFGCN5. Further mapping of selected genes indicated that the acetylation was located in the promoter region. Also, this low-resolution mapping suggests a weak correlation with gene expression, suggesting that PFGCN5 is recruited to the promoters of genes and regulates gene expression in the malaria parasites.

(ACMCIP Abstract)

## 572

### THE *PLASMODIUM* SES PROTEIN EXHIBITS A SPIRAL LABELING PATTERN ON THE SPOROZOITE SURFACE AND APPEARS TO PLAY A ROLE IN MOSQUITO SALIVARY GLAND INVASION

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The *Plasmodium* sporozoite has dual infectivity for mosquito salivary glands and vertebrate host tissues. Although it is a key developmental stage of the malaria parasite, relatively few sporozoite surface proteins or secreted proteins have been identified and characterized. The recently characterized Sporozoite and Erythrocytic Stage (SES) protein is expressed in both the erythrocytic stage parasites and on the sporozoite surface. In *P. gallinaceum*, the SES protein (PgSES) is 63.2 kDa and has three

conserved regions of unknown function that are present in seven other *Plasmodium* spp. representing human, non-human primate, and rodent malaria. PgSES is preferentially expressed in salivary gland sporozoites versus hemolymph and oocyst sporozoites. Interestingly, the PgSES protein exhibits a spiral surface labeling pattern on the sporozoite and overlays a known surface antigen, the circumsporozoite protein, with only minor co-localization. Studies assessing the role of PgSES in sporozoite invasion of mosquito salivary glands have shown that anti-PgSES antibodies injected intrathoracically into parasite-infected *Aedes aegypti* mosquitoes block sporozoite invasion by 49-61% when compared to controls. Currently, additional studies assessing the function of both PgSES and PfSES in the sporozoite and erythrocytic stages are being conducted.

## 573

### CONTROL OF COCCIDIOSIS IN POULTRY WITH LIVE VACCINES AS A MODEL FOR THE CONTROL OF MALARIA

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Coccidiosis is a common protozoan disease of commercial chickens and turkeys. This disease has been well controlled for over five decades with anticoccidial drugs but with declining efficacy because of the emergence of drug resistance against existing drugs and with no new drugs on the horizon. However, with the introduction of the concept of uniform exposure, several live vaccines of multiple *Eimeria* spp have also been used successfully for the past 2 decades on at least 10 billion commercial birds. These live vaccines do not merely complement the use of medications but can also be used synergistically in the control of coccidiosis. They may also serve as useful tips for the control of malaria a disease caused by fellow Apicomplexan parasites of coccidia. 1. Comparable performance between vaccine and medication allows the rotating or selective use of either vaccine or medication. 2. Drug sensitive vaccine strains can be used to salvage drug resistant anticoccidials (or antimalarials in malaria) by the displacement of field strains, as reported previously 3. Use of vaccine first followed with a brief period or medication to mitigate possible vaccine reaction, as reported previously, can be a synergistic use of vaccine and medication. 4. Like malaria, there is no cross-species or cross-strain protection observed in coccidiosis. 5. Strangely, 3 or 4 more commonly used commercial vaccines containing 4 to 7 species of *Eimeria* spp can be used in rotation and perform equally well with vaccines containing fewer species. 6. Antigenic gene transfer from a pathogenic species (or strains) into a less pathogenic *Eimeria* spp, as reported previously, may be used to reduce the number of species or strains required in vaccines. 7. To date, no dead vaccines have been shown to successfully control coccidiosis in commercial poultry.

(ACMCIP Abstract)

## 574

### VALIDATION OF ASSAYS RELEVANT TO IMMUNOGENICITY ASSESSMENT OF CSP-DNA VACCINE IN GHANA

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Malaria is one of the most significant public health problems in developing countries and is argued to be a major indirect cause of poverty. The emergence of multiple drug-resistant parasites and insecticide-resistant mosquitoes has shifted focus to the development of an anti-malaria vaccine. Such a vaccine may include multiple-antigens from the pre-

erythrocytic, erythrocytic and gametocytic stages of the parasite for complete immunity. A phase 1 CSP-DNA vaccine trial is planned in Ghana, in collaboration with the Naval Medical Research Center and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health. The immunization strategy involves first priming with CSP-DNA vaccine and then boosting with a MVA-recombinant vaccine to enhance both T-cell and antibody responses. To validate standardized ELISPOT, ELISA and IFA assays, a baseline study was done using PBMC and plasma samples from 40 malaria exposed Ghanaians and 5 non-exposed US volunteers. The data indicated that both the magnitude and responder proportions to overlapping CSP vaccine peptide antigens were higher in the Ghanaians compared to the US volunteers. In the ELISPOT assay, we considered a response positive if the number of spots in PBMC incubated with the PfCSP peptide pool was (1) statistically significantly greater than the response to medium alone (2) two-fold higher and (3) at least 25 greater than the medium control in absolute SFC count. Using this criteria, fourteen of 40 Ghanaians responded to 1 or more CSP peptide pools whilst none of the non-exposed US volunteers did. ELISA Antibody titres to recombinant CSP at OD<sub>0.5</sub> ranged from 160 to 3000, whilst that in the US volunteers were less than 50, clearly indicating higher anti-CSP responses in the Ghanaians. IFA titres to sporozoite were also higher in the Ghanaians (ranging from <10 to 640, mean titre >80) compared to the non-exposed US volunteers (mean titre <10). The study has allowed us to develop algorithms for assessment of the proportion as well as magnitude of antigen induced interferon  $\gamma$  responses by ELISPOT and anti-CSP antibody responses by ELISA. This is a critical component of immunogenicity evaluation in the upcoming phase-1 CSP-DNA vaccine trial as well as subsequent vaccine trials in Ghana.

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### MOUSE POTENCY ASSAYS FOR MEASURING RECOMBINANT PROTEIN VACCINE STABILITY

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Potency testing is done on GMP batches of purified, lyophilized recombinant protein malaria vaccines to ensure both the longevity of the vaccine in the vial, and to quantify any lot-to-lot variation. To measure potency, groups of 10 mice are injected with vaccines formulated in adjuvants intended for human use, at a range of antigen doses. Serologic responses are measured by a standardized ELISA. The dose group is chosen in which approximately half of the animals meet a statistically defined criterion of seroconversion. This dose, plus the next highest and next lowest dose group, are repeated quarterly for the first year, and annually thereafter. Each vaccine formulation has been found to require individual dose optimization to choose the best three dose groups to follow over time. Although the ELISA itself has been increasingly standardized, the statistical approach to defining seroconversion has reduced the impact of inevitable variation over time in the assay itself. This strategy, in conjunction with physicochemical characterization, has enabled us to demonstrate a long shelf-life for our candidate malaria vaccine products.

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### USE OF ADENOVECTOR ARRAYS FOR HIGH THROUGHPUT SCREENING OF NOVEL MALARIA ANTIGENS FROM GENOMIC SEQUENCE DATA

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We have evaluated the potential of arrays of adenovirus vectors for the identification of novel malaria vaccine candidates from genomic sequence

data. In the BALB/c *Plasmodium yoelii* (Py) mouse model, immunization with irradiated sporozoites or with unirradiated sporozoites followed with drug treatment confers robust protective immunity against sporozoite challenge. We and others believe that this immunity is mediated by T cell responses to multiple pre-erythrocytic stage antigens, including the well characterized antigens such as PyCSP and PyHep17, as well as, as yet unknown antigens that are expressed in the sporozoite and liver stages of parasite development. We hypothesized that these novel antigens can be identified by an *in vitro* immune screening system and when identified, could represent potent pre-erythrocytic stage vaccine candidates. In our initial studies, we transduced A20/2J cells with adenovectors expressing PyCSP and used these as APCs in an IFN  $\gamma$  ELISpot assay, with splenocytes from mice immunized with *P. yoelii* CSP plasmid DNA or *P. yoelii* sporozoites as effectors. Strong PyCSP specific responses were recalled from splenocytes isolated from immune mice but not from unimmunized mice. Subsequently, we transduced A20 cells with adenovectors expressing uncharacterized *Plasmodium* genes (generated using the Gateway system), and with PyCSP in parallel as a positive control, for evaluation by IFN  $\gamma$  ELISpot. Positive recall immune responses with splenocytes from sporozoite immunized mice have been demonstrated. *In vivo* studies to assess the potential protective efficacy of selected antigens is anticipated. We envisage that the *Plasmodium falciparum* orthologues of the most promising antigens will represent high priority antigens for the development of a vaccine against malaria.

(ACMCI Abstract)

## 578

### PILOT-SCALE PRODUCTION OF THE *PLASMODIUM VIVAX* TRANSMISSION BLOCKING VACCINE CANDIDATE PVS28

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The development of a novel malaria vaccine that blocks transmission of *Plasmodium vivax* in the mosquito host is being investigated. The ookinete specific proteins, Pvs25 and Pvs28 which are expressed by the parasite in the mosquito midgut are targets of transmission blocking antibodies. A human Phase I study with *Saccharomyces* derived Pvs25H on alum induced limited antibody and transmission blocking responses. We hypothesize that the inclusion of Pvs28 would enhance this response. Therefore, we have developed a scalable process for pilot-scale production of Pvs28. Pvs28 was expressed in the methylotrophic yeast *Pichia pastoris* using a synthetic codon optimized gene in which putative N-linked glycosylation sites were replaced by conserved amino acid substitutions. After screening Pvs28 transformants, a production clone was identified and fermentation conditions were optimized for pH, temperature and methanol feed in chemically defined media in 5L fermentation runs. Fermentation broth was harvested by microfiltration in serial with ultrafiltration and diafiltration. Clarified, dialyzed and concentrated supernatant was purified using immobilized metal affinity chromatography, hydrophobic interaction chromatography, Q membrane and size exclusion chromatography. The final bulk substance was characterized biochemically and biophysically for purity, integrity and as well as conformation. The process was successfully tech transferred to a contract pilot-plant facility where a 60L fermentation and downstream purification was performed in accordance with cGMP. The yield of cGMP Pvs28 bulk substance was ~50 mg/L fermentation broth. Pvs28 bulk substance is now available for human Phase I studies.

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## RE-INGESTION OF *PLASMODIUM BERGHEI* SPOOROZOITES AFTER DELIVERY INTO THE HOST BY MOSQUITOES

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Malaria-infected mosquitoes feeding on a mammalian host inject sporozoites into the skin to induce a malaria infection. The numbers of sporozoites ultimately able to reach the liver are important determinants of the characteristics of the ensuing blood infection. Because mosquitoes not only inject sporozoites into the host but concomitantly ingest blood to obtain a bloodmeal, some sporozoites are re-ingested by the feeding mosquito. We studied transmission of fluorescent *Plasmodium berghei* sporozoites injected into mice by individual *Anopheles stephensi* mosquitoes and were able to quantify the numbers re-ingested by the mosquito. To distinguish between sporozoites ingested into the midgut with the bloodmeal vs. exogenous sporozoites already on the exterior of the midgut, we developed a procedure that allowed us to successfully quench fluorescence from exterior sporozoites with no discernable effect on fluorescence of sporozoites within the midgut lumen. To further confirm that sporozoites we observed within the midgut lumen were salivary gland sporozoites re-ingested during feeding rather than endogenous oocyst sporozoites already residing within the abdomen, we used two procedures previously shown to differentiate between these classes of sporozoites: assessment of sporozoite gliding motility (characteristic of sporozoites of salivary gland but not oocyst origin) and assessment of the circumsporozoite precipitation reaction (also characteristic of sporozoites of salivary gland but not oocyst origin). In both cases, we found that the luminal sporozoites fit the phenotype of salivary gland rather than oocyst sporozoites, thus confirming that they had been re-ingested by the feeding mosquito. Our results showed that the numbers of sporozoites re-ingested by mosquitoes are comparable to numbers previously reported to be delivered directly into mice. Thus, re-ingestion of sporozoites likely plays a significant role in transmission dynamics of malaria by mosquitoes, and may account for failure of some sporozoite-infected mosquitoes to induce blood infections.

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## DIVERSE *WOLBACHIA* STRAINS CAN INFECT *ANOPHELES GAMBIAE* CELLS

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*Wolbachia* are maternally-inherited endosymbionts associated with cytoplasmic incompatibility (CI). CI provides *Wolbachia*-infected females with a reproductive advantage relative to uninfected females, promoting the spread of *Wolbachia* into uninfected host populations. This ability has led to the proposed use of *Wolbachia* to spread transgenic traits into *Anopheles* populations for malaria control. *Wolbachia* infections are common in many mosquitoes, but have never been observed in any *Anopheles* species, leading to the hypothesis that *Anopheles* mosquitoes are incapable of harboring infection. We used an *in vitro* system to evaluate the ability for *Anopheles gambiae* cells to harbor diverse *Wolbachia* infections. We successfully established *Wolbachia* infections (wRi and wAlbB) in the immunocompetent *Anopheles gambiae* cell line Sua5B. Infection was confirmed by PCR, antibiotic curing, DNA sequencing and direct observation using fluorescence *in situ* hybridization. Infections have been stable for >30 passages with no decrease in infection levels. Our results indicate that there is no intrinsic genetic block to *Wolbachia* infection in *A. gambiae* cells, suggesting that establishment of *in vivo* *Wolbachia* infections in *Anopheles* mosquitoes may be feasible.

(ACMCIP Abstract)

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## ENVIRONMENTAL MANAGEMENT FOR MOSQUITO CONTROL IN SOME SELECTED STATES IN THE UNITED STATES WITH RECOMMENDATIONS FOR NIGERIA

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In both the developed and developing nations of the world, vector borne diseases such as malaria constitute a major threat to health. World wide, mosquitoes are responsible for the transmission of malaria and the factors responsible for mosquito breeding are found in the physical environment. Malaria is one of the greatest health problems in Nigeria. The environmental factors contributing to malaria vector breeding in Nigeria include the absence of proper systems of solid and liquid waste disposal, heavy rainfall and the poor drainage systems. In view of the high mortality and morbidity rates due to malaria in Nigeria, the author undertook research into environmental management for malaria vector control. This study considered the naturalistic methods of mosquito control. Source reduction methods used for mosquito control in the united states have achieved a high degree of efficiency due to financial and manpower resources, well organized mosquito control staff and awareness by the public of the basic principles of public health. However, the best mosquito control is achieved when various method of mosquito control are integrated into the overall pro gram. At present, Nigeria could operate an efficient environmental management for mosquito control as is found in the United States by incorporating some of the basic environmental control practices used for mosquito control in the united states. There is therefore, an immediate need to replicate these studies in Nigeria with a view to determining which of the control strategies will be more applicable to Nigeria considering her unique socio-cultural characteristics.

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## FIRST REPORT OF TRANSGENIC MOSQUITOES IN LATIN AMERICA

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Interference with the mosquito vectorial capacity is an important strategy for malaria control that is being explored. One approach is to introduce into the mosquito, a gene whose product is harmful to the parasite, but innocuous to the mosquito. Previously we demonstrated that the *Anopheles gambiae* carboxypeptidase (AgCP) promoter strongly and rapidly induced the secretion of the bee venom phospholipase A2 (PLA2), in the midgut of transgenic *An. stephensi* mosquitoes, as reported previously. Transgenic mosquitoes expressing PLA2 inhibited *Plasmodium berghei* oocyst formation by 68-99%. Furthermore, transmission of the parasite from transgenic mosquitoes to naïve mice was dramatically reduced. Reported experiments to measure the fitness of transgenic mosquitoes have shown that PLA2 expression significantly reduced their egg production (58-75% fewer eggs). Recent reports showed a similar fitness reduction when PLA2 was driven by the *An. gambiae* peritrophin 1 promoter and based on electron microscopical evidence they concluded that phospholipase damages the mosquito midgut epithelia, possibly reducing nutrient absorption. Based on reported observation that PLA2 enzymatic activity is not required for inhibition of oocyst formation, we constructed a mutant PLA2 gene carrying a point mutation that abolished enzymatic activity. This gene was cloned into the *piggyBac* transposable element to generate transgenic Brazilian mosquitoes. Recently we were successful to generate the first transgenic mosquitoes in Latin America (*Aedes fluviatilis*), which can open the possibilities of studying parasite-

vector interactions under our conditions. We are currently studying the blocking ability of the transgenic mosquitoes towards *P. gallinaceum*. With this study we expect to add the powerful blocking ability of the bee PLA2 without compromising mosquito fitness.

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### A COMPARATIVE ANALYSIS OF ANOPHELES STEPHENSI AND A. GAMBIAE THROUGH BAC SEQUENCING AND PYROSEQUENCING

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We are studying the genomics and evolution of *Anopheles* mosquitoes through comparison between *A. stephensi* and *A. gambiae*, two medically important species in the subgenus *Celia*. The genome of *A. gambiae* has been sequenced, which offers a good reference for comparison. We have sequenced 15 BAC clones from *A. stephensi*, and identified orthologous regions in the *A. gambiae* genome. Pair wise comparison for comparative gene prediction and annotation has been performed using VISTA. Majority of coding sequences predicted in *A. gambiae* can be identified in *A. stephensi* and gene order is general maintained at the scale of individual BAC clones. On the other hand, rearrangement breakpoints region are also identified. In addition, the substitution-based evolutionary rate between the two species was calculated using PAML. VISTA comparison between annotated *A. gambiae* sequences and *A. stephensi* BACs revealed a large number of "conserved non-coding sequence region (CNS)", which are good candidates for regulatory elements, new exons, and new genes. RT-PCR analysis indicated that these CNS consisted of many missed or mis-annotated genes in *A. gambiae* database. We also used pyrosequencing to obtain 78 Mbp *A. stephensi* sequences (~1/3 of the genome) in 100 bp fragments. These sequences map back onto the sequenced *A. stephensi* BACs at expected rate. There are 4 Mbp pyrosequences that are homologous to *A. gambiae* fragments. These data may be used as "landing pads" to sequence *A. stephensi* genes. We have also identified of novel transposable elements and possible horizontal transfer events from these comparative analysis.

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### SEQUENCE ANALYSIS OF THE DOMAIN II SODIUM CHANNEL IN ANOPHELES FUNESTUS

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The discovery of insecticide resistance in *Anopheles funestus* in South Africa has prompted an urgent need to understand the molecular mechanisms conferring pyrethroid resistance in *An. funestus*. Biochemical analysis and synergist assays have previously identified the enhanced oxidativ capabilities of monooxygenases as the primary mode of detoxification. DDT and pyrethroids resistance in many insects has been associated with substitution from leucine (TTA) to phenylalanine (TTT) in the gene that encodes the voltage-gated sodium channel. To establish whether this mutation is present or not in southern African *An. funestus*, the S6 transmembrane segment of domain II region of the voltage-gated sodium channel gene was cloned and sequenced in a pyrethroid resistant strain (FUMOZ-R) and a susceptible strain (FANG) of *An. funestus*. A comparison of the aligned nucleotide cDNA sequences obtained for the resistant and susceptible strains revealed that only two nucleotide changes differentiated them. At the amino acid level, 96% identity was found between these sequences and those of the voltage-gated sodium channel of *An. gambiae*. The point mutation change involving a single A-T

base change encoding leucine to phenylalanine that had been detected in *An. gambiae* and other insects with kdr-like resistance was absent in the pyrethroid resistant strain of the *An. funestus* studied. Analysis of the genomic sequences revealed the presence of two introns; a large intron upstream, 1003bp for the susceptible strain and 1005bp for the resistant strain with seven nucleotide changes differentiating the two strains and a second intron downstream, 68bp for both strains with no polymorphism was detected. The findings obtained here suggest that kdr is not associated with pyrethroid resistance in this strain of *An. funestus*.

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### MOLECULAR BASIS OF THE ESSENTIAL AMINO ACID ABSORPTION IN VECTOR MOSQUITOES

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Metabolism of essential amino acids in organisms depends upon a specific network of plasma membrane transporters. A few sodium-dependent essential amino acids transporters, similar to broad substrate spectra system (B<sup>0</sup>), were cloned from mammals and insects. The screening of completed genomes revealed a number of B<sup>0</sup>-like homologs comprising a set of 4-11 genes-per-lineage expansions in the Sodium Neurotransmitter symporter Family (SNF = SLC6), denoted as a subfamily of Nutrient Amino acid Transporter, as reported previously. To determine the variety of phenotypes and structure-function relationship we aim to clone and characterize representative sets of NATs from simple genomic models including two vector mosquitoes and fruit fly. Presently we have characterized 6 SNF-NATs including several novel phenotypes which perform sodium-driven absorption of essential amino acids and some metabolites with specific preferences to indole-, phenyl-, or thiole-branched substrates. Two B<sup>0</sup>-like transporters with virtually uniform carrier efficiencies for all Essential amino acids plus some D-isomers were also characterized from *Anopheles* and *Drosophila*. The comparison of characterized transporters revealed a high evolutionary plasticity of individual NAT phenotypes but a conserved synergetic role of NAT populations. The consensus of electrochemical and morphological characteristics suggests that NATs comprise an active pipeline for absorption and redistribution of essential amino acids. The interference with such a crucial and lineage-specific molecular mechanism is proposed as rational vector management strategy.

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### OBSERVATIONS OF ANOPHELES GAMBIAE WITH MIXED RDNA ARRAYS CONTAINING BOTH MOPTI AND SAVANNA TYPES

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Different rDNA sequences are associated with sub-forms of *Anopheles gambiae*. Analyses of these have been reported using PCR and PCR-RFLP based on a polymorphism in a *Hha* I restriction site. During a survey of laboratory colonies using the PCR-RFLP method, we observed stocks that contained males and females yielding fragments indicative of both Mopti (M) and Savanna (S) rDNA intergenic spacers (IGS). Sequences of the IGS rDNA of male individuals had a mixture of arrays associated with both M and S types. In addition to the diagnostic *Hha* I restriction enzyme site, two nucleotide positions were conserved for the M and S types in contiguous regions. Both results demonstrate that M and S IGS types can both occur within the rDNA arrays in laboratory *Anopheles gambiae* stocks, possibly on one chromatid. In order to determine the inheritance of these arrays, crosses of individual males having the mixed arrays to females of M and S types were performed. These yielded only progeny with mixed M and S rDNA indicating that the DNAs associated with these mixtures may not be located exclusively on the X chromosome

as previously reported. We report the results of crosses similar to those previously reported assigning the rDNA exclusively to the X chromosome. In contrast to previous experiments which used Southern hybridization, we used a PCR technique. We also developed a primer set that produces distinct fragment sizes for the M and S rDNA arrays without restriction digestion, and we demonstrate that the results are similar to those of an existing PCR-RFLP method.

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### EVOLUTION OF *HERVES* TRANSPOSABLE ELEMENT IN *ANOPHELES GAMBIAE* IN AFRICA

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Understanding behavior of active transposable elements in natural populations of *Anopheles gambiae* is important to understand the consequences of using transposable elements as gene drive agents. *Herves* is a Class II transposable element isolated originally from *An. gambiae*. It is a member of the *hAT* super-family of transposable elements which includes the gene vectors *hobo* from *Drosophila melanogaster* and *Hermes* from *Musca domestica*. *Herves* is found in all members of *An. gambiae* species complex (except *An. bwambae* for which data is unavailable). *Herves* was found in all *An. gambiae* s.s samples across Africa with a low copy number of 3-7. The nucleotide sequence polymorphism data also indicates that the introduction of *Herves* into *An. gambiae* is not a recent event and predates the radiation of the species complex. Higher sequence polymorphism was observed in non-coding region as compared to the coding region of *Herves* transposase in all the locations of study. Surprisingly, a high level of structural integrity was seen with a high frequency of complete open reading frames. A high degree of conservation was also observed in amino acid sequences in the region of analysis; less than 10% of the sample had stop codons resulting in early termination of the *Herves* transposase protein. Nucleotide sequence diversity from different locations across Africa will be presented. The role of selection on evolution of *Herves*, similarities and differences with other Class II transposable elements and the implications of this study towards using transposable elements as gene drive agents will be discussed.

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### PATTERN OF GENOME REARRANGEMENTS IN MALARIA MOSQUITOES

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The chromosomal model of speciation by suppression of recombination suggests that genome rearrangements promote differentiation by acting as a genetic filter between populations. Genomic regions of low recombination, such as the areas around inversion breakpoints, may contain genes important for adaptations, speciation, and evolution of vectorial capacity. The availability of polytene chromosomes in malaria mosquitoes provides the opportunity to develop physical maps suitable for detailed analysis of evolutionary changes in the genome structure. *Anopheles gambiae*, *An. funestus*, and *An. stephensi* belong to different series of the subgenus *Cellia*. We have developed a low resolution physical map for *An. stephensi* and compared it with the existing genome maps of *An. funestus* and *An. gambiae*. The correspondence of chromosomal elements between the three malaria vectors has been established. We have found preservation of synteny but substantial shuffling of gene order along corresponding chromosome arms due to paracentric inversions. The results suggest that whole arm translocations have been taking place during evolution of subgenus *Cellia*. Three-way analysis has assigned the rearrangement events to one of the three lineages. We have calculated the

sizes of conserved segments and inversion distances between the species. The development of a high resolution physical map will allow us to identify possible "hot spots" of inversion fixation and characterize genes located in the genomic regions enriched with the breakpoints.

