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## DRUG DISCOVERY FOR CHAGAS' DISEASE

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Chagas' disease, a neglected tropical disease prevalent throughout the Americas, is a major cause of cardiomyopathy. Nifurtimox and benznidazole are the only therapies currently available. Both drugs have serious side effects and limited efficacy. The Sandler Center developed the vinyl sulfone cysteine protease inhibitor K777 for the treatment of Chagas' disease. K777 irreversibly inhibits cruzain, a key protease required for viability of the parasite *Trypanosoma cruzi*. The inhibitor prevents cruzain autoprocessing within Golgi cisterns. K777 recently allowed us to elucidate a biological role for cruzain in immune evasion. Cruzain hinders macrophage activation by the proteolytic disruption of an NF- $\kappa$ B P65 mediated signaling pathway allowing *T. cruzi* survival and replication. Therapeutic intervention with the cysteine protease inhibitor prevents normal cruzain expression to the cell membrane of the pathogenic amastigote and leads to host immune cell activation. Studies of K777 have proven that the targeting of cruzain can be done selectively and effectively enough to cure *T. cruzi* infection in acute and chronic models of infection and also ameliorate cardiac damage in dogs. We have documented the efficacy of K777 against various *T. cruzi* strains that represent a spectrum with various tissue tropisms, and even against nifurtimox and benznidazole -resistant parasites. To identify alternative chemotypes with efficacy, we are currently exploring several classes of cruzain inhibitors. A second approach to our drug discovery efforts targets the C14 $\alpha$ -demethylase of *T. cruzi*. The lead compound LP10 that disrupts ergosterol biosynthesis showed efficacy in an animal model of disease. By high throughput screening in combination with cell-based assays and animal trials, aided by NMR, crystallography, and molecular modeling, we have selected several top-ranking molecules with high trypanocidal capacity for further development.

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## OPTIMAL DOSING OF MILTEFOSINE IN LEISHMANIASIS PATIENTS

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Pharmacokinetics and -dynamics (PK/PD) of miltefosine in children with visceral leishmaniasis (VL) remain ill-characterized. In a large phase 4 trial in India, the number of treatment failures was significantly higher in the pediatric population than in adults given a similar dosage of 2.5 mg/kg. Based on this and the previous finding that the mean steady-state concentration in children was almost half of that reached in adults, we hypothesized that the current linear mg/kg dosage is too low for children and that a dose based on allometric scaling might result in a similar exposure to miltefosine between children and adults. A population PK analysis was performed comparing various body size models, based on pooled PK data from three separate studies, including Indian children, Indian adults and European adults. An allometric dosing-formula for miltefosine was proposed. Exposure to miltefosine after the current dose and the proposed new dosing algorithm were compared between adults and children by Monte Carlo-simulations. Modeling and simulations were performed with software packages NONMEM, R and Pirana. The population PK model with allometric power scaling fitted best to the pooled miltefosine data. Moreover, allometric scaling by fat-free mass (FFM) reduced unexplained between-subject variability (BSV): linear scaling by total weight (WT) or FFM, and allometric scaling by WT or FFM resulted, respectively, in a BSV of 50%, 43%, 35% and 32% for CL, and 43%, 37%, 38% and 34% for V. We proposed an allometric miltefosine dose, scaled with a power 0.75 from a standard adult (60 kg) receiving 150 mg

(Dose = 150\*(Weight/60)\*\*0.75). Simulated exposure to miltefosine was similar between adults receiving 2.5 mg/kg and children receiving the new allometric dose. More importantly, only 74-78% of the children receiving the currently used linear dose of 2.5 mg/kg achieved a similar minimal systemic exposure as 90% of adults receiving 2.5 mg/kg. In conclusion, the currently applied dose of 2.5 mg/kg results in a significantly lower exposure to miltefosine in children than in adults. We recommend the use of an allometric dose formula for miltefosine for leishmaniasis, which results in a similar exposure to miltefosine between adults and children. More data are urgently needed on both PK and PD of miltefosine in VL, certainly in children, to further improve the treatment of this fatal neglected disease.

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## HIT-TO-LEAD AND LEAD OPTIMIZATION OF NOVEL SMALL MOLECULES FOR THE TREATMENT OF HUMAN AFRICAN TRYPANOSOMIASIS

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Human African trypanosomiasis (HAT), or sleeping sickness, is a neglected tropical disease that is transmitted through the bites of tsetse flies infected with the kinetoplastid parasite *Trypanosoma brucei* and is fatal if left untreated. As existing chemotherapies are old, scarce, highly toxic, and encounter parasite resistance, there exists an urgent need for new drugs. As part of our ongoing program to identify new drug candidate chemotypes, we have screened ca. 50,000 novel small molecules in a high throughput *T. brucei* whole cell assay. We report here the identification, hit-to-lead and initial lead optimization efforts opposite one of the chemotypes found by this screening effort. Compounds were first optimized for trypanocidal activity, selectivity vs. mammalian cell toxicity, and *in vitro* ADME properties over the initial screening hits. Several lead compounds were progressed to *in vivo* efficacy and pharmacokinetic assays in rodents.

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## SHORT-COURSE MULTI-DRUG TREATMENT FOR VISCERAL LEISHMANIASIS IN INDIA

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Most of the available drugs used as monotherapy for visceral leishmaniasis (VL) are toxic, not well tolerated, require long treatments or are expensive. Better treatment modalities are needed. We conducted a randomized, controlled, non-inferiority trial ( $\Delta = -7\%$  between combinations and standard treatment) in Bihar, India, to compare standard treatment (amphotericin B infusion alternate days for 30 days) with three drug combinations: single injection of 5 mg/kg liposomal amphotericin B (L-AmB) and 7-day miltefosine; L-AmB and 10-day paromomycin; miltefosine and paromomycin for 10 days. Patients were hospitalized for 15 days if on combination therapy or 31 days for standard treatment (end of treatment, EOT). Clinical assessments were performed at EOT, day 45 and 6 months after the start of treatment. Definitive cure was defined as

no sign/symptom of VL and parasitologically cured to the last follow-up. A total of 634 patients were randomly assigned and received amphotericin B (n=157); L-AmB and miltefosine (n=160); L-AmB and paromomycin (n=158); or miltefosine and paromomycin (n=159). 627 patients were included in the per protocol analyses. There were eight relapses, two in each group. The efficacy rates were: amphotericin B 93.0% (93.0% CI 87.50-96.27); L-AmB and miltefosine 97.5% (93.32-99.20); L-AmB and paromomycin 97.5% (93.24-99.19); miltefosine and paromomycin 98.7% (95.06-99.78). Combination therapies were well tolerated and had fewer adverse events. In conclusion, all three combination treatments were highly effective and safe. Due to shorter duration of treatment, combinations can increase compliance as well as reduce emergence of drug resistance.

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### FORMATION OF CYCLODEXTRIN INCLUSION COMPLEXES WITH A BENZIMIDAZOLE DERIVATIVE: THEIR CHARACTERIZATION AND *IN VITRO-IN VIVO* TRYPANOCIDAL ACTIVITY

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*Trypanosoma cruzi* is the etiological agent of Chagas' disease, a chronic illness affecting many people principally in Central and South American countries. According to the World Health Organization, an estimated 20 million people are infected with this parasite and about 25% population of Latin America is at risk of being infected. Treatment of Chagas' disease is still unsatisfactory. Nifurtimox and benznidazole have been widely used as trypanocidal agents, however, both have significant activity only in the acute phase of the disease and, when associated with long term treatments, give rise to severe side effects. Additionally, *T. cruzi* resistance to these nitroderivatives constitutes an important factor in the low rate of cure in treated patients; therefore, there is an urgent need to develop new antiparasitic leads with improved pharmacological and pharmacokinetic characteristics. In this sense our research group found a benzimidazole derivative (G2) in preliminary *in vitro* trypanocidal screening. However, in these studies G2 showed poor aqueous solubility, which hampered the subsequent *in vivo* experimental trials. Furthermore, the lack of water solubility reduces flexibility for drug administration. To overcome these drawbacks, increasing the aqueous solubility of G2 was performed through the formation of inclusion complexes with cyclodextrins (CD). In this work, we report the change of the solubility profile of G2 when is complexed with three different CD, the physicochemical characterization of the complexes and their *in vitro-in vivo* activity against *T. cruzi*. Additionally, we show toxicity results in human lymphocytes and erythrocytes.

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### *IN VITRO* EVALUATION OF SOME NOVEL IMIDO-SUBSTITUTED 1,4-NAPHTHOQUINONE DERIVATIVES AS ANTITRYPANOSOMAL AGENTS

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Chagas disease is a tropical disease, caused by the protozoan *Trypanosoma cruzi* and transmitted by triatomine bugs. It commonly occurs in poor and rural areas of Central and South America. Chagas disease is expanding beyond its endemic area as a result of migration from

and to the endemic countries. Currently used drugs have been reported to have undesirable side effects including gastrointestinal, neurological and mutagenic effects. In addition, problems such as bone marrow depletion, skin rashes, weight loss and dizziness have been reported for these drugs. Consequently, there is a search for safer drugs with more selective mode of action. Several classes of drug-like molecules have been studied for their antitrypanosomal activity. One of the most interesting ones is the quinone family of compounds. This class of compounds incorporates several diverse structural types including the naphthoquinones, which are known to possess a number of useful biological activities. We have developed some imido-substituted 1,4-naphthoquinones as a unique class of compounds with antitrypanosomal activities. Cytotoxic activities on Balb/C 3T3 mouse fibroblasts cell lines revealed excellent selectivity index for four of these compounds. Initial attempt to understand the mechanism(s) of action of these compounds appears to point to possible modulation of tubulin polymerization.

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### ANTILEISHMANIAL ACTIVITY OF NOVEL ARYLIMIDAMIDES

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We recently showed that the arylimidamide DB766 (2,5-bis[2-(2-propoxy)-4-(2-pyridylimino)aminophenyl]furan) possessed excellent activity against several *Leishmania* species in intracellular assays and displayed good efficacy in murine and hamster models of visceral leishmaniasis when given orally, as reported previously. A series of DB766 analogs have since been prepared and tested for their effect on intracellular *Leishmania in vitro*. Several classes of these analogs exhibited potency similar to that of DB766. Compounds possessing isosteric substitution of fluorine for hydrogen in the alkoxy linker moiety were active, as exemplified by DB1961 (2,5-bis[2-(1,3-difluoropropan-2-yloxy)-4-(2-pyridylimino)aminophenyl]furan, IC<sub>50</sub> = 98 ± 33 nM). Molecules bearing an unsymmetrical linker showed potent activity, illustrated by DB1967 (2-[2-(2-propoxy)-4-(2-pyridylimino)aminophenyl]-5-[4-(2-pyridylimino)aminophenyl]furan, IC<sub>50</sub> = 93 ± 28 nM). 35DAP081, an arylimidamide compound possessing a terphenyl linker, also displayed sub-micromolar potency (3,4"-bis-[N-(2-pyridylimidoyl)]amino-*m*-terphenyl, IC<sub>50</sub> = 260 ± 130 nM). A new class of arylimidamides also showed submicromolar *in vitro* antileishmanial activity. Furthermore, compounds in this class do not possess overt toxicity to mice when administered at a dose of 30 mg/kg/day × 5 by the intraperitoneal route. Members of this new class of arylimidamides are being tested in our murine model of visceral leishmaniasis in comparison to the reference arylimidamide DB766 and the oral antileishmanial drug miltefosine.

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### STUDY OF THE POTENTIAL RESISTANCE OF *LEISHMANIA AMAZONENSIS* TO A THIOSEMICARBAZONE DERIVATIVE

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The chemotherapy of leishmaniasis is based until now in drugs which are not totally efficient and present severe side effects and in some case are able to induce resistance to treatment. This resistance could be related to the volume of the drug (dose and frequency) and the time of administration, among other factors. The mechanism of resistance have been associated to the increased expression of a transmembrane protein (Pgp), that act as a efflux pump for a wide spectrum of drugs and

depends on energy (from ATP) and must be phosphorylated to be active. As part of our research program on chemotherapy against diseases caused by trypanosomatids we have been studying several thiosemicarbazones and semicarbazones derivatives, which have a medical interest because of their capacity to inhibit the growth of several pathogens. Studies concerning its biological activity show that these compounds are active against trypanosomatids, such as *T. cruzi*, *T. brucei* and *Leishmania* sp. In the present work, it was used a thiosemicarbazone [(3-methoxy-4-hydroxy-estiryl)-thiosemicarbazone], that showed to be very active against *L. amazonensis* promastigotes and Pentamidine as a reference drug. Parasites were grown in Schneider's medium, pH7.2, temperature of 26°C and resistance was induced by the compounds in the presence of the compounds for several passages in culture. During this process, it was evaluated the potential acquired resistance by new screenings in each passage (new LD50), besides the assay of infectivity of the parasites through complement lysis test (to detect metacyclic forms) and *in vitro* infection. The results showed that a significant increase in the LD50 was observed at passage number 10 and the parasites were able to maintain its infectivity, even after several passages in culture.

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### COMPARISON OF REAL-TIME PCR ASSAYS FOR DETECTING *TRYPANOSOMA CRUZI* DNA IN CLINICAL SAMPLES

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Chagas disease is caused by the parasite *Trypanosoma cruzi* and is characterized by chronic infection of the heart. Vectorial transmission is the main route of infection in endemic areas. Other routes of infection include congenital transmission, blood transfusion, organ transplantation and oral ingestion of foods contaminated with the parasite. Diagnosing Chagas disease is challenging, in part because of the varying clinical manifestations during different phases of the disease. Serology is the preferred method for patients in the chronic phase, whereas PCR can be successful in acute and congenital cases. Quantitative PCR can be especially powerful to monitor changes in parasite blood levels during re-activation or drug treatment. Here we present data comparing three TaqMan PCR assays (one previously published and two new methods) and a well-established conventional PCR targeting the kinetoplast minicircle. Included in the analysis were DNA extracted from 191 EDTA blood samples, 12 heart biopsies, 9 umbilical cord blood samples, 1 skin tissue sample and 2 CSF samples. The sources of the samples were patients with suspected exposure to *T. cruzi* through organ transplantation, bug contact or laboratory accidents, and from immunosuppressed patients with suspected re-activation. The assays had differing sensitivities (ranging from 63% to 97%) and specificities (96% to 100%). The most sensitive assay was a TaqMan PCR targeting the kinetoplast minicircle; however, that assay also had the highest number of false positives. The published TaqMan assay, targeting the TCZ microsatellite region, was 84% sensitive and 98% specific in this evaluation. The third TaqMan assay was designed to be very specific but unfortunately had the lowest sensitivity. These data strongly suggest at least two PCR assays with different performances should be used to obtain accurate results and are consistent with the conclusions from the recent multicenter study by the Consortium for standardization and validation of clinical use of PCR for *T. cruzi* DNA detection in Chagas disease.