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### MACROGEOGRAPHIC PATTERNS OF CHROMOSOMAL POLYMORPHISM IN THE MOLECULAR FORMS OF *ANOPHELES GAMBIAE* IN RELATION TO ENVIRONMENTAL HETEROGENEITIES IN CAMEROON

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We are carrying out a survey on *Anopheles gambiae* complex in Cameroon, with the main aim to characterise the ecological niches of M and S molecular forms and to define their patterns of inversion polymorphisms in relation to environmental heterogeneities. From August to December 2005, we collected about 5,000 *An. gambiae* s.l. in 305 villages over a north-to-south transect throughout the Country, ranging from northern dry savannas to southern equatorial forest. PCR-RFLP identifications of ca. 4,200 specimens showed a strong prevalence of *An. arabiensis* in northern Cameroon (mean annual rainfall < 1,200mm); moreover, above 11°N (< 600mm) it was the only member of the complex. Elsewhere, *An. gambiae* s.s. is widespread and increases its relative frequency in southern more humid areas. Most of the specimens belonged to the S-form (2,150/2,298 identified so far). M-form was very scarce in the northern half of the Country, where its presence seemed associated to permanent water collections (lakes, dams). The relative frequency of M-form mosquitoes increased southwards, but their distribution remained patchy, suggesting very different population dynamics than S-form. Northern populations showed 2R chromosomal arrangements typical of SAVANNA chromosomal form (2R b, 2R d and 2L a), decreasing in frequency southwards; rare carriers of the MOPTI-specific 2R bc arrangement were also recorded. Unexpectedly, 7 heterozygous 2R j/+ specimens were recorded in 7 different villages: this is the first report of inversion 2R j in Cameroon. More southwards, both molecular forms were homokaryotypic, mainly carrying the standard 2R + -- 2L + arrangement. As observed in other western Afrotropical areas, in Cameroon both molecular forms of *An. gambiae* seem to use inversion polymorphisms as tools for ecotypic adaptations rather than as tools for driving speciation phenomena.

Further genetical and ecological investigations are planned to assess whether / how the two molecular forms differentially exploit the same ecological context through niche partitioning.

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### PHYLOGEOGRAPHY OF THE SOUTHEAST ASIAN SUNDAICUS COMPLEX INFERRED BY DIFFERENTIAL EVOLUTION MARKERS

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Mitochondrial (mtDNA) and nuclear ribosomal (rDNA) DNAs are widely used to infer species and population history in the *Anopheles* genus. Our work based on the ITS2 (rDNA), Cyt-b and COI (mtDNA) sequences allowed to describe the phylogeography and the species status of 15 populations of *Anopheles sundaicus* s.l., a malaria vector along the Southeast Asian coasts. Our study reported a lower diversity among ITS2 sequences than the mitochondrial ones. The ribosomal marker was unable to reconstruct phylogenetic relationships, whereas analyses of both mtDNA markers discriminated three allopatric species within the Sundaicus Complex. *Anopheles sundaicus* s.s. was recognized in northern Borneo, the continental distribution of *An. epiroticus* was enlarged southward to Malaysian Peninsula, and a new distinct entity named *An. sundaicus* species E was found in Indonesia. The evaluation of ITS2 intra-individual variability provided new insights in the phylogeography of the species complex by detecting past introgression events. COI and Cyt-b evolution rates were estimated respectively at 2.31% and 1.82% per Myr providing information at two different steps of the species story. Cyclical sea-level variations, which implied the creation of islands and tropical refugia during the Pleistocene epoch, are assumed to play a role in speciation events and the current distribution of the three species. According to all the markers, *An. sundaicus* s.s. diverged from the two other species most likely by isolation on the Borneo Island. Continental and Indonesian species were also isolated but the proximity of the Indonesian and Peninsular Malaysian refugia allowed further contacts during sea-level fluctuation.

## 591

### CHROMOSOMAL AND MOLECULAR GENETICS OF ANOPHELES GAMBIAE COMPLEX IN MALI

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Eight cross-sectional entomological surveys were carried out in two villages of Mali (Banambani and N'Gagakoro-Droit) from June 2000 March 2004. Two mosquito-catching methods (human landing catch and spray catch) were compared. The main objective was to measure and analyze genetic variations in *An. gambiae* populations using chromosomal and microsatellite markers under different conditions and relate them to malaria epidemiology and transmission. The vector population and the malaria transmission were studied in the two villages. The vector population was composed of *Anopheles gambiae* s.s. and *Anopheles arabiensis*. The distribution of the *An. gambiae* s.s. and *An. arabiensis* was significantly different between Banambani and N'Gagakoro-Droit ( $\chi^2=76.23$ ;  $P<0.000001$ ). In N'Gagakoro-Droit *An. gambiae* s.s. represented 95.2% vs 84.0% in Banambani. *An. arabiensis* which represented 16.0% of the *An. gambiae* s.l. vector population in Banambani was much less prevalent in N'Gagakoro-Droit (4.8%). The transmission pattern differed markedly between the two villages. The highest man-biting rates were observed during the month of August. The mean of the EIR after day catches in Banambani over the period of study is 2.38 infective bites per man vs 0.83 in N'Gagakoro-Droit.

Banambani has a 2.9 fold greater EIR than N'Gagakoro-Droit based on day catches. The mean of the EIR after Night catches in Banambani over the period of study is 13.64 infective bites per man vs 6.31 in N'Gagakoro-Droit. Banambani has a 2.2 fold higher EIR than N'Gagakoro-Droit based on night catches.

In contrast the highest infection rates and the entomological inoculation rates were observed at the end of the rainy during the month of October.

## 592

### CROSS-GENOME COMPARISON OF CELL DEATH REGULATION IN MOSQUITOES

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Cell death (apoptosis) is an important component of host/vector immune-response mechanism and has been implicated in the life cycle of various pathogens in mosquitoes. Transcriptionally regulated pro-apoptotic genes such the *reaper/hid* genes in *Drosophila* also hold the promise of successful mosquito population control strategies because of their tissue-ablation activity. However the understanding of cell death regulation in mosquito was hindered by the fact that major insect cell death regulators, namely the *reaper/hid/grim* (RHG) like IAP-antagonists identified in *Drosophila*, were not present in the annotated mosquito (*Anopheles gambiae*) genome. Using a systematic bioinformatics approach, we have identified several candidates of *reaper/hid/grim*-like IAP-antagonists in both the *Anopheles* and *Aedes* mosquito genomes. One of them, *micelob\_x* has been characterized as indeed an IAP-antagonist. Cross genome comparison of the RHG genes revealed some very interesting findings, which should not only contribute to our understanding of immune-response and cell death regulation in mosquitoes but also to the design of controlled tissue-ablation strategies.

(ACMCIP Abstract)

## 593

### CHARACTERIZING THE ROLE OF Aedes Aegypti EARLY AND ABUNDANT TRYPSINS IN DENGUE VIRUS INFECTIVITY OF THE MOSQUITO MIDGUT

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Dengue virus (DENV) is the most significant arbovirus afflicting humans today. Currently, there is no vaccine or effective anti-dengue therapeutic and mosquito control programs are the primary strategy for dengue disease prevention, but DENV continues to persist globally. Understanding the mechanisms surrounding vector competence is critical for the development of novel control strategies. Empirical evidence has suggested that tryptic enzymes may be involved in DENV midgut infectivity, possibly by proteolytic processing of exposed regions of infectious viral particles which would condition them for efficient midgut infection. We hypothesized that *Aedes aegypti* early (ET) and abundant (AT) trypsin are integral in conferring vector susceptibility to DENV infection. We tested two immunization regimens to invoke specific immune responses against ET and AT. In our infectivity assays, sera from immunized mice are mixed with cultured DENV, fed to *Aedes aegypti* and midgut infection rates are determined. A DNA immunization regimen using a mammalian expression plasmid containing ET and AT resulted in low, but detectable specific antibody titers and had minimal effects on DENV midgut infection rates. However, a DNA prime/recombinant alphavirus boost strategy (recombinant double-subgenomic Sindbis virus expressing ET or AT) resulted in higher specific antibody titers. In infectivity assays, sera from the AT-immunized mice resulted in approximately a two and half-fold increase in *Aedes aegypti* midgut infection rates and the relative infection intensity was also increased. Surprisingly, the effects observed from these experiments demonstrated that AT specific antibodies did not reduce infection rates as hypothesized but instead significantly increased DENV infection. Our results suggest that trypsins do not proteolytically activate the virus but that trypsin activity within the midgut actually limits virus infectivity. From these data, we predict that variable expression or activity

of trypsins may contribute towards vector competence differences across worldwide strains of *Aedes aegypti*.

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### IMPROVED MEASUREMENT OF THE DISTRIBUTION FORMS OF ANOPHELES GAMBIE SENSU STRICTO FORMS AND THEIR RELATION TO THE ENVIRONMENT

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The distribution of molecular and chromosomal forms of *Anopheles gambiae* sensu stricto is well characterized for their broad distribution in much of Africa, and all Mali. In particular, the M molecular (Mopti) form tend to be in dryer areas to the north of the Mali and the S molecular form (Savanna and Bamako chromosomal forms). There are some obvious departures from this. Attempts to get improved ties between environment and genotypes had been hindered by a lack of good environmental classification. Using remote sensing we recently completed a landscape classification for Mali in an effort to better understand the underlying factors that affect the distribution of *Anopheles gambiae* forms in this West Africa country. In association with this study we collected mosquitoes and scored for molecular and chromosomal form. A parsimony method was used to estimate the haplotype of the mosquito chromosomal configurations and then estimated their frequency at each collection site.

The resulting landscape classification of Mali has eight classes and has a high accuracy (94%). A 5 kilometer area around each collection site was extracted and each class counted in order to estimate the habitat. A Principal Components Analysis was performed on the landscape characterization. The resulting principal components were regressed on the form composition at each collection site. We observed R square (>75%) and small root-mean-square error of prediction (RMSEP < 30) for most forms and haplotypes. We will present the resulting principal components regressions and the most parsimonious models for each for forms.

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### QTL ANALYSIS OF THE GENETIC BASIS FOR AUTOGENY IN AEDES ALBOPICTUS

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While most mosquitoes require a blood meal to initiate oviposition, some are autogenous and are able to produce an egg batch without a blood meal. *Aedes albopictus* has become well established in many US states after introduction from Asia about 20 years ago, and remains a threat for viral transmission. This species breeds around human dwellings and also readily blood feeds on animals, but it also is known that some individuals display facultative autogeny in that they normally take a blood meal prior to oviposition, but retain the ability to produce a first egg batch without a blood meal if necessary. We studied the genetic basis for facultative autogeny by crossing an *Aedes albopictus* autogenous female and a male from anautogenous strain. Our autogenous strain originated from Japan and was lab selected for high autogeny, while our anautogenous strain originated from Sri Lanka. F<sub>2</sub> progeny were obtained by sibling mating. Females of these progeny were fed 2% sugar solution for five days; thereafter, the developmental stage and size of the first ovarian follicles were recorded and number of follicles developing to mature egg size were counted. Wing length was measured as a proxy for adult body size. DNA was then extracted from the individual carcasses and DNA marker genotypes were determined. We previously reported tentative QTL

positions based on a limited DNA marker set. Here, we report results of a detailed QTL analysis that includes markers developed from *Aedes aegypti* and SNP markers developed from *Aedes albopictus*.

## 596

### IDENTIFYING ANOPHELES GAMBIAE GENES THAT AFFECT PLASMODIUM OOCYST DEVELOPMENT

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Malaria is a devastating disease caused by *Plasmodium* parasites that is transmitted to humans exclusively by *Anopheline* mosquitoes. Mosquitoes vary greatly in their ability to transmit malaria, but the molecular basis for these differences is only starting to be understood. Insects mount strong innate immune responses against *Plasmodium* and other pathogens. Many genes involved in mosquito innate immunity have been characterized based on their sequence homology to known *Drosophila melanogaster* immune genes. Previous studies indicate that *in vitro* cultured *P. gallinaceum* ookinetes can develop to mature oocysts when injected into *Drosophila*. A genetic screen has identified 25 *Drosophila* mutant lines that significantly reduced the flies ability to support oocyst development. We have identified homologues for six of these genes in *Anopheles gambiae*, and confirmed that their mRNAs are expressed. We are using dsRNA-mediated gene silencing to determine whether these genes are also important for the development of *P. berghei* (murine malaria) oocysts in *A. gambiae*. Preliminary data indicate that silencing of the mosquito homologue of the *Drosophila* L82F gene does prevent *P. berghei* oocysts development in *A. gambiae*.

## 597

### CHARACTERIZATION RNAI-BASED EFFECTORS SEQUENCES THAT TARGET DENGUE VIRUSES IN AEDES AEGYPTI

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Dengue viruses (DENVs) are medically important arthropod-borne viral pathogens of humans. The peridomestic mosquito, *Aedes aegypti*, is the principal vector of DENV serotypes 1-4 during epidemics. DENVs enter the mosquito when the adult female takes a blood meal from a viremic individual. We hypothesize that triggering the RNA interference (RNAi) pathway in relevant tissues of DENV vectors may be an important way of stopping the infection of DENVs and negatively impacting virus transmission. RNAi is an innate immune pathway in insects, including mosquitoes, that is triggered by double stranded RNA (dsRNA) and leads to the degradation of RNAs with long stretches of sequence identity to the dsRNA trigger. We are characterizing RNAi-based effectors sequences that target the four DENV serotypes for use in the development of transgenic mosquitoes resistant to DENV infection. We have targeted the NS5 gene of the DENV genome. The NS5 gene of DENVs is ~2700 nt and encodes a multifunctional protein that includes RdRp activity. We have identified a region of the NS5 coding region of DENV2 that allows maximal silencing efficiency when used as a trigger for RNAi. Nine different 300 nt segments of the DENV-2 NS5 gene were RT-PCR amplified and used as templates for *in vitro* transcription of effector sequences and formation of dsRNA. One µliter of DENV2-specific dsRNA (1µg/ul) was injected into Higgs White Eye (HWE; Puerto Rico, Rexville D) mosquitoes. Two days later mosquitoes were orally infected with DENV2 (Jamaica 1409; 10<sup>7</sup> pfu/ml). At 7 and 12 days post infection (dpi), mosquitoes treated with either NS5 dsRNA or -Gal dsRNA were assayed for infectivity. All nine 300 nt NS5 segments significantly reduced DENV2 infection. NS5-1, 2, and 3 dsRNAs targeting the N-terminal region (2'-O- methyl-transferase domain) appeared to be less effective inhibitors of DENV2 at 12 dpi than NS5-5, 6, 7, 8, and 9 dsRNAs targeting the RdRp. To determine if these DENV-2 NS5 dsRNA have a high level of interference across multiple DENV serotypes, HWE

mosquitoes were injected with target dsRNA and then challenged with DENV4. At 7 dpi, but not 12 dpi, NS5-5, 6, 7 and 8 dsRNAs showed only modest inhibition of DENV4 indicating the importance of having dsRNA from each DENV serotype. Hybrid NS5 dsRNAs derived from the four DENV serotypes are currently being developed to generate an effector molecule that will target multiple DENV serotypes.

## 598

### FIELD-READY METHOD FOR DETECTING RUBIDIUM-MARKED ANOPHELENE MOSQUITOES

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We present a mark-release-recapture technique that can be used with a hand-held, portable X-ray fluorescence (XRF) spectrometer practical for field-use. Third instar *Anopheles gambiae* larvae and pupae were reared in water containing RbCl at concentrations of 0-1000 ppm Rb<sup>+</sup>. Larvae survived to the adult stage at concentrations as high as 1000ppm Rb<sup>+</sup> but did suffer increased pupal and adult mortality at the higher concentrations. The effect of RbCl on adult longevity was recorded and survival curves determined. The presence of Rb<sup>+</sup> in adults was determined using XRF, and we were able reliably to detect Rb<sup>+</sup> above background in females until at least 10 days post-emergence and males up to 6 days post-emergence when cultured at concentrations that resulted in minimal pupal or adult mortality. Rb<sup>+</sup> loss with time was observed and is presumed to be via diuresis. Incubating the pupae without RbCl decreased the level of Rb<sup>+</sup> in adults suggesting that Rb<sup>+</sup> can be lost through diffusion into the rearing water. The low cost of labeling with RbCl and the field portability of the spectrometer provide a useful means for labeling mosquitoes for numerous tracking experiments in which marking has been problematic via breeding site marking or in laboratory culture.

## 599

### POPULATION STRUCTURE OF THE CULEX PAPIENS VECTORS OF WEST NILE VIRUS IN EASTERN NORTH AMERICA

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To determine whether the genetic diversity of mosquito vectors of West Nile virus is more structured along a north-south (N-S) than an east-west (E-W) axis in eastern North America, we reared *Culex pipiens* families from egg rafts taken in nine sites along both axes in 2005 and have analyzed them using 12 microsatellite markers. The N-S axis included mosquitoes from Quebec through South Carolina, while the E-W axis included those from Illinois through Massachusetts. PCR-based protocols were applied to individual mosquitoes and processed further by a DNA sequencer. The copulatory structures of six male mosquitoes from each family were examined. In 2005, genetic differentiation ( $F_{ST}$ ) based on partial data from populations along the N-S axis of our samples was 0.09, exceeding 0.04 which represented populations at the E-W extremes. In 2004,  $F_{ST}$

between the N-S populations was 0.086, greatly exceeding 0.02 for E-W populations. Although genetic differentiation differs somewhat from year to year, *Cx. pipiens* populations are more structured along a N-S than an E-W axis.

## 600

### DAILY TEMPERATURE PROFILES IN AND AROUND WESTERN KENYAN LARVAL HABITATS OF ANOPHELES GAMBIAE AS RELATED TO EGG MORTALITY

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Small batches of eggs from *Anopheles gambiae* mosquitoes collected in houses near Kisian, Kenya and from the Kisumu laboratory strain were bathed for 10 min in water whose temperature was precisely controlled. Separate treatments varied in 1° increments from 40 - 48 °C. Hatchability was normal (> 70%) at exposure temperatures < 41°C. For wild eggs, a slight but significant increase in mortality occurred upon brief heating between 42 - 44°C. Few eggs hatched after 10 min at 45°C and none hatched above this temperature. A very similar pattern occurred for the Kisumu laboratory eggs, except it was shifted downwards by 1°C. Varying exposure times proved egg mortality was time-dependent above 40°C; few eggs survived 2 h of exposure at 42 °C, and none survived 1 h of exposure at 43 °C. Thus, the upper limit of temperature immunity for these eggs was 40°C. Daily temperature profiles were recorded by an infrared thermometer on seven different days in and around three types of typical *An. gambiae* larval habitats at Kisian: puddle in a maize drainage ditch, puddle in road, and borrow pit. Temperatures of water never exceeded 35°C. Wet or damp mud rarely and only briefly exceeded 40°C; thus, water and mud would be highly conducive to egg survival and development. However, dry soils frequently reached 40 - 50°C for several h. Eggs stranded on dry surfaces would experience substantial mortality on hot, sunny days, due likely to protein denaturation. Therefore, soil temperature (and indirectly hydration) is critical to *An. gambiae* egg survival.

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### AN EXTRACELLULAR METALLOPROTEASE EXPRESSION IN Aedes Aegypti: ROLE IN BLOOD DIGESTION, MIDGUT MATRIX REMODELING, EGG HATCHING, AND IMMUNITY

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Blood feeding induces many genes in mosquito midguts. These are mostly serine proteases involved in digestion of the ingested blood components. The mosquito genome also has a set of metalloproteases, the biological functions of most of which remain unknown. To identify and characterize these metalloprotease genes expressed in *Ae. aegypti*, we created a unique cDNA library using mRNA extracted from blood-fed midguts after food has been completely digested. The retroviral vector (pLPCX) library was useful in screening in a variety of eukaryotic cells. Here we report full-length cDNA sequence of a gene that codes for a metalloprotease, expressed in the midgut of *Aedes aegypti*. The predicted open reading frame (ORF) codes for a 29 kD protein. A 21 amino acid signal peptide and absence of transmembrane domain or GPI anchor, indicated that the protein is secreted. Amino acid sequence homology and phylogenetic analysis classified the protein as a member of Matrix Metallo Proteases (MMPs) from various blood-feeding and non-blood-feeding arthropods. The analysis also predicted that the protein is a zymogen, and is activated by enzymatic cleavage of a 37 amino acid propeptide from the N-terminus. Suitably spaced four Cys residues indicated that the protein is stably folded by two disulfide bonds. Arrangements of three His residues in mature polypeptide suggested Zinc ion is required for enzymatic activity of the mature protein. Analysis of temporal and



spatial gene expression profiles indicated the gene is not expressed in early stages of development, with virtually no expression in larvae and pupae. High-level expression in female midguts indicated the gene is involved in blood digestion. The presentation will discuss the enzyme's role in blood digestion and remodeling of extracellular matrix due to the stress caused by blood ingestion. It will also describe the gene's role in egg hatching and immunity against infection.

## 602

### ENTOMOLOGIC AND SMALL MAMMAL STUDIES FOLLOWING A LA CROSSE ENCEPHALITIS OUTBREAK IN TRANSYLVANIA COUNTY, NC 2005

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A La Crosse encephalitis (LACE) outbreak was reported in Transylvania County, North Carolina during the fall of 2005. Focused entomologic and small mammal surveys were performed at or near case residences during September and October of that year. *Aedes albopictus* and *Ochlerotatus japonicus* were the two most abundant species, accounting for 89% of the total collections. *Ochlerotatus triseriatus* adults were present in low numbers (n=25) and represented only seven percent of the total. Small mammals were collected from three case residences to obtain La Crosse virus (LACV) seroprevalence data. Sera from 19 sciurid mammals (e.g., eastern gray squirrels and chipmunks) were screened for LACV neutralizing antibodies. Preliminary results suggest enzootic transmission of LACV occurred at the LACE case residences. The results of pending plaque-reduction neutralization tests (La Crosse, Keystone and Jamestown Canyon viruses) and cytochrome B blood meal analyses of blood-engorged mosquitoes will be included. The implications of these data and the need for future studies addressing the role of *Oc. japonicus* in LACV transmission will also be discussed.

## 603

### MALE SPERM CAPACITY AND MATING BIOLOGY OF THE DENGUE VECTOR, *Ae. AEGYPTI*

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Understanding mosquito mating biology is essential for studies of mosquito behavior, genetics, population structure, and genetic control. We investigated baseline parameters of the mating biology of the dengue mosquito, *Aedes aegypti*. The effect of age and body size on spermatozoa number in several strains of *Ae. aegypti* was examined. Spermatozoa number in *Ae. aegypti* males increased significantly up to 10 days after emergence and leveled off until 20 days old. Body size was a major predictor of total spermatozoa number with the total number of spermatozoa in male reproductive organs significantly greater in large vs. small males within the same age group. Body size effects on insemination rates and other aspects of mosquito mating biology will be presented.

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### GROWTH AND DEVELOPMENT OF *ANOPHELES GAMBIAE* IN MOVING WATER

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Growth, development, and successful eclosion of larvae from *Anopheles gambiae* house-caught near Kisian, Kenya were measured in 35 cm diam. basins where water was either still or propelled with a large stir-bar at ca. 10 cm/sec. Production of adults held in Lake Victoria water changed every morning was equal to that for water from puddles characterized as typical *An. gambiae* habitat. River water produced few and smaller adults, however, larvae were capable of living in river water for > 12 days. Slow to moderate stirring did not reduce adult productivity significantly. Collectively, these findings challenge the idea that this important vector of malaria cannot exploit moving water as habitat.

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### HOST CHOICE IN MOSQUITOES COLLECTED IN A PERI-URBAN AREA OF WESTERN TENNESSEE, 2002-2003

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The source of blood meals in over 2,000 blooded mosquitoes collected from February, 2002 through November 2003 in Shelby County, Tennessee, in and around the city of Memphis were determined. Members of the genus *Culex* and *Anopheles quadrimaculatus*, predominated in the collections. Members of the *Culex pipiens* L complex mosquitoes and *Culex restuans* were found to feed predominately upon avian hosts, though mammalian hosts made up a substantial proportion of the blood meals in these species. No significant difference was seen in the host classes in mosquitoes classified as *Cx. pipiens*, *Cx. quinquefasciatus* or hybrids between these species. *An. quadrimaculatus* and *Culex erraticus* fed primarily upon mammalian hosts. Three avian species (the American Robin, the Common Grackle and the Northern Cardinal) made up the majority of avian derived blood meals identified, with the American Robin representing the most preferred avian host. The American Robin, Field Sparrow, House Finch and Grey Catbird were represented in the blood meals at frequencies greater than predicted based upon abundance or abundance corrected for body size or surface area. A temporal analysis of the feeding patterns of the major *Culex* species did not support a shift in feeding behavior away from robins to mammals late in the summer. However, a significant degree of temporal variation was noted in the proportion of robin-derived blood meals when the data were analyzed by semi-monthly periods throughout the summers of 2002 and 2003. This pattern was consistent with the hypothesis that the mosquitoes were preferentially feeding upon fledglings.

## CHARACTERIZATION OF *ANOPHELES GAMBIAE* HABITATS IN MALI AND CAMEROON

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Projects investigating the potential for genetic malaria control strategies require a good understanding of the population structure and habitat characteristics of wild mosquitoes. Broad patterns of spatial variation for gene arrangements in the malaria vector, *Anopheles gambiae*, suggest association with certain habitat types. We have been investigating how to make those characterizations more precise. Using a remote sensing approach incorporating an intensive ground reference strategy, a new 500 m MODIS classification product of Mali, West Africa has been developed for use in analysis of genotype frequencies in the study of gene flow within and between populations of *An. gambiae*. The new product serves as a verification and substantial refinement over existing land cover maps of the region, and was produced using 550 ground control points collected across Mali between 2003 and 2005. Ground reference incorporated vegetation structure analysis, Leaf Area Indices (LAI) measurement, and soil measurement at selected locales to increase the environmental representation of the study area. The ground referencing was carried out concurrently with collections of genetically distinct specimens of *An. gambiae*, and the resulting product allow for advanced characterization and analysis of the relationship between environmental variables and the geographic distribution of *An. gambiae* populations. We have recently begun to extend this study to include Cameroon, in Central Africa, with the hopes that information from a region with substantial ecological and climatic divergence from Mali will enable results to be extended across a continental scale. At this time, the product is being developed using data from 120 ground control points collected in Cameroon, and will be refined as future field campaigns yield additional ground reference data.

## CO-OCCURRENCE OF "EAST" AND "WEST" AFRICAN KDR MUTATIONS IN *ANOPHELES GAMBIAE* S-FORM (DIPTERA: CULICIDAE) IN WEST AFRICA

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Knock-down resistance (kdr) to DDT and pyrethroids in *Anopheles gambiae* s.s. is associated with two alternative point mutations at amino acid position 1014 of the voltage-gated sodium channel gene, resulting into either a Leucine-Phenylalanine ("kdr-west"), or a Leucine-Serine ("kdr-east") substitution. The former mutation is widespread in S-form populations from West Africa and has been recently reported from Uganda. The latter substitution was originally recorded in Kenya and has recently been found in Uganda, Gabon, and Cameroon. We here report the results of the genotyping of the kdr locus from several

S-populations collected in Senegal (2001), Burkina Faso (2000), Ivory Coast (1998), Benin (2002), Nigeria (2001), Equatorial Guinea (2004), and Angola (2002-03). The samples have been identified to molecular form by PCR-RFLP. The kdr-genotypes have been determined using the HOLA approach and confirmed by sequencing. The "kdr-west" allele was found in all populations analyzed with frequencies ranging from 2% to 100%. Interestingly, both resistance alleles were observed in samples from Equatorial Guinea (Ngonamanga: (N= 12; kdr-west= 20.8%; kdr-east= 25.0%), and north western Angola (Cabinda: N=60; kdr-west= 16,7%; kdr-east=30,8%; Kikudo: N=53; kdr-west= 1,9%; kdr-east=1,0%). In all these samples the kdr-genotypes were in Hardy-Weinberg equilibrium. The presence of the kdr-east allele in the S-populations from Angola and Equatorial Guinea shows that this allele is more widespread in West Africa than previously thought. It is also worth noting that the populations collected in the two villages of Angola showed different frequencies of both resistance alleles, in spite of the fact that they were less than 100 km apart and seem to be in similar ecological conditions. The implications of the above findings will be discussed.

## DEVELOPING A LOW COST REPELLENT TO REDUCE MALARIA IN THE AMERICAS - RESULTS OF TWO FIELD TRIALS IN GUATEMALA AND PERU

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Some major malaria vectors in South America exhibit reduced susceptibility to insecticides and a tendency to bite both inside and outdoors, early in the evening before people retire under bednets. The use of an insect repellent in the early evening may therefore provide significant protection against malaria. The greatest hindrance to the wider use of insect repellents in Latin America is their cost and, to address this issue, a low-cost (a projected \$ 0.045 per day in Peru) insect repellent was developed that reduces the proportion of costly actives by combining them with inexpensive oil-based fillers and fixatives. This combination extends the effective period of the repellent by slowing the evaporation of repellent volatiles. The repellent chosen was a mixture of p-Menthane-diol (PMD) and lemongrass oil (LG), two actives that are derived from plant sources and considered more acceptable than DEET to many rural people in Latin America. To test the efficacy of this repellent two trials were staged - one on the Pacific coast of Guatemala and one in the Peruvian Amazon. Each test was a balanced human-landing design in which the repellent(s) were compared with a positive control (DEET) and a negative control. In the Guatemalan trial, mosquito fauna comprised 19 species, including local malaria vectors, with a biting pressure of >100 landings/hour. The three PMD+LG repellents provided >98% protection for five hours after application. The 15% DEET control provided significantly lower protection at 93.67% (p<0.0001). In the Peruvian trial, 18 species of mosquito fauna were collected, but 86% of all collections were *Anopheles darlingi*, with a biting pressure of >46 landings/hour. The PMD+LG repellent provided >96% protection for six hours after application. The 20% DEET control provided significantly lower protection at 63.63%.

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### SPATIO-TEMPORAL VARIATIONS IN THE DISTRIBUTION OF ANOPHELINE LARVAL HABITATS IN WESTERN KENYA HIGHLANDS

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We investigated the spatio-temporal variations in the distribution of anopheline larval habitats in western Kenya highlands over a 4-yr period in relation to changes in land use. The distribution of *Anopheles gambiae* complex larvae were mainly confined to valley bottoms both during dry and wet season. Larvae of *A. gambiae* were frequently found in farmlands and the other man-made habitats in the areas where riparian forests had been cleared and natural swamps turned into farms, while larvae of *A. funestus* were mainly found in permanent habitats. The association between land cover type and occurrence of anopheline larvae was statistically significant. The distribution of anopheline positive habitats varied significantly between the months over the study period. Mean density of *A. gambiae* larvae was significantly higher during the month of May 2004 compared to the other months. Similarly, the density of *A. funestus* was significantly higher during the month of February 2004 compared to the other months. Over the 4-yr period, land use and land cover (LULC) major changes affected 3.13 out of 13,493.00 ha, mostly in the valley bottoms. From the overall changes, 31%, 19%, 19% and 17% occurred into pastures, forest, shrubs and farms respectively. The reversal of LULCs into farmland and pastures constitutes 71% and 18% of the changes. Land cover under farmland increased by 0.3% while that under pasture and natural swamps decreased by 0.8% over the 4-yr period. The area under forest cover decreased by 0.5%. These results suggest that land use changes in the study area influence the distribution of anopheline larval habitats and consequently malaria transmission in western Kenya highlands.

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### HOST MORTALITY AS AN EPIDEMIOLOGIC MECHANISM OF THE NEW WORLD SUCCESS OF WEST NILE VIRUS

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West Nile virus (WNV) currently is the most important and most widespread cause of mosquito-borne disease in North America. Widespread WNV-associated mortality among wild birds, especially corvids contrasts with the absence of avian mortality during WNV outbreaks in the Old World. I demonstrate that WNV-associated mortality in birds may facilitate the ongoing WNV outbreak in North America by increasing the force of transmission (infectivity per infected bird) and by preventing the accumulation of immune hosts. This epidemiologic mechanism is illustrated by simple quantitative arguments and by computer simulation. I will present an analysis of these observations in the context of WNV epidemiology in North America and in Central Europe and the Mediterranean.

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### WEST NILE VIRUS INFECTION IN MOSQUITOES IN THE MID-SOUTH USA, 2002-2005

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West Nile Virus (WNV) was first detected in the Tennessee Valley and in Alabama in August, 2001. In the summer of 2002, intensive viral activity was seen, but in subsequent years viral activity settled into a more endemic pattern. Here, we report the results of an extensive analysis of viral activity in the endemic mosquito fauna in the Mid-South from the period spanning 2002 (when the first viral activity in mosquitoes was detected) through 2005. Eight mosquito species were confirmed as infected with WNV during 2002. However, viral activity was only detected in four species (*Culex salinarius*, *Culex erraticus*, *Coquillettidia perturbans* and *Aedes vexans*) in multiple years. The greatest number of positive pools were identified in *Culex erraticus* and *Cx. salinarius*. Despite being specifically targeted for collection, *Aedes albopictus* was only found to be infected during the epidemic year of 2002, suggesting that under endemic transmission conditions its role as a bridge vector in the region may not be significant. Virus positive pools of *Cx. erraticus* were identified from hibernating mosquitoes and early season collections in 2005, implicating this species in over-wintering of the virus in Alabama. Molecular analysis of individuals initially identified as members of the *Culex pipiens* L complex suggested that alleles characteristic of *Cx. pipiens* predominated in mosquitoes collected in Huntsville, AL, while alleles in the Auburn, AL population were predominately characteristic of *Cx. quinquefasciatus*. The sympatric zone between the two species thus appears to be located primarily between Huntsville and Auburn, a distance of 160 miles.

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### PRELIMINARY MICROARRAY ANALYSIS FOR THE IDENTIFICATION OF DENGUE-REFRACTORINESS CANDIDATE GENES IN MOSQUITO VECTORS

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*Aedes aegypti* is the primary vector for the yellow fever and dengue fever viruses; it is also an excellent laboratory model for investigating other mosquito borne diseases. In this report we present preliminary results of our efforts to identify genes that are differentially regulated in *Ae. aegypti* in response to exposure to Dengue-2 JAM1409 virus between two laboratory strains: DS3 (susceptible) and Moyo-In-Dry (refractory). Our microarray platform consists of spotted PCR amplicons representing ~9,500 unique cDNAs identified as part of the *Ae. aegypti* whole-genome sequencing project. Two point times were examined for this initial study: three hours and three days post feeding on both dengue-infected and uninfected blood meals. Transcriptional differences were evaluated in midgut tissues. Using stringent criteria, we identified twenty-eight genes at three hours and twelve genes at three days that showed significant differential expression between the DS3 and Moyo-In-Dry strains. Pending release of the annotated *Ae. aegypti* genome sequence will facilitate identification of putative gene functions. In addition, qRT-PCR validation of our microarray results will be presented. Additional experiments are ongoing to examine a more detailed time course following an infected blood feeding, and the potential for dengue serotype-specific gene response by the mosquito. Our goal is to identify clusters of co-expressed genes up- or down-regulated at different time points in response to the virus and correlate this response with dengue vector competence.

### A GEOGRAPHICAL SAMPLING STRATEGY FOR FIELD SURVEYS IN AN URBAN AREA USING HIGH-RESOLUTION SATELLITE IMAGERY

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Field evaluations for studying the epidemiology of vector-borne diseases like dengue in urban areas are often restricted to selection of households and buildings for field surveys. Therefore, the resulting sampling frame may exclude specific locations within the urban environment that contain vector habitats and thus may bias the results. A sampling strategy was developed for field surveys in an urban area using high-resolution satellite imagery. The site selected was Puntarenas, a city affected by dengue on the pacific coast of Costa Rica, for which high-resolution satellite imagery was available from the Advanced Spaceborne Thermal Emission and Reflection Radiometer (ASTER, 15 m spatial resolution) and QuickBird (0.6 m and 2.4 m spatial resolution for panchromatic and multispectral bands, respectively). Grids obtained from the ASTER imagery and a cover map generated from the QuickBird multispectral bands were used to determine the optimal grid area of 100 km<sup>2</sup>, which contain 13±6 houses. A final grid 42 by 42 pixels (100.8 x 100.8 m) was created using the multispectral Quickbird imagery, and cells that had an area less than 90% within one specific locality of Puntarenas were excluded. The remaining cells were grouped according to locality and a random sample (10%) was selected from each. These cells would be used for field sampling of mosquitoes and larval habitats by evaluating the entire area within the geographical limits of each cell. To assess the suitability of the selected grid cells, the proportion of tree area ("tree" class Kappa = 0.91) was extracted for the individual cells from the QuickBird cover map. The mean percentage of tree cover in each locality and total area was compared between the selected sample cells and the total cells of the Puntarenas image. Overall, the sample adequately represented the total area and most of the individual localities in terms of tree cover. In 8 of 10 localities the difference between the estimate (sample) and the real percentage of tree cover was less than 3%. These results show that high-resolution satellite imagery and geographical information systems are useful in evaluating urban areas and selecting sections for field sampling of larval and adult mosquitoes that are practical, representative, and will reduce bias.

### SPATIAL ANALYSIS OF SPILL-OVER EFFECTS OF INSECTICIDE-TREATED MATERIALS IN A CLUSTER-RANDOMIZED TRIAL AGAINST Aedes Aegypti MOSQUITOES IN TRUJILLO, VENEZUELA

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A cluster-randomized trial of insecticide-treated curtains and water jar covers against *Aedes aegypti* mosquitoes was done in Trujillo, NW Venezuela. Entomological indices declined from baseline in both intervention and control clusters. Control houses positive for *Aedes* at baseline were more likely to be negative one month later if they were

within 50m of an intervention house. The results were interpreted as the intervention's effect 'spilling over' from intervention to control clusters. Here we extend the analysis to a) use the number of positive containers per house as the outcome (Breteau Index, BI), b) include the effect of all intervention houses, rather than only the nearest one, c) directly estimate the scale of spill-over effect, and d) estimate the difference between intervention and control arms. The study area extended 1.4km north-south and 0.8km east-west. No buffer areas were used, and most of the 18 clusters were separated by less than 10m from their nearest neighbouring cluster. The median cluster extent was 119m north-south, and 167m east-west. In the baseline survey of 1091 houses, the between-house spatial correlation of BI was estimated to reduce by half over a distance of 110m: similar to the cluster dimensions. At the final follow-up, 9 months after baseline, 730 houses were surveyed. Preliminary results from the spatial model estimate an 18% reduction in BI in intervention versus control arm (95% CI 58% reduction to 14% increase). The intervention effect was estimated to reduce by half over a distance of 8.7m (95% CI 0.5-20m). This implies that the decrease in BI in an intervention house is the same as, for example, the decrease in BI in a control house within 8.7m of 2 intervention houses, or within 17.4 meters of 4 intervention houses. Mass effects of this magnitude would be important in areas of high housing density, especially in the likely circumstance that not all houses in an intervention area deploy the intervention correctly or at all.

### THE IMPACT OF ENVIRONMENTAL CHARACTERISTICS AND ENGINEERED SYSTEMS ON ANOPHELINE LARVAL-PREVALENT WATER BODIES IN THE URBAN COMMUNITY OF MALINDI, KENYA

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Environmental characteristics and municipal-engineered systems were studied to determine their impact on anopheline prevalence in urban water bodies in the urban community of Malindi, Kenya. Previously collected spatial, entomological, environmental, and municipal-engineering data were used to evaluate the dynamics of malaria vector propagation in urban Malindi. Logistic regression analyses were done to determine, the associations between environmental features and engineered systems on the one hand, and anopheline larval-prevalent water bodies on the other hand. Statistical interactions were also tested. The probability of finding anopheline larvae in water bodies was found to be significantly affected by engineered systems, specifically distance to water system, after controlling for environmental variables. The presence of culicine larval mosquitoes was also found to increase the probability of finding anopheline larval mosquitoes in urban water bodies. Two significant statistical interactions were identified: 1) water body size by culicine larvae presence, and 2) water body substrate type by distance to piped water system. This suggests that regression models that only examine main predictor effects may obscure complex relationships between predictor variables and outcome variables; in particular, those analyses that attempt to evaluate the impact of ecological variables on entomological variables. Engineered systems, such as water systems, have impacts on the presence of mosquitoes, but further studies are needed to evaluate the mechanisms by which they reduce mosquito burden. These results should guide mosquito control efforts in developing countries.

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**ORIGIN OF BLOOD MEALS IN INDOOR RESTING MALARIA VECTORS IN A RICE IRRIGATION SCHEME IN EASTERN KENYA**

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Blood meals were obtained from indoor resting malaria vectors in five villages in a Rice Irrigation Scheme in Eastern Kenya and tested by sandwich ELISA to determine host preferences and their human blood index (HBI). A total of 2,286 *Anopheles arabiensis* and 145 *An. funestus*, were collected and tested. Overall, the enzyme-linked immunosorbent assay (ELISA) identified 95 and 100% of the samples tested in *An. gambiae* s.l. and *An. funestus*, respectively. Of these, human IgG was detected in 73.5% ( $n = 1,595$ ) of *An. arabiensis* and 100% ( $n = 145$ ) of *An. funestus*. Most indoor females of *An. arabiensis* had fed on cows (24.3%) and 2.2% on goats. Indoor-collected female *Anopheles funestus* had mainly fed on people (100%), but taken at least some of their blood (33.3%) from goats. In all the 5 villages that had blood fed mosquitoes, *An. arabiensis* had a human Index less than 0.8, except Mbui Njeru where the HBI exceeded 0.95. The *Plasmodium falciparum* sporozoite infection rates were 0.5% for *An. arabiensis* and 2.8% for *An. funestus*. These results emphasize that *An. funestus* is highly anthropophilic, while *An. arabiensis* is a mixed feeder and moderately anthropophilic. The results further demonstrated active transmission of *P. falciparum* sporozoites by the primary vector species. This study suggests that the use of insecticide-treated nets will be effective for controlling biting mosquitoes inside houses in the rice irrigation Scheme of Eastern Kenya.

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**BRANCHING PROCESS MODEL FOR THE EARLY STAGES OF A TRANSPOSON INVASION IN A DIPLOID POPULATION**

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A continuous-time multi-type branching process is investigated to model the early stages of invasion of a transposable element in a diploid host population. The model assumes (1) in the early stages of element spread the number of organisms carrying the transposable element is small and hence all matings involve at least one host not carrying the element, (2) replicative transposition rate decreases dependent on the number of transposable elements within each host, and (3) the fitness of each host decreases with the number of transposable elements it contains. These assumptions are used to determine whether the branching process is subcritical, critical or supercritical, and hence under what conditions the transposable element has a nonzero probability of reaching a nontrivial equilibrium frequency in the host population. Extinction probabilities are calculated for the element under a variety of conditions. The host population is shown to be significantly more receptive to the invasion of a new element during periods of modest population growth. The model has the benefit of being analytically tractable while still having the stochasticity of a Markov chain. It is also applicable to diploid hosts whose genomes are often analyzed for the presence of transposable elements, such as *Drosophila melanogaster*. The model is applied to the hypothesized use of a transposable element to drive an effector gene into a diploid host population, and specifically to drive an antimalarial gene into an *Anopheles gambiae* mosquito population. The model favors a transgenic release immediately following the dry season when the *An. gambiae* host population begins to grow. Following a hypothetical release, the expected number of infected hosts is calculated after one cycle of seasonal population growth and decline. Recommendations are made regarding the characteristics required for the transposable element to prosper throughout this cycle and how these vary as the number of transgenic hosts released is altered.

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**TREATMENT OF NEOSPORA CANINUM AND GIARDIA INTESTINALIS WITH NITAZOXANIDE AND OTHER THIAZOLIDES INTERFERES IN FUNCTIONAL ACTIVITY OF PROTEIN DISULFIDE ISOMERASE**

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The thiazolide nitazoxanide (2-acetyloxy-N-(5-nitro 2-thiazolyl) benzamide; NTZ) exhibits a broad spectrum of activities against a wide variety of intestinal and tissue-dwelling helminths, protozoa, and enteric bacteria infecting animals and humans. The drug has been postulated to act via reduction of its nitro group by nitroreductases including pyruvate ferredoxin oxidoreductase (PFOR). In this study, we investigated the efficacies of NTZ and other thiazolides on *Neospora caninum* tachyzoites *in vitro*. We employed real-time PCR-based monitoring of tachyzoite adhesion, invasion and intracellular proliferation, and the studies were complemented by TEM. We also investigated 29 modified versions of NTZ. These modifications included the replacement of the nitro group on the thiazole ring with other functional groups, with the goal of rendering this class of drugs applicable for use in food animals, and the differential positioning of methyl groups on the salicylate ring. We show that the thiazole-associated nitro group is not necessarily required for the action of NTZ, and that methylation of the salicylate-ring at the ortho-position can result in complete abrogation of the anti-parasitic activity against *N. caninum*. These findings clearly indicate the presence of alternative or other targets than PFOR, and that not the thiazole- but the benzene ring is important in interacting with these targets. By affinity chromatography of *N. caninum* extracts on epoxy-agarose-coupled drugs, we identified *N. caninum* protein disulfide isomerase (NcPDI) as a NTZ-binding protein, and thus as a potential target. NcPDI was functionally expressed in *E. coli*, and indeed those thiazolides that are active against *N. caninum* also impair the functional activity of NcPDI *in vitro*. Similar results were obtained for *Giardia intestinalis*, which expresses 5 PDI-isoforms, of which we demonstrated that GIPDI2 and GIPDI4 are functionally inhibited by a range of thiazolides. These results suggest, that PDIs represent important targets for NTZ and other thiazolides in both intracellular as well as extracellular protozoan parasites. We have now generated NTZ-resistance *G. intestinalis* clones, which are being analyzed for differential protein expression. These studies will provide further insights into the mechanisms of action of thiazolides against protozoan parasites, as well as on the molecular basis of drug resistance.

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**AN IN-VITRO THREE DIMENSIONAL CULTURE MODEL OF ENTAMOEBIA HISTOLYTICA**

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*Entamoeba histolytica* is a major cause of colitis among returning travelers and is endemic in third world countries. Three dimensional tissue culture using immortalized cell lines grown in a microgravity environment produces intestinal tissue-like structures ("organoids"), with a polarized outer cuboidal cell layer demonstrating microvilli, tight junctions, and desmosomes. These organoids can be used for the study of enteric infections. *Entamoeba* were grown axenically in TYI medium. HCT-8 cells were maintained in RPMI medium (10% FBS, 1mM sodium pyruvate, 50U PCN/50µg Streptomycin per 500ml). Collagen scaffolds were added to 10 ml vessels, then  $10^5$  cells were added. Organoids were grown for eight days with media changed every four days. On day 8, the medium was changed to M199S (15% Bovine serum), then  $10^5$  amoeba were added. Vessels were sealed to simulate anaerobic conditions. Organoids were harvested for histology at 30 minutes, 180 minutes, and 15 hours. Organoids demonstrated typical villous intestine-like epithelium. *Entamoeba* infection was demonstrated at all time points, increasing up to

15 hours. Amoeba invaded the epithelial layers and caused visible tissue destruction reminiscent of *in vivo* ulcers. In conclusion, the microgravity organoid culture system provides an effective model for *Entamoeba histolytica* infection that approximates many aspects of *in vivo* infection. Sustained infection at 15 hours, tissue invasion and destruction were demonstrated. Further work that the model is suited for includes gene expression profiles of both host and parasite cells, apoptosis staining, and investigating potential therapeutic interventions.

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### EXTRACELLULAR LIPOPROTEIN INFLUENCES VIRULENCE FUNCTIONS IN *ENTAMOEBIA HISTOLYTICA*

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*Entamoeba histolytica* is the causative agent of amoebic dysentery and liver abscess. It is surpassed only by malaria, Chagas' disease and leishmaniasis as a worldwide cause of death due to parasitic infection. *E. histolytica* trophozoites encounter host cells and macromolecules during infection, and it is postulated that these host components may trigger signal transduction events in the parasite that lead to changes in virulence. With the rate at which the components of signaling pathways are being discovered in *E. histolytica*, the new challenge is to understand the temporal and spatial regulation of signaling events in these cells. In other eukaryotes, lipid rafts, cholesterol-rich plasma membrane domains, compartmentalize signaling events in the plasma membrane. Recently, *E. histolytica* cells were shown to possess raft-like plasma membrane domains which were important for virulence. Since *E. histolytica* trophozoites cannot be cultivated in the absence of lipoproteins, which are a significant source of cholesterol and cholesterol esters, it is likely that the pathogen relies on a host source of cholesterol *in vivo*. Therefore we examined the effect of extracellular lipoprotein on virulence functions of *E. histolytica in vitro*. Incubation of trophozoites with increasing concentrations of lipoprotein enhanced two important virulence functions, adhesion to model epithelial monolayers and phagocytosis of human red blood cells, in a concentration-dependent manner. Interestingly, incubation with human lipoprotein inhibited secretion of a cysteine protease in a dose-dependent fashion. Preliminary data also show that incubation with lipoprotein enhances staining with the raft-preferring fluorescent lipid analog, DiI<sub>16</sub>, suggesting that extracellular cholesterol sources may influence raft structure and/or abundance in the pathogen. Together these data indicate that extracellular cholesterol may influence pathogenicity of *E. histolytica*. Examination of the effects of lipoprotein on additional virulence functions is currently underway.