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### ADMINISTRATION OF TOMATO EXPRESSING INTERLUKIN 12 CONTROLS *TRYPANOSOMA CRUZI* INFECTION IN ORALLY TREATED MICE

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Chagas Disease (American Tripanosomiasis) is an anthrozoootic illness by the *Trypanosoma cruzi* parasite. The disease is endemic in the Americas and there are 10 -15 million existing cases. A third of infected people develop cardiomyopathy or mega-digestive syndrome. Interleukin IL-12 is a cytokine that is induced early when a host is challenged by a pathogen. Additionally, IL-12 is required to elicit a Th1 immune response, which is responsible for eliminating *T. cruzi* parasite through the activation of T lymphocyte. In previous studies, oral administration of tomatoes expressing murine IL-12 allowed the control of a *Mycobacterium tuberculosis* infection. Here we tested if this treatment could control an infection with *T. cruzi* in mice. Balb/c mice were infected with 500 *T. cruzi* parasites via intraperitoneal and they were treated during 30 days with daily doses tomatoes pure expressing IL-12 (240 ng IL-12) via oral. The control group received tomatoes without cytokine. Every three days, parasitemia was recorded in both groups. The density of inflammatory cells in cardiac tissue was measured in tissue section stained with hematoxylin using Multispec software 3.0. Amastigote nests were also counted from cardiac tissue. Administration of tomatoes expressing IL-12 significantly decreased the parasitemia in treated mice. These also presented significantly less cardiac inflammation than the control group and had fewer amastigote nests. Results show that oral administration of tomatoes expressing IL-12 at least partially control *T. cruzi* infection in mice, which opens new opportunities for the control of Chagas Disease.

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### EVALUATING THE IMPACT OF AN INTERVENTION ON PEDIATRIC MALARIA CASE-MANAGEMENT PRACTICES IN PUBLIC HEALTH FACILITIES IN KENYA

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An intervention to improve effective case management of febrile children under 5 years was implemented in public health facilities in Nyanza Province, Kenya between 2008 and 2009. An evaluation of the intervention using pre & post intervention cross sectional health facility surveys was conducted in one district of the Province. The surveys included: audit of the health facility; health worker structured interviews; exit interviews with caretakers of sick children under 5 years of age. The primary outcome indicator was the proportion of febrile children managed in accordance with national recommended treatment guidelines. At baseline 33 government health facilities, 48 health workers and 560 febrile children consultations were evaluated. At follow-up the same health facilities were surveyed and 36 health workers and 423 febrile children consultations evaluated. Our findings show : 1) the proportion of health workers who had received any malaria case-management training increased from 46% to 75% (P=0.01); 2) the proportion of health workers who received the intervention specific training was 61% 3) the proportion of febrile children with uncomplicated malaria treated with the first-line antimalarial drug, artesunate-lumefantrine (AL), at health facilities where AL was in stock increased from 74% to 84.6% (P=0.007) 4) The proportion of caregivers who knew the correct AL duration increased from 67% to 80% (P=0.009). However, when the analyses were restricted to health workers who received the intervention training versus those not trained, there were no significant differences. In conclusion, although there were significant improvements in case management, these could not be attributed to the intervention.



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### ANTIPLASMODIA ACTIVITIES OF METHANOLIC EXTRACT OF *ANOGEISSUS LEIOCARPUS* AND ITS PATHOLOGICAL EFFECT ON MALARIA PARASITE INFECTED MICE

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Methanolic extract from *Anogeissus leiocarpus* has been considered locally to have the same antimalarial activities as artemisinin combination therapy newly introduced by WHO. Therefore this work studies the *in vivo* antiplasmodia activities of extract of *A. leiocarpus* and its pathological effect on some ectopic organ of malaria parasite infected mice. Mice used for this study were infected with *Plasmodium berghei* and divided into 5 groups. The first group was not infected with parasite. The second group was infected with parasite and was not treated with antimalarial drugs. The third group was infected and treated with artesunate at 5mg/kg body weight. The fourth and fifth groups were infected and treated with 100 and 200mg/kg body weight of extract of *A. leiocarpus* respectively. Thick and thin films were prepared and used for malaria parasite counts. High density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride levels were determined from plasma. MDA and Catalase levels were also determined from the plasma and homogenates from kidney, liver and heart. The full white blood counts were also determined. The parasite density was significantly higher ( $P < 0.05$ ) in group infected with the malaria parasite but without treatment than other infected groups which were treated. The rate of parasite clearance was higher in the group treated with artesunate than the groups treated with *A. leiocarpus*. MDA level was significantly higher in serum, liver and heart of mice infected with artesunate than mice in other groups. This could be as a result of increase in lymphocyte, neutrophils, eosinophils and basophils levels in group treated with artesunate. Catalase level was significantly higher in the homogenate from liver and heart of the mice treated with 200 mg/kg body weight of *A. leiocarpus* than other groups. LDL and total triglyceride were significantly higher in group treated with artesunate than other groups, while HDL was significantly higher in the two groups treated with *A. leiocarpus* as compared with the group treated with artesunate. This study shows that extract of *A. leiocarpus* has antimalarial activities with minimal adverse effect as compared with artesunate.

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### EFFECTIVENESS AND TREATMENT ADHERENCE TO ARTEMETHER/LUMEFANTRINE UNIT DOSE AGE SPECIFIC PRE-PACKS VERSUS BLISTER PACKS IN THE TREATMENT OF UNCOMPLICATED MALARIA IN UGANDA

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Prompt and adequate treatment of clinical malaria episodes remains one of the key elements of malaria control and this partly depends on effectiveness of the drugs and patients' compliance to treatment. Uganda adopted Artemether/Lumefantrine (AL) 6 dose unit dose age specific pre-packs as first line treatment for uncomplicated malaria, however, concerns about the costs and stock-outs of these packages have been raised. This has led to a need for equally efficacious alternatives drugs in order to reduce these problems. We are currently conducting a randomized, open label trial to compare the effectiveness and treatment adherence to AL unit dose age specific pre-packs to AL blister packs plus instruction leaflets for the treatment of uncomplicated malaria. An interim analysis including 100 participants (target sample size = 702) is presented here. Children aged 4 months to 5 years with history of fever/axillary temperature > 37.5 and a positive malaria blood smear were randomized to receive one of the study regimens and were followed for 28 days. Participants were

assessed for treatment outcomes over 28 days according to modified World Health Organization criteria. Of 100 participants enrolled in the study, 94% completed follow-up and were assigned a treatment outcome; 6 participants were withdrawn or lost to follow-up. By day 28, clinical failure (CF) occurred in 21% of the children and parasitological failures (PF) occurred in 42% of the participants. All treatment failures occurred between days 14 and 28. At least one adverse event was reported in 53% of the participants, but no serious adverse events occurred. Treatment failure rate (CF + PF) unadjusted by genotyping was 65% for the unit dose age specific pre-pack group compared with 55% for the blister packs plus instruction leaflet group. The adherence to unit dose age specific pre-packs was 92% compared with 96% to the AL blister packs plus instruction leaflets. Complete results, including assessment of parasite isolates by genotyping, and full results of safety will be presented.

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### IMPROVING RELATIVE BIOAVAILABILITY AND PROPHYLACTIC EFFICACY OF ORAL WR299958 BY REDUCING PARTICLE SIZE USING AN ULTRA-SONICATOR

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Particle size is an important determinant of gastrointestinal absorption in human and animal species by oral administration. Although the use of particle size reduction to increase bioavailability of compounds has been reported in the literature, the effect of a reduction in particle size on the bioavailability of WR299958, a new antimalarial compound, is unclear. Suspension and emulsion formulations of WR299958 were made using a homogenizer and an ultra-sonicator, respectively, and the particle sizes of each formulation were measured by a LA-950 laser particle size analyzer. The mean particle size of the suspension and emulsion formulations were measured and showed the particle size of 102.2 and 0.085  $\mu\text{m}$ , respectively. The two new suspension and emulsion formulations of WR299958 at various doses were administered intragastrically to infected- or uninfected-mice for efficacy and pharmacokinetic (PK) evaluations. For the PK assessment, the plasma and liver samples were collected and drug concentrations were analyzed by LC-MS/MS. For the efficacy test, blood was taken from the mice through tail nicks and the parasitemia was determined by flow-cytometry. The results indicated that the particle size reduction resulted in significant differences in PK and efficacy evaluations. If bioavailability of 100% was set for the emulsion formulation, the relative bioavailability of WR299958 for the suspension formulation was only 30.8% *in vivo*. With the same oral dosage, the peak concentration of the emulsion formulation ( $C_{\text{max}}$ ) was 14.75 ng/ml in mice which was 2.32 times higher than the peak concentration of the suspension group at 6.20 ng/ml. Similarly, the area under the curve (AUC) of 60.33 ng-h/ml after administration of the emulsion was 3.27 fold higher than in animals treated with the drug in suspension with 18.60 ng-h/ml. The initial efficacy of these formulations was also tested and full causal prophylaxis in mice treated with emulsion WR299958 was two-fold stronger than that of the suspension in *Plasmodium berghei* sporozoite-infected mice. Although the bioavailability of WR299958 was significantly increased by using the emulsion formulation with a nanoparticle size, the drug bioavailability remains very poor. Therefore, further improvement in the oral bioavailability of WR299958 will require additional work.

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### A NEW METHOD SUITABLE FOR HIGH THROUGHPUT SCREENING OF *PLASMODIUM FALCIPARUM*

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The standard *in vitro* protocol currently used for assessing susceptibility of drugs against *Plasmodium falciparum* is based on the incorporation

of radioactive 3H-hypoxanthine. This methodology relies on the use of 96-well plates and together with the inherent problems of the use of radiolabelled material, makes this assay unsuitable for use in a *Plasmodium* high-throughput whole cell screening. Alternative methodologies, amenable for use in a high-density format (384-well) and preferably non-radioactive, are required to tackle a whole cell screening of *P. falciparum* in a high scale. We have implemented conditions for growing *P. falciparum* cultures in a 384-well format. Using optical microscopy, we have demonstrated that growth rates observed in these conditions are equivalent to the ones occurring in a 96-well format. Parasite lactate dehydrogenase activity (PfLDH) is a good surrogate of *P. falciparum* growth and can be used to determine susceptibility to antimalarial drugs. APAD+ (acetyl pyrimidine adenine nucleotide) is an analogue of NAD+ cofactor. During enzymatic L-lactate oxidation, APAD+ is used 300 times more efficiently by PfLDH than by its human counterpart. To determine parasite growth in a semiautomatic way, we have adapted a colorimetric method that takes advantage of the described structural differences of *P. falciparum* and human lactate dehydrogenase enzymes. This assay is non-radioactive and suitable for use in a high-density format without the need for filtration or centrifugation steps, making it useful for low technology settings. The new method has been validated using known antimalarial compounds. Drug sensitivity results (IC50) obtained with this protocol compared well to that of the traditional isotopic method.

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### EFFECT OF ARTESUNATE ON DISPOSITION OF ORALLY ADMINISTERED AMODIAQUINE IN PATIENTS WITH UNCOMPLICATED MALARIA

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The emergence of drug resistance in *Plasmodium falciparum* has necessitated that *falciparum* malaria be treated with Artemisinin-based Combination Therapy (ACT). Amodiaquine (AQ) is one of the drugs used in combination with artesunate for malaria treatment. We assessed the pharmacokinetics of AQ and the effect of artesunate on its disposition in patients with malaria. A liquid chromatographic method was developed for analysis of AQ and its metabolite, desethylamodiaquine (AQm). Twelve patients positive for malaria parasite were randomized to receive either AQ or AQ plus artesunate (AS). The doses were AQ 600mg once daily and fixed-dose AQ/AS daily for 3 days. Blood samples were collected before and at 0.5, 1, 2, 4, 6, 12, 24, 48, 72, 144 and 336 hr after drug intake. Plasma was separated and used to assay for AQ. The analytical method was highly sensitive and specific. Calibration curves were linear ( $r^2 > 0.99$ ) in the range of 100 - 1000 ng/ml for AQ and AQm. The intra-assay coefficients of variation were 1.87-4.94% for AQ and 0.49-5.34% for AQm. While inter-assay coefficients of variation were 1.67-6.37% for AQ and 2.49-6.89% for AQm. The mean values of Peak Plasma Concentration,  $C_{max}$  ( $22.7 \pm 0.01$  vs  $20.43 \pm 0.12$  ng/ml) and Area Under the Plasma Concentration-time Curve, AUC ( $59.63 \pm 0.05$  vs  $57.52 \pm 0.24$  ng/ml) of AQ were significantly ( $P < 0.05$ ) higher in the AQ alone compared with AQ/AS group. Terminal half life,  $t_{1/2}$  was longer ( $2.83 \pm 0.02$  vs  $2.69 \pm 0.01$  hr) and oral clearance, Cl/F ( $9729.54 \pm 7.61$  vs  $9857.24 \pm 42.5$  ml/h) was significantly lower in AQ/AS when compared to AQ group ( $P < 0.05$ ). The mean values of peak time of plasma concentration ( $T_{max}$ ) of AQ in the two groups were the same at 2hr. There were however no statistically significant differences in the values of  $T_{max}$ ,  $C_{max}$ ,  $t_{1/2}$ , Cl/F and AUC of AQm in both treatment groups ( $P > 0.05$ ). Artesunate significantly affected the disposition of the parent drug, amodiaquine but not the metabolite, desethylamodiaquine when orally administered in combination in patients with malaria.

### THE ANTIMALARIAL EFFICACY OF PRIMAQUINE: THE ROLE OF CYTOCHROME P450-MEDIATED METABOLITES

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Primaquine (PQ) is currently the only FDA-approved drug to treat relapsing malaria, but PQ causes hemolytic toxicity in glucose-6-phosphate dehydrogenase deficient individuals. Metabolic activation of PQ appears necessary for antimalarial efficacy and hemotoxicity. The link between cytochrome P450 (CYP450)-mediated metabolism of PQ and causal prophylactic efficacy was investigated in a murine (ICR stain) *Plasmodium berghei* (ANKA strain) sporozoite challenge model. 1-Aminobenzotriazole (ABT) was used to irreversibly inhibit multiple CYP450 isoforms to determine the effect of inhibition on antimalarial efficacy. Mice were treated with ABT two hours prior to oral dosing with a single (37.5 mg/kg) or 3 day (25 mg/kg and 40 mg/kg x 3 days) curative dose of PQ. Comparator groups were treated with the same curative doses of PQ without ABT. Parasitemia was monitored over 31 days as an indicator of protective action or failure in this lethal model. ABT blocked the prophylactic activity of PQ, suggesting that CYP450 metabolites of PQ contribute significantly to exo-erythrocytic efficacy. To compare plasma exposures of PQ and metabolites  $\pm$  ABT, terminal bleeds were conducted on the day that mice succumbed to infection in the ABT treatment groups. A pharmacokinetic and metabolism profile of PQ  $\pm$  ABT in non-infected mice was constructed to interpret results attained in the murine malaria model. Taken together, these results have prompted further exploration of the CYP450 pathway as being critical to PQ's efficacy. Experiments are underway to investigate the effect of ABT on radical cure (i.e. anti-hypnozoite activity) of PQ in a Rhesus monkey model of relapsing malaria.

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### NOVEL BORON-CONTAINING SMALL MOLECULES DEMONSTRATE POTENTIAL FOR MALARIA THERAPY: EXCELLENT *IN VIVO* EFFICACY IN MURINE *PLASMODIUM BERGHI* MODELS AND FAVORABLE PHARMACOKINETICS

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Recent suggestions of resistance to artemisinin-based combination therapies in Southeast Asia underscore the ongoing need for discovery of new chemical entities for treatment of *falciparum* malaria. New therapeutics need to be orally active, effective in short-course therapy, inexpensive to produce, and safe for use in developing world populations. We have discovered a series of novel boron-containing small molecules with excellent *in vitro* potency against *Plasmodium falciparum* and have evaluated pharmacokinetic properties and *in vivo* efficacy of several potent scaffolds. Greater than a thousand members of the Anacor compound library were screened *in vitro* against cultured W2-strain *P. falciparum* at a single concentration of 10  $\mu$ M. Activities of hit compounds were then titrated and numerous compounds with IC50 values  $< 500$  nM were observed. The most potent compound, AN3661 (IC50 = 26 nM against W2-strain *P. falciparum*) showed high plasma clearance (4513 mL/h/kg) with reasonable oral bioavailability when dosed to mice at 30 mg/kg IV and PO. AN3661 demonstrated *in vivo* efficacy after oral treatment

in a 4-day murine model of *P. berghei* infection, where parasitemia was detected by flow cytometry on Day 4 (ED<sub>90</sub> < 3 mg/kg). In addition, AN3661 showed 100% cure when dosed twice-daily for 4 days at 100 mg/kg, in a 42-day model, with no parasitemia detected after 42 days. Lower doses of AN3661 significantly extended mouse survival, although didn't cure. The high required dose for cure may be attributed to high clearance in mice. Analogues of AN3661 are being synthesized to optimize pharmacokinetic properties. Initial PK analysis of the new designs revealed significant reduction of plasma clearance in mice. In summary, novel boron-containing small molecules offer promising potential as new orally-active antimalarials.

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### POPULATION SCREENING FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCIES IN ISABEL PROVINCE, SOLOMON ISLANDS, USING A MODIFIED ENZYME ASSAY ON FILTER PAPER DRIED BLOODSPOTS

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Glucose-6-phosphate dehydrogenase deficiency poses a significant impediment to primaquine use for the elimination of liver stage infection with *Plasmodium vivax* and gametocyte clearance, because of the risk of life-threatening haemolytic anaemia that can occur in G6PD-deficient patients. Although a range of methods for screening G6PD deficiency have been described, almost all require skilled personnel, expensive laboratory equipment, freshly collected blood, and are time consuming; factors that render them unsuitable for mass-screening purposes. We have adapted a published WST8/1-methoxy PMS method to assay G6PD in a 96-well format using dried blood spots, and used it to undertake population screening alongside a malaria survey undertaken in Isabel Province, Solomon Islands. The assay was validated by comparing it to biochemical screens and a recently marketed rapid diagnostic test. The overall prevalence of G6PD deficiency was determined to be 20.3% by mass-screening approximately 8541 people from 41 villages in Isabel Province, Solomon Islands. Comparative testing with biochemical and rapid diagnostic test indicated that results obtained by filter paper assay were accurate. The assay enabled simple and quick semi-quantitative population screening in a malaria-endemic region. The study indicated a high prevalence of G6PD deficiency in Isabel Province and highlights the critical need to consider G6PD deficiency in the context of *P. vivax* malaria elimination strategies in Solomon Islands, particularly the potential role of primaquine mass drug administration.

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### AN LC-MS BASED METHOD FOR THE MICRO-SAMPLING AND MEASUREMENT OF COMMON ANTIMALARIAL DRUGS *IN VIVO*

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Rodent PK models often require population sampling due to volume constraints. Furthermore, efficacy models can be confounded by excessive blood drawing in unhealthy animals, depending on the experimental end-point. To circumvent these issues, and facilitate measurement of drug levels in efficacy models on single living animals, an LC-MS based method for the micro sampling (15µl) and extraction of PK samples collected *in vivo* was developed and the limits of detection and quantification for a number of common antimalarials, including drugs from the 8 and 4-aminoquinoline classes, were compared. In this method, a droplet

of blood is dried on paper (GE Healthcare FTA DMPK-B) at the time of collection and extracted into acetonitrile. Multiple combinations of solvent and paper type were tested, and the combination with maximum signal for extracted drug was chosen. Liquid liquid extraction from whole blood methods were also compared to the solid liquid extraction used in the microsampling technique to assess extraction efficiency. This procedure can be used to increase sampling rates in population PK models, collect single animal PK data, or to correlate drug levels to endpoints in efficacy models with little or no perturbation on the animal model itself. Although drug levels (Mefloquine) as low as 30-50 ng/ml can be measured reproducibly in this method, compared to single digit ng/ml values in currently used liquid liquid extraction protocols, the method is being refined to try to enhance sensitivity as the volume required for reproducible sampling in many animal models is a critical and often limiting factor.

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### THE USE OF A PRODRUG APPROACH TO MINIMIZE POTENTIAL CNS EXPOSURE OF NEXT GENERATION QUINOLINE METHANOLS WHILE MAINTAINING EFFICACY IN *IN VIVO* ANIMAL MODELS

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Among the drug development programs currently under way at the Walter Reed Army Institute of Research is an effort to produce an analog of mefloquine (MQ) that is less susceptible to penetration of the blood-brain barrier (BBB) while maintaining levels of efficacy that are equal to or greater than MQ. To that end, a library of MQ analogs was synthesized to explore the relationship between a range of physiochemical properties and efficacy/BBB permeability (1,2). One of the compounds from this library, WR308245, initially generated interest due to its promising *in vitro* efficacy and toxicity values. However, its MDCK-MDR1 permeability values suggested high BBB penetration. *In vivo* mouse PK confirmed this finding with drug levels in the brain approximately six times that of MQ at the C<sub>max</sub> after IV dosing. Another drug in this class of analogs, WR319670, was found to have *in vivo* activity against *Plasmodium berghei* in a mouse model of anti-malarial efficacy while exhibiting poor activity levels in hypoxanthine and SYBR Green *in vitro* efficacy screens. These data suggested that the activity of WR319670 in the *in vivo* model was due to drug metabolism, i.e., that WR319670 behaved as a prodrug. Based on the structure of WR319670 and common Phase I biotransformations, it was postulated that one of the metabolites would be WR308245. *In vivo* mouse PK confirmed the formation of WR308245 upon IV administration of WR319670. In addition, drug brain levels for both WR319670 and WR308245 achieved a C<sub>max</sub> of approximately 1/3 that of MQ. In order to support the assertion that the *P. berghei* efficacy of WR319670 would translate to human malaria parasites, the drug's efficacy was tested in an Aotus monkey *P. falciparum* model. The Aotus model demonstrated sufficient efficacy to confirm the correlation between the *P. berghei* and *P. falciparum* models and, consequently, the use of a prodrug approach to minimize drug brain levels while achieving antimalarial efficacy.



### IN VITRO AND IN VIVO METABOLIC AND PHARMACOKINETIC PROFILES OF WR283194, A NOVEL ANTIMALARIAL DRUG CANDIDATE

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The imidazolidinedione (IZ) class of compounds has a long history with many pharmaceutical and industrial uses including herbicides, polymers, antibacterials, and anticonvulsants. Several compounds of the class have also demonstrated causal activity in *in vivo* malaria models, and the IZ compounds have been reported to be the first class of compounds demonstrated to possess activity exclusively against liver stage malaria (Guan et. al, 2002). The novel antimalarial drug candidate WR283194, of the imidazolidinedione class, was evaluated for its *in vitro* metabolic characteristics and *in vivo* pharmacokinetic (PK) properties in Rhesus monkeys. *In vitro* metabolic stability assays predict stability >60 min in both human and mouse liver microsomes, and drug-drug interaction screening against a panel of CYP isoenzymes and known substrates showed no significant interaction with each of the five primary abundance CYPs (3A4, 1A2, 2C9, 2C19, and 2D6). In a three day dosing model (PO 2.5 mg/kg), the average C<sub>max</sub> was determined to be 6546 ng/ml with a T<sub>max</sub> of 8 hrs in plasma and an average V<sub>D</sub> of 2225 ml/kg. The compound cleared relatively rapidly, with an average half life of 4.6 hrs and an observed average AUC<sub>inf</sub> of 156,411 hr\*ng/ml. Preliminary data identified WR283246 as a major metabolite in Rhesus plasma and red blood cells. Metabolite ID studies in progress will help understand the compound's metabolic profile. These studies help define the pharmacokinetic and metabolic characteristics of compounds in the IZ class to guide future antimalarial drug efforts.

### DHA INHIBITS HUMAN ERYTHROID CELL DIFFERENTIATION BY ALTERING THE GATA SWITCH

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WHO recommends to avoid artemisinin treatment during the first trimester of pregnancy, because animal models showed a significant depletion of embryonic red cells, which occurs only during a specific days of gestation. We recently demonstrated for the first time that DHA, which is the *in vivo* metabolite of many artemisinin derivatives, inhibits human erythroid cell differentiation, as well. We showed that DHA specifically targets the pro- and basophilic erythroblasts during *in vitro* erythroid cell differentiation of CD34+ stem cells. By using K562 cells differentiated toward the erythroid lineages by chemical inducers and by comparing the effects of several artemisinins, we confirmed that DHA is the most toxic compound of this drug family. Significant reduction by DHA was observed not only of cell growth, but also of erythroid cell maturation, as shown by the changes in cell viability, cell cycle progression, GpA expression, inhibition of  $\gamma$ -globin gene and GATA-1 mRNAs. In addition, we observed that the toxicity is related to pathways which regulate the haemoglobin synthesis. In fact, DHA rapidly induces the release of Cytochrome C from the mitochondria, which in turn, activates Caspase-3 and, together with the HSP70 down regulation, induces the GATA-1 cleavage and the up-regulation of GATA-2. In conclusion, altering the GATA switch, DHA modifies the fate of the erythroid cell: it prevents the erythroid cell differentiation and simultaneously causes the arrest of cell growth and, eventually, the cell death through apoptosis. This dual effect is clearly dose-dependent. In conclusion, our results support WHO recommendations and the urgent need to better define the risk-benefit of the use of artemisinins treatment for malaria during the first trimester of human pregnancy.

### PERMEABILITY OF ANTIMALARIAL DRUG CANDIDATES USING CACO-2 AND MDCK CELL LINES

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Malaria figures amongst the major health and developmental challenges in the world. There is a need for orally active, liver stage antimalarials without CNS adverse effects. The use of cell lines such as CACO-2 and MDCK in permeability assays serve as surrogate indicators of absorption and transport; with the two approaches often used interchangeably. The growth period for the CACO-2 cells is at least 2 weeks before their trans-epithelial electrical resistance (TEER) reaches its optimal level of 300-500 ohms. On the other hand, MDCK cells reach their optimal TEER (>800 ohms) in 4 days; making MDCK more desirable in terms of time needed to cultivate and maintain them. We sought to characterize both approaches in support of our antimalarial drug development paradigm. Accordingly, the bi-directional transport using both CACO-2 and MDCK cells was evaluated for over 20 candidate antimalarial compounds and the permeability coefficient (Papp) values were calculated based on liquid chromatography/tandem mass spectrometry (LC-MS/MS) analyses. The result showed that the Papp results were similar in CACO-2 and MDCK permeability assay with low and medium permeable antimalarial compounds. However, there were variations of Papp result and efflux ratio between the CACO-2 and MDCK approaches. This could be due in part to P-gp mediated efflux in apical-basolateral or basolateral-to-apical transport, as well as, the tight junctions found in the MDCK permeability model. While the use of MDCK cells may be a "fast-growing" alternative to CACO-2 cells for measuring compound CNS transport, the later assay may still desirable permeability model for measuring intestinal transport of antimalarial candidates.

### IN VITRO AND IN VIVO METABOLIC PROFILE OF TWO DEOXO-IMIDAZOLIDINEDIONE ANALOGS WITH ANTIMALARIAL PROPERTIES

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A series of newly synthesized Deoxo-Imidazolidinedione (IZ) analogs are being evaluated by the U.S. Army for development as candidate antimalarials. This study provides *in vitro* and *in vivo* metabolic profile based on mass spectrometry analyses for two IZ analogs: WR308449 and WR308597. For the *in vitro* efforts, compounds were incubated for up to two hours in the presence of pooled microsomes originated from human, monkey or rat livers. The samples were extracted by protein precipitation and analyzed by LC-Trap/MS. The *in vivo* metabolic profile work analyzed samples from a mouse pharmacokinetic study following a single 50 mg/kg oral dose. Plasma and liver tissues were collected for up to 48 hours. Plasmas were extracted and analyzed as described above, while the liver tissues were homogenized prior to extraction. *In vitro* microsomal incubations of WR308449 yielded four putative metabolites while WR308597 yielded two putative metabolites. For WR308449, hydroxylation (+16) appears to be the major metabolite compared to bis-oxidation (+32), methylation (+14), and metabolite 388 (-40). For WR308597, hydroxylation (+16) appears to be the major metabolite followed by bis-oxidation (+32), regardless of the specie evaluated. *In vivo*, WR308449 and WR308597 yielded more metabolites than the *in vitro* microsomal assays. WR308499 had 10 metabolites in plasma and 11 metabolites in liver, while WR308597 had 5 metabolites in plasma and 10 metabolites in liver. The *in vivo* metabolites of WR308499 and WR308597 included masses consisting with glucuronidation (+176), product of phase II metabolism. Based on the *in vitro* and *in vivo* results obtained, metabolic

profiles were postulated for WR308499 and WR308597. The relative contribution of the putative metabolites to efficacy and/or toxicity is yet to be characterized. The findings contribute to the development of new IZ analogs with desired attributes and facilitate characterization of their metabolic pathways.