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### DNA METHYLATION HAS LIMITED EFFECT ON GENE EXPRESSION IN *ENTAMOEBIA HISTOLYTICA*

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In higher eukaryotes DNA methylation regulates a number of important biological functions including silencing of gene expression, chromatin structure, parental imprinting, development, and protection from retrotransposons. In the early diverging eukaryote *Entamoeba histolytica*, some roles of DNA methylation have been characterized including the identification of a DNA methyltransferase and genomic DNA methylation and regulation of the *E. histolytica* heat shock protein 100 (EHsp100) gene. Additionally, it has been shown that *E. histolytica* grown with 5-azacytidine (5-azaC), a potent inhibitor of DNA methyltransferase, is

severely attenuated in virulence. However, the overall extent of DNA methylation and its subsequent effect on gene expression in this parasite are currently unknown. In order to identify the genome-wide effects of DNA methylation in *E. histolytica*, we used a short oligonucleotide microarray (Affymetrix) representing ~95% of all annotated amebic genes and compared the expression profile of *E. histolytica* (HM-1:IMSS) parasites with those treated with 23µM 5-azaC for up to one week. Under these conditions the growth of the parasites was not affected, although they were markedly attenuated in virulence. Surprisingly, however, only ~1.5% of genes were transcriptionally modulated under these conditions. A total of 76 genes were upregulated and 67 genes down-regulated (1.75 fold changed; p-value <0.05). Sodium-bisulfite sequencing of genes up regulated by 5-azaC treatment confirmed that these genomic regions were methylated indicating that in fact these genes are endogenously silenced by DNA methylation. This work represents the first genome-wide analysis of DNA-methylation in *E. histolytica* and indicates that DNA methylation has relatively limited effects on gene expression in this parasite.

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### EVALUATION OF THE TECHLAB'S *E. HISTOLYTICA* ANTIGEN AND ANTIBODY POINT-OF-CARE TESTS FOR THE RAPID DIAGNOSIS OF AMEBIASIS

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*Entamoeba histolytica* is a protozoan parasite and the causative agent of amebiasis in humans. Intestinal amebiasis causes debilitating diarrhea and dysentery, while extraintestinal amebiasis occurs when the parasite migrates from the intestinal tract to organs throughout the body, most commonly the liver. *E. histolytica* must be specifically identified since non-pathogenic strains of *Entamoeba* spp. colonize the human intestine at 3-10 fold the rate of *E. histolytica*, making *E. histolytica*-specific diagnostics of particular value. Accurate diagnosis of infection by fecal microscopy, antigen testing, PCR, or IFA leads to successful treatment and full recovery. Recently, TechLab Inc, Blacksburg, Virginia has developed *E. histolytica* antigen and antibody tests for point-of-care rapid diagnosis of amebiasis. These tests were compared to a commercialized *E. histolytica*-specific antigen detection test (TechLab *E. histolytica* II test) and with an in house anti-amebic antibody ELISA test respectively. The *E. histolytica* antigen point-of-care test had a sensitivity of 97%, a specificity of 100% compared to *E. histolytica*-specific antigen detection test by ELISA with a correlation of 99%. The *E. histolytica* antibody point-of-care test had a sensitivity of 100% and a specificity of 95% compared to an in house anti-amebic antibody ELISA test with a correlation of 97%. The point-of-care tests are easy to use, rapid and sensitive for detection of *E. histolytica*-specific antigen in stool specimens and antibodies in stool and serum samples respectively. These two tests together promise to be useful at the bedside to diagnose amebiasis in humans.

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### FIRST RECORD OF HUMAN *CRYPTOSPORIDIUM FELIS* INFECTION IN TWO HIV-POSITIVE PATIENTS IN HAITI

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A survey of intestinal parasites was conducted in 2000 in HIV-positive patients attending the clinic of GHESKIO Centres in Port-au-Prince (Haiti). Oocyst detection in the stools was performed using the modified Ziehl-Nelsen. Species identification was done using PCR and RFLP analysis, and sequencing of rDNA 18S fraction. In addition, the viability of isolates was performed by oral infestation of NMRI-suckling mice. Among 1,529 stool specimens examined, 158 were positive for *Cryptosporidium*

oocysts. Using genotyping analysis, 69 isolates were identified, 41 as *Cryptosporidium hominis*, 26 as *C. parvum* and 2 as *C. felis*. These two isolates were found in a 61 year old immunocompromised (CD4 = 73/mm<sup>3</sup>) VIH-positive woman, and in a 12 year old boy, VIH-infected through vertical transmission, both followed for severe chronic diarrhoea. Despite the provision of medical care, the two patients died a few weeks later. The viability of one *C. felis* isolate was experimentally confirmed. To date, human *C. felis* infection was reported in immunocompromised patients from USA, Peru, Thailand, India, and from 5 European countries (France, Italy, Portugal, Switzerland, UK). This is the first report of human *C. felis* infection described in the Caribbean region.

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### DIRECT TRANSMISSION OF *CRYPTOSPORIDIUM CANIS* BETWEEN CHILDREN AND A DOG

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The role of dogs in the transmission of human cryptosporidiosis is not clear. Even though a few epidemiologic investigations have identified contact with dogs as a risk factor in the acquisition of *Cryptosporidium* infection in children and AIDS patients, this has not been supported by results of other studies. More recently, molecular epidemiologic studies have shown that dogs are infected with *C. canis*, whereas humans are mainly infected with *C. hominis* and *C. parvum*. Even though *C. canis* have been reported in some patients, a recent study by our group in Peru has shown concurrent *C. hominis* infection in some *C. canis*-infected patients, indicating that person-to-person transmission of *C. canis* in humans may occur. In this study, we have followed weekly a cohort of children living in a periurban slum in Lima, Peru for *Cryptosporidium* infection. One girl had three episodes of cryptosporidiosis at 13, 25 and 29 months of age during an 18-month period. The second episode was diagnosed by the detection of *C. canis* on May 3, 2005, and was associated with transient diarrhea for two days. The examination of additional specimens collected from the girl for 15 days yielded no further positive stools. However, the screening of a 6 year old male sibling during the period showed *C. canis* infection on May 10. Among 18 fecal specimens taken from a dog in the house, three specimens collected on May 12, May 17 and May 25 had *C. canis*. This is the first documented transmission of cryptosporidiosis between humans and dogs.

(ACMCIP Abstract)

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### MOLECULAR EPIDEMIOLOGY OF *CRYPTOSPORIDIOSIS* IN CHILDREN IN KENYA

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To investigate the molecular epidemiology of cryptosporidiosis in Kenya, residual *Cryptosporidium*-positive stool specimens from diarrheic children submitted for routine etiologic diagnosis were genotyped by PCR-RFLP analysis of the small subunit rRNA gene and subtyped by DNA sequencing of the 60 kDa glycoprotein (GP60) gene. Approximately 300 specimens from children aged under 5 years were studied. *Cryptosporidium hominis* was the most common genotype (identified in almost 80% of the

specimens), followed by *C. parvum*, *C. felis*, *C. canis*, *C. meleagridis* and *C. muris*. Eight *C. hominis* and *C. parvum* GP60 subtype families were identified, consisting of a total of 26 subtypes. The *C. parvum* specimens consisted of mainly the anthroponotic subtype families IIc and IIe, with the existence of additional two new subtype families and the lack of the zoonotic subtype family IIa. The four common *C. hominis* subtype families (Ia, Ib, Id and Ie) were all seen in Kenyan children, with an almost equal distribution among them and a high heterogeneity within Ia and Id. These results demonstrate that intensive transmission of *Cryptosporidium* spp. occurs in children in Kenya and anthroponotic transmission plays an important role in cryptosporidiosis epidemiology.

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### IN VITRO, MODULATION OF INTERFERON- $\gamma$ RESPONSE BY INDUCING SODIUM ANTIMONY GLUCONATE RESPONDER ISOLATE IN T-CELLS OF NON-RESPONDER VISCERAL LEISHMANIASIS

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This study is the first line evidence for a significant up regulation of IFN- $\gamma$  and insignificant down regulation of IL-4 on stimulation of T-cells of visceral leishmaniasis patients unresponsive to antimony by a responder *Leishmania donovani* isolate. Since IFN- $\gamma$  is pre-dominant cytokine in anti Leishmanial defense, it was speculated that a non-responder *Leishmania* isolate might decrease IFN-g and a reverse of relationship for IFN- $\gamma$  in non-responders for a responder isolate. T-lymphocytes of 34 confirmed non-responder patients to Antimony were cultured and stimulated separately *in vitro* by *Leishmania* parasites isolated from responsive and unresponsive patients in two same sets. Parallel cultures were set up for 10 apparently healthy subjects. Initial culture examination of the parasite showed difference among the isolates and confirmed that *Leishmania donovani* from VL patients who later resulted into unresponsiveness of VL patients to antimony displayed different growth rate and were fast grower when compared to a responder isolate. To see if these differences could alter cytokine release pattern, in non-responder, IFN- $\gamma$  and IL-4 ex-pression was investigated using FACS-caliber. When the T-cells of non-responders were stimulated with responder isolate, the ex-pression by CD4+T-cell for IFN- $\gamma$  was increased. This was accompanied with an insignificant down regulation of IL-4 on stimulation of T-cells of non-responders by a responder isolate. The possibility that responder *Leishmania* parasites can act by up-regulating IFN- $\gamma$  needs further exploration. An immunotherapeutical strategy to investigate the IFN- $\gamma$  switch on mechanism in VL patients might be based on IFN- $\gamma$  producing CD4+ cells, triggered by responder parasite and their use in treatment of VL patients in combination to Antimony.

(ACMCIP Abstract)

### AMPLIFICATION OF REPEATED SEQUENCES BY PCR FOR DIFFERENTIATION OF *SCHISTOSOMA HAEMATOBIIUM* FROM RELATED SCHISTOSOMES

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For PCR-based differentiation of *Sschistosoma haematobium* from related schistosome species we undertook a wide search for suitable repeated sequences and corresponding primers. Clones (813) representing repeated sequences were selected from genomic libraries of *S. haematobium*, *S. bovis*, *S. mattheei*, *S. curassoni*, *S. margrebowiei* and *S. intercalatum*. Clones (250) cross hybridizing specifically with *S. haematobium* DNA underwent further selection by sequence-based criteria. Of 63 PCR primers designed from 28 selected sequences, none enabled differentiation by intra-repeat or inter-repeat amplifications, suggesting a high structural similarity of repeated sequences in *S. haematobium* group members. Differentiation of *S. haematobium* from the other species examined, excluding *S. margrebowiei*, was accomplished by PCR employing one primer from a newly identified repeat, Sh110, and a second one from the schistosoml splice-leader sequence. This differentiation tool should facilitate accurate evaluation of transmission potential of *S. haematobium* by monitoring snail hosts shared with other members of its group.

### CHARACTERIZATION OF SNAIL IMMUNE GENES THAT ENCODE PEPTIDOGLYCAN RECOGNITION PROTEINS AND GRAM-NEGATIVE BACTERIA BINDING PROTEIN, THE KEY HOMOLOGOUS MOLECULES CONTROLLING THE UPSTREAM TOLL/IMD SIGNALLING PATHWAYS

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Discovery of Toll/Imd pathways in arthropods has revolutionized our knowledge of evolutionary immunology. Recent studies demonstrate that peptidoglycan recognition proteins (PGRPs) and gram-negative bacteria binding protein (GNBP) play an essential role in regulation of the upstream Toll/Imd signaling pathways; PGRP short secreted forms (e.g., PGRP-SA) and GNBP are involved in activation of the Toll pathway, whereas long forms (e.g., PGRP-LC) control the Imd pathway. The existence of homologous pathways in other major invertebrate phyla (e.g., Mollusca) remains to be clarified although the pathways are believed to be evolutionarily conserved. Recently, we have cloned two *PGRP* genes and one *GNBP* gene from the snail *Biomphalaria glabrata*, the intermediate host of parasite *Schistosoma mansoni*, and we therefore named them as *BgPGRP* and *BgGNBP*, respectively. The *BgPGRP* genes encode a short secreted PGRP (*BgPGRP-SA*; 183 aa) and a long form PGRP (*BgPGRP-LA*; 512 aa), respectively. Additionally, it was found that the *BgPGRP-LA* (long form) produces two isoforms, *BgPGRP-LA1* (466 aa) and *BgPGRP-LA2* (428 aa), by the process of alternative splicing. Our study revealed that the *BgGNBP* gene encodes a secreted protein (424 aa). To gain insight into the functional role of *BgPGRPs* and *BgGNBP*, we have examined the expression of *BgPGRP-SA*, *BgPGRP-LA* and *BgGNBP* in the snails after exposure to a variety of microorganism, including trematodes *S. mansoni*, *Echinostoma paraensei*, bacteria, and fungi. The expression patterns obtained by quantitative PCR are complex. Our ongoing study of snail PGRPs and GNBP will broaden our understanding of evolution of invertebrate immunity and the defense responses of *B. glabrata* to pathogens, particularly to *S. mansoni*, one of the causative agents of human schistosomiasis.

### TRAIT-MEDIATED BIOLOGICAL CONTROL OF SCHISTOSOMIASIS BY A FACULTATIVE MOLLUSCIVORE

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Schistosomiasis is a parasitic disease caused by aquatic trematode worms, resulting in considerable human morbidity in the tropics. A group of potential biological control agents of Schistosomiasis, molluscivorous fish, have been shown to reduce the abundance of vector snail species in many areas. However, no effective biological control agent native to East Africa has been successfully implemented in this region, which has the highest human incidence of the disease. One widespread East African fish that was once widely touted for its potential to serve this function is a common haplochromine cichlid, *Astatoreochromis alluaudi*. Experimental introductions of wild-caught *A. alluaudi* into artificial water bodies initially proved successful, but subsequent generations of this fish failed to reduce snail populations. It has been hypothesized that competition with other fish species in the wild drives young *A. alluaudi* to consume snails, a less preferred food source, while in the absence of competition they select more soft-bodied prey items. The decrease in snail consumption by *A. alluaudi* noted from previous introductions is likely the result of developmental plasticity in the pharyngeal jaw apparatus. We hypothesized that *A. alluaudi* that develop in the absence of competitors will select a diet dominated by soft-bodied prey and thus will not develop the necessary jaw apparatus for easily crushing and consuming snails as adults. We are testing this hypothesis by examining diet choice and jaw morphology development in *A. alluaudi* in the absence of competitors in a replicated tank experiment. Our results indicate a preference for soft-bodied prey during the development of young *A. alluaudi*, leading to jaw morphology less well-suited for snail consumption. Future research will consider the effects of the presence of competitors on diet selection in developing *A. alluaudi*, and will hopefully shed light on the potential for biological control approaches that include an understanding of ecological processes in determining their outcomes.

### Schistosomiasis mekongi Control Project towards Elimination in Cambodia

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Despite the fact that human schistosomiasis has been known in Cambodia for decades, the enormous impact of schistosomiasis on the health of the local population was only recognized in 1993. Operational research provided the necessary information on parasite epidemiology and the associated morbidity in order to develop adequate control measures. As a result of a Rapid Assessment Method and parasitological survey in the suspected transmission areas in Cambodia, comprising all villages along the banks of Mekong River and its tributaries, schistosomiasis occurs in the provinces of Stung Treng and Kratie. About 80 000 people are estimated to be exposed to the risk of infection. In this area, baseline data showed prevalence of up to 70% in school-age children in 1995. *Schistosoma mekongi* transmission occurs in rocky banks of the river due to the characteristics of the intermediate host, the snail *Neotricula aperta*. A seasonal cycle is observed, consisting of a period of transmission in the dry season, from February to April. Schistosomiasis control in Cambodia has been based on universal treatment campaigns since 1995 and resulted in a dramatic fall in the prevalence of the infection from 70% to 5% and in morbidity control. However, transmission still occurs and 80 000 people are estimated to be at risk of infection, even if the disease and the infection have been satisfactorily controlled. The impoverished nature of the region makes the possibility of sanitation unfeasible, and external



support is still needed to enable the substantial reduction of risk behaviors. The new challenge in schistosomiasis control in endemic areas along the Mekong River is to consolidate the results, to establish a sensitive and reliable surveillance system, and to adapt control strategies towards elimination of *Schistosomiasis mekongi* in Cambodia. When elimination is aimed, case detection is a problem, as the commonly used clinical and parasitological diagnostic procedures may lack sensitivity to detect low intensity infection in the low endemic situation. The result of ELISA showed high sensitivity and usefulness for mass screening. These tests could be used in low transmission areas, and their further development into field applicable formats is encouraged.

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### FACTORS INFLUENCING ONE-YEAR RISK OF SCHISTOSOMA JAPONICUM INFECTION IN HUMANS AFTER TREATMENT, SAMAR PROVINCE, THE PHILIPPINES

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The objective of this study was to describe the predictors of 12-month cumulative incidence (risk) of human infection with *Schistosoma japonicum* one year after treatment with Praziquantel in 50 villages of Samar Province, the Philippines. A total of 25 villages with predominantly rain-fed farms and 25 villages with some irrigation system were selected from 75 eligible villages. A total of 2,290 participants provided from one to 3 stool samples both at baseline and one-year follow-up and were treated with Praziquantel at baseline. Stools from the study subjects were examined for *S. japonicum* eggs using Kato-Katz thick smear. A Bayesian three category outcome hierarchical cumulative logit regression model with adjustment for age, sex, occupation and measurement error of the Kato-Katz technique was used for analysis. The crude 12-month risk of infection was 13.3% (95% CI: 12.0 - 14.8), which is considerable compared to the baseline prevalence proportion of 23.5% (95% CI: 21.7 - 25.2). The adjusted odds ratio (OR) for 11-16-year-old age group (reference: 0-10-year-old age group) was found to be higher than other age groups for both males (OR: 2.50; 95% Bayesian Credible Interval: 1.03 - 5.92) and females (5.11; 1.60 - 17.43). Participants living in upper coastal plains (reference: elongated valley region) have highest risk of infection (30.45; 7.81 - 184.57). The effect of villages' irrigation status on incidence of human infection was inconclusive. The one-year risk of infection was very high when compared to the baseline prevalence which reflects several years of exposure to contaminated environment. The low treatment coverage in the rest of the village residents and the high prevalence of infection in several animal reservoirs of schistosomiasis may be responsible for this. The greater risk among adolescents has been reported before and is likely due to a combination of greater exposure, and maturity associated development of immunity to reinfection after cure. Higher transmission in the upper coastal plain region may be associated with stream flows and snail habitats, but further analysis is needed. The effectiveness of alternative control strategies such as more efficient mass treatment programs, control of infection in mammalian definitive hosts and/or changing the snail intermediate host environment is likely required for effective control of human infection.

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### AGREEMENT ANALYSIS OF STOOL EXAMINATION USING KATO-KATZ THICK SMEAR METHOD IN DIFFERENT SCHISTOSOMIASIS JAPONICA ENDEMIC AREAS IN CHINA

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Kato-Katz thick smear stool examination is the most widely used method to determine fecal egg counts in field surveys. However, several studies have demonstrated that this method may miss some infections, especially light ones. In order to explore the reproducibility of Kato-Katz in endemic areas with low prevalence, we analyzed the data from 3 different schistosomiasis japonica endemic areas where the prevalence is 19.37%, 6.31% and 2.77% respectively in three provinces in China. Observation data of egg counts were analyzed using weighted kappa statistic ( $K_w$ ) or interclass correlation coefficient (ICC) considering the characteristics of data to assess the agreement of data that are provided by multiple raters. Here we analyzed Inter-specimen variation (day-to-day) and intra-specimen variation (between slides and within sample). Inter-specimen variation was assessed categorically (stool positive or negative) by  $K_w$  statistics, and the result shows that K value of field-1 was 0.4927, 0.4171 of field 2 and 0.0208 of field3. ICC statistics was used to evaluate intra-specimen variation. ICC values of the first sample were 0.9265 of field-1, 0.5602 of field-2, and 0.0047 of field-3; ICC values of the second sample were 0.9334 of field-1, 0.5029 of field-2, and 0.1217 of field-3. The inter-specimen agreement was fair to good in field-1 and field-2, while poor in field-3. The intra-specimen agreement was excellent in field-1, fair to good in field-2, but still poor in field-3. Although we have excluded the artificial error by observers, the inter-specimen consistency of stool examination still can not reach a high-agreement level ( $K > 0.6$ ) and the intra-specimen agreement is not steady in different areas. The result that intra-specimen consistency is better than inter-specimen agreement may suggest the poor agreement of stool examination comes primarily from the day-to-day variation of eggs in stool. In addition, we noticed that the endemic areas with lower prevalence have lower agreement, this may relate to the disease intensity. Our study suggests the agreement of Kato-Katz should be evaluated before making any control policies based on the results of stool examinations, or maybe a better way is to find new approaches to field diagnosis for scientific studies and control programs. To sum up, our findings have substantial implications for the diagnosis and control of schistosomiasis japonica, especially in low endemic areas.

## 633

### ACTIVE SURVEILLANCE FOR AVIAN INFLUENZA IN MIGRATORY BIRDS IN THE FLYWAYS FROM CHINA TO AFRICA

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It has been hypothesized that migratory birds spread Influenza A/H5N1 pathogen into naïve countries via migratory flyways. Active surveillance in migratory birds in Ukraine, Egypt and Kenya establishes an early warning system to reduce the threat of highly pathogenic avian influenza (HPAI) transmission to humans in endemic areas. Surveillance was conducted over a migratory season in 3 sites along the flyways from China to Africa. The US Naval Medical Research Unit No. 3 (NAMRU3) has teamed up with

ornithologists in Ukraine, Egypt and Kenya to develop an avian influenza surveillance network. Birds are either humanely trapped for banding and descriptive observations or sampled from live bird markets or hunters. Cloacal swabs are collected and placed in cryovials with viral transport media and deposited directly into a liquid nitrogen tank. Our collaborators in Kenya screen bird samples with real time PCR and send them to NAMRU3 for further characterization. In November 2005 and January 2006, a NAMRU3 team was invited to Ukraine to screen cloacal swabs for influenza A using a portable real time PCR machine. Samples from all sites were tested for influenza A matrix and those that were PCR positive for influenza A were further screened for H5 and N1 genes. All influenza A matrix positives were also inoculated into embryonated hen's eggs and subtyped by hemagglutination inhibition. Finally, each H5 PCR positive samples was sequenced (HA gene) to determine specifically if the strain was highly pathogenic. A total of 4775 birds were caught and registered: 2605 from Egypt, 1803 from Ukraine and 367 from Kenya; 4332 samples were screened by PCR. Influenza A matrix was detected in 484 birds. These positive samples were subsequently screened for the presence of the H5 gene and seven were positive; four from Kenya, one from Ukraine and two from Egypt. These samples were further characterized by sequencing and none were found to contain a highly pathogenic H5N1 strain. In conclusion, influenza A is present in migratory bird populations in Ukraine, Egypt and Kenya but is considered to be within expected levels compared with the general migratory bird population; however, low pathogenic avian influenza (LPAI) H5 strains may mutate to HPAI in infected countries. Our detection of H5 virus indicates that this surveillance network is useful for early warning along migratory bird flyways.

### 634

#### PHYLOGENETIC ANALYSIS OF PERUVIAN ENCEPHALOMYOCARDITIS VIRUS

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Encephalomyocarditis virus (EMCV) is a single-stranded positive-sense RNA virus with a genome of about 7.8 Kb which belongs to the genus **Cardiovirus**, family **Picornaviridae**. Although it is considered a rodent virus, it infects a wide range of vertebrates like swine, elephants and captive primates. Previously we reported two EMCV human infections in the Peruvian cities of Iquitos and Cusco. Here we

describe the genetic analysis of the Peruvian isolates. 246 nucleotides of the 3D polymerase coding region of the viruses were RT-PCR amplified and sequenced. The sequences were aligned, compared with GenBank EMCV submissions and analyzed phylogenically. Sequences of the two Peruvian isolates are identical. Phylogenetically they are genotype B and share 97% homology with animal isolates from Cypriot, Belgium and Italy. The sequences presented here are the first EMCV sequences from clinical human cases.

### 635

#### MODIFIED SHELL VIAL CULTURE PROCEDURE FOR ARBOVIRUSES

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Isolation of arboviruses from patients' low titer sera can be difficult. In some circumstances PCR has been shown to be more sensitive than cell culture techniques for the identification of arboviruses. Here we compared the isolation efficiency of Dengue, Oropouche, VEE, Yellow Fever, Mayaro, Group C, Ilheus and West Nile viruses using a modified shell vial culture (MSVC) protocol to a standard cell culture protocol (SCC). The MSVC and SCC protocols were compared using five dilutions (10000, 100, 10, 1 and 0.1 PFU) for each of the following viruses: Den-1, Den-2, Den-3, Den-4, Oropouche, VEE, Yellow Fever, Mayaro, Group C, Ilheus and West Nile. Five isolate sera for each virus, were also compared by the two methods

using five sera dilutions (1:5, 1:10, 1:50, 1:100 and 1:500). In addition, 7 sera that were positive to Den-3 by PCR and negative by SCC were processed by MSVC. The MSVC protocol was consistently 1-2 logs more sensitive than the SCC protocol for all Flaviviruses and Bunyaviruses tested (Den-1, Den-2, Den-3, Den-4, Yellow Fever, Ilheus, West Nile, Oropouche and Group C). For the Alphaviruses (VEE and Mayaro) the two methods were equally sensitive. For the Flaviviruses and Bunyaviruses sera isolates the MSVC was consistently 1-2 sera dilutions more sensitive than SCC. Both methods were approximately equally sensitive for the Alphavirus sera isolates. Five of 7 Den-3 PCR positive, SCC negative, specimens yielded Den-3 virus by MSVC. In conclusion, overall, the MSVC protocol is more sensitive for virus isolation than the SCC protocol and it may, possibly, be approaching the sensitivity of PCR.