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### ANTIMALARIAL ACTIVITY OF INDIVIDUAL ENANTIOMERS OF 8-AMINOQUINOLINES

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8-Aminoquinolines are a group of important antiparasitic agents with broad activity and excellent efficacy against malaria, leishmania and *Pneumocystis jirovecii* pneumonia. However, a serious limitation to widespread use of this class of compounds is that they produce reversible methemoglobinemia and hemolysis in individuals who suffer from glucose-6-phosphate dehydrogenase deficiency. Primaquine (1), the only drug currently approved in this class, is utilized as the racemate. Previous studies using animal models have shown that an enantiomer of primaquine or NPC1161C (WR233078) (2), another drug candidate of this class, has a better therapeutic index than the racemate. In order to further study these observations, we resolved primaquine and two 8-aminoquinolines (3 (WR225448) and 4 (WR247705)) with potent antimalarial activity, and evaluated them for antimalarial activity using a mouse model infected with *Plasmodium berghei*. Comparison of these results with those data we previously reported for 2 indicated that the (-)-(*R*)-enantiomer had better activity than the (+)-(*S*)-enantiomer or the racemate, except for primaquine in which the converse was true. The racemate, (-)-(*R*)- and (+)-(*S*)-enantiomer of primaquine, 2 and 3 were not toxic at the highest dose (16 mg/kg/day) tested. However, the racemate and both enantiomers of 4 were toxic at this dose and the (-)-(*R*)-enantiomer was more toxic than the (+)-(*S*)-enantiomer.

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### A MATHEMATICAL MODEL TO DESCRIBE THE ARGININE CATABOLISM IN MALARIA

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Decreased nitric oxide (NO) is associated with severe disease in *falciparum* malaria. Possible etiologies for low NO in malaria are decreased plasma L-arginine concentrations, the substrate for NO production, and NO quenching by cell-free hemoglobin (Hb) released during hemolysis. L-arginine infusion has been shown to increase vascular NO in moderately severe malaria but the optimal dosing regimen remains unclear. A mathematical model was developed, building on the work of others to describe L-arginine catabolism in endothelial cells in malaria. The model included the time course of hemolysis and subsequent release of arginine and cell free Hb. This model was used to investigate the optimal dosing schedule of arginine infusion in malaria to achieve maximal production of NO. Additional simulations were conducted in order to predict the extra- and intracellular concentrations of arginine, ornithine and citrulline as well as the cumulative NO molecules reach vascular muscle cells in both healthy volunteers (HV) and those with moderate severe malaria (MSM). The model described adequately the data collected from our previous study in Timika, Indonesia. The cumulative NO molecules produced by endothelial cells was significantly increased with supplementation of extracellular arginine. The choice of dose (3, 6 or 12 g) was less important than the duration of the infusion over which the dose was administered. Additionally, an increase in cell free Hb decreases cumulative NO molecules reaching vascular smooth muscle cells in an approximately inverse

exponential manner. In conclusion, the model provided an adequate description of the time course of arginine catabolism in HV and MSM and results were in agreement with the current *in vivo* and *in vitro* data. The administration of arginine is schedule-dependent, i.e. how the arginine is administered is at least as important as how much arginine is administered, and this should be taken into account in the design of future clinical trials.

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### COMMUNITY BASED PHARMACOVIGILANCE, A WAY FORWARD FOR STRENGTHENING THE PHARMACOVIGILANCE SYSTEM IN AFRICAN UNDERSERVED AREAS: EXPERIENCE IN SARAYA HEALTH DISTRICT IN RURAL SENEGAL

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Following the Abuja Conference, many strategies have been put in place for malaria control in Senegal. ACTs were implemented for the treatment of uncomplicated malaria and Intermittent Preventive Treatment for Pregnant women (IPTp) with Sulfadoxin Pyrimethamin (SP) was also adopted. In addition, from November 2006, Intermittent Preventive Treatment for infants (IPTi) has started in an operational research held in Saraya and two other districts. The objective of the study was to assess the monitoring of pharmacovigilance at the community level. Saraya district is located in southeastern Senegal, a rural area where patients' ability to have access to health facilities is extremely limited. Health staff (14) and community health workers (30) have been trained in passive and active pharmacovigilance. Trainings were reinforced by supervision; follow up meetings, social mobilization and information through community radio broadcasts from July 2007 to December 2009. In a 38000 estimated population, 7067 ACT treatments were administered and 53 notifications of adverse events completed; 21/53 notifications that were completed by health staff were related to ACT administration; among them 7/21 were put under observation. The other 32 notifications were brought to the attention of the health staff by the community (Community Health Workers, matrons, volunteers, leaders); they concerned SP in IPTi (12/53), Ivermectin (14/53), Cotrimoxazole (3/53), Mebendazole (1/53), Anti inflammatory (1/53), unknown drug (1/53). In conclusion, to successfully implement a pharmacovigilance program, it is fundamental not only to reinforce health staff training but to involve communities by engaging leaders, families, schools, and traditional healers. It is also urgent to ensure the validity of information related to Pharmacovigilance.

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### A STRUCTURE BASED DRUG DESIGN APPROACH TO REPURPOSE DRUGS AGAINST *PLASMODIUM FALCIPARUM* HSP90 (PFHSP90)

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*Plasmodium falciparum* heat shock protein 90 (PfHsp90 or PfHsp86 PF07\_0029) is essential for the development of the parasite during the intra-erythrocytic cycle and has the potential to serve as a drug target and circumvent antimalarial resistance when targeted in combination. In fact studies in *Candida albicans* have shown that Hsp90 inhibitors are able to reverse resistance to common antifungal agents such as cyclosporine A and echinocandins, as reported previously. Based on the conservation of the Hsp90 binding pocket and its central role in folding resistance associated proteins, our central hypothesis is that PfHsp90 inhibitors can reverse antimalarial resistance. Furthermore, evidence from the literature suggests that ATP mimetic inhibitors of the Hsp90 ATP-binding pocket target the phosphorylated "active form" of the protein in abnormal cancer cells. Regulation of PfHsp90 by phosphorylation by casein kinase II has



been previously reported suggesting that such a mechanism of selectivity of Hsp90 inhibitors for infected cells may be in place and may account for specificity of these inhibitors. Based on this hypothesis, we used the anticancer inhibitor of Hsp90 PU H71 for activity against malarial Hsp90. PU H71 inhibits ATP binding on the PfHsp90 GHKL domain and provides inhibition of parasite growth in cell culture in the nanomolar range. In addition, PU H71 exhibits synergistic activity with mefloquine and is able to reverse chloroquine resistance in the chloroquine resistance parasite line W2. Crystallization of PfHsp90 with PU H71 was achieved in order to understand the interactions of PU H71 with the PfHsp90 binding pocket and to further optimize this inhibitor for malaria. In conclusion, we are presenting a synergistic inhibitor of malaria hsp90 that can reverse resistance. Crystal data are being used to further optimize this inhibitor for malaria. Doses at which synergy is obtained are likely not toxic and will prevent emergence of resistance.

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### **IN VIVO ANTIMALARIAL, SERUM LIPID PROFILE AND HEMATOLOGICAL EVALUATIONS OF *ANOGEISSUS LEIOCARPUS* (DC.) GUILL. AND PERR. IN *PLASMODIUM BERGHEI* INFECTED MICE**

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Malaria is a public health problem most especially in the tropical countries where majority bear the burden of the disease. It is one of the six killer diseases in the world to-day and it has been estimated that 40% of the world's population is at risk and 500 million people suffer from the disease annually. Symptoms of malaria include fever, shivering, vomiting, anemia (caused by hemolysis), hemoglobinuria, retinal damage and convulsions. *Anogeissus leiocarpus* is an evergreen tree native to the savannas of tropical Africa. The inner bark is used as a chewing stick in Nigeria and extracts of the bark is used locally to treat malaria and show antibacterial properties. *Plasmodium berghei* strain NK 65 was used to infect mice grouped into five and left to establish for 7 days. On the seventh day, groups A, B and C were treated with Artesunate 5mg/kg, *A. leiocarpus* 100mg/kg and *A. leiocarpus* 200mg/kg respectively while groups D and E were negative control and uninfected mice respectively. The parasite density was monitored daily for five days and on the 5th day, haematological and serum lipid profile parameters were assessed. Treatment of malaria infected mice with artesunate 5mg/kg and *A. leiocarpus* reduced the parasite density compared with the negative control. Also treatment with artesunate and *A. leiocarpus* extracts increased the packed cell volume and red blood cell count which decreased in the negative control mice. Neutrophil and lymphocyte counts of the treated infected mice were brought to the levels of the uninfected mice. SOD level was significantly higher in the homogenate from liver and heart of the mice treated with 200mg/kg of *A. leiocarpus* than other groups. LDL, Total cholesterol, triglyceride were significantly higher in mice treated with artesunate than other groups, while HDL was higher in the group treated with 200mg/kg *A. leiocarpus*. The study justifies the traditional use of the extract of *A. leiocarpus* as an active antimalarial and has antianaemic as well as an antioxidant properties.

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### **EVALUATION OF THE ANTI-MALARIA, HAEMATOLOGICAL AND ANTI-OXIDANT PROPERTIES OF METHANOLIC EXTRACTS OF *TERMINALIA AVICENNIoidES* IN *PLASMODIUM BERGHEI BERGHEI* INFECTED MICE**

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Various extracts of *Terminalia avicennioides* (Combretaceae) are used in Nigeria to treat ailments such as rheumatic pain, helminthiasis, gastric and peptic ulcers. *T. avicennioides* has also been shown to have significant activities against *Salminella typhi*, *S. paratyphi* and *Vibrio cholera*. This study investigated the *in vivo* anti-malarial effects of different methanolic crude extracts of *T. avicennioides* in *Plasmodium berghei berghei* infected mice. The haematological and oxidative statuses of the mice were also evaluated. 25 mice in 5 different groups were used for this study. The parasites density of *P. berghei* infected mice was monitored daily for five days (by thin and thick blood films stained with Leishman's stain) upon treatment with artesunate (5mg/kg body weight) and *T. avicennioides* (100 and 200mg/kg body weight). Parameters to assess haematological and oxidative status were measured after five days. The parasite density of artesunate and *T. avicennioides* treated malaria parasite positive (MP+) mice decreased compared to untreated MP+ mice. The decreases seen in the haemoglobin (Hb) and red blood cell (RBC) count, as well as the increased neutrophil and decreased lymphocyte counts, of the untreated MP+ mice was restored to normal control levels in the artesunate and medicinal plant treated MP+ mice. Serum MDA levels of the treated MP+ mice were significantly ( $p < 0.05$ ) lower than untreated MP+ mice, while increases were observed in serum and liver superoxide dismutase (SOD) activities of the treated MP+ mice. In conclusion, oxidative stress during acute malaria infection, including depletion of antioxidants and increased plasma lipid peroxidation, has been documented. Oxidized molecule thus produced may play a role in the pathogenesis of malaria. The restoration of oxidative status, as well as, haematological parameters to normal values may reduce the severity of malaria infection.

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### **RELATIONSHIP BETWEEN AGE AND BODY WEIGHT GROUPS IN CHILDREN WITH *FALCIPARUM* MALARIA RECEIVING ARTEMETHER-LUMEFANTRINE (AL) AND ANALYSIS OF AL EFFICACY AND SAFETY ACCORDING TO BODY WEIGHT GROUPS**

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Artemether-lumefantrine (AL) is the current standard of care for uncomplicated *Plasmodium falciparum* malaria. Dosing by body weight is recommended but can not be used if weighing scales are unavailable e.g home-based management. Therefore age by body weight (BW) data can provide some guidance for optimal dosing when information on weight is not available. Data from a randomized, multicenter, investigator-blinded study in 899 infants and children ( $\geq 5$ kg and  $< 35$ kg) with uncomplicated *falciparum* malaria in five African countries (Benin, Kenya, Mali, Mozambique, and Tanzania) was analyzed. AL was dosed according to body weight groups (BWG): BWG1, 5kg to  $< 15$ kg (1 tablet/dose); BWG2, 15kg to  $< 25$ kg (2 tablets/dose); or BWG3, 25kg to  $< 35$ kg (3 tablets/dose). The primary analysis population included 477, 277, and 58 patients in the

BWG1: BWG2: and BWG3 respectively. PCR-corrected cure rate at day 28, the primary end point, was similar across all BWGs: BWG1 98.3%, BWG2 97.8% and BWG3 98.3%. Median times to parasite clearance and fever clearance were comparable between all BWG groups. There were no unexpected differences in safety or tolerability between the three BWG groups. Age by BW data showed that 80% (10-90th percentile) of the patients in the BWG1, BWG2 or BWG3 were aged 10-50 months (median 27, range 0-70; mean 29.3±15.4), 46-100 months (median 71 months, range 31-148, mean 71.7±22.4) and 90-147 months (median 122 months, range 78-152, mean, 119.3±20.5) respectively. In conclusion, the efficacy of AL in treating uncomplicated *falciparum* malaria is similar across body weight dosing groups in infants and children, with no clinically relevant differences in safety or tolerability. Analysis of age by BW group in this population of children from sub-Saharan African countries showed there to be only a minor overlap of age ranges between BWGs based on the 10-90th percentiles.

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### ANTI-MALARIAL ACTIVITY OF CEM-101, A FLUOROKETOLIDE ANTIMICROBIAL, IN BOTH BLOOD STAGE AND PRESUMPTIVE CAUSAL PROPHYLACTIC MOUSE MODELS

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CEM-101 is a new broad spectrum macrolide that has completed Phase 1 trials that acts to inhibit protein synthesis through binding to bacterial ribosomal RNA. A comparator drug, azithromycin, causes a delayed death effect *in vitro* *Plasmodium falciparum* blood stage assays and demonstrates antimalarial activity against liver stage parasites. CEM-101 was recently shown to be active *in vitro* against *P. falciparum* in extended incubation assays which measure the potency of inhibitors that demonstrate delayed death effects. CEM-101 is also active against blood stages in *P. berghei*-infected mice. Dose-response for CEM-101 was characterized in both blood stage treatment and causal prophylactic *P. berghei*-infected mice models using 3 day PO or SC dosing. Efficacy was measured by number of mice with delayed parasitemia and mice that were malaria free at day 31. Antimalarial liver stage activity was assessed in mice infected with luciferase expressing *P. berghei* parasites using an *in vivo* imaging system. For blood stage infections, the minimum curative SC dose was 40 mg/kg/d X 3 days, while 80 mg/kg/d X 3 was the minimum active dose for PO route. In the *P. berghei* causal mouse model, CEM-101 was curative at 40 mg/kg/d X 3 days with SC or PO dosing. No systemic toxicity was observed with SC or PO dosing as high as 160 mg/kg/d X 3 days. No demonstrable antimalarial activity against liver stage parasites was observed by *in vivo* imaging analysis of luciferase-expressing *P. berghei* with PO dosing at 40 mg/kg/d X 3 days. While drug activity against liver stage parasites could not be measured by *in vivo* imaging, no blood stage infection was detected in mice dosed as low as 40 mg/kg/d X 3 days and the minimum active dose was 20 mg/kg/d X 3 days. In conclusion, CEM-101 shows 100% prophylactic activity in causal mouse malaria models with PO dosing at 40 mg/kg/d X 3 days and 3/5 mice remain parasite free at 20 mg/kg/d X 3 days. The *in vivo* imaging analysis of liver stage parasites suggests that CEM-101 does not affect parasite growth at 40 mg/kg/d X 3 days. Based on *in vitro* blood stage drug assays and the mechanism of inhibition of this class of compounds, the lack of demonstrable liver stage activity was probably due to dosage. These results suggest that CEM-101, like azithromycin, demonstrates a delayed death effect; that is, developing liver stage merozoites are effectively non-viable blood stage parasites.