### 636

#### COMPARATIVE ANALYSIS OF BRAZILIAN VACCINIA VIRUS STRAINS

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Although the WHO declared global smallpox eradicated in 1980, concerns over emergent poxvirus infections have increased. Increased reports of Cowpox virus infections in Europe, the 2003 U.S. multi-state outbreak of Monkeypox virus and the emergence of Vaccinia virus (VV) in Brazil have raised interest in efforts to diagnose, treat and control infections caused by orthopoxviruses. Considering that poxviruses affecting humans are largely zoonotic, exploring the genetic diversity among poxviruses will illuminate the genetic and evolutionary aspects of poxvirus infections, ecology and epidemiology. In previous decades, VV strains largely infecting cattle and their handlers have been repeatedly isolated in Brazil. Genetically distinct populations of VV are circulating in the country and even within the same outbreak. To further investigate the evolution of these viruses, a genetic analysis is presented here that compares Brazilian VV strains isolated from multiple hosts and different geographic areas. The analysis compares the diversity of essential and non-essential genes involved in DNA replication, virion structure and viral pathogenicity. Results obtained indicate that these genetic elements possess nucleotide similarity between 96% and 100% when compared to reference VV strains, Buffalopox and a Horsepox isolated in Mongolia. Phylogenetic analyses were performed using both parsimony and bayesian methods with the programs PAUP\* and MrBayes. Brazilian VV strains separated into two groups that did not correspond to host or geographic origin. Despite genetic similarities, variation among strains consistently suggests that multifactorial events best explain the genetic diversity present in Brazilian VV, including the possible existence and continued circulation of autochthonous poxviruses.

### 637

#### IMPACT OF SELECTIVE LYMPHOID DEFICIENCIES ON ENCEPHALITIS AND VIRUS PERSISTENCE IN THE MURINE BRAIN (VEEV)

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$\gamma\delta$  T cells displaying a surface phenotype consistent with memory cells can consistently be found in the central nervous system (CNS), but their role in protection against invading viruses and other microorganisms is not understood. We show here a definitive role for these cells in clearance of a pathogenic arthropod-borne alphavirus, Venezuelan equine encephalitis virus (VEEV), from the CNS in mice previously immunized with a chimeric, live-attenuated alphavirus. This study demonstrates a strict requirement

for  $\alpha\beta$  TCR-bearing T cells but not for  $\gamma\delta$  TCR-bearing T cells in protection from lethal encephalitis in response to a recombinant alphavirus vaccine. A significant finding is the observation that  $\gamma\delta$  T cells are critical for complete viral clearance of the VEEV challenge strain from the brain of vaccinated mice after experimental exposure. In asymptomatic mice lacking  $\gamma\delta$  T cell function, we consistently isolated persistent viral variants from the brains, reflecting the incomplete clearance of VEEV or alternatively, the selection for low virulence VEEV mutants.

### 638

#### RECOMBINANT ALPHAVIRUSES ARE SAFE AND USEFUL SEROLOGICAL DIAGNOSTIC TOOLS

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The identification of Venezuelan equine encephalitis virus (VEEV) currently requires biosafety level-3 facilities and select agent certification for handling of VEEV viruses used to produce antigens, reference anti-sera or viral stocks. Rapid identification of VEEV infection is required to respond to outbreaks of encephalitis in humans as well as horses and can be useful for epidemiological surveillance. Alphavirus (Sindbis)-based recombinant viruses that express VEEV structural proteins are attenuated in animal models and thus represent an alternative to the handling of virulent, infectious virus. Virus and viral antigens from recombinant Sindbis/VEE constructs engineered to express structural proteins from different VEEV subtypes were evaluated as diagnostic reagents in VEEV-specific serological assays, e.g., plaque reduction neutralization test (PRNT), hemagglutination inhibition (HI) and complement fixation (CF) tests. Chimeric viruses and antigens were as effective as the parental virus at identifying infection of humans, horses, and rodents in serological assays and their production in cell culture is comparably as simple as described for VEEV.

### 639

#### PEPTIDE-CONJUGATED PHOSPHORODIAMIDATE MORPHOLINO OLIGOMERS INHIBIT ALPHAVIRUS REPLICATION AND PREVENT LETHAL ENCEPHALITIS IN VEEV-INFECTED MICE

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Vaccination of large populations for every viral pathogen that is a newly emerging disease or is potentially weaponizable is impossible. The development of traditional antiviral drugs requires an extensive screening process and usually results in the identification of only a single specific agent. Therefore, the development of a platform technology that could be used to treat various diseases caused by bioterrorist attacks or emerging viral infections is highly desirable. Recently developed peptide-conjugated phosphorodiamidate morpholino oligomers (P-PMO) have many advantages over traditional antisense oligomers and function by sequence-specific steric blockage. We hypothesize that P-PMO directed against essential RNA synthesis and/or translational control elements are potential candidates to effectively treat infections by RNA viruses, and can be synthesized rapidly in response to a viral outbreak. We have used alphaviruses as representative of RNA viruses that could emerge as a considerable threat to public health, or be developed as bioweapons. Alphaviruses exhibit robust replication in both cell culture and animals, and, as such, provide a robust system to investigate the antiviral efficacy and specificity of P-PMO. We have 1) achieved potent inhibition of both Sindbis virus and a variety of VEE viruses in cell culture with P-PMO

targeted to the 5'â€™ area of the genomic RNA and 2) were able to prevent VEEV-caused lethal encephalitis utilizing a VEEV mouse model.

### 640

#### ALPHAVIRUS-BASED VACCINES AGAINST RIFT VALLEY HEMORRHAGIC FEVER VIRUS

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Rift Valley fever virus (RVFV) is a representative member of the Bunyaviridae family that is in continuous circulation in the livestock-raising regions of Africa. The introduction of sheep and cattle, which are not native to this continent, into intensive farming, has resulted in the emergence of massive mosquito-borne epidemics. Death rates are very high for domestic animals. Moreover, the fatality rate for fetuses in pregnant livestock is 100%, and the fatality rate for newborn lambs is 90%. The virus has spread to new geographic areas, as evidenced by an extensive epidemic in Egypt in 1977. Most recently, RVFV caused a massive epidemic in sub-Saharan Africa in 1997-98 and spread across the Red Sea to Saudi Arabia and Yemen, causing devastating disease outbreaks in sheep and cattle. RVFV is also a significant human pathogen with an approximate 1% mortality. There is no treatment for humans or vaccine for the livestock that are such important amplifiers of the virus. Our goal is to create a new type of preventive recombinant vaccine against RVFV infection. This vaccine will combine the safety of inactivated and subunit vaccines and the efficacy of live attenuated vaccines and will be based on Sindbis and Venezuelan equine encephalitis virus replicons and viruses expressing structural proteins of RVFV. They will efficiently protect against RVFV infection, and it will be possible to manufacture them on an industrial scale.

We are developing i) the optimal strategy for expression of structural proteins of RVFV by recombinant Sindbis and VEE viruses, ii) the system for delivery of recombinant genomes into antigen-presenting cells and optimal presentation of the RVFV-specific antigens, and iii) the manufacturing procedure for the large-scale production of the recombinant viruses. We believe that final constructs and procedures will be a basis for an alternative strategy of rapid response to emerging viral infections that will require development of vaccines and their large-scale production for immediate use. Vaccine candidates that protect mice efficiently against the lethal RVFV infection will be presented.

### 641

#### RELATIONSHIP OF A NEW GROUP OF SOUTH AMERICAN PHLEBOVIRUSES TO RIFT VALLEY FEVER VIRUS: A PHYLOGENETIC STUDY

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The Phlebovirus genus of the family Bunyaviridae consists of 68 antigenically distinct virus serotypes, with high genetic diversity. By complement fixation assay (CF), a group of previously uncharacterized phleboviruses isolated from Brazil and Panama, namely, Belterra, BeAn 578142, and Icoaraci were found to be related to Rift Valley fever virus (RVFV). To further examine the relationship between 6 phleboviruses isolated from Brazil and Panama (BeAn 416992, Salobo, Belterra, Icoaraci, Frijoles and Joa) and RVFV. The infectious characteristics of these viruses were studied by plaque assay, indirect fluorescence assay (IFA), and experimental hamster infections. RT-PCR was also performed to amplify a portion of the S, M and L segments of the genome of each virus, for phylogenetic analyses. Plaque assay and IFA showed no apparent difference among these viruses. In vivo studies revealed the infectious characteristics of the 6 isolates were different from RVFV. The nucleotide and deduced amino acid identities among the 6 isolates were higher

than the identities when they were compared with RVFV, Punta Toro (PT) virus, sandfly fever Sicilian (SFS) virus and Toscana (TOS) virus used in this study. Phylogenetic analysis showed the Brazilian phleboviruses (BeAn 416992, Salobo, Belterra and Icoaraci) fell together in the same subclade; Joa virus isolated in Altamira, Para state, Brazil, and Frijoles virus from Panama formed another subclade. However, the 6 isolates always clustered together. In conclusion, these 6 isolates constitute a new group of phleboviruses different from RVF, PT, SFS, and TOS viruses. Their positions in the phylogenetic tree closely correlate with their geographical distributions. These findings will serve as the genetic basis for further studies of the evolution and epidemiology among the genetically diverse phleboviruses.

## 642

### ESTIMATING THE POSSIBLE IMPACT OF INFLUENZA PANDEMIC ON HEALTHCARE DEMAND AND CAPABILITY IN PERU

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The next influenza pandemic is inevitable and countries must be prepared to face it. Estimates of the possible impact are crucial to determine the priorities for the design of the preparedness plan of every country. The objective of this study was to estimate the possible impact of influenza pandemic on healthcare demand and capability in Peru. We developed a model based on FluAid software to estimate the possible impact of a first wave of influenza pandemic and FluSurge software to estimate the possible impact on healthcare capability. We reviewed data from the 2005 National Population Census and from the 2005 Health Sector infrastructure and resources Census. We used two scenarios: the 1918-1919 and the 1950-1960 pandemics. We assumed a single wave of 12 weeks length and an attack rate of 25% with confidence interval of 15% -35%. According to the 1918-1919 scenario, 90,635 (51,899 - 185,330) deaths, 511,603 (161,096 - 700,709) hospitalizations and 1,295,242 outpatient attendances would be expected. Meanwhile, in the 1950-1960 scenario, 11,027 (6,312 - 22,545) deaths, 62,240 (19,596 - 85,244) hospitalizations and 3,622,079 (2,798,646 - 4,883,516) outpatient attendances would be expected. In the 1918-1919 scenario, 267% of all public hospital beds, 2071% of ICU beds and 2306% of mechanic ventilators would be required. On the other hand, in the 1950-1960 scenario, 37% of all public hospital beds, 274% of ICU beds and 305% of mechanic ventilators would be required. In conclusion, this model has a number of limitations, possibly over or under-estimating the impact of the next influenza pandemic. However, it has provided important information that has helped us identify the infrastructure and human resources needed for the design of the influenza preparedness plan for the country.

## 643

### PRODUCTION OF LA CROSSE VIRUS-LIKE PARTICLES

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Transient expression strategy is being used to produce LACV virion structural components that allow assembly of LACV virus-like particles (VLPs) as potential vaccine candidates. Constructs expressing LACV (strain Human/78) structural proteins have been developed. The open reading frames (ORFs) of LACV M segment (G2/NSm/G1) and S segment (N/NSs) were subcloned into the eukaryotic expression vector pCAGGS under control of the chicken  $\beta$ -actin promoter (pCA-M1Pk and pCA-S1Pk, respectively). For production of LACV-like vRNA molecules, we have used a human RNA polymerase I based promoter/terminator expression plasmid (pHH21) originally developed for the production of influenza virus-like vRNAs. Expression constructs have been produced that encode an antisense GFP ORF terminated by LACV M segment 5' and 3' non-coding vRNA UTS sequences (pME-GFP). A LACV RNA-dependent RNA polymerase (RDRP) protein expression plasmid has also been constructed (pCAL1Pn); VLP formation was assessed with or without concomitant

RDRP protein expression (as viral RNP complexes within the virion have associated RDRP protein). Transfected cell culture supernatants were examined for the presence of released GFP encoding vRNA into culture supernatants by RT-PCR. As controls, pME-GFP transfected cells, or cells transfected with pME-GFP and either pCA-M1Pk or pCA-S1Pk alone were also tested. Only those cells that expressed M, S, and GFP vRNA genes released detectable GFP vRNA into supernatants suggesting N protein-dependent encapsidation of the GFP vRNA into particles formed through self-assembly of the G1 and G2 proteins into VLP-like complexes. Inclusion of the polymerase construct had no effect on GFP vRNA release into the supernatant.

## 644

### EVIDENCE OF A NEW GENOTYPE OF OROPOUCHE VIRUS (OROV) IN ARGENTINA, 2005

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Oropouche virus (OROV) circulating in Latin America has been classified in three different phylogentic groups according to the nucleotide sequences obtained for the nucleocapsid (N) genes of several OROV strains isolated from different hosts, time period and geographic areas in Central and South Americas. These groups include: Genotype I (Trinidad and Brazil), Genotype II (Perú and Brazil) and Genotype III (Panamá and Brazil). On Early 2005, several febrile cases suggestive of dengue infection were detected in the province of Jujuy, Argentina. All samples were found negative for Dengue 1-4 by virus isolation in C6/36 cells. Further studies were performed in order to identify other etiologic agents. A total of 10 serum samples obtained on days 1 to 4 after onset of symptoms, were submitted to RNA extraction and used for OROV N gene amplification by RT-PCR technique. RT-PCR products were purified, directly sequenced and evaluated by phylogenetic analysis using Neighbor Joining (NJ) and Maximum Parsimony (MP) implemented in the Mega 2.1 and PAUP 4.0, respectively. A total of 8 samples were positive by RT-PCR. Phylogenetic analysis of 5 N gene nucleotide sequences showed that OROV from Argentina forms a separate group which represents a different genotype from the previously reported and temporarily classified as genotype IV. Nucleotide sequences diverged from 3.9 to 6.6 % in comparison to OROV sequences from Panamá, Perú and Brazil. Our results suggest the circulation of OROV by the first time in Argentina, associated to febrile cases at the north-western region of the country where Dengue viruses have been circulating in the last years, as well as the identification of a new OROV genotype.

## 645

### EVIDENCE FOR SEGMENT REASSORTMENT IN LA CROSSE VIRUS FIELD ISOLATES FROM WISCONSIN AND MINNESOTA

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The evolutionary success of La Crosse virus (LACV, family *Bunyaviridae*) is due to its ability to adapt to different conditions through intramolecular genetic changes and segment reassortment. Vertical transmission of LACV in mosquitoes increases the potential for segment reassortment. Studies were conducted to determine if segment reassortment was occurring in naturally infected *Ochlerotatus triseriatus* mosquitoes from Wisconsin and Minnesota in 2000 and 2004. Mosquito eggs from various sites in

Wisconsin and Minnesota were collected. They were reared in the lab and the adults were tested for LACV antigen by immunofluorescence. RNA was isolated from the abdomen of infected mosquitoes and portions the small (S), medium (M) and large (L) segments were amplified by RT-PCR. PCR products were sequenced and a consensus sequence was formed for each sample. Overall, 44 viruses were analyzed (6 viruses from 2000, 34 viruses from 2004, and 4 virus isolates from 1970, 1978, 1979, and 1981). Phylogenetic analysis revealed that 15 of the 44 (31.8%) viruses are reassortants, suggesting that significant LACV segment reassortment is occurring in nature.

## 646

### MOLECULAR DETERMINANTS FOR RECEPTOR SPECIFICITY OF *PLASMODIUM VIVAX* DUFFY BINDING PROTEIN

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The invasion of *Plasmodium* merozoites into human red blood cells is a key step of clinical disease and is a target of therapeutic intervention. This invasion step is reliant upon specific interactions between erythrocyte receptors and parasite ligands, including the Duffy-binding like erythrocyte binding protein (DBL-EBP) family. Each member of this family contains one or more cysteine-rich Duffy binding like (DBL) domains. *P. vivax* has only one member of the DBL-EBP family, the Duffy binding protein (DBP) which interacts with the Duffy blood group antigen. In the *P. vivax* DBP, region II of the DBL domain is responsible for receptor recognition and the residues between cysteines 4 and 6 have been implicated as the minimal region necessary for receptor recognition. We have previously analyzed this region of the DBL domain of the *P. vivax* DBP using site-directed mutagenesis to identify specific residues important for receptor binding. Only a subset of these functionally important residues map to the predicted receptor binding site of the homologous *Plasmodium knowlesi* DBP. *P. knowlesi*, a simian malaria, has three DBL-EBP family members, the  $\alpha$ ,  $\beta$ , and  $\gamma$  proteins, each of which contain a single DBL domain. Although the four proteins share approximately 70% homology with each other, the *P. vivax* DBP and the *P. knowlesi*  $\alpha$  protein bind to human erythrocytes, while the *P. knowlesi*  $\beta$  and  $\gamma$  proteins do not. We have used a phenotype conversion approach along with information from the recently elucidated crystal structure of the *P. knowlesi* protein to clarify the mechanism of this specificity and to further validate the residues necessary for binding to the Duffy receptor.

(ACMCIP Abstract)

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### PROTEIN TRAFFICKING TO THE *P. FALCIPARUM* DIGESTIVE VACUOLE

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*Plasmodium falciparum* development inside the infected red blood cell involves the formation of specialized organelles including the digestive vacuole (DV), an acidic lysosomal-like compartment whose functions include the degradation of hemoglobin and the detoxification of heme. Hemoglobin is ingested through a double membrane invagination of the parasite membrane and the parasitophorous membrane through cytosomes. Vesicles budding from the cytosome appear to traffic to and fuse with the DV, releasing a single membrane-enclosed vesicle. When and how the DV develops, and how hemoglobin-degrading proteins and membrane transporters involved in drug resistance are targeted to this organelle, remains largely unexplored. To investigate this, we have developed a transgene expression system that produces fluorescent fusion proteins for imaging. The choice of promoters has proven to be critical for proper targeting to the DV and for suitable expression. All of the known

DV proteins appear to all be expressed within a similar developmental timeframe. Using live imaging, as well as immunofluorescence microscopy with antibodies against BiP, ERD2, Exp1 and NT1, we are able to monitor expression and targeting of DV protein fusions throughout the entire intraerythrocytic cycle. The known DV proteins all appear to be targeted to particular sites on the parasite membrane and then on towards the DV, possibly by cytosome-mediated endocytosis. Jasplakinolide and cytochalasin D treatments have helped to support these findings by showing that the cytosomal process is actin dependent and by enabling the trapping of fluorescent proteins on the parasite membrane at specific locations. The targeting of known DV proteins was investigated using computational biology and serial truncations were created to screen for a common conserved motif. A candidate motif is currently being tested using site-directed mutagenesis. Using this motif and the timing of expression as search criteria, we are also testing several potential DV protein candidates. This information should help in the identification of additional DV proteins and provide new insights into DV biology, which could help unravel mechanisms of drug action and resistance in *P. falciparum* malaria.

(ACMCIP Abstract)

## 648

### CLONING OF A CDNA ENCODING A *PLASMODIUM YOELII* INTEGRAL MEMBRANE PROTEIN LOCATED IN THE PARASITOPHOUS VACUOLE MEMBRANE

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Blood stage proteins of malaria parasites serve as important vaccine targets for malaria. We have isolated a gene encoding an integral membrane protein from a *Plasmodium yoelii* blood stage cDNA library. The gene corresponds to the gene locus PY 02667, has partial homology to the gene encoding the integral membrane protein of *P. chabaudi* Ag3008 and the *P. falciparum* early transcribed membrane protein PFE1590w. Peptides corresponding to the gene product were also identified in a blood stage merozoite rhoptry proteome analysis. Two predicted transmembrane domains and a signal peptide were identified in the protein structure. In Southern hybridization data a single band of approximately 4.5 kb from EcoR1 digests hybridized to *P. yoelii*, *P. berghei*, and *P. chabaudi* genomic DNA but not to *P. falciparum* genomic DNA. To further characterize the new gene, cDNA was cloned into the plasmid pRSETA and the full length recombinant protein expressed in *Escherichia coli* BL21DE3 cells. The purified recombinant protein generated mouse antisera that reacted with an approximately 35-40 kDa protein from purified recombinant protein preparations and from *P. yoelii* and *P. berghei* extracts by western blotting. The antisera localized the native protein to the parasitophorous vacuole membrane (PVM) and in clefts of schizont-infected erythrocytes by immunoelectron microscopy. The protein may have a role in merozoite invasion and PVM formation. Studies are currently in progress to identify the role of this novel protein in PVM formation and remodeling.

(ACMCIP Abstract)

## 649

### RELEASE OF THE INTERNAL SUBPOPULATION OF MAJOR SURFACE PROTEASE (MSP) OF *LEISHMANIA CHAGASI* UPON STIMULATION BY MATRIGEL™ MATRIX

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The major surface protease (MSP) is a virulence factor expressed on all *Leishmania* spp. studied to date. It constitutes 1% of total cellular proteins in metacyclic promastigotes. Much MSP is on the surface membrane, but up to one-third of MSP is located intracellularly and some isoforms are released into extracellular medium. Factors that regulate localization of the three MSP subpopulations (surface-located, internally-located, released) are poorly understood. The purpose of this study was to determine factors that regulate this differential localization in virulent parasites. We previously showed that the half life (T<sub>1/2</sub>) of surface-located MSP increases three-fourths during growth of virulent *L. chagasi* promastigotes from logarithmic to stationary phase, as reported previously. In contrast, the T<sub>1/2</sub> of surface-located MSP in L5 changed only minimally during promastigote growth, suggesting the growth-associated regulation of surface-located MSP occurs only in virulent *L. chagasi*. Interestingly, the T<sub>1/2</sub> of both L5 logarithmic and L5 stationary MSP was similar to that of logarithmic phase virulent *L. chagasi* MSP. Methyl- $\beta$ -cyclodextrin, a lipid-raft disrupting reagent, enhanced the release of surface-located MSP from virulent *L. chagasi* into extracellular medium in a dose-dependent manner. Matrigel™ Matrix is a soluble basement membrane extract of EHS tumor which is extensively used in the study of tumor metastasis. The combination of Matrigel™ Matrix and 37°C was used to mimic the environments encountered by promastigotes upon inoculation into a mammalian host by a sand fly vector. The stable subpopulation of internal MSP from virulent promastigotes was released by incubation in Matrigel™ at 37°C but not at room temperature. These data suggest that different subpopulations of MSP are localized and regulated separately. We hypothesize that MSP subpopulations play different functions during *Leishmania* spp. infection of sand fly vector and mammalian host.

(ACMCIP Abstract)

## 650

### ARGININE METABOLISM IN MACROPHAGES DETERMINES THE OUTCOME IN EXPERIMENTAL VISCERAL LEISHMANIASIS

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Progressive disease in the hamster model of visceral leishmaniasis (VL), caused by *Leishmania donovani*, in contrast to infection in mice, mimics the progressive disease observed in untreated humans. During progressive infection in hamsters, despite a vigorous Type 1 cellular immune response, there was no expression of nitric oxide synthase 2 (NOS2), which is the primary mechanism for control of infection in mice. The absence of NOS2 expression was not related to NOS2 gene deletion or a non-functional protein, and the IFN- $\gamma$  signaling pathway in hamster macrophages was intact. The metabolic fate of arginine plays a significant role in determining the phenotype of activated macrophages. The generation of NO by the action of NOS2 on arginine leads to microbicidal activity, whereas the metabolism of arginine by arginase diverts the metabolic pathway away from NO-mediated antimicrobial activity, and leads to the generation of polyamines, which could promote parasite growth. We therefore hypothesized that the innately low NOS2 expression in hamster macrophages would lead to default toward an arginase-driven alternatively activated state in macrophages, with the combination of low NOS2 and high arginase expression resulting in impaired parasite

killing. We found that hamsters with VL had, in contrast to the low NOS2 expression, increased levels of splenic arginase I mRNA and enzyme activity compared to uninfected animals ( $p=0.005$  and  $p=0.016$ , respectively). In contrast, there was no increase in arginase activity in the spleen tissue of infected compared to uninfected mice. Furthermore, we found that the polyamines putrescine, spermidine, and spermine, downstream products of arginase activity, were increased in the spleens of infected compared to uninfected hamsters ( $p<0.008$ ), but not in infected mice. *L. donovani*-induced production of polyamines was also evident in an *in vitro* model of infected hamster macrophages ( $p<0.03$ ). Culture of *L. donovani* infected macrophages with the selective arginase inhibitor nor-NOHA reversed the arginase/NOS2 balance (dose-dependent enhanced NO production,  $p=0.0001$ ) and increased killing of the intracellular parasite ( $p=0.02$ ). Thus, the arginase-driven metabolic pathway in this model, with low NO and high polyamine production, results in the inability to control the infection. The targeting of this pathway has therapeutic potential for this and other intracellular pathogens.