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### ANIMAL MODELS FOR SCREENING OF EXOERYTHROCYTIC ANTIMALARIAL DRUGS

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AFRIMS has used both mouse and monkey models for screening new antimalarial compounds from Walter Reed Army Institute of Research. Currently, novel antimalarials are screened first in the *Plasmodium berghei* (ANKA strain)-ICR mouse malaria model at AFRIMS and/or the new *in vivo* imaging system (IVIS) at Walter Reed Army Institute of Research. The active compounds are selected for testing in the *P. cynomolgi*-Rhesus monkey (*Macaca mulatta*) relapsing malaria model. The infection has been achieved through standard intravenous inoculation doses of 1 x 10<sup>5</sup> and 1 x 10<sup>6</sup> sporozoites from *Anopheles dirus* mosquitoes for the mouse and monkey models respectively. In 2008-2010, >200 new compounds and human approved drugs were screened in >80 mice experiments, and >20 compounds were tested in the monkey model in 5 experiments. More than 25 compounds showed causal prophylaxis activity in the mouse model and these included WR283205, WR296580, WR301855, WR299548, Clindamycin, Mirincamycin-*Trans*, Mirincamycin-*Cis*, Mirincamycin-racemic mixture, WR308499, WR308597, WR308271, WR308272, WR308145, WR308482, WR308658, Primaquine (+), Primaquine (-), WR279825, WR299958, WR319568, Tafenoquine (TQ), WR319590, CEM101, WR319673, WR319718 and WR282315. In addition, the mouse model was further validated with known antimalarials, Atovaquone (AT), Azithromycin (AZ), TQ, and Primaquine (PQ) to determine standard minimum effective oral (PO) doses. The minimum effective doses (MEDs) of AT, AZ, PQ and TQ for the oral three-day regimen (Days -1, 0 and 1), two-day (0, 1) and one-day (Day 0) were 0.5, 0.5 and 2.5 mg/kg; 120, 160 and 240 mg/kg; 25, 30 and 35 mg/kg; and 3.0, 2.5, and 7.5 mg/kg, respectively. In the monkey model, active tissue schizontocidal compounds in combination with chloroquine treatment were Tinidazole 300 mg/kg/PQ 0.3 mg/kg, PO for 7 days and twice weekly for 4 weeks; WR299548 (Decoquinat) 15 mg/kg, IM for 7 days; WR308499 at 30 mg/kg, IM for 3 days; PQ (-) at 0.6 mg/kg, PO for 7 days; and PQ (+) at 1.3 mg/kg, PO for 7 days. There are several compounds in the process for screening in both models and updated results will be discussed.

### NOVEL INHIBITORS OF *PLASMODIUM FALCIPARUM* DIHYDROOROTATE DEHYDROGENASE EXHIBIT ANTI-MALARIAL ACTIVITY IN MURINE MODELS

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Dihydroorotate dehydrogenase (DHODH) catalyzes the rate-limiting step in the *de novo* pyrimidine biosynthetic pathway, in which dihydroorotate is formed through a coupled redox reaction utilizing a mitochondrial respiratory chain ubiquinone. *Plasmodium falciparum* is unable to salvage pyrimidines and must rely on *de novo* biosynthesis for survival. DHODH represents the ultimate target of atovaquone via that agent's disruption of the electron transport chain, and it offers a viable target for additional chemotherapeutics. A high-throughput screen and subsequent medicinal chemistry program identified two promising series of compounds: 5-benzimidazolyl-N-alkylthiophene-2-carboxamides and 5-(4-phenyl)-imidazolyl-N-alkylthiophene-2-carboxamides. Compounds from each of these series demonstrated double-digit nanomolar *in vitro* potency against DHODH from *P. falciparum*, *P. vivax*, and *P. berghei*, with selectivity for the parasite enzymes over human DHODH. The activity against the *P. falciparum* enzyme was well correlated with *in vitro* potency against the *P. falciparum* 3D7 and Dd2 parasites. Several of the most potent compounds demonstrated good tolerability and oral exposure in the mouse, as well as ED50 values in the 4-day murine *P. berghei* (N strain) model of 10-15 mg/kg/day with oral b.i.d. dosing. Furthermore, oral b.i.d. dosing of the benzimidazole compound Genz-667348 at 100 mg/kg/day in the *P. berghei* (ANKA strain) model resulted in sterile cure, as defined by absence of recrudescence during a 30-day period following the cessation of dosing. This compound exhibited comparable activity in the *P. falciparum* humanized NOD-*scid* mouse model. An iterative lead optimization process is continuing, and closely related analogs with good potency and improved ADME properties are currently under investigation.

### THAI MULTIDRUG-RESISTANT (MDR) C2A STRAIN OF *PLASMODIUM FALCIPARUM* ADAPTED FOR USE IN THE *AOTUS LEMURINUS* IN VIVO MODEL

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*Aotus* monkeys infected with adapted human *Plasmodium falciparum* strains have been used for more than 40 years to study the pathogenesis, efficacy and pharmacokinetics of antimalarial compounds. The emergence of resistant malaria strains makes adaptation of recent strains from geographically relevant areas especially important. A recent preliminary report indicates that a multidrug-resistant (MDR) C2A strain originally obtained in Thailand may have been successfully adapted. Six splenectomized animals were inoculated with 5,000,000 parasites IV from a donor animal previously inoculated with a preserved aliquot of the 2008 Level VIII C2A. Daily parasite densities were obtained until >

100,000 parasites/ $\mu$ L. Animals randomized to each of the three treatment arms [mefloquine (MQ) 40 mg/kg orally X 1, artesunate (AS) 33 mg/kg orally daily X 3, or MQ 40 mg/kg orally X 1 plus AS 33 mg/kg orally daily X 3] were then started on their respective treatments. Parasitological and clinical responses were followed for 100 days. Animals in which primary treatments failed were administered the rescue regimen of AS+MQ. All 6 animals showed parasite patency at Day 1- 2 and reached peak parasite levels of > 100,000 parasites/ $\mu$ L by Day 9-11. One animal given AS+MQ was cured. The regimens administered to four of five of the remaining animals failed to adequately clear their parasitemia (late treatment failures) and required treatment the rescue treatment. All had final clearance by Day 23-28. The remaining MQ treated animal failed to clear by Day 9 and required rescue treatment (late parasitological failure). The rescue treatment was curative. *In vitro* IC50 and IC90 values obtained via a labeled hypoxanthine assay showed preserved to increased values of 29.6  $\pm$  1.3 and 138.8  $\pm$  8.7 ng/ml respectively when compared to the standard lab strain C2A values of 20.9  $\pm$  1.0 and 93.9  $\pm$  4.7 ng/ml. In conclusion, the current strains of the Thai MDR C2A strain has been successfully adapted to growth within splenectomized *Aotus* monkeys.

### CEM-101, A NEW FLUOROKETOLIDE WITH ANTIMALARIAL ACTIVITY

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CEM-101(CEM) is a new fluoroketolide antibiotic under clinical development for the treatment of community-acquired bacterial respiratory tract infections. In this study, we evaluated its *in vitro* and *in vivo* activity against different species of *Plasmodium* parasites to see if there is a potential for use in the treatment of malaria. CEM was tested by the semi automated microdilution assay against intra-erythrocytic forms of *P. falciparum* derived from asynchronous cultures of the strain NF54, essentially as described previously. Parasite growth over 120h was measured by the incorporation of radiolabelled [3H]hypoxanthine (in hypoxanthine-free culture medium) added after 96h of drug incubation and 24h prior to the termination of the test. Because of its slow mode of action, CEM was followed for 120 hrs vs the usual 72h assay. *In vitro P. falciparum* data for CEM in the 120h assay (96h + 24h). CEM showed an IC50 against NF54 of 2.4ng/ml compared to artesunate with an IC50 of 3.3ng/ml, clindamycin 5.3ng/ml and chloroquine 4.7ng/ml. Based on its promising *in vitro* activity, CEM was tested in the murine *P. berghei* model as described previously. CEM was first studied after a single dose of 100 mg/kg either in DMSO or HPMV. In the second experiment CEM was given at a dose of 100 mg/kg daily for 4 days in both vehicles. Following the single dose, CEM showed antiparasitic activity of 80.05% and 81.45% and mouse survival in days was 15.2 and 12.7 days, respectively. Following daily doses of 100 mg for 4 days CEM showed antiparasitic activity of 99.79% in both vehicles. The mice survived for 30 days in both experiments and thus CEM is considered to be fully curative in this model. CEM has been shown to have excellent *in vitro* and *in vivo* activity against *Plasmodium* species. This data would support future studies to determine CEM potential for the treatment of blood stage malaria in combination with a fast-acting antimalarial. It may also have additional benefits because of its activity as an antibiotic.



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### PRIMAQUINE AND TAFENOQUINE IN THE *PLASMODIUM CYNOMOLGI* CAUSAL PROPHYLACTIC MALARIA MODEL

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The relapsing malaria model consisting of *Plasmodium cynomolgi bastianellii* (B strain) in the rhesus is a valuable tool for identifying causal prophylactic drug candidates against *P. vivax* in humans. Historically, the 8-aminoquinolines (8-AQs) primaquine and tafenoquine have been protective at oral doses administered on days -1, 0 and 1 against sporozoites inoculated on day 0, presumably due to drug action against pre-erythrocytic stages. However, recent data suggest that the historically effective dosing regimens are not protective in the modern model. At historically effective doses of the two 8-AQs on days -1, 0, and 1, development of parasitemia was delayed slightly when compared with the untreated animals, but was not prevented. Delay in parasitemia averaged 5 days for the primaquine group (1.78 mg/kg/day) and 3 days for the tafenoquine group (0.316 mg/kg/day) when compared to the controls (vehicle only). Increasing the dose of tafenoquine to 6.0 mg/kg/day has provided complete protection to date, study day 53. While increasing the length of primaquine dosing from 3 to 10 days also provided protection to one monkey in the group, the other developed parasitemia on day 49. In this model, the primary attack is observed in untreated monkeys between days 8-10, the monkeys are treated with chloroquine for 7-10 days, and relapse occurs approximately 10 days after the last chloroquine dose. In the case of drugs with long elimination half-lives, such as tafenoquine, a lengthy delay in development of parasitemia may be attributed to drug still in the system. For primaquine, with a 2 hr elimination half-life in monkeys, other possibilities must be considered; the most likely being hypnozoite latency period. Comparison of 8-AQs to atovaquone-proguanil, an antimalarial with no antihypnozoite activity, will be presented, as will details of the analysis of plasma drug concentration-time data to determine dosing-exposure profiles and plasma drug levels associated with protection.

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### SPATIAL AND TEMPORAL PATTERN OF ANTI-MALARIA ANTIBODY RESPONSES AS EVALUATION OF HUMAN EXPOSURE IN THE WESTERN KENYAN HIGHLANDS

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Assessment of exposure to malaria at different altitudes and transmission intensities will inform the implementation and evaluation of malaria control programs. Recently anti malaria antibodies to merozoite surface protein 1 (MSP-1) have been described as the best immunological marker for estimating malaria exposure as a proxy for transmission intensity across various altitudes. The purpose of this study was to determine if the spatial and temporal patterns of antibody (Ab) responses are consistent with varying transmission intensities in the highlands of western Kenya. We measured total IgG levels to *Plasmodium falciparum* MSP-119 in an age stratified cohort (1= ≤1, 2=2-3, 3= 4-14, 4=15-45) of 900 participants from uphill and valley bottom residents at highland site during a low transmission and high malaria transmission season. Total IgG levels to salivary gland peptide gSG6 -P1 were also measured to determine whether micro-heterogeneity exposure to *Anopheles* bites correlates with MSP-1 IgG titers. Significantly higher proportions of sero-positives and total IgG titers were observed in valley bottom residents and in high transmission

season. Age stratified cohort revealed intriguing differences; higher titers in 1 yr olds, a decrease in 2-3yr olds with a non significant increase in 4-14 yr old before rising to significantly higher levels in the 15-45yr olds. No significant differences between age groups 1, 2, and 3 across all parameters compared except for seasonal variation, however significant differences were observed between each younger age group and group4. In conclusion, this data confirms a highly heterogeneous malaria exposure at this highland site possibly due to clustered vector densities around major breeding sites near valley bottoms. Whether the high level of Ab in infants is as a result of exposure or exclusively due to maternal antibodies is yet to be elucidated.

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### INFLUENCE OF EXPOSURE TO *ANOPHELES* BITES ON THE DEVELOPMENT OF ACQUIRED ANTIBODY RESPONSE TO *PLASMODIUM FALCIPARUM* IN CHILDREN

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Numerous ecological and epidemiological factors could modulate the anti-malaria immunity. Among these factors, the exposure to *Anopheles* bites, especially by active components of *Anopheles* saliva, could play a key role on the development of human immune response to *Plasmodium falciparum*. We investigated here the influence of exposure to *Anopheles* bites on the acquired antibody (Ab) response specific to *P. falciparum* whole schizont extract (WSE) and to CSP vaccine candidate, in children (1-9 years) living in malaria area. A multi-disciplinary and longitudinal study was conducted in two Senegalese villages where intensity of exposure to *Anopheles* bites was clearly different: Mboula, presenting low exposure (BHN =3) versus Gankette, with high exposure (BHN = 120). IgG, IgG1, IgG3 response directed to WSE and CSP antigen were determined before (June), at the peak (September) and after (December) the period of malaria exposure. In Mboula, the peak of exposure was followed by increase of anti-WSE IgG levels whereas low and constant specific IgG response was observed in Gankette. Interestingly, anti-WSE and anti-CSP IgG1 levels were higher in Mboula, whereas specific IgG3 response predominated in Gankette. Specific IgG1 response appeared therefore observed mainly in area presenting low exposure to *Anopheles* bites, whereas IgG3 isotype predominate in high exposure area. In addition, Ab response to WSE and CSP antigens decreased progressively with the season of exposure to *Anopheles* bites and this decrease appeared dependent to IgG1/IgG3 balance and to the level of exposure. Altogether, these results show that the development of anti-malaria Ab response was profoundly different according to areas where the level of *Anopheles* bites exposure was dissimilar. This influence of exposure to bites appeared to differently regulate the balance between specific IgG1 and IgG3 isotype levels, known to be associated with anti-malaria protective immune response. One hypothesis is that the influence of *Anopheles* saliva could be involved in the observed anti-malaria immune regulation.

### STUDIES ON ABO BLOOD GROUPS, HAEMOGLOBINOPATHIES AND G6PD GENOTYPES, AND *PLASMODIUM FALCIPARUM* INFECTION IN KPONE-ON-SEA, GHANA

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Erythrocyte variants such as the ABO blood groups, haemoglobinopathies and G6PD genotype are known to be associated with naturally acquired immunity against malaria. Despite some evidence of their protection, other epidemiological studies have provided evidence to the contrary, therefore their associations with malaria at Kpone-On-Sea, a coastal fishing village with high malaria incidence, was investigated. The design was cross-sectional, 592 individuals were randomly selected from whom 0.5ml of blood was collected and human DNA extracted using DNeasy Kit (Qiagen, USA). Blood groups and haemoglobinopathies were determined by standard agglutination method and cellulose acetate haemoglobin electrophoresis respectively. G6PD genotypes were determined by a PCR-based method using primers 5'-CCTGTCCCTCTGCCACA-3' and 5'-GGGGGTCTCAAGAAGTAC-3', followed by restriction of the amplified product with Hsp 92II enzyme. Parasitaemia was determined using microscopy. Among the study participants, 60.5% were females and 39.5% males. The distribution of the blood groups O, A, B and AB were 44.76%, 20.61%, 31.25% and 3.38% respectively. The prevalence of HbAA, HbAC, HbAF, HbAS, HbSC and HbSS were 71.28%, 8.11%, 1.18%, 16.89%, 1.35% and 1.01% respectively. Of all study participants, 50.68%, 35.81%, 8.11%, 1.69% and 3.72% were G6PD homozygous normal, hemizygous normal, heterozygous deficient, homozygous deficient and hemizygous deficient respectively. Only 72 individuals among the total study participants were parasitaemic. The geometric mean parasite density was 829.7 parasites/ $\mu$ l of blood (95%CI, 574.0-1199.40). Blood group O was not associated with reduced parasitaemia ( $t = -0.546$ ,  $P = 0.587$ ). HbAS was not associated with reduced parasitaemia ( $t = -1.262$ ,  $P = 0.212$ ). HbAC was not associated with reduced parasitaemia ( $t = 0.189$ ,  $P = 0.851$ ). The heterozygous G6PD deficiency was also not associated with reduced parasitaemia ( $t = 0.437$ ,  $P = 0.664$ ). Sample collection occurred in a period following a long dry season, resulting in low parasite prevalence rates being recorded, therefore the need for more studies to further explore the associations of these RBC variants and parasitaemia in the area. A more sensitive diagnostic technique such as PCR should be used in future studies to determine parasitaemia. There may be a clinal trend in the distribution of HbS and HbC in the country so the need for nationwide screening.

### ANTIBODIES THAT INHIBIT BINDING OF *PLASMODIUM FALCIPARUM* INFECTED ERYTHROCYTES TO CSA ARE ACQUIRED DURING PREGNANCY AND CORRELATE WITH ANTI-VSA AND ANTI-VAR2CSA

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Pregnant women are infected by *Plasmodium falciparum* presenting with unique adhesion properties that allow them to specifically bind Chondroitin sulphate A proteoglycan in the placenta. Acquisition of

protective immunity over successive pregnancies is attributed to antibodies that block the adhesion of infected erythrocytes to CSA. In this study we analysed plasma samples of women of various parity enrolled from their first trimester of pregnancy till delivery in the ongoing STOPPAM project in Benin. The plasma level of anti-VSA antibodies and adhesion inhibitory activity were measured on two parasite lines selected for CSA binding on Bewo cells (FCR3 and HB3). Specific antibodies to var2csa were measured on recombinant proteins of the DBL5 domain and the full-length extracellular part of the VAR2CSA. The majority of primigravidae had low levels or no anti-adhesion antibodies at enrollment compared to multigravidae. Women who experienced a detected parasitemia during the follow up significantly increased their levels of anti-VSA, anti-var2csa as well as their plasma inhibitory activity. However a difference in the kinetics of antibody production was observed between primigravidae and multigravidae following an infection. Women infected with HIV displayed an antibody acquisition pattern similar to that of HIV negative primigravidae. Overall, a significant correlation was found between the plasma level of anti-VSA and anti-var2csa IgG and the plasma anti-adhesion activity. The results from this study suggest that the anti-adhesion antibodies play a significant role in the protective immunity acquired against pregnancy malaria and should be considered a priority in strategies aiming at developing a vaccine against this pathology.

### DEVELOPMENT OF ANTIBODY RESPONSES AND RESTRICTED GLOBAL DIVERSITY OF *PLASMODIUM FALCIPARUM* ERYTHROCYTE MEMBRANE PROTEIN-1 IN MALARIA ENDEMIC REGIONS

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Clinical cases due to *Plasmodium falciparum* malaria in areas of high stable transmission reduce with age partially due to acquired humoral immunity to parasite proteins exposed on the surface of infected erythrocytes. To better understand the development of immunity to *P. falciparum*, we measured antibodies among a cohort of Kenyan children and adults to surface antigens expressed by the trophozoite stages of *P. falciparum* using five *P. falciparum* parasite isolates from different geographic origins. The isolates were selected for adhesion to ICAM-1, thought to be an important receptor for endothelial adhesion. Furthermore, we quantified the importance of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) as a target of acquired antibodies by using transgenic parasites with altered expression of PfEMP1. IgG was measured by flow cytometry. Most adults had IgG antibodies that reacted with the surface of infected erythrocytes, and all isolates were well recognized by serum antibodies. In contrast there was very low to no antibody reactivity in children aged below three years. PfEMP1 appeared to be the dominant target of antibodies among adults and children. Results suggest that there is restricted global diversity or common antigenic determinants in PfEMP-1 antigens and antibody reactivity increases with age and/or exposure. Further studies on PfEMP-1 are required to define the common epitopes for development as correlates of immunity and potential blood stage vaccines.

## PHENOTYPE AND ACTIVATION LEVELS OF DENDRITIC CELLS (DC) AND MONOCYTES IN PREGNANCY-ASSOCIATED MALARIA DURING A FOLLOW-UP IN BENIN

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Dendritic cells (DC) are important both in amplifying the innate immune response, and in initiating adaptive immunity and shaping the type of T helper (Th) response. Although the role of DC in immune responses to many intracellular pathogens has been delineated and research is underway to identify the mechanisms involved, relatively little is known concerning the role of DC in immunity to malaria. We evaluated the immunophenotype of antigen presenting cells (APC) in peripheral blood of pregnant Beninois women from the area of Cote, southwestern Benin, where we are conducting a longitudinal prospective study of 1000 mothers. Pregnant women are enrolled  $\leq 24$  weeks of pregnancy and followed at each ante-natal visit until delivery. Cellular immunological assessments have been performed with samples from a subgroup of 149 women at enrolment and 106 at delivery, with or without active *Plasmodium falciparum* infection detected by a rapid diagnostic test. Immunophenotyping of APC and their level of activation (HLA-DR, CD86 expression) are being evaluated using flow cytometry. *P. falciparum* infection was associated with DC altered maturation in pregnant women, as reflected by lower frequencies of MDC and PDC and their downregulated expression of HLA-DR but not CD86, whether in early pregnancy or at delivery. DC of pregnant women with anaemia were present at low frequency during pregnancy. In conclusion, HLA class II expression on DC is fundamental for presenting antigens to T cells and inducing their activation. Therefore, impaired DC activation upon malaria infection in pregnant women may result in a deficient and delayed adaptive immune response to the parasite and/or to other pathogens. Therefore, through an inhibitory effect on DC, *P. falciparum* may impair cell mediated immunity in pregnant women leading to a reduce response against the parasite itself and possibly rendering pregnant women more susceptible to other infections.

## INHIBITORY HUMORAL RESPONSES TO THE PLASMODIUM FALCIPARUM VACCINE CANDIDATE EBA-175 ARE LINKED TO ERYTHROCYTE RECEPTOR USAGE

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*Plasmodium falciparum* utilizes multiple ligand-receptor interactions for invasion. The invasion ligand EBA-175 is being developed as a major blood-stage vaccine candidate. It is located in the apical micronemes

of merozoites and mediates parasite invasion of host erythrocytes in a sialic acid dependent manner. In this study, we seek to address the ability of naturally acquired antibodies raised against the EBA-175 RII erythrocyte binding domain to inhibit parasite invasion, in relationship to its sialic acid dependence. To address this hypothesis, we have taken two primary approaches. We have determined the presence of antibodies to the PfEBA-175 RII domain by ELISA in individuals from malaria endemic areas of Senegal with high or low transmission. We have tested the plasma of those individuals for their specific EBA-175 inhibitory potential by performing invasion assays using *P. falciparum* EBA-175 KO transgenic parasites. We have also affinity purified antibodies to the EBA-175 RII domain from pooled patient serum for the invasion inhibition of uncultured Senegalese parasite isolates in *ex vivo* assays. Our results suggest that naturally acquired anti-EBA-175 RII antibodies significantly inhibit invasion of Senegalese parasites and this inhibition is dependent on the sialic acid dependence of the parasite strain. This work has implications for vaccine design based on EBA-175 in the context of alternative invasion pathways.

## IMPACT OF MATERNAL CYTOKINE GENE POLYMORPHISMS ON MOTHER AND FETUS BIOLOGICAL AND IMMUNOLOGICAL PARAMETERS IN THE CONTEXT OF PLACENTAL MALARIA

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Some single mutations in cytokines genes are related to modifications of the protein production. For cytokines involved in the regulation of the antibody production, particular gene polymorphisms influence the antibody levels. We investigated the consequences of some cytokine gene polymorphisms on the antibody levels of mothers at delivery, and on fetal immunity, in the context of *P. falciparum* placental malaria infection. We hypothesized that if some maternal cytokine gene polymorphisms lead to an increased production of maternal specific antibodies, they could help to lower the *in utero* sensitization of the fetus to plasmodial antigens, and contribute to delay the occurrence of the first malaria attack in early life. Six-hundred pairs of mothers and children were recruited in southwest Benin, where malaria is endemic. At delivery, peripheral blood was drawn from mothers, as well as corresponding cord blood. Eleven percent of mothers had a placenta infected with *P. falciparum*. From the maternal genomic DNA, 5 mutations occurring in genes coding for IL-4, IL-10 and IL-13 were genotyped by quantitative PCR. High frequencies were observed for genotypes IL-4-590 TT (61.8%), IL-4 +33 CT (50.5%), IL-10-1082 AA (52.5%), IL-10-592 AC (51.3%) and IL-13-1055 CT (51.0%). We evaluated the influence of these mutations on the ability of maternal mononuclear cells to produce the cytokines of interest, following stimulation by mitogens. Finally, we determined maternal and fetal plasmatic levels of IgM, IgG and cytophilic isotypes IgG1 and IgG3 directed against recombinant proteins from the MSP1, MSP2, MSP3, AMA1 and / or GLURP antigens, which are candidates for inclusion into a multivalent vaccine against malaria. The analysis of the relationships between i) maternal cytokine gene polymorphisms, ii) maternal cytokine and related antibody production, and iii) fetal specific antibody production, may help to understand the strength of the mother and child immunological interactions during pregnancy, depending on the presence or not of a plasmodial placental infection.



### INFLUENCE OF IPT ON THE ACQUISITION OF ANTI-VAR2 CSA ANTIBODIES IN HYPOENDEMIC ZONE

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The susceptibility of pregnant women to malaria is mainly related to an immuno-modulation related to pregnancy, an adhesion of *Plasmodium* strains to chondroitin-A sulfate of syncytiotrophoblast and the selection of strains resulting in it. In an endemic zone, studies showed that susceptibility to malaria depended on gravidity. In a hypo-endemic zone this placental antimalarial immunity is very slow in taking shape because transmission is not a continuous process and susceptibility seems to be more related to age than parity. Senegal adopted in June 2003, on the recommendations of WHO, the (IPT) through the sulfadoxine-pyrimethamine (SP) combination. However, IPT is somewhat limited because of some factors which could be related to the development of *P. falciparum* resistance to SP and to the low percentage of women who take 2 SP doses. The general objective of this study is to determine the impact of TPI on the acquisition of antimalarial antibodies. 101 women were recruited between the first and the second term of pregnancy from September to December 2008. For each woman, blood sample collections were performed at inclusion and delivery as well as following each fever attack. After centrifugation, IgGs Anti-P MSP1, GLURP and Var2CSA (DBL5) were proportioned in the serums through ELISA test. Among the 101 women included in the study, 18 disappeared as they did not deliver at the hospital maternity. The anti-MSP1 and anti-GLURP antibodies were determined in 101 women at inclusion and 83 women at delivery. Specific Anti-P VAR2CSA were proportioned in 82 women both inclusion and delivery. During all the follow-up exercise, only one woman presented a thick drop positive. The IgGs anti MSP1 and anti GLUR did not undergo any significant variations between inclusion and delivery. However, a reduction in the percentage of women presenting these IgGs at delivery was observed. With regard to anti DBL5 IgGs, no significant difference between the primigravida and the multigravida was noted both at inclusion and delivery. On the other hand, a significant reduction of these IgGs was noted between inclusion and delivery. In conclusion, these results confirm that IPT reduces malaria incidence in pregnant women. The reduction in the rate of the anti CSA antibodies at delivery and the lack of significant difference between multigravida and primigravida seem to delay immunity acquisition against malaria during pregnancy.