(ACMCIP Abstract)

## 651

### CHARACTERIZATION OF SCHISTOSOMA MANSONI CONSTITUTIVE ANDROSTANE RECEPTOR

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A *Schistosoma mansoni* homologue of vertebrate CAR/PXR/VDR group nuclear receptor, termed SmCAR was isolated from a *S. mansoni* adult worm cDNA library. SmCAR, a 702 amino acid protein, exhibits a typical domain organization of nuclear receptor superfamily members. Like its orthologues from invertebrates, SmCAR contains a P box sequence of ESCKA in the DNA binding domain. The P box is important in determining the DNA binding specificity for nuclear receptors. SmCAR mRNA is expressed in every stage of *S. mansoni* life cycle with an elevated expression level in the egg and cercaria stages. SmCAR protein was demonstrated to be widely distributed in adult worms by immunolocalization studies, being found in the subtegument in both male and female worms and in the ovaries, vitellaria and eggs in female worms. In vitro DNA binding assays demonstrated that SmCAR binds to the hsp27 ecdysone response element (EcRE) as well as the female-specific schistosome p14 gene upstream region. SmRXR1 but not SmRXR2, two *S. mansoni* homologues of vertebrate retinoid X receptors (RXRs) was able to heterodimerize with SmCAR as demonstrated by yeast-two hybrid analyses, and *in vitro* pull-down and co-immunoprecipitation assays. Using chimeras consisting of the DBD of SmCAR and the ligand binding domain (LBD) of mouse (m) CAR, we show that despite a different P box, SmCAR DBD shares DNA binding specificity with mCAR. However, the SmCAR DBD does have some DNA binding properties specific to SmCAR. Studies of the chimeras also demonstrated that the SmCAR DBD is able to heterodimerize with the DBD of human RXR, allowing high affinity DNA binding. Based on this study and previous results, we conclude that SmCAR may recognize its cognate hormone response element via two mechanisms: binding to DNA monomerically or heterodimerizing with SmRXR1. These studies form the basis for an in depth understanding of the role of SmCAR in schistosome biology.

(ACMCIP Abstract)

## 652

**ARTEMISININ DERIVATIVES LOCALIZE WITHIN DIGESTIVE VACUOLE-ASSOCIATED NEUTRAL LIPID BODIES IN *PLASMODIUM FALCIPARUM***

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Despite extensive study, the antimalarial mechanism of action of artemisinin (ART) remains poorly defined. Most evidence indicates that ART potency in *Plasmodium falciparum* is due to cleavage of the endoperoxide bridge by ferrous iron, initiating the formation of a series of cytotoxic intermediates. In the malaria parasite, ART metabolites alkylate proteins and heme, and this may ultimately lead to cell death. Another hypothesis is that ART acts by the specific inhibition of the *P. falciparum* sarco-endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA; PfATP6). To provide insight into possible targets of ART, we used microscopic imaging techniques to study the cellular distribution of novel fluorescent ART derivatives in living parasites. We show that parasites rapidly accumulate fluorescent signal within neutral lipid bodies (NLBs) that are closely associated with the exterior of the digestive vacuole (DV). The specific cellular distribution is entirely dependent on the presence of the endoperoxide moiety of the ART derivatives. Pretreatment with the SERCA inhibitor thapsigargin did not affect the intensity of the fluorescent signal, suggesting that ART-SERCA binding is not involved in the observed localization. NLBs are stores of phospholipid metabolites formed in the DV. They are known to promote  $\beta$ -hematin formation *in vitro* and may play a role in heme detoxification in the parasite. As heme is a known target of ART alkylation, heme-ART adducts may interact with these neutral lipid environments. Our findings suggest that accumulation of ART metabolites in parasite lipids may be an important component of the antimalarial mechanism of action of ART-based compounds.

## 653

**INHIBITION OF YEAST HEXOKINASE ACTIVITY BY ARTEMISININ: AN *IN VITRO* MODEL OF DRUG-PROTEIN BINDING**

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Derivatives of artemisinin (ART), an endoperoxide-containing sesquiterpene lactone, are becoming potent mainstays of malarial chemotherapy. Although the endoperoxide moiety is required for efficacy against *Plasmodium*, an unequivocal mechanism of parasitocidal action has not been determined. The ability of ART to covalently bind to protein and heme is well-documented. Alkylation is believed to occur through reductive scission of the peroxide group, leading to a series of cytotoxic free-radical intermediates. ART may have a specific target, such as the sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA)/PfATP6, or may function as a non-specific alkylating agent. Using a coupled-enzyme spectrophotometric assay, we demonstrate that ART, in the presence of iron(II), inhibits the phosphotransferase activity of a model nucleophile-rich enzyme, yeast hexokinase (HX). Inhibition of HX is concentration-dependent with respect to both ART and iron(II). The necessity of reduced iron suggests that the decrease in enzymatic activity is dependent on iron(II)-catalyzed cleavage of the ART endoperoxide bridge. Complete and partial protection of HX activity is achieved by pre-incubation with the substrates ATP and mannose, respectively, indicating preferential interaction of ART with the nucleotide binding site. Dialysis is unable to restore activity to HX inhibited by ART, suggesting covalent binding as a

possible mode of enzyme inhibition. Structures of putative ART-protein adducts will be investigated by mass spectroscopy.

## 654

**A MOLECULAR METHOD FOR DETECTING POINT MUTATIONS IN THE ACTIVE SITE OF *PLASMODIUM FALCIPARUM* ADENOSINE TRIPHOSPHATASE 6, THE PUTATIVE TARGET FOR ARTEMISININ**

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Till recently, chloroquine and fansidar constituted the national policy treatments for uncomplicated malaria in Uganda and most African countries. However, in order to combat increased drug resistance, artemisinin combination therapy has now been widely recommended. It is proposed that the artemisinins work by binding and inhibiting *Plasmodium falciparum* ATPase6. Significantly, experience with the ACTs especially in south East Asia suggests that artemisinin combinations may limit the emergence of drug resistant parasites better than aminoquinolines or antifolates alone. Nevertheless, *in vivo* clinical studies of drug efficacy carried out one year ago show that 4% of malaria cases in Uganda were resistant to artemisinins. Thus, as ACTs replace chloroquine and fansidar by national policy, it is necessary to develop practical procedures for monitoring the likely emergence and spread of artemisinin resistant *P. falciparum* strains. In the work reported here, we have analyzed the genotypes of PfATPase6 in parasites from 300 malaria cases that were diagnosed and treated in the years 1999 to 2004 at three field sites in Uganda, a period just prior to policy introduction of co-artem (lumefantrine + artemether). Nucleic acid encoding PfATPase6 regions important in artemisinin binding and related structure-function relationships was amplified by 'nested' DNA polymerase chain reaction. To detect mutations at key amino acids of PfATPase6, the amplified DNA was digested by restriction enzymes and the fragments sized by agarose gel electrophoresis. Two fragments of DNA, which between them, contain most of the codons of amino acids located within the putative active site of pfATPase6 were readily amplified from the field samples. Interestingly, using this procedure based on restriction fragment length diversity, we did not find mutations at codons for the key residues Lys260, Gln266 and Asn1039. Nucleotide sequencing of pfATPase6 gene DNA from at least 15 clinical isolates confirmed the above findings and suggested that mutations at these and several other reportedly important amino acid residues have not emerged in our study sites. Thus, we have developed a simple molecular procedure for diagnosing mutations at several residues of pfATPase6 that have been reported to be important in artemisinin action.

(ACMCI Abstract)

## 655

**PFSUB2 MATURASE, A NEW TARGET FOR DRUG DESIGN: ANALYSIS OF THE POLYMORPHISM OF PFMSP1 AND PFAMA1 MATURATION SITE AND OF THE CATALYTIC SITE OF PFSUB2 IN WILD-TYPE ISOLATES OF *P. FALCIPARUM***

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Invasion of red blood cells by malaria parasites is an essential step currently investigated for vaccines or drugs design. Proteolytic shedding of two major surface proteins MSP1 and AMA1 proteins leads to receptor-ligand disengagement and invasion. These proteins are trimmed by the same maturase, PFSUB2. The development of drugs to block this process needs to question the ability of the parasites to evade using genetic polymorphism. This is the aim of this work. Our study provides first data on polymorphism of the catalytic site of PFSUB2 and of the maturation sites of PFAMA1 and PFMSP1 of wild isolates of the parasites. After

informed consent, blood samples were collected from patients attending dispensaries of the suburbs of Dakar and suffering from mild malaria. Sequences were obtained for the three genes on both strands after direct sequencing of PCR products. Sequences were aligned and single nucleotide polymorphisms were confirmed by re-amplification and re-sequencing of the ADN. For MSP1, 35 strains were analyzed. The MSP1 maturation site harboured a weak polymorphism. For AMA1, 7 out of 15 isolates had mutations in four locations in 3' part of the maturation site. For PfSub2, 34 strains were analysed. Only intronic polymorphic regions were found, which suggests strong conformational constraints on the molecule. Various intronic mutations were found revealing the diversity of the parasites collected. Despite the low level of transmission in this area, this high diversity of the isolates was also confirmed by the study of MSP-1 and MSP-2 polymorphism blocks. These data highlight the low level of polymorphism of the catalytic area of three genes of interest. These results confirm the interest of PfSub2 as potential target for the synthesis of new drugs. Studies on the *in vitro* activity of inhibiting peptides are ongoing.

(ACMCIP Abstract)

## 656

### EFFECTS OF INTERNAL DELETIONS OF HYDROXYMETHYLPTERIDINE PYROPHOSPHOKINASE-DIHYDROPTEROATE SYNTHASE FROM *P. FALCIPARUM*

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Folates are synthesised de novo in most bacteria, in many eukaryotic microorganisms and in plants. The biosynthetic pathway starts from GTP and involves six enzyme activities leading to the synthesis of tetrahydrofolate (THF). The bifunctional enzyme hydroxymethylpteridine pyrophosphokinase-dihydropteroate synthase (HPPK-DHPS) is a target for sulfadoxine, which is a part of the combination drug Fansidar, used in antimalarial chemotherapy. The full length HPPK-DHPS as well as a separate HPPK part can complement an HPPK knock-out of *E. coli*. In contrast, the full-length gene is necessary to complement a DHPS knock-out. The HPPK part of the plasmodial bifunctional enzyme is much larger than corresponding bacterial HPPK enzymes. The extra amino acids in the plasmodial enzymes are contained within two extensive insertions both of ca 100 amino acids. The first insertion is shared with rodent malaria and *Plasmodium vivax* while part of the second insertion is unique for *P. falciparum*. The *P. falciparum*-specific part could be deleted without affecting the complementation of either HPPK or DHPS knockouts. However, a small decrease in enzyme activity was noticed by careful kinetic analysis. Further deletions into the part common to all plasmodia however led to diminished activity of either or both enzyme activities. In the first insertion only very small deletions could be tolerated for complementation of DHPS but not of HPPK. When only the HPPK part was used, only the *P. falciparum*-specific part could be removed and still complement the HPPK defect. Expression studies showed that deletions in the parts common to all parasites led to diminished efficiency of expression. The conclusion is that apart from a *P. falciparum*-specific amino acid sequence, the extended HPPK is necessary for both activities of the bifunctional enzyme.

## 657

### GENOME-WIDE STRUCTURE AND EXPRESSION CHANGES IN RESPONSE TO SINGLE-STEP CHLOROQUINE AND QUININE SELECTION IN *PLASMODIUM FALCIPARUM*

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Lab-based drug selections have been key to understanding gene adaptations. Single-step (single high dose treatment) and stepwise (incremental doses) selection studies have demonstrated the plasticity of the *Plasmodium* genome, resulting in both sequence and copy number changes. It has been suggested that some parasite strains of *P. falciparum* exhibit enhanced genomic adaptability, reflecting, for example, consequences of defective DNA repair processes that could augment acquisition of beneficial polymorphisms. Until recently, it has been impossible to monitor the kinds and frequencies of mutations that arise in response to drug selection except in a few favored candidate drug resistance genes. However, advances in comparative genome hybridization (CGH) technology allow a high-resolution view of structural changes genome-wide. Here we identify genetic changes in the 106/1 line of *P. falciparum* that have accompanied point mutations in *pfprt* selected by chloroquine or quinine. We have combined CGH analysis with transcription microarrays to search for changes in the expressed genome that could reveal additional drug response adaptations or effects that compensate altered PfCRT function. Our results highlight the nature of the 106/1 genome to rapidly adapt to drug selection at multiple loci including *pfprt* and *pfmdr1*, as well as novel gene-exon excisions on chromosome 10. We observe that genomic structural changes effectively alter transcription, and explore the nature of drug-induced evolution in cultured parasites. Multidimensional approaches that integrate various levels of genomic and phenotypic information will be crucial to understanding mechanisms of genome adaptation and the evolution of drug resistance.

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### USE OF THE QUANTITATIVE MSP-1 HETERODUPLEX TRACKING ASSAY TO DISTINGUISH *PLASMODIUM FALCIPARUM* REINFECTION FROM FAILURE

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In antimalarial clinical trials, it is necessary to distinguish true drug failure (recrudescence) from reinfections. Since most patients carry multiple variants, failure is usually assumed when any of the variants detected by nested PCR in the recurrent infection were present pre-treatment. However, this method may misclassify outcomes. First, in an individual host, nested PCR cannot detect minority variants composing <20% of the population, and if the recurrent infection is due to a drug's failure to clear this undetected variant, the infection will be wrongly classified as a reinfection. Second, some patients might be cured by a drug, but reinfected by a variant identical to one found initially, and be misclassified as a failure. The use of Heteroduplex Tracking Assays (HTAs) lessen the likelihood of these misclassifications in two ways. First because they are quantitative, HTAs can detect minority variants comprising ~1% of the population. In a single patient with 10 variants, the HTA band intensity correlated well with its representation in colony PCR ( $R^2=0.93$ ). Second, since HTAs are highly reproducible across gels, the prevalence of each variant in a geographic area can be determined; from this, the probability that each individual recurrent episode was due to a treatment failure can be calculated. For example, in an IPT trial in Malawi, there were 30 recurrent episodes of which 16(53%) were definitely failures and 4 (13%) were definitely new infections. 10 shared a single highly prevalent variant (present in >10% of the population) and could be either



failures or reinfections. Assuming binomial probability, the likelihood that an individual recurrence represents a failure is  $(1-y)^x$ , where  $y$  is the prevalence of the shared band and  $x$  is the number of bands present in the recurrent infection. The mean probability of failure was 0.407 for these 10 recurrences, suggesting that 67%  $[(16 + 4.07)/30]$  of the recurrences were probably failures. This is the first *a priori* method for determining failure rate based on probability of reinfection.

## 659

### CONSUMPTION OF BATS IS A RISK FACTOR FOR EBOLA VIRUS INFECTION AMONG RURAL CAMEROONIAN ADULTS

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Despite the major public health importance of the filoviruses Ebola (EBO) and Marburg (MAR), the dynamics of transmission from animal reservoirs to humans is poorly understood. Previous studies have found that antibodies to filoviruses in Central African human and animal populations are common, but the transmission mechanisms for these putatively widespread infections are unknown. To explore risk factors for transmission of EBO and MAR to humans, we administered a detailed behavioral questionnaire and collected serum samples from individuals in nine rural villages in rural Cameroon. Samples were tested by ELISA for IgG antibody to EBO-Zaire (EBO-Z), EBO-Ivory Coast (EBO-IC), and MAR. Of the 1706 tested individuals, 135 (7.9%) were positive for EBO-Z, 43 (2.5%) were positive for EBO-IC, and 30 (1.8%) were positive for MAR. Seroprevalence rates varied widely between study sites, and men had significantly higher seroprevalence of MAR than women ( $p=0.05$ ). In bivariate analysis, hunting as a secondary occupation was a significant predictor of MAR seropositivity ( $p=0.01$ ). In adjusted analysis, logging as a primary occupation (OR: 5.51, 95% CI: 1.38, 21.96) and eating bats at least once a month (OR: 4.81, 95% CI: 1.51, 15.32) were significant predictors of EBO-Z seropositivity. These results suggest that consumption of bats, either directly or through exposure during preparation, may represent a mechanism for EBO-Z infection in humans, and that non-human primates or other animal intermediaries may not be necessary for transmission to occur.

## 660

### CHARACTERIZATION OF MARBURG VIRUS FROM A RECENT OUTBREAK IN ANGOLA

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A recent outbreak of Marburg Hemorrhagic Fever (MHF) in the Uige Province of Angola marks the largest and most recent outbreak of Marburg virus (MARV) to date. At the conclusion of the outbreak, the Ministry of Health in Angola reported a total of 252 cases, including 227 deaths. This case fatality rate of 90% is the highest observed during a MHF outbreak thus far and is more typical of past Ebola Hemorrhagic fever outbreaks. Sequencing of the glycoprotein (GP), nucleoprotein (NP)

and polymerase (L) genes of multiple clinical specimens indicated a very high level of conservation; therefore, one of the isolates (MARV-ANG) was selected for further *in vitro* and *in vivo* characterization, in comparison to the closely related strain, MARV-Musoke (MUS). Infection of Vero-E6 cells resulted in a strong cytopathic effect (CPE) for MARV-ANG starting at 24 hours post infection (pi) with destruction of the monolayer by day 5 pi, whereas MARV-MUS infected cultures did not show evidence of CPE until day 7 pi. Interestingly, MARV-ANG replication and particle release was delayed by more than 24 hours and end titers were lower in comparison to MARV-MUS. This finding was also confirmed by the delayed expression of viral antigen for MARV-ANG. Subsequently, nonhuman primates (*Rhesus macaques*) were infected with MARV-ANG and the clinical course and outcome were compared with that of other MARV strains. MARV-ANG showed an increased disease progression in these animals and led to death within 5-6 days compared to 7-8 days for ZEBOV and 9-10 days for MARV-MUS. Histopathological analysis revealed strong liver damage associated with MARV-ANG replication in contrast to previous results with other filoviruses. In conclusion, MARV-ANG seems to be associated with an increased *in vitro* cytopathogenicity and *in vivo* virulence. Increased cytopathogenicity is associated with reduced virus production due to early and massive cell destruction. Thus, this data is in line with the observed high case fatality rate and seemingly higher virulence for humans reported during the outbreak in Uige.

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### CHARACTERIZATION OF MARBURG VIRUS FROM A RECENT OUTBREAK IN ANGOLA

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A recent outbreak of Marburg Hemorrhagic Fever (MHF) in the Uige Province of Angola marks the largest and most recent outbreak of Marburg virus (MARV) to date. At the conclusion of the outbreak, the Ministry of Health in Angola reported a total of 252 cases, including 227 deaths. This case fatality rate of 90% is the highest observed during a MHF outbreak thus far and is more typical of past Ebola Hemorrhagic fever outbreaks. Sequencing of the glycoprotein (GP), nucleoprotein (NP) and polymerase (L) genes of multiple clinical specimens indicated a very high level of conservation; therefore, one of the isolates (MARV-ANG) was selected for further *in vitro* and *in vivo* characterization, in comparison to the closely related strain, MARV-Musoke (MUS). Infection of Vero-E6 cells resulted in a strong cytopathic effect (CPE) for MARV-ANG starting at 24 hours post infection (pi) with destruction of the monolayer by day 5 pi, whereas MARV-MUS infected cultures did not show evidence of CPE until day 7 pi. Interestingly, MARV-ANG replication and particle release was delayed by more than 24 hours and end titers were lower in comparison to MARV-MUS. This finding was also confirmed by the delayed expression of viral antigen for MARV-ANG. Subsequently, nonhuman primates (*Rhesus macaques*) were infected with MARV-ANG and the clinical course and outcome were compared with that of other MARV strains. MARV-ANG showed an increased disease progression in these animals and led to death within 5-6 days compared to 7-8 days for ZEBOV and 9-10 days for MARV-MUS. Histopathological analysis revealed strong liver damage associated with MARV-ANG replication in contrast to previous results with other filoviruses. In conclusion, MARV-ANG seems to be associated with an increased *in vitro* cytopathogenicity and *in vivo* virulence. Increased cytopathogenicity is associated with reduced virus production due to early and massive cell destruction. Thus, this data is in line with the observed high case fatality rate and seemingly higher virulence for humans reported during the outbreak in Uige.

## EMERGENCY VACCINATION RESPONSES DURING LARGE MEASLES OUTBREAKS: EARLY INTERVENTION LEADS TO A HIGH PROPORTION OF AVERTED CASES

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The current World Health Organization (WHO) recommendations for response during measles epidemics focus on case management rather than a reactive vaccination intervention, which may occur too late to have an impact on morbidity and mortality and has a high cost per case prevented. Previous studies in European settings suggested that intervening with a vaccination campaign may not stop epidemics, as transmission is so rapid, but may result in reduced morbidity and subsequent mortality. We present the results of a series of studies aimed at examining the spatial spread of measles epidemics in African urban high-burden settings, and the potential number of cases averted depending on the timing of the intervention. We used data collected during the investigation and response to a large measles outbreak in Niamey, Niger in 2003-2004. Using an individual-based computational model, we determine the potential impact of different intervention strategies in terms of the number of cases potentially averted. We then developed a general model to extend the analysis to other epidemic settings. We estimate that a median of 7.6% [95%CI: 4.9, 8.9] of cases were potentially averted as a result of the outbreak response, which vaccinated 57% (84,563/148,595) of children in the target age range (6-59 months), 23 weeks after the epidemic started in Niamey. We demonstrate that intervening early (up to 60 days after the start of the epidemic) and expanding the age range to all children aged 6 months to 15 years may lead to a much larger (up to 90%) reduction in the number of cases. Extending these results to other settings yields similar trends. In conclusions, our results suggest that intervening earlier even with lower target coverage (e.g., 70%), but over a wider age range, may be more effective than intervening later with high coverage (>90%) in urban resource-poor settings like Niamey. This has important implications for the implementation of vaccination interventions in epidemic contexts. Although responding to measles outbreaks depends upon other competing public-health priorities and the capacity of the country's health services and relief agencies, this research suggests that there is sufficient time to intervene with an acceptable benefit, in contradiction to the current recommendations.

## LATE OUTCOMES OF RIFT VALLEY FEVER IN KENYA: IJARA CLINICAL SURVEY

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Transmission of Rift Valley Fever Virus (RVFV), a high priority emerging pathogen, is both focal and episodic in nature, and is closely tied to flooding rainfall events. However, there may be sporadic transmission in enzootic areas that perpetuates and/or extends the range of RVFV transmission to humans. While most RVFV-infected humans have symptoms of limited duration and appear to make a full recovery, we hypothesized that the late-onset, non-hemorrhagic complication of RVFV retinitis could be associated with permanent, measurable injury to the eye. We performed a stratified cross-sectional survey sample in Masalani, Ijara District, Kenya, to test for evidence of prior RVFV infection

and resultant long-term medical consequences, including retinitis. 270 survey participants aged 1-86 years were selected via randomized cluster sampling in four sections (two rural/two town) of Masalani Division. These completed questionnaire administration regarding non-animal exposures, animal exposures, housing, recent or remote RVFV-related symptoms, complete physical examination, eye examination, and phlebotomy. In preliminary anti-RVFV IgG ELISA results, 22/149 (15%) of subjects tested were seropositive. Nine percent of children < 15 yr were seropositive, with the youngest seropositives aged 1, 4, 6, and 7 years. Twenty percent of adults were seropositive with the oldest aged 64 years. There were 13 cases of significant retinal disease, including retinal scarring, vasculitis, and maculopathy, in the surveyed population; all were adults > 21 years old. Seven were RVFV seropositive and 6 were seronegative. The Odds Ratio (OR) of late eye disease association with RVFV exposure (seropositivity) was 5.95 ( $P < 0.003$ ). In conclusion, the young seropositive children identified in our survey indicate ongoing interepidemic transmission of RVFV within Ijara District, Kenya. RVFV seropositivity was not specifically associated with any general physical exam outcomes, but there was a strong association between RVFV exposure and risk for chronic forms of retinal disease.

## SUCCESSIVE OUTBREAKS OF VIRAL HAEMORRHAGIC FEVERS (CCHF AND RVF) IN MAURITANIA, 2003

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We describe two successive outbreaks of CCHF and RVF in Mauritania between February and November 2003. The urban CCHF outbreak began in Nouakchott on early February 2003. Between Feb 12th and March 14th a cluster of 28 cases was noticed with a case fatality ratio equal to 25%. This outbreak was mainly nosocomial as the majority of the cases occurred at the National Hospital Centre (NHC) in Nouakchott where patients with fever and hemorrhagic syndrome was hospitalized. In September 2003 we received 20 samples from suspected cases of hemorrhagic fever from the NHC in Nouakchott. Strikingly, diagnosis led to a mixed pattern of both CCHF (5 IgM+ persons) and RVF infections (2 IgM+ persons). Subsequently, we undertook a large investigation in Nouakchott and other areas where animals were tested IgM+ for RVF in September. In total, 128 persons were tested, 25 and 4 of them was recently infected by RVFV and CCHFV respectively. For RVFV infection, the case fatality ratio was 32.1%. It is noteworthy that the RVF outbreak was associated with flooding conditions in the southern part of the country. A phylogenetic study showed that the RVFV strains isolated during this epidemic belong to the Central-East African lineage. This is the first evidence of the occurrence of this genotype in Mauritania and Western Africa. The S gene sequences of these strains are closely related to those of RVFV strains isolated 2 years ago in Chad (Central Africa). Our findings confirm genetic exchange between strains from different lineages in Central-East Africa and West Africa. These overlapping circulations of VHF viruses in 2003 give also new insights to the conditions of re-emergence and therefore measures to be taken to control viral hemorrhagic fevers (VHF) diseases and avoid human deaths in sub-saharan countries. They also stress the need of prompt and differential laboratory diagnosis during all seasons and for all known endemic VHF viruses.