### THERAPEUTIC TARGETING OF NUCLEIC ACID-SENSING TOLL-LIKE RECEPTORS PREVENTS CEREBRAL MALARIA

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Toll-like receptors (TLRs) and their outputs, pro-inflammatory cytokines, and pro-inflammatory mediators. With this in mind we assessed E6446, a small molecule antagonist for TLRs 7 and 9. Herein, we describe the protective effect and mechanism of action of E6446 on *Plasmodium berghei* ANKA induced cerebral malaria (CM). *In vitro*, E6446 inhibited the activation of human and mouse E6446 in a dose dependent manner. Furthermore, therapy with E6446 diminished the *in vivo* cytokine responses of dendritic cells to TLR9 ligands or *Plasmodium* infection and prevented severe signs of CM, such as limb paralysis, brain vascular leak and death. Therefore, we

provide novel insights into how TLRs are involved in malaria pathogenesis and show that interference with nucleic acid sensing TLRs is a promising strategy to prevent deleterious pro-inflammatory responses mediating malaria severity. have been implicated in the pathogenic basis of malaria. We had previously shown that the nucleic acid sensing TLR9 is a key receptor that initiates pro-inflammatory responses during malaria leading to septic shock symptoms. We, thus, believe that interference with TLR function will, in all likelihood, render better clinical outcomes by preventing excessive release

### ANTIBODIES TO PLASMODIUM FALCIPARUM BLOOD-STAGE ANTIGENS BUT NOT CIRCUMSPOROZOITE PROTEIN PERSIST IN THE ABSENCE OF MALARIA TRANSMISSION

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As the campaign for malaria eradication widens, more areas will achieve low or absent transmission of *Plasmodium falciparum*. However, little data exists on how absence of transmission affects the immune responses to malaria. Such data are important for consideration of a population's epidemic risk after successful interventions and for assessment of potential differences in vaccine immunogenicity and efficacy. We documented possible interruption of malaria transmission in two highland areas of Kenya from 2007-2008. To characterize changes in immunity in this population, we measured antibody frequencies to eight *P. falciparum* vaccine candidate antigens, just before interruption of transmission and one year later. Testing for immunoglobulin G (IgG) antibodies was performed by multiplex cytometric bead assay (CBA) and ELISA in 1000 randomly selected individuals from the two sites (Kipsamoite, n = 457, Kapsisiywa, n = 543) in May, 2007 and July, 2008. Antigens tested included AMA-1, CSP, EBA-175, GLURP, LSA-1, MSP-1, MSP-3, and TRAP. None of the 1000 individuals had an episode of clinical malaria during this time period. For all antigens, antibody frequencies increased with age and were higher in the area of historically higher malaria transmission (Kapsisiywa). In both areas, frequencies of IgG antibodies to antigens other than CSP showed minimal decreases over the one-year period of absent transmission, but frequencies of IgG antibodies to CSP decreased significantly (CSP, Kipsamoite, 30.6% vs. 22.1%; Kapsisiywa, 38.9% vs. 24.3%, P < 0.0034, P < 0.0001 respectively). In conclusion, interventions that dramatically reduce or eliminate malaria transmission have differential effects on IgG antibodies to *P. falciparum* antigens. IgG antibodies to blood-stage antigens persist in the absence of malaria transmission, but antibodies to the pre-erythrocytic antigen CSP, the antigen used in the most successful malaria vaccine to date, wane rapidly.

### THE EFFECT OF INTERMITTENT PREVENTIVE TREATMENT (IPT) DURING PREGNANCY WITH SULPHADOXINE-PYRIMETHAMINE (SP) ON PLASMODIUM FALCIPARUM-SPECIFIC IGG ISOTYPIC ANTIBODY LEVELS IN PAIRED MATERNAL-CORD BLOOD

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A successful regime of IPT could decrease exposure to malaria during pregnancy and antibody titres to malarial antigens could decline, leaving women more susceptible to malaria and consequently decrease transplacental transfer of immunity to malaria in their newborns. We

investigated the influence of IPTp/SP on the levels of *Plasmodium falciparum* specific IgG and its subclasses (1 - 4) in mothers and their newborn babies. IgG levels to *P. falciparum* crude blood stage antigens were determined using Enzyme-linked Immunosorbent Assay (ELISA) in 270 paired maternal-cord blood samples collected at delivery from women who attended the Mutengene medical centre, Cameroon from March-October, 2007. The use of SP/dosage were documented. All four IgG subclasses were transferred across the placenta. The mean values and hierarchy of cord/maternal concentration ratios of IgG subclasses were as follows: IgG1 (1.03) > IgG3 (0.98) > IgG4 (0.92) > IgG2 (0.81) indicating a preferential high transfer rate for IgG1 and a low for IgG2. Also, IgG1 levels were significantly higher ( $t = -7.223$ ;  $p < 0.001$ ) in cord ( $3.62 \pm 0.38$ ) than its corresponding maternal blood ( $3.53 \pm 0.40$ ). Women who took two or more SP doses ( $3.42 \pm 0.55$ ) had lower ( $t = 2.791$ ;  $p = 0.006$ ) plasma levels of *P. falciparum* specific IgG compared to those who had taken one dose ( $3.60 \pm 0.40$ ) during pregnancy. Similarly, neonates from women who had two or more SP doses ( $3.40 \pm 0.56$ ) had lower ( $t = 2.428$ ;  $p = 0.015$ ) plasma levels of IgG compared to those from who had taken one dose ( $3.56 \pm 0.40$ ) during pregnancy. In addition, IgG1 levels were significantly lower ( $t = 2.596$ ;  $p = 0.01$ ) in cord blood of neonates born to mothers who had taken two or more doses ( $3.54 \pm 0.49$ ) compared to those whose mothers had taken one dose ( $3.68 \pm 0.35$ ). Two or more doses of SP taken during pregnancy is associated with lower anti-malarial IgG levels in the mother and IgG I levels in newborns are particularly affected. Future studies are needed to evaluate the impact of IPTp/SP on development of maternal and infant immunity in malaria endemic areas.

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### CYTOKINE PROFILES AND HEMATOLOGICAL CHANGES ACCOMPANYING CLINICAL DISEASE IN *PLASMODIUM VIVAX* AND *P. FALCIPARUM* UNCOMPLICATED MALARIA

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The balance between pro- and anti-inflammatory cytokines may be important in malaria presentation and outcome. Over the past few decades, a literature has emerged that argues for most of the pathology seen in malaria being explained by activation of the inflammatory system, with the balance between the pro and anti-inflammatory cytokines being tipped towards the onset of systemic inflammation. However, the respective roles played by the different cytokines in humans during acute malaria episodes remain unclear. The aim of this study was to investigate the hematological changes and cytokine profiles in a group of patients infected with *Plasmodium vivax* and *P. falciparum* at the day of diagnosis before treatment (D0) and 2 weeks later (D15). As a result, a complete blood count and plasma cytokines levels were measured in patients suffering from an uncomplicated *P. falciparum* (n=24) and *P. vivax* (n=45) malaria and uninfected individuals (n=12). In our study, at the day of diagnosis patients with *P. falciparum* and *P. vivax* had thrombocytopenia, leucopenia and an increased number of band cells returning to normal levels at D15. The parasitemia was similar in *P. falciparum* ( $4604 \pm 4630$  parasite/ul) and *P. vivax* ( $3020 \pm 3411$ ) infections. The cytokines IL5, IL7, IL13, MCP-1 and GM-CSF was absent from most plasma samples at the day of diagnosis. In contrast, high levels of IL6, IL8, IL17, TNF $\alpha$ , IFN $\gamma$  and Mip-1b were present in nearly all individuals. In *P. falciparum* plasma levels of IL12 and IL1 $\beta$  was elevated at D0 and IL4 at D15. Interesting IL-10 levels were high at D0 in both *P. falciparum* and *P. vivax* and decreased at D15. Our preliminary data shows that the cytokine profile in *P. falciparum* and *P. vivax* uncomplicated malaria is similar with elevated plasma levels of both pro and anti-inflammatory cytokines. Analysis on the levels of nitric oxide and inflammatory markers such as acute phase proteins and their correlation with the cytokine profile are in progress.

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### GENETIC MARKERS AND RISK OF MALARIA INFECTIONS: GENETIC-EPIDEMIOLOGY STUDY IN A LOW MALARIA ENDEMIC AREA OF SRI LANKA

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Malaria transmission is seasonal and unstable in the dry zone of Sri Lanka and the levels have steadily declined over the past 15 years. This is a follow up of an immuno-epidemiological study conducted in 8 villages in the district of Moneragala, a known endemic area of Sri Lanka with transmission of predominantly *P. vivax* (>80%) and *P. falciparum*. The original study was a cohort study with active case detection of 1,951 individuals during 1992/93. All clinical data including malaria attacks and parasite densities were recorded during that period. In year 2006, 1133 of these individuals were traced, blood collected and past history of malaria during last 15 years recorded. DNA extracted from whole blood and SNPs in selected genes related to humoral immune-response investigated. Serum separated for serological investigations and titers of antibodies against; AMA1, MSP1, MSP2, NANP, Pv\_AMA1 and Pv\_MSP1 together with total IgE determined. SNP data analyzed in relation to past history of malaria attacks and serum antibody levels. A total of 169 SNPs were typed in 1008 study subjects. After sample and genotype quality control, 118 SNPs in all subjects were analyzed. Allele frequencies in 2 SNPs in 2 genes found to be significantly different between those who have experienced repeated malaria attacks and those with apparent protection ( $p < 0.05$ ; Chi-square test). When antibody levels were classified into low-high binary trait, significant association was found in 4 SNPs for AMA1; 2 for MSP1 (none for MSP2); 8 for NANP; 3 for Pv-AMA1; 7 for Pv-MSP1; and 9 for IgE. None of the SNPs had any significant association with all tested antibodies. Preliminary evidence is in favour of a genetic basis for susceptibility to or protection against malaria infection in this population, which may or may not have links with the generation and/or maintenance of anti-malarial antibodies, the levels of which appear to be maintained in spite of low malaria transmission levels.

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### CELLULAR IMMUNOLOGICAL RESPONSES IN PREGNANCY-ASSOCIATED MALARIA

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Pregnancy-associated malaria (PAM) due to *Plasmodium falciparum* is detrimental to both mother and child. Ongoing anti-PAM vaccine development focuses on the induction of antibodies targeting VAR2CSA, a parasite-derived protein expressed on the surface of infected erythrocytes that sequester in the placenta, since naturally-acquired anti-VAR2CSA IgG titres increase in a gender-specific and parity-related way, and PAM shows a concomitant parity-related decrease in incidence. These findings imply a protective function for antibody responses. In contrast, a defined role for VAR2CSA-specific T cell responses is unclear and remains largely unexplored. We are conducting a longitudinal, prospective study of 1000 pregnant mothers in Korogwe, north-eastern Tanzania. For a subgroup of mothers with and without evidence of *P. falciparum* infection, *ex vivo* frequencies of the T cell, B cell, monocyte, regulatory T cell and dendritic cell populations are being measured at inclusion and at delivery.

Cytokine activity of isolated peripheral blood mononuclear cells is assessed following short-term stimulation *in vitro* with either VAR2CSA-specific reagents or *P. falciparum*-infected red blood cells. Cord blood mononuclear cells isolated at delivery are assessed in a similar way in order to determine the extent of sensitization to *P. falciparum* antigens *in utero*. For comparative purposes, *P. falciparum*-infected women are matched to uninfected women based on age, gestational age and gravidity. We have completed assays on samples collected at inclusion and data analysis is ongoing. The collection of samples at delivery is still ongoing. The focus of the results presented will be on the *ex vivo* phenotyping of T regulatory cells and the *in vitro* T cell responses to VAR2CSA-specific reagents. We will compare and contrast our cellular immunological findings with those from an identical study that is being conducted in parallel in southern Benin, in an area where malaria transmission is both more intense and perennial rather than seasonal.

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### EFFECT OF ARTEMETHER ON THE EXPRESSION OF GENES INVOLVED IN THE MOSQUITO IMMUNE RESPONSE TO PLASMODIUM INFECTION

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Malaria is a vector-borne disease that still remains, to our days, as one of the major causes of mortality worldwide. At present, there is still no effective prevention and control measure, so it is necessary to develop new strategies for controlling Malaria transmission. We are trying to find out the effective drug, which can not only kill the malaria parasite in the human host, but also block the development of malaria parasite in the mosquito. Recently, Anti-malarial drugs have played a key role in controlling the spread of malaria. Although the effect of anti-malarial drugs on mosquito immunity has been recently improved, nothing is known about the impact of artemether, one of artemisinin derivatives, on mosquito immunity. Artemether which is the recommended first-line treatment is a potent and quick acting anti-malarial, used for treating chloroquine resistant *falciparum* malaria, including cerebral malaria. In order to characterize the influence of artemether on the mosquito immune system, we have analyzed the effect of artemether on *Anopheles stephensi* six important immune-related genes expression using semiquantitative PCR, and the activity of PO enzyme in uninfected and *Plasmodium yoelii* infected *Anopheles stephensi*. We have demonstrated for the first time that fed on *Anopheles stephensi* 632ng/ml artemether, according to pharmacokinetic study, we chose the highest the plasma concentration of artemether in human bodies. Our results showed artemether significantly down-regulated the expression of serine protease1 (AsSP1), serine protease2 (AsSP2), serine protease inhibitor(AsSNP), nitric-oxide synthase(AsNOS), thioester-containing protein 1(AsTEP1), Prophenoloxidase(AsPPO), and PO enzyme activity, which are necessary for interrupting *Plasmodium* development during infection *Anopheles stephensi*, in different degrees. We found that artemether could increase *Plasmodium* oocyst counts 1-3 times to untreated *Anopheles stephensi*. These results suggest that artemether might act on *Anopheles* serine proteases cascade and synthesis of nitric oxide at the transcriptional level. Understanding the mechanism artemether of action in mosquito vector and vertebrate hosts will reveal biological details that can be fruitful for novel malaria control strategies such as those based in transmission-blocking vaccines.

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### EVALUATION OF MALARIA PREVENTION STRATEGY DURING PREGNANCY IN NDOLA, ZAMBIA

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Malaria in pregnancy is associated with many negative outcomes for the pregnant woman, fetus and neonate. Intermittent Preventive Treatment during pregnancy (IPTp) using 3 doses of Sulfadoxine-Pyrimethamine

(SP), Insecticide Treated mosquito Nets (ITN) and Indoor Residual Spraying (IRS) are the main strategies used to prevent malaria. The aim of this study was to evaluate the effectiveness of these strategies on the reduction of malaria prevalence in pregnant women, five years after their implementation in Ndola, Zambia and to make recommendations on how prevention can be improved. We had ethical approval from Tropical Disease Research Centre and Stellenbosch University's Human Research Ethics Committee. A questionnaire on socio-demographic information, history of malaria during current pregnancy and malaria prevention strategies used, was administered to 450 consecutive patients admitted in the labour ward of 3 local clinics. Information was collected from the antenatal cards concerning the last menstrual period, date of taking each dose of SP, gravidity, and HIV status. A blood slide to detect *Plasmodium* was collected from each woman in labour ward. 2.4% of participants had a positive blood slide at term and 15.8% reported malaria during pregnancy. All the participants took at least one dose of SP, 87.6% completed the stipulated three doses. The mean gestational age for delivery of each dose was 22.1 (SD 4.6), 29.1 (SD 4.4) and 34.4 (SD 3.9) weeks for the first, 2nd and 3rd dose respectively. 79.5% had an ITN, but only 74.1% used it regularly. Only 23.4% used commercial insecticide. In conclusion, the measured malaria prevalence was remarkably low although, the self-reported malaria rate was still high. The national target for IPTp access was exceeded, but the timing of delivery of each dose of SP needs improvement and so is the utilization rate of ITN with more sensitization of health workers and the community. The national policy on use of quinine in pregnancy may need revision with view of using it throughout pregnancy due to current SP resistance level. IRS had been completed in all the 3 clinics catchment areas.