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**TWO NOVEL ASSAYS FOR MOLECULAR DETECTION OF CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS**

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Crimean-Congo hemorrhagic fever virus (CCHFV) is the causative agent of Crimean-Congo hemorrhagic fever (CCHF) a highly contagious human disease with a fatality rate up to 60%. CCHFV has been reported in Africa, a number of Middle Eastern countries and parts of Europe and China. We present two novel assays for detection of this pathogen both in reference and field-based laboratories: Real-time PCR facilitates rapid and sensitive detection of a large spectrum of viruses. However, the remarkable genetic variability in CCHFV interferes with detection by primers and probes. Even single mismatched nucleotides may be detrimental, rendering existing CCHFV PCR assays insufficient in selected cases. By comparative genome analysis we identified a conserved region within the CCHFV s-segment that was appropriate for application of a novel design strategy for primers and probes. In total, the assay uses three fluorescent detector oligonucleotides to cover the whole known spectrum of virus diversity. Detection is carried out in a standard LightCycler system. The second assay is based on PCR amplification of a different s-gene fragment with modified and biotin-linked primers. Amplification products are hybridized to low density arrays carrying 21 different capture probes, including universal and strain-specific sequences. Probes cover the whole spectrum of known CCHFV strains. The arrays are based on plastic photo-slide frames and are processed in a simple and purely manual procedure. Bound PCR fragments are visualized with the naked eye using a blue precipitating peroxidase substrate. The assay protocol has been optimized for usage in field laboratories with minimal technical equipment. Both assays were successfully validated using 11 different CCHFV strains from all endemic regions worldwide. Hybridization patterns on the arrays facilitated discrimination between all virus strains. Sensitivities of both assays were consistently high at 14 and 25 copies per reaction, respectively. A competitive internal control is included in each assay to identify sample-derived PCR inhibition. We believe both assays will be useful for diagnosing CCHF in the laboratory and in the field. The assays are currently established by virological reference centers in Europe and Africa, for confirmation of CCHFV infections, and for typing of virus isolates.

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**A ROLE FOR THE CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS (CCHFV) NUCLEOPROTEIN IN MEDIATING PARTICLE ASSEMBLY AND RELEASE**

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Crimean-Congo haemorrhagic fever virus (CCHFV) is a member of the family Bunyaviridae, genus Nairovirus. CCHFV is transmitted by tick vectors and is the causative agent of human disease in more than 30 countries in Asia, Africa and Europe. The global distribution of CCHFV is closely linked to the geographical location of its vector, most commonly *Hyalomma* sp. ticks. As seen in other bunyaviruses, the viral genome is comprised of a tri-segmented, negative-sense RNA genome which encodes four structural viral proteins; the RNA-dependant RNA polymerase (L), the surface glycoproteins Gn and Gc, and the viral nucleocapsid protein (NP). In contrast with other bunyaviruses, relatively little is known about the molecular biology of CCHFV. The NP is known to encapsidate the viral genome and is an important component of the ribonucleoprotein (RNP)

complex. A strong humoral immune response is directed at the NP. Given that bunyaviruses lack a matrix protein equivalent, it was hypothesized that the NP would fulfill the role of mediating viral assembly and/or release from cells. The main objectives of this study were to clone and express the CCHFV NP and assess its role in the maturation and budding of viral particles. To address these objectives, the NP was cloned into a eukaryotic expression vector and expression was evaluated by western blot analysis and both immunofluorescent and confocal microscopy. Confocal microscopy demonstrated a cytoplasmic localization of the NP when expressed alone. When the viral glycoproteins were co-expressed, the NP was redistributed and could be detected in both the Golgi apparatus and the ER, similar to NP localization during CCHFV infection. Western blot and electron microscopy analysis identified NP-driven formation and release of virus-like particles (VLPs). Co-expression of Gn and Gc resulted in the incorporation of the glycoproteins into the VLPs and increased stability of VLPs. VLPs formed by both NP alone and NP/Gn/Gc were similar to authentic CCHFV virions and demonstrated the same sensitivity to proteinase K digestion. Our data indicate an important role for the NP in mediating particle maturation and release, suggesting a matrix protein-like function for the NP. In addition to providing important information about mechanisms of virus assembly, VLPs provide an attractive avenue for both diagnostics and vaccine development in that the immunogenicity associated with VLPs is typically quite high.

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**STAT SIGNALING REGULATES PLASMODIUM BERGHEI INFECTION IN ANOPHELES GAMBIAE MOSQUITO**

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The role of Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling in the mosquito immune response to bacteria and *Plasmodium* was investigated. A single STAT gene has been identified in all insect genomes, including *Drosophila* and culicine mosquitoes, but *A. gambiae* has two STAT genes (AgSTAT-A and AgSTAT-B) that share 64% homology in their amino acid sequence. AgSTAT-B maps to Chr3 (38B), has six introns, three of which have the same location as in *Drosophila*-STAT and has higher homology to STATs from other mosquito species suggesting that it represents the ancestral form of STAT. In contrast, AgSTAT-A is located in ChrX (4B), is intron-less and its amino acid sequences is more divergent from all other mosquito STATs, suggesting that this second gene was the result of a retrotransposon event. Both STATs and other components of the JAK/STAT cascade (such as SOCS and NOS) are expressed in the *A. gambiae* Sua 5.1 cell line. SOCS and NOS mRNA expression is induced by bacterial challenge, and this response can be suppressed by silencing either AgSTAT-A or B. STAT-A silencing also down regulates the expression of STAT-B suggesting that STAT-A is upstream to STAT-B. *In vivo* silencing of AgSTAT-B down regulates mRNA expression of SOCS and NOS mRNA, but surprisingly this decreased the number of developing oocysts, indicating that this pathway also regulates expression of a factor required for *Plasmodium* infection.

(ACMCIIP Abstract)

### A NOVEL ANTIVECTOR *PLASMODIUM FALCIPARUM* TRANSMISSION-BLOCKING ANTIBODY REVEALS HETEROGENEOUS OOKINETE INVASION STRATEGIES

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Antivector malaria transmission-blocking vaccines prevent ookinete-to-oocyst transition by targeting mosquito midgut receptors. Previous studies determined that the lectin Jacalin inhibits ookinete midgut invasion by binding to glycan receptors on unidentified glycoproteins. Here we report on the identification of the polypeptides bearing these oligosaccharides. Through a glycoproteomic analysis, we identified the membrane-bound *Anopheles gambiae* Aminopeptidase N (AgAPN1) as a putative receptor. Polyclonal anti-AgAPN IgG inhibited significantly *Plasmodium berghei* oocyst development in diverse mosquitoes. Because inhibition was incomplete, we combined both antibody and salivary and midgut peptide 1 (SM1), which also exhibits incomplete blocking, in a *P. berghei* bloodmeal and observed a slight increase in inhibition, suggestive of overlapping inhibitory mechanisms. However, in contrast to SM1, anti-AgAPN1 IgG blocked *P. falciparum* development significantly; thereby partitioning murine from human parasite midgut invasion models. The results provide evidence for a novel, conserved antivector transmission-blocking antigen and for diverse ookinete invasion modalities in the mosquito.

(ACMCI Abstract)

### VARIANT-SPECIFIC BINDING OF RECOMBINANT *PLASMODIUM VIVAX* DUFFY BINDING PROTEIN TO HUMAN ERYTHROCYTES

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*Plasmodium vivax* requires binding to the Duffy Antigen for Chemokines (DARC) on the erythrocyte surface in order to penetrate the cell. The parasite ligand responsible for binding to DARC has been identified within region II of the *P. vivax* Duffy Binding Protein (PvDBPII). The gene encoding this portion of the protein is highly polymorphic, thus binding affinity to DARC may be affected. Moreover the exact region of PvDBPII that binds DARC is unknown. Five common haplotypes of PvDBPII were expressed, properly refolded, and examined for their binding to erythrocytes by flow cytometry. Two haplotypes, differing by only one amino acid residue, showed 2.0 to 4.1 greater relative binding compared to the other haplotypes that differed at multiple residues. A 15kD C-terminal portion of PvDBPII was purified and showed similar or greater binding relative to the full-length PvDBPII. These data indicate variant-specific binding of PvDBPII to DARC that may affect the efficiency of parasite invasion of erythrocytes. Moreover, the principal binding motif of PvDBPII appears to reside within the C-terminal 15kD region of the molecule, the consideration of which could be important to the development of a subunit vaccine.

(ACMCI Abstract)

### MOLECULAR BASIS OF PLACENTAL MALARIA - STRUCTURAL REQUIREMENTS OF CHONDROITIN SULFATES FOR BINDING PFEMP1, THE INFLUENCE OF PROTEIN POLYMORPHISMS ON BINDING SPECIFICITY, AND IDENTIFICATION OF OPTIMAL INHIBITORS OF PARASITE ADHESION

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Adhesion of parasite-infected erythrocytes (IEs) to the microvasculature of various organs is an important pathogenic feature of *Plasmodium falciparum*. During pregnancy, novel *P. falciparum* variants accumulate in the placenta through adhesion to chondroitin sulfate (CS) A, and other receptors, with severe consequences for mother and baby. CSA has also been identified as a cell surface receptor for adhesion to endothelial cells. The parasite adhesive ligand is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is a highly polymorphic protein encoded by the var multigene family and is an important target of protective antibodies. The major PfEMP1 variant mediating adhesion to CSA is encoded by var2csa-type genes. In this study, we have further defined the molecular basis of the interaction between IEs and CSA and placental tissue and established key features common to different CSA-binding variants expressing var2csa-PfEMP1. Furthermore, we have identified oligosaccharides with optimal adhesion-inhibitory activity. We examined direct binding of native PfEMP1 to oligosaccharides and polysaccharides, adhesion of different parasite variants to CS polysaccharides with differing degrees of sulfation and composition, and inhibition of IE adhesion to purified CSA and placental tissue using various oligo- and polysaccharides with defined composition and sulfation. Var2csa-PfEMP1 is highly polymorphic, and we determined whether this influences recognition by acquired antibodies and the structural requirements for binding CSA. Our findings have important implications for the development of therapeutic inhibitors, for structural studies of CSA-binding domains, and for understanding the molecular basis of malarial disease.

(ACMCI Abstract)

### EVALUATION OF IMPACT OF MALARIA RAPID DIAGNOSTIC TESTS (RDTs) ON HEALTHCARE WORKER PRESCRIBING PRACTICES - TANZANIA, MARCH 2005

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Tanzania recently adopted but has not yet implemented artemether-lumefantrine (AL, Coartem<sup>®</sup>) as the first-line treatment for uncomplicated malaria. Because of its considerable expense, the Ministry of Health and Social Welfare is considering strategies to decrease unnecessary use of AL. Clinical diagnosis of malaria, widely used in Tanzania and other sub-Saharan African nations, is sensitive but not specific, and overdiagnosis and overtreatment is substantial. Rapid diagnostic tests (RDTs) can improve diagnosis but their true impact on healthcare worker (HCW) prescribing practices is unknown. During March-April 2005, we conducted a cross-sectional health facility-based survey among ill patients attending 6 rural dispensaries using clinical diagnosis in Mkuranga district, Tanzania, to investigate HCW diagnostic and prescribing practices and determine the impact of RDTs on these. We collected baseline data on patients and RDT

quality using standardized exit interviews and malaria blood smears. RDTs were implemented and during the following 8 weeks records of diagnoses and prescriptions were reviewed. Interviews with HCWs and patients were conducted at weeks 4 and 8. Among the 595 patients presenting to the dispensaries at baseline, 31.9% (95% confidence interval [CI]: 28.2-35.7) were positive by blood smear. Compared to microscopy, the sensitivity and specificity of clinical diagnosis were 74.5% and 45.0%, and of RDTs were 93.2% and 88.6%, respectively. Among those with a clinical diagnosis of malaria (n=365), almost all of whom were prescribed antimalarials, 54.8% (95% CI: 49.7-59.9) were negative by RDT. Data collected during the 8 weeks post-implementation suggest that RDT results were used to determine patient treatment with antimalarials. Among 2519 patients having an RDT performed, antimalarials were appropriately prescribed or not prescribed for 87.7% (95% CI: 86.4-89.0). Antimalarials were prescribed for 17.9% (95% CI: 16.0-19.7) of patients with a negative RDT result, a statistically significant reduction compared with pre-implementation overtreatment (54.8%) ( $p < 0.0001$ ). These results suggest that HCW prescribing practices can be improved with the use of RDTs and yield a substantial reduction in unnecessary use of antimalarial drugs. Countries changing malaria treatment policy should consider implementing RDTs to improve rational use of more expensive antimalarial drugs.

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### ACCEPTANCE AND USAGE OF MALARIA RAPID DIAGNOSTIC TESTS AT DISPENSARY LEVEL BY PRESCRIBERS AND PATIENTS - TANZANIA, MARCH 2005

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Prescriber and patient satisfaction and acceptance of diagnostic procedures are essential elements in implementing prompt and effective treatment and improving rational use of drugs. To strengthen the diagnosis of malaria and enhance rational antimalarial drug use, the Tanzanian Ministry of Health and Social Welfare is considering using malaria rapid diagnostic tests (RDTs). Compared to clinical algorithms, RDTs could improve the appropriateness of malaria diagnosis and treatment but only if prescribers are willing to use the results and patients accept those results. Understanding both prescriber and patient response to and usage of RDTs will improve the implementation of RDTs. During March - April 2005, we conducted a cross-sectional health facility-based survey at 6 rural dispensaries in Mkuranga District, Tanzania, to examine the impact of RDTs on prescribing behaviors and malaria case management (results discussed elsewhere) and to assess prescribers' and patients' perceptions about the use of RDTs. Baseline data were collected using standardized patient exit interviews, RDTs (Paracheck), and malaria smears. RDT quality assessment was conducted on a sample of tests using smears from the baseline survey and laboratory controls. Following RDT implementation in the dispensaries, a log of diagnoses and prescriptions by prescribers was collected. Interviews were conducted at 4 and 8 weeks post-implementation with 21 prescribers and 355 patients. Findings indicated that RDTs were well-received by both patients and providers who perceived the test as an easy-to-use tool that improved malaria diagnosis and treatment. Prescribers appreciated having additional data to confirm their clinical diagnoses. Patient satisfaction was high, with patients requesting the test. Staff noted that they had a higher than normal number of patients coming to the facilities during the time of the RDT testing. Prescribers voiced concerns that use of RDTs added to the work burden in understaffed dispensaries. Initially, community members feared that the RDT was an HIV test but this fear subsided over time. Providers and patients recommended that all facilities have access to RDTs. These preliminary findings suggest that RDT use would be feasible at dispensary level and accepted by both providers and patients.

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### EVALUATION OF TWO RAPID DIAGNOSTIC TESTS (RDTs) FOR MALARIA IN A LONGITUDINAL COHORT IN KAMPALA, UGANDA

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In Africa, treatment of malaria is often presumptive, leading to overuse of antimalarials. When reliable microscopy is unavailable, management based on rapid diagnostic tests (RDTs) may provide a practical alternative. We are evaluating RDTs in a cohort (age 1-10) enrolled in a longitudinal antimalarial efficacy trial in Kampala. When a child presents with fever, s/he is evaluated with a blood smear and two RDTs, detecting histidine-rich protein 2 (HRP2; Paracheck, Orchid Biomedical Systems) and parasite lactate dehydrogenase (pLDH; Parabank, Zephyr Biomedicals). Our goal is to evaluate the diagnostic accuracy of RDTs, using expert microscopy as the gold standard, and to identify factors associated with false-positive and false-negative results. An interim analysis is presented here. To date, of 736 fever episodes, 215 (29%) were positive for malaria by microscopy. Considering readings by a single trained technician, results for the HRP2 and pLDH tests were, respectively: positive predictive value (PPV) 88% and 99%; negative predictive value 96% and 93%; sensitivity 92% and 83%, and specificity 95% and 100%. Both tests were more sensitive at higher parasite densities: above 5000 parasites/ $\mu$ L, the HRP2 test detected 98% of cases, and the pLDH test 96%; below 5000 parasites/ $\mu$ L, HRP2 sensitivity dropped to 71% and pLDH to 40%. Of 33 false positive HRP2 results, 16 (48%) occurred within 4 weeks and 24 (73%) within 8 weeks of a previous malaria episode. Of 16 false negative HRP2 results, 7 (44%) occurred in cases of mixed or non-falciparum malaria. RDT accuracy did not vary significantly with age or gametocytemia. We will conduct PCR analysis to assess whether RDT-microscopy discordant results may be associated with subpatent parasitemia or with mixed infections. Preliminary results demonstrate that in Kampala, RDTs may be useful when microscopy is not available. HRP2 tests showed superior sensitivity, but the higher PPV of pLDH tests may prove useful for directing the use of new, more costly antimalarial therapies in areas with high transmission intensity.

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### THE USE OF MICROSATELLITES IN MALARIA GENOTYPING IMPROVES THE ABILITY TO CORRECTLY DISTINGUISH NEW INFECTIONS FROM RECRUDESCENCE

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Genotyping methods for *Plasmodium falciparum* drug efficacy trials have not been standardized and may fail to accurately distinguish recrudescence from new infection, especially in high transmission areas where polyclonal infections are common. We investigated whether genotyping based on merozoite surface proteins 1 and 2 (*msp1* and 2) would be improved by the addition of 4 microsatellite markers. Fluorescent-tagged primers were designed to amplify 4 microsatellite loci using a single round of PCR, and products were sized using capillary electrophoresis. This method was validated using multiple controls prepared to mimic clinical filter-paper samples and containing known concentrations of between 1 and 6 laboratory parasite clones. The four microsatellite markers, *msp1*, and *msp2* identified the correct alleles for all single clone samples. When samples contained multiple clones, both methods occasionally missed minority alleles but at similar rates. However, microsatellite results were less likely to contain alleles that did not correspond to clones present

than results of *msp1* and 2. In order to evaluate these methods on clinical isolates, we genotyped 15 samples from patients with recurrent parasitemia within 28 days following therapy with sulphadoxine-pyrimethamine (SP) in Burkina Faso. In this region, *dhfr* 59R is the major mutation known to predict resistance to SP; patients without this mutation would not be expected to fail therapy. Classifying outcomes based on the results of *msp1* and 2 alone, 4/7 patients presenting with parasites containing the *dhfr* 59R allele experienced recrudescence vs. 2/8 patients presenting with wild-type alleles. (57% vs. 25%,  $p=0.31$ ). When outcomes were reclassified adding microsatellite data, 4/7 patients with *dhfr* 59R were considered recrudescence vs. 0/8 patients with wild-type alleles (57% vs. 0%,  $p=0.03$ ). The addition of 4 microsatellite markers to genotyping using *msp1* and 2 adds information which may more accurately estimate the true risk of drug resistance and improve the ability to identify risk factors for drug resistance.

## 674

### THE CLINICAL PRESENTATION OF MALARIA IN AFRICAN PREGNANT WOMEN: CORRELATION OF SYMPTOMS AND SIGNS WITH *PLASMODIUM FALCIPARUM* PARASITAEMIA

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The burden of malaria in pregnant women continues being a huge public health problem in endemic regions. However, little is known on the clinical presentation of non-complicated malaria episodes during pregnancy in areas of stable endemicity. This information is of relevance for decision-making and clinical management in areas where health facilities are scarce. Since August 2003 until November 2005 data from all pregnant women who attended the maternity clinic of the Manhiça Health Center, southern Mozambique, referring any clinical complaints, were collected through the established health-facility based morbidity surveillance system. A capillary blood sample for parasitaemia and haematocrit determination was collected if at least one pre-defined clinical criteria suggestive of malaria was met. The clinical criteria included were axillary temperature  $\geq 37.5^{\circ}\text{C}$ , referred history of fever in the last 24 hours, pallor, arthromyalgias, headache and history of convulsions. During this period 4440 visits were made by pregnant women. Of them, 3129 visits corresponded to those with criteria for blood collection with complete results. The more sensitive sign/symptoms during pregnancy like axillary fever  $\geq 37.5^{\circ}\text{C}$  and/or history of fever in the last 24 hours (64.7%; 2023/3129, Se=80%; [77-82] CI 95%), arthromyalgias (74.8%; 2339/3129, Se=82%; [79-84] CI 95%) and headache (86.5%, 2706/3129, Se=92%; [89-93] CI 95%), showed a low specificity. About one third (26.9%; 842/3129) of pregnant women with criteria for blood collection were parasitaemic and about 60% (56.1%; 1755/3129) were anaemic. 22.6% (708/3129) of visits attended were on the first trimester. These results show that, most of women attended at the maternity clinic presented signs or symptoms suggestive of malaria but only in one third parasitaemia, identified by microscopy, could be found and, one fourth of the women were in the first trimester. Safety profile of most antimalarials is unknown during the first trimester, and some of them have clear severe adverse effects. There is a need to improve parasitological diagnosis of malaria during pregnancy to avoid unnecessary treatment in these women, as well as, to develop new antimalarials to achieve a good safety profile for non-complicated malaria treatment.

## 675

### A RAPID ASSESSMENT ON THE HOME MANAGEMENT OF MALARIA IN ZAMBIA: THE CARETAKER AND THE COMMUNITY HEALTH WORKER

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Malaria is endemic in Zambia causing a large percentage of mortality and morbidity. To curb the effects of malaria, timely and effective anti-malarial therapy is a required. The Roll Back Malaria (RBM) aims to ensure access to effective treatment within 24 hours of onset of symptoms. Even though effective treatment in form of combination therapy has been adopted in Zambia, only patients with access to health facilities are able to benefit from this treatment. In order to facilitate apt treatment, the concept of Home Management of Malaria (HMM) is being encouraged. This primarily involves, use of community health workers (CHWs) in administering the antimalarials at community level where geographical access is a problem. A cross sectional study was conducted in 2004 in 23 clinics in 6 districts representing 4 of the 9 provinces. A statistically calculated total of 1,220 community members and 66 active CHWs in each area, were sampled to assess knowledge, attitudes and practice. Knowledge of signs and symptoms for simple malaria in the community was found to average at 89.5% (95% CI 83.8- 95). CHWs showed greater knowledge of signs and symptoms of both uncomplicated and complicated malaria compared to the caregivers. If a child had fever (malaria), 69.4% of the caretakers sought care at the clinic first, 23.7% reduced temperature by tepid sponging or an analgesic, 7.2% went to a CHW, 1.9% gave traditional medicine while 1.7% gave an anti malarial at home. Caretakers 30 years and below took sick children to the CHW while older counterparts preferred to use traditional or alternative therapy. CHW utilization was almost zero in urban areas. About 78.2% of the caretakers were mothers, 12.3% were fathers and 9.5% were other relatives. In conclusion, the findings show an appreciable knowledge of signs and symptoms among caregivers and CHWs studied. This has implications in deployment of Artemether Lumefantrine at community level. Currently community health workers dispense the less effective sulphadoxine pyrimethamine.

## 676

### RAPID ASSESSMENTS OF MALARIA CONTROL STRATEGIES IN TANZANIAN REFUGEE CAMPS - JANUARY 2006

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To improve malaria control in Tanzanian camps, the Centers for Disease Control and Prevention and Prevention (Centers for Disease Control and Prevention) and the Office of the United Nations High Commissioner for Refugees (United Nations High Commissioner for Refugees) conducted a rapid assessment of malaria control in 6 refugee camps during January 2006. Malaria was reported to be the leading cause of morbidity and mortality. Laboratory testing was not routinely utilized to confirm clinical diagnosis of malaria, despite available laboratories. Case management was problematic. Of the 39 charts reviewed from admitted malaria patients, 82.7% were treated inappropriately; errors included: lack of blood smear results, incorrect timing of dosages or inappropriate route of drug administration. We reviewed 2,293 patient records in the outpatient department (OPD) but the records contained too little information to determine if antimalarials were used according to standard treatment guidelines. Ninety-three patients diagnosed with malaria (5.0%) did not receive any antimalarials. We also examined 659 antenatal care (ANC) records in 2005 to assess the rates of Intermittent Preventive Treatment (IPT). Although rates were reported to be between 70-100% for two doses, 146 (22.2%) women did not receive a 2<sup>nd</sup> dose of sulphadoxine-

pyrimethamine (SP) when it was due and 41 (6.2%) did not receive any doses of SP, although they met the criteria of when to receive one. Multiple recording systems were used resulting in inconsistent dose documentation. Stockouts of antimalarials were reported in half of the camps. Prevention efforts were also inadequate. Vector control strategies, such as indoor residual spraying (IRS), had not been conducted in any camp within the last three years. Insecticide-treated net (ITN) distribution occurred in all camps, but the camps lacked accurate data reflecting ITN retention or usage. We conducted a convenience survey of 355 households to determine ITN usage and found that only 35.4% of those under nets were young children. Recommendations included improving clinical management of patients and increasing clinical supervision to ensure appropriate drug therapy and IPT, standardizing laboratory quality control measures, improving ITN retention and usage rates, re-initiating IRS, and strengthening logistics for pharmaceutical supplies. Without instituting improvements in malaria control strategies, malaria will continue to needlessly jeopardize the health of refugees.

## 677

### SEOUL VIRUS REPLICATION AND ANTIVIRAL RESPONSES DIFFER BETWEEN MALE AND FEMALE NORWAY RATS

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Reported human hantavirus infections, as well as field observations of several rodent species, indicate that more males than females are infected with hantaviruses. In addition to behavioral differences, immunological differences exist between the sexes. We hypothesize that sex steroids affect dimorphic expression of genes that modulate immune responses against Seoul virus. To test this hypothesis, we bred adult female Long Evans rats and assigned pups to the following experimental groups: 1) intact-sham males; 2) intact-sham females; 3) neonatally gonadectomized (gdx) males (gdx on PND 2-4); 4) neonatally gdx females; 5) adult gdx males (gdx on PND 60); and 6) adult gdx females. Animals were inoculated with the ID<sub>90</sub> dose of Seoul virus in adulthood and immune responses and virus replication were monitored 0, 1, 3, 15, 30, or 40 days p.i. Gonadally intact male and female rats differed in the amount of virus present in the lungs. Among intact females, the quantity of viral RNA in the lungs peaked at 15 days p.i. and declined thereafter resulting in almost no virus present in lungs by Day 40 p.i. Among intact males, there was a temporal shift in the amount of viral RNA, in which the number of Seoul virus copies peaked at 30 days p.i. and remained high 40 days p.i. Neither adult nor neonatal removal of the ovaries affected virus replication in lungs of female rats. Removal of the gonads in males, either during the neonatal period or in adulthood, dramatically reduced viral persistence 40 days p.i. Microarrays and real-time RT-PCR were used to assess the expression of genes that encode for immunological proteins. Of particular interest, the expression of type I interferons (IFN $\alpha$  and IFN $\beta$ ) was significantly lower in intact males compared with intact females 3-40 days p.i. and was primarily caused by downregulation of gene expression in male rats during infection. Similar to IFN $\beta$ , the expression of Mx2 was consistently downregulated in males as compared with females during Seoul virus infection. Whether gonadectomy of male and female rats alters the expression of these antiviral genes is currently under investigation. These data provide novel information about the causes of sex differences in viral pathogenesis and suggest that dimorphic antiviral responses to infection may underlie sex differences in hantavirus persistence.

## 678

### PROINFLAMMATORY AND REGULATORY RESPONSES MAY MEDIATE SEOUL VIRUS PERSISTENCE IN NORWAY RATS

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Hantaviruses are noncytopathic and cause two diseases in humans, hantavirus pulmonary syndrome (HPS) and hemorrhagic fever with renal syndrome (HFRS). Human pathology is hypothesized to be caused by sustained, elevated levels of proinflammatory cytokines. Hantaviruses are transmitted by rodents and each hantavirus has coevolved with a specific rodent host. In contrast to humans, hantaviruses maintain a persistent infection in their rodent hosts without causing pathology. The mechanisms mediating hantavirus persistence in rodents are unknown, but may involve suppression of host proinflammatory immune responses. Norway rats (*Rattus norvegicus*) were inoculated with Seoul virus or vehicle and proinflammatory, anti-inflammatory, and regulatory responses were measured in the spleen and lungs at various time points post-inoculation (p.i.). Expression of IL-1 in the spleen was cyclical during infection and was inversely correlated with Seoul virus copy number; when virus replication was high (i.e., 15-30 days p.i.), IL-1 mRNA was low. Expression of IL-1 in the lung, however, was consistently suppressed during the Seoul virus infection, with these differences being most pronounced at Day 30 p.i. Similar, but less dramatic, trends also were observed for IL-6 and TNF in both the spleen and lungs. To determine whether elevated production of anti-inflammatory cytokines reduce proinflammatory cytokine expression, IL-10 and IL-1ra expression were examined. Expression of IL-10 was downregulated and IL-1ra mRNA remained unchanged in both the spleen and lungs during infection and neither was correlated with proinflammatory cytokine expression or Seoul virus replication. Elevated regulatory responses can contribute to viral persistence; expression of the regulatory T cell marker, foxp3, was elevated in the lung during the persistent phase of infection (i.e. Day 30 p.i.), but remained unchanged in the spleen, suggesting a possible role of regulatory T cells in mediating Seoul virus persistence locally at the site of elevated virus replication. Taken together, these data suggest that proinflammatory and regulatory responses are altered during infection and may contribute to Seoul virus persistence in Norway rats.

## 679

### NEITHER TLR-3 NOR RIG-I ARE SUFFICIENT FOR RECOGNITION OF THE PATHOGEN-ASSOCIATED MOLECULAR PATTERN (PAMP) THAT INITIATES AN INNATE IMMUNE RESPONSE TO SIN NOMBRE HANTAVIRUS

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The pathogenesis associated with SNV is considered to be, at least in part, mediated by a maladaptive immune response. Endothelial cells, the native targets of hantavirus infection, respond to viral infection by inducing interferon-stimulated genes (ISG) that lead to the expression of soluble mediators of innate and adaptive immunity, which may be important in pathogenesis. Our previous studies identified several interferon-stimulated genes (ISG) that are induced shortly after SNV infection or exposure to replication-defective virus particles treated with minimal doses of UV light (UV-SNV). Thus, hantavirus particles are themselves capable of inducing transcription of ISG through a yet-to-be defined pathogen-associated molecular pattern (PAMP). Toll-like receptor 3 (TLR3) and the RNA helicases RIG-I and MDA-5 are two PAMP receptors that recognize dsRNA and induce ISG in response to infection with many viruses. In efforts to identify a putative PAMP receptor involved in the ISG response to SNV particles, we exposed the human hepatoma cell line (Huh7) and a RIG-I-defective clone (Huh7.5) to UV-SNV and assessed induction of ISG. UV killed SNV particles are able to induce ISG in both cell lines whereas treatment with Poly I:C or Sendai virus leads to ISG induction in the parental cell line only and these

ISG profiles are differentially regulated as compared to inductions by Poly I:C and SeV. Overexpression by transient transfection with TLR3 and RIG-I constructs renders cell lines responsive to Poly I:C and SeV respectively, but does not enhance UV-SNV-mediated ISG responses. Finally, knockdown of RIG-I via RNA silencing, and knockdown of the RIG-I and MDA-5-pathway intermediary IPS-1 inhibited SeV-mediated ISG responses, but did not alter UV-SNV-mediated responses. These results suggest that neither RIG-I/MDA-5 nor TLR3 are sufficient for the induction of the antiviral response to SNV particles.

## 680

### EVALUATION OF A PRAIRIE DOG ANIMAL MODEL FOR MONKEYPOX VIRUS INFECTION

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Human monkeypox, a zoonotic disease, causes a rash-like illness clinically similar to smallpox. Human monkeypox was first identified outside Africa during a US outbreak in 2003. All US human monkeypox cases were linked to exposure to prairie dogs that had been previously exposed to West African rodents.

There are two distinct clades of monkeypox virus (MPXV), West African/US and Congo Basin, with unique human disease presentations associated with each clade. To better understand possible differences between the pathogenesis associated with the two MPXV clades, we developed an animal model using the prairie dog (*Cynomys ludovicianus*) and a West African/US clade MPXV isolate. Prairie dogs were inoculated with 10<sup>5</sup> plaque forming units administered by either intranasal (IN) or scarification (SC) routes. Temperatures, weights, and samples (oral, nasal, and ocular swabs; feces, blood) were collected every third day. Although all animals survived (euthanized day 35), infected animals demonstrated signs of MPXV infection including lesions, elevated temperature, inappetence, inactivity, and hypopigmentation at lesion sites. Both infection routes caused disseminated disease as evidenced by lesions upon the extremities and face. Disease signs were first observed between days 6-9 (SC) and days 9-12 (IN); disseminated lesions began to resolve by day 21. Samples were examined for the presence of MPXV DNA (Real-Time PCR) and viable virus (tissue culture inoculation). Several day 21 samples still possessed viable virus, with oral swabs containing the highest viral load. By day 28, infectious viral shedding had ended; however certain samples contained low levels of viral DNA. Both the IN and SC inoculation methods resulted in an animal model demonstrating systemic infection. Comparison of these findings to a duplicate study performed with a MPXV Congo strain will provide greater understanding of MPXV disease pathogenesis and help characterize differences between the MPXV clades.

## 681

### PERSISTENCE OF ATTENUATED VARIANTS OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS (VEEV) IN THE MURINE BRAIN

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Arboviruses such as Venezuelan equine encephalitis virus (VEEV) cause lethal encephalomyelitis and are prevalent worldwide. To examine the role of specific effector mechanisms involved in clearance of VEEV from the central nervous system and in prevention of lethal encephalitis, we vaccinated  $\gamma\delta$  T cell-deficient mice with chimeric Sindbis-VEE virus and challenged mice with virulent VEEV. While naïve wild-type mice are susceptible to VEEV infection, and develop lethal infection of central

nervous system (CNS), pre-immunized mice survive the infection and clear the virus from the brain. In contrast, in vaccinated and challenged  $\gamma\delta$  T cell-deficient mice, VEEV invaded the CNS and was not cleared from the brains. However, these animals tolerated a high level of viral replication in the brain over an extended period of time. More surprisingly, virus persisted in the brains of  $\gamma\delta$ -T cell deficient mice despite a moderate level of inflammation and cellular infiltration to the site of infection. Furthermore, we isolated attenuated variants of VEEV from the brains of these asymptomatic mice at 28 days following challenge either via cell culture inoculation or, alternatively, by intracranial infection of suckling mice. Sequencing of these recovered viruses has revealed several genetic changes that are present irrespective of the isolation method, excluding the possibility of mutations associated with cell culture adaptation and suggesting that these mutations are of biological relevance. The results of reverse genetics studies testing the role of these mutations on the attenuation of VEEV will be presented. The potential epidemiological importance of this finding is unknown; nevertheless, our results indicate the ability of VEEV to persist in the host with selective immune deficiencies in the T cell compartment, allowing the speculation about the potential for re-emergence of virulent virus in the same host.

## 682

### VENEZUELAN EQUINE ENCEPHALITIS VIRUS CANDIDATE VACCINE (V3526) PROTECTS HAMSTERS FROM CHALLENGE BY BOTH MOSQUITO BITE OR INTRAPERITONEAL INJECTION

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Several studies have indicated that a virus introduced by mosquito bite may be more pathogenic than the same virus introduced by needle inoculation. Although preliminary studies indicate that the candidate Venezuelan equine encephalitis virus (VEEV) vaccine (V3526) protects animals from challenge after needle inoculation of the challenge virus, there were concerns that the presence of mosquito saliva, or changes in the virus due to replication in a mosquito immediately before injection into the vertebrate host, may allow the virus to overcome the protective effects of prior vaccination with V3526. Therefore, we determined if hamsters vaccinated with V3526 were protected from challenge with the virulent Trinidad donkey strain of VEEV. All mock-vaccinated hamsters died or were euthanized when moribund within 5 days after intraperitoneal challenge (n = 10) or after feeding by VEEV-inoculated *Ochlerotatus taeniorhynchus* (n = 15). In contrast none of hamsters vaccinated with the V3526 vaccine candidate and then challenged intraperitoneally (n = 10) or by bite from a VEEV-inoculated *Oc. taeniorhynchus* (n = 15) died or became ill. Therefore, vaccination with the V3526 candidate vaccine should provide protection from virus introduced by mosquito bite.

## 683

### CLINICAL MANIFESTATIONS ASSOCIATED WITH HTLV-I INFECTION: A CROSS-SECTIONAL STUDY

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Human T-lymphotropic virus type I (HTLV-I) causes HTLV-I-associated myelopathy/tropical spastic paraparesis and adult T cell leukemia in a small percentage of infected individuals. HTLV-I infection is increasingly associated with clinical manifestations. To determine the prevalence of clinical manifestations in HTLV-I infected individuals, we conducted a cross-sectional study of 115 HTLV-I-infected blood donors without myelopathy and 115 age- and sex-matched seronegative controls. Subjects answered a standardized questionnaire and underwent physical examination. Compared with controls, HTLV-I-infected subjects were more likely to report arm or leg weakness (OR = 3.8, 95% CI: 1.4 - 10.2; OR = 4.0, 95% CI: 1.6 - 9.8, respectively), hand or foot numbness (OR = 2.1, 95% CI: 1.1 - 3.9; OR = 4.8, 95% CI: 2.0 - 11.7, respectively), arthralgia (OR =



3.3, 95% CI: 1.7 - 6.4), nocturia (OR = 2.7, 95% CI: 1.04 - 6.8), erectile dysfunction (OR = 4.0, 95% CI: 1.6 - 9.8); and to have gingivitis (OR = 3.8, 95% CI: 1.8 - 7.9), periodontitis (OR = 10.0, 95% CI: 2.3 - 42.8) and dry oral mucosa (OR = 7.5, 95% CI: 1.7-32.8). HTLV-I-infection is associated with a variety of clinical manifestations, which may occur in patients who have not developed myelopathy.

## 684

### CD8+ T CELLS RECOGNIZE BUT DO NOT ELIMINATE *T. CRUZI* FROM NONLYMPHOID TISSUE

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Mice chronically infected with *Trypanosoma cruzi*, the causative agent of Chagas disease, maintain a substantial, parasite-responsive population of effector memory CD8+ T cells. However, CD8+ T cells isolated from skeletal muscle of these mice appear to be deficient in effector function. To date we have largely ruled out a role for TGF- or CD4+CD25+ regulatory T cells in mediating this peripheral tissue dependent downregulation of T cell function. Herein we propose a model in which chronic phase CD8+ T cell priming, immunoregulation, and parasite persistence are all anatomically coincident. To support this model, we have confirmed that adipose tissue is a site of parasite persistence and demonstrated that a dysfunctional CD8+ T cell response occurs in this tissue as well. CD8+ T cells infiltrating adipose tissue display an effector memory surface phenotype (CD44<sup>hi</sup>, CD11a<sup>hi</sup>, CD62L<sup>lo</sup>), but are generally incapable of producing interferon- when polyclonally stimulated. In contrast to splenic CD8+ T cells in these mice, which exhibit no signs of recent activation, a significant fraction of CD8+ T cells in skeletal muscle, heart, and adipose tissue upregulate CD69, indicating recent antigen encounter. Additionally, a proportion of CD8+ T cells isolated from these same locations express programmed death-1 (PD-1), providing a possible mechanistic explanation for their observed dysfunction. Importantly, these phenotypic changes are likely a genuine indication of antigen encounter and not just a general characteristic of effector memory cells trafficking through nonlymphoid tissue as CD8+ T cells isolated from lungs of these mice do not express increase levels of CD69 or PD-1. Thus we propose that *T. cruzi*-specific CD8+ T cells are primed in sites where parasites persist, but a PD-1/PD-L1 interaction contributes to attenuation of a potentially parasite-clearing immune response.

(ACMCIP Abstract)

## 685

### LEISHMANIA CHAGASI T CELL ANTIGENS IDENTIFIED THROUGH A DOUBLE LIBRARY SCREEN

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Control of human visceral leishmaniasis in endemic regions is hampered by limited accessibility to medical care and emerging drug resistance. A protective vaccine would be of tremendous value. *Leishmania* species protozoa express multiple antigens recognized by the vertebrate immune system, and it is likely that a vaccine will require a panel of antigenic proteins. We generated a cDNA library from the intracellular amastigote form of *Leishmania chagasi*, the cause of South American visceral leishmaniasis. We employed a two-step expression screen of the library to systematically identify potentially protective antigens. The first step was aimed at identifying the largest possible number of clones producing a protein of significant size. For this we used a pool of sera from Brazilians with documented visceral leishmaniasis. Two hundred forty two positive clones underwent a second step screen for their ability

to cause proliferation of T cells from immune mice. After removal of clones encoding heat shock proteins, six unique clones were identified in the second screen. These encode glutamine synthetase, a transitional endoplasmic reticulum ATPase, elongation factor 1 $\gamma$ , kinesin K-39, repetitive protein A2, and a hypothetical conserved protein. Sera from VL patients with parasitologically confirmed disease in bone marrow aspirates were able to recognize those antigens, whereas non-exposed subjects were negative. These antigens could be useful for either serologic diagnosis or as components of a subunit vaccine.

(ACMCIP Abstract)

## 686

### SILENCING OF LAMININ $\Gamma$ -1 GENE BY RNA INTERFERENCE BLOCKS *TRYPANOSOMA CRUZI* INFECTION

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It is thought that *Trypanosoma cruzi*, the protozoan that causes Chagas' disease, modulates the extracellular matrix network to facilitate infection of human cells. However, direct evidence to document this phenomenon is lacking. Here we show that *T. cruzi* gp83 ligand, a cell surface trans-sialidase like molecule that the parasite uses to attach to host cells, increases the level of laminin  $\gamma$ -1 transcript and its expression in mammalian cells, leading to an increase in cellular infection. Stable RNA interference (RNAi) of host cell laminin  $\gamma$ -1 knocks down the levels of laminin  $\gamma$ -1 transcript and protein expression in mammalian cells causing a dramatic reduction of cellular infection by *T. cruzi*. Thus, host laminin  $\gamma$ -1, which is regulated by the parasite, plays a crucial role in the early process of infection. This is the first report showing that knocking down the expression of a human gene by RNAi inhibits the infection of an intracellular parasite.

(ACMCIP Abstract)

## 687

### STAT1 PLAYS DISTINCT ROLE IN DETERMINING OUTCOME OF *L. DONOVANI* INFECTION IN C57BL/6 AND BALB/C MICE

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We recently showed that STAT1 is involved in pathogenesis of *Leishmania donovani* infection in BALB/c mice. STAT1<sup>-/-</sup> BALB/c mice show increased resistance to *L. donovani* despite mounting a poor Th1 response and develop minimal or no liver pathology. Here, we show that STAT1<sup>-/-</sup> C57BL/6 mice also mount a poor Th1 response and produce levels of IFN- $\gamma$  when infected with *L. donovani* but they are highly susceptible to infection unlike STAT1<sup>-/-</sup> BALB/c mice. STAT1<sup>-/-</sup> C57BL/6 mice contain significantly high number of parasites in their liver and spleen as compared to WT C57BL/6 mice which resolve infection by day 60. STAT1<sup>-/-</sup> C57BL/6 mice also develop significant liver pathology characterized by the development of poorly formed granulomas containing parasite filled macrophages. These findings indicate that STAT1 plays distinct role in regulating host response to *L. donovani* in "non-cure" BALB/c and "cure" C57BL/6 mice. While STAT1 mediates susceptibility in BALB/c mice, it is critical for host resistance to *L. donovani* in C57BL/6 mice. Ongoing studies in our laboratory are investigating the immune mechanisms regulated by STAT1 in susceptible BALB/c and resistant C57BL/6 mice during *L. donovani* infection.

(ACMCIP Abstract)

### STABLE RNAI OF HUMAN THROMBOSPONDIN-1 (TSP-1) INHIBITS THE EARLY PROCESS OF *TRYPANOSOMA CRUZI* INFECTION

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Interactions between *Trypanosoma cruzi* and the extracellular matrix play an important role in cellular invasion. TSPs are unique members of the ECM in that they have been described as 'matricellular' proteins because they modulate cell function but they do not play a direct role in the structure of the extracellular matrix. Since nearly all cells that *T. cruzi* infects are surrounded by basement membranes, of which several TSP isoforms are important constituents, the ability of the parasite to effectively regulate and interact with TSP-1 is critically important for its passage through the membrane barrier. Here we show that *T. cruzi* increases the levels of TSP-1 expression in host cells during early infection. Stable RNAi of host cell TSP-1 knocks down the levels of TSP-1 transcripts and protein expression in mammalian cells causing inhibition of *T. cruzi* infection. Addition of TSP-1 to these cells restores infection. Overexpression of TSP-1 in cells up regulates *T. cruzi* infection. Thus, host TSP-1, regulated by the parasite, plays a crucial role in early infection. This is the first report showing that a human parasite modulates TSP-1 expression to facilitate infection.

(ACMCIP Abstract)

### DEFENSIN A-1 EXPRESSION IS UP-REGULATED IN HUMAN CELLS IN RESPONSE TO EARLY *TRYPANOSOMA CRUZI* INFECTION AS A TRYPANOCIDAL MECHANISM TO DECREASE CELLULAR INFECTION.

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Mammalian defensins play a fundamental role in the initiation of immune responses to microbial pathogens. Here we show that early cellular infection by *Trypanosoma cruzi*, the causative agent of Chagas' disease, up-regulates the expression and secretion of defensin  $\alpha$ -1, which displayed a trypanocidal role. Microarray analysis of the whole human epithelial cell transcriptome upon short infection of cells by *T. cruzi* indicates that the parasite up-regulates the levels of transcripts of defensin  $\alpha$ -1. Exposure of defensin  $\alpha$ -1, at concentrations not toxic for host cells, significantly reduced the viability of infective trypomastigote forms of *T. cruzi*. Transmission and scanning electron microscopic analysis of trypomastigotes exposed to defensin  $\alpha$ -1 for a short time revealed pore formation, membrane disorganization and cytoplasmic vacuolization. Furthermore, pre-incubation of trypomastigotes with exogenous defensin  $\alpha$ -1 followed by exposure to a primary culture of human epithelial cells significantly reduced trypanosome binding to human cells, including entry and parasite load at 72 hr. Membrane depolarization of invasive trypanosomes abolished the trypanocidal activity of defensin  $\alpha$ -1, indicating that the mechanism of defensin  $\alpha$ -1 mediated trypanosome killing is membrane-voltage dependent. These results indicate that the induction of defensin  $\alpha$ -1 expression in mammalian cells during early *T. cruzi* infection modulates parasite load as a result of defensin  $\alpha$ -1 trypanocidal activity. We conclude that defensin  $\alpha$ -1 gene expression and peptide secretion is an effective host innate immune response to control *T. cruzi* infection. We suggest that novel therapeutic approaches, mimicking defensin  $\alpha$ -1 mediated innate immune response to early *T. cruzi* infection in host cells, can be used to control Chagas' disease.

(ACMCIP Abstract)

### PEROMYSCUS YUCATANICUS PROTECTIVE IMMUNITY INDUCED BY EXPERIMENTAL SUBCLINICAL INFECTION WITH LEISHMANIA (LEISHMANIA) MEXICANA

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The Yucatan peninsula of Mexico is a well known endemic area of localized cutaneous leishmaniasis (LCL) caused predominantly by *Leishmania (L.) mexicana*. Subclinical infection rate in humans reported is of 18.9% in the State of Campeche. *P. yucatanicus* has been adapted to captivity and experimental infection studies with *L. (L.) mexicana* are possible. Moreover, this rodent is highly polymorphic as the human being. It is unknown how *P. yucatanicus* immune system with subclinical infection could control the parasite. Therefore the main purpose was to induce a protective immune response through subclinical infection of *P. yucatanicus* inoculated with *L. (L.) mexicana*. Group A (experimental-subclinical) was inoculated with  $1 \times 10^2$  and group B (positive-clinical control) with  $2.5 \times 10^6$  promastigotes (n=18, per group). They were followed-up for 20 weeks. At the end they were sacrificed to determine lymphoproliferative response to ASL from splenocytes, and nitrite oxide production by macrophages from skin from inoculated area. No one of the group A (subclinical) develops signs of Leishmaniasis; stimulation index to ASL was significant ( $p < 0.05$ ) in this group; nitrite oxide production was observed in both groups. This is the first report supporting protective immunity induced by subclinical infection in a primary reservoir of *L. (L.) mexicana*.

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### QUANTITATIVE ANALYSIS OF THE BEHAVIORAL INTERACTIONS OF *ANOPHELES GAMBIAE* S.S. WITH INSECTICIDE-TREATED BEDNETS

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The Observer software was used to record behavioral details of the interactions of adult female *Anopheles gambiae* with bednet materials either untreated or containing delta-methrin (Permanet) or permethrin (Oleset). These 30 min tests were conducted within: 1) 30 cm diam x 30 high cylindrical cages made entirely of netting, and 2) a small rectangular wind-tunnel with the upwind end of the working section either fully or 1/2 covered with ITN material or all untreated netting. Equivalent host cues were released upwind of all netting types. No evidence was found that vapors from ITN materials are sensed by females. The main effects of the insecticides were reduction in time sitting on treated surfaces, enhanced flying and agitation after sitting on insecticide but with no directional bias away from the insecticide source, muscular tightening and postural abnormalities, followed by an extended period of intermittent knockdowns leading always to death within a few h of commencement of a test. Subtle differences were detected between insecticide types as revealed by comparative ethograms, e.g., irritability and knockdown were quicker for Oleset than Permanet. However, we conclude that both nets are highly effective and they act mainly as contact toxins and not by causing avoidance.