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### IMPLEMENTING A CAMPAIGN TO DISTRIBUTE NINE MILLION FREE LLIN'S TO CHILDREN UNDER FIVE YEARS IN TANZANIA

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Tanzania launched a national voucher program in 2004 to provide pregnant women and infants with subsidized insecticide-treated nets (ITNs). Three years later, 24.8% of Tanzanian children <5 years of age were sleeping under an ITN (only 12.9% of the lowest wealth quintile). In 2008, the Ministry of Health and Social Welfare (MoHSW) initiated a national campaign to rapidly and equitably deliver a free long-lasting insecticidal net (LLIN) to every child <5 years of age in Tanzania. The ITN Cell, a Swiss-funded unit within the MoHSW's National Malaria Control Program (NMCP), coordinated the campaign. Government contractors trained and facilitated local government officials to supervise village-level volunteers to conduct a house-to-house registration of all children under 5 years. The registration formed the basis for the LLIN factory order and delivery to village level. Caregivers brought their registration coupons to LLIN issuing posts during a 3-day period. Five district-representative rapid household surveys (two-stage cluster sampling) assessed household ownership of an ITN and ITN use among children <5 approximately one month following a hang-up campaign. Nine donors contributed to the national campaign, purchasing 9.2 million Olyset LLINs (4x6x7 ft) at a cost of \$7.51/LLIN, including delivery and all campaign-associated activities. The campaign started March '09 and ended May '10. Household (n=1,483) surveys found ITN ownership of at least one ITN ranged from 61-82%. Overall, use among children <5 was 48.0% and 62.2% in the first and second zones, respectively. ITN use generally increased across all wealth quintiles, but regional variation was detected. Despite providing free LLINs to all children <5 years of age and substantially increasing household ownership,



use did not rise as high as anticipated. The campaign addressed issues of equity, but not across all regions. Additional strategies will be needed to address the gap between ITN ownership and use.

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### A SIMPLE COLORIMETRIC TEST FOR THE RAPID DETECTION OF TYPE 2-PYRETHROIDS ON BED NETS AND ON SPRAYED WALLS

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Insecticide treated nets and indoor residual spraying of insecticides (IRS) are used as the major modes of intervention in the fight against malaria. Measuring the actual amount of deposits of insecticides on nets and on walls is essential for evaluation of quality control of the applied intervention as per instruction. Currently such information can only be provided by costly, chromatography techniques or through technically demanding bioassays, both requiring sophisticated laboratory facilities. We have developed a rapid, field friendly / cost effective colorimetric test that can be carried out by individuals without specialised scientific training to estimate the amount of type 2 pyrethroids on the bed nets as well as to check for the compliance of IRS, as reported previously. These tests rely on the detection of cyanide using three inexpensive reagents. The tests are equally sensitive for deltamethrin,  $\alpha$ -cypermethrin and  $\lambda$ -cyhalothrin. Various types of the tests can be developed depending on choice of reagents and assay format e.g. microtitre plate, test tube, dipstick. Our simple test is performed in situ and leads to the formation of an orange-red colour whose depth will indicate semi quantitatively the amount of type 2 pyrethroid on the bed net and has been validated by measuring the amount of the extracted insecticide from parts of bed nets with HPLC, as reported previously. No interference to the formation of this colour has been found from soaps, possible degradation products of deltamethrin, insecticide binders, non-fast colour bleaching off the nets or charcoal. Prototype KITS of our test have recently undergone field evaluation in Rwanda and Tanzania. The final KIT will be widely available in the near future.

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### PERCEPTIONS ON THE USE OF INSECTICIDE TREATED NETS IN PARTS OF THE IMO RIVER BASIN OF NIGERIA: IMPLICATIONS FOR PREVENTING MALARIA IN PREGNANCY

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This study aimed at assessing perceptions on use of Insecticide Treated Nets (ITNs) in parts of the Imo River Basin, Nigeria and its implications in preventing malaria in pregnancy. Data was collected using focus group discussions, key informant interviews and structured questionnaires. Results showed high awareness on the benefits of ITNs. Factors affecting use of ITNs included its high cost, perceptions of chemicals used to treat them as having dangerous effects on pregnancy, low utilization of antenatal care, husband's lack of interest in malaria prevention and perceptions that adolescent girls are at low risk of getting malaria. The implications of these findings include demystifying the negative perceptions on the chemicals used for net treatment and subsidizing the cost of ITNs to increase access. These findings provide important lessons for malaria programmes that aim at increasing access to ITNs by pregnant women in developing countries.

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### EVALUATION OF SENEGAL'S FIRST NATIONWIDE CAMPAIGN TO DISTRIBUTE LONG-LASTING INSECTICIDE-TREATED NETS (LLINS)

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In 2009, the first national LLIN distribution campaign in Senegal resulted in the distribution of 2.3 million LLINs in two phases. Door-to-door teams visited all households to administer vitamin A and mebendazole, and to give a coupon to children <5 to later redeem for an LLIN. We conducted a nationwide two-stage cluster survey, with clusters selected within regions by probability proportional to size sampling, followed by GPS-assisted mapping, simple random selection of households in each cluster, and administration of a questionnaire on PDA. The questionnaire followed the Malaria Indicator Survey format, with rosters of household members and bednets, and questions on campaign participation. We surveyed 3,302 households representing 33,222 people. At least one insecticide-treated net (ITN) was present in 82% of all households, 89% of households with a child < 5 years and 57% of households without a child < 5 years. Just over half (53%) of ITNs had been received during the campaign. In 60% of households at least one ITN was hanging the previous night. Considering possible indicators of universal coverage, 40% of households had at least one ITN per two people, 22% had at least one ITN per sleeping space and 34% of the general population slept under an ITN the night before the survey. In addition, 45% of children < 5 years, and 49% of pregnant women had slept under an ITN. Most (92%) of guardians of eligible children had heard about the campaign, 34% from a health agent, 26% from a neighbor and 22% by radio. Campaign coverage was 93% for mebendazole, 95% for vitamin A, and 83% for LLINs. Almost all (91%) LLINs received during the campaign remained in the household; of those not remaining 74% had been given away and none were reported sold. The nationwide integrated LLIN distribution campaign successfully reached its target population. It allowed household ITN ownership to surpass the RBM target of 80% set for 2010 and contributed substantially to universal coverage, though work remains to reach Senegal's goal of 80% utilization in the general population.

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### MODELING AND SIMULATION TO EXPLORE THE FACTORS INFLUENCING THE IMPACT OF TREATMENT OF PLASMODIUM FALCIPARUM CARRIERS WITH ARTEMETHER-LUMEFANTRINE ON DISEASE TRANSMISSION

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Computer modeling of malaria is important for assessing key factors that may impact the effectiveness of a proposed intervention strategy. Though the range of model complexity can be quite wide, relatively simple models that can run on laptop computers are informative and provide insights that

were not intuitively apparent. This is highlighted for the evaluation of a study to assess the impact of detecting and treating asymptomatic carriers (AC) of *Plasmodium falciparum* with artemisinin combination therapies (ACT) through scheduled community screening campaigns (CSC). A deterministic model of parasite vector and host populations dynamics developed by Okell et al (2008) in order to explore the impact of ACTs on malaria prevalence, was coded in Matlab (Newton, USA). The model was modified to represent different settings of malaria transmission (intensity and seasonality) and use of AL as treatment of AC and of clinical malaria episodes. Simulations of CSC were assessed for number and interval that showed the greatest impact on malaria reduction in these different settings. Conditions that significantly extend any effect of this intervention were assessed. The transmission intensity in the simulated region was the most important factor affecting reduction in malaria incidence after the intervention. The timing of CSCs and the interval between them were also important criteria. Short intervals between CSCs allowed the capture of cases that would have been missed earlier due to disease latency. If transmission intensity is low and markedly seasonal, a single round of intervention can show persistent effects for multiple years, which gradually taper off. Perennial transmission would not allow a sustained effect. The simulation results identified factors that have the greatest impact on study results in a study of treating asymptomatic carriers. The timing of CSCs relative to the pattern of malaria transmission is important for maximizing the intervention impact. The intervention will have immediate effect in regions with marked seasonality and moderate transmission intensity.

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### SYSTEMATIC SCREENING FOR AND TREATMENT OF ASYMPTOMATIC CARRIERS OF *PLASMODIUM FALCIPARUM* MALARIA WITH ARTEMETHER-LUMEFANTRINE (AL) IN A COMMUNITY SETTING TO REDUCE DISEASE TRANSMISSION: A CLUSTER RANDOMIZED, SINGLE-CENTER, CONTROLLED, 12-MONTH PROSPECTIVE STUDY IN AFRICA

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Treatment of asymptomatic carriers (AC) of *Plasmodium falciparum* with artemisinin combination therapies should reduce the parasite reservoir and impact transmission. A 12-month follow-up, open label, controlled, prospective, cluster randomized study was designed to evaluate the effect of such an intervention at community level on the malaria pattern in a Sahelian country with a 6 month transmission season. The study population will consist of all consenting inhabitants of randomized clusters. A total of 18 clusters (ca 8000 subjects) will be randomized (1:1) to an intervention and a control arm. Community screening campaigns (CSCs) will be conducted: Systematic screening of the study population by RDT and microscopy (MC), followed by AL treatment (intervention) or screening of a population subset by MC alone (delayed reading), without treatment (control). Three CSCs will be performed at around bi-monthly intervals before the malaria transmission season, with a final CSC at study end (no treatment). Universal bed-net coverage will be provided. RDT confirmed malaria episodes occurring at any time during the study (in either arm) will be treated with AL (or alternative treatment). Primary objectives are to assess (1) the number of confirmed malaria episodes per person-year in pediatrics (i.e. <5 years of age) over

12 months and in intervention vs. control arm (2) the direct benefit in ACs by measuring their hemoglobin level change from baseline to D 28 in treated (intervention) vs. not-treated (control) AC diagnosed at first CSC. Secondary objectives: assessment of incidence of confirmed malaria episodes in the whole population and in individuals diagnosed as AC; mortality and hospitalization (all-cause and for malaria); severe malaria, proportion of asymptomatic carriers and gametocyte carriers (confirmed by MC) over time, adverse events, serious adverse events. If the strategy is proved effective, it may be considered by public health policymakers to make a significant contribution to the multifaceted approaches of surveillance strategies being implemented by malaria control programs across Africa.

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### THE EFFECT OF ARTEMETHER-LUMEFANTRINE ON GAMETOCYTE CARRIAGE IN *PLASMODIUM FALCIPARUM* MALARIA

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*Plasmodium falciparum* gametocytes are not affected by most antimalarials, except the amino-8-quinolines. The artemisinin-based combination therapies (ACTs) have been shown to reduce gametocyte carriage and transmission in areas of low endemicity. The effect of artemether-lumefantrine (AL) as per current dosing recommendation on gametocyte carriage was analysed in data pooled from 7 studies (2 in Africa, 3 in South-Asia, 1 in South America and 1 in non-immune travellers from Europe and Colombia). In all studies there was a marked reduction of the gametocyte carriage in patients treated with AL. This can be explained by a direct gametocytocidal effect of artemether and its metabolite as well as the rapid killing of asexual stages of *P. falciparum*. We will present an analysis of published data, comparing the effect on gametocyte carriage of AL with other ACTs such as dihydroartemisinin-piperazine, artesunate-amodiaquine and artesunate mefloquine. We will also describe the potential factors (age, gender, baseline parasite count, anaemia etc) associated with gametocyte carriage following treatment with these ACTs.

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### MALARIA VECTOR CONTROL USING INDOOR RESIDUAL SPRAYING WITH DDT IN ARUSHA REGION, TANZANIA: A COMPARISON OF COMMUNITY AND GOVERNMENTAL VIEWS ON THE PERCEIVED BARRIERS PREVENTING A HIGH LEVEL OF COMMUNITY UPTAKE AND WIDER IMPLEMENTATION

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Malaria causes over a million deaths worldwide annually. The World Health Organisation (WHO) estimates that 91% of these deaths occur in Africa. In Tanzania, malaria accounts for 30% of the national diseases burden posing a significant impediment to social and economic development. Indoor residual spraying (IRS) with dichloro-diphenyl-trichloroethane (DDT) was successfully used worldwide to reduce malaria transmission from

1940-70. Perceived health worries about DDT led to its decline in use. The Government of Tanzania banned DDT (1991) and severe restrictions were placed on DDT by the Stockholm Convention on Persistent Organic Pollutants (2001). In 2006, the WHO realised the potential of IRS with DDT to combat malaria and endorsed/actively promoted its use. Despite WHO recommendations, the Government of Tanzania has failed to reintroduce IRS with DDT, even though advocated in the National Guidelines for Integrated Malaria Vector Control (NGIMVC) (2008). The aims and objectives of this study were to assess the public's current knowledge of malaria, the community and governmental perceived barriers to the reintroduction of IRS with DDT, and community ideas of how to reintroduce IRS with DDT to Arusha Region. Qualitative research, including 16 interviews with community members, 1 focus group discussion and 5 interviews with government officials was undertaken. Data was analysed using thematic analysis. Community members had good knowledge about malaria transmission and vector control. All interviewees claimed that IRS was not used but believed the government should reintroduce IRS with DDT with adequate education and precautions. Several government officials claimed IRS was being performed but that before DDT could be used it needed to be approved by the Ministry of Health, despite the strong advocacy of DDT use in the NGIMVC. With the use of IRS with DDT in conjunction with other techniques (insecticide treated nets, public education) provided through a Malaria Control Unit, Tanzania could reduce the burden of malaria.

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### TRACKING WEEKLY NET USE IN KONGWA, TANZANIA

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The use of long-lasting insecticidal nets (LLINs) is one of the principal interventions to prevent malaria in young children. Prioritizing young children for net use is important to achieve mortality reductions, particularly during transmission seasons. The aim of this study was to measure patterns of net use within households before, during and after the rainy season in a rural area of Tanzania. Data collection was carried out from January to July 2009 as part of the PRET+ antibiotic study, a randomized, community-based trial to determine the effect of a single mass administration of Azithromycin on community prevalence of malaria, diarrheal diseases, and other diseases, including longitudinal surveillance of 1040 households on a weekly basis. Households were asked to list all children 6 years of age or younger and their mothers who had slept under a net the previous night. Weekly data was compiled for each member of the household and analyzed by age and for correlation with the rainy season, which lasted from mid-January through April. In July, an exit survey was conducted with each household, in which behavioral questions and more detailed questions about net shape and size were asked. Net ownership among households was 51.5%, and among these, net use was very high. Reported use of nets rose quickly as the rainy season began and remained high through the end of the study period in July. Younger children (0-2 years) were prioritized, reaching a steady rate of 93% use throughout the study period. Children 3 and 4 years old had use rates in the 80% range, while 5 and 6 year olds reached only 50-60% use. Net use was not affected by the net's shape, by the number of nets in the household, or the presence of holes in the net. Ninety-three percent of net-owning households reported noticing fewer fevers among their children since they obtained a net, and 94.5% said they used nets in order to prevent malaria. In this area of Tanzania, net use is very high among net-owning households, especially during the rainy season and continuing through the post-rainy season high transmission period. The youngest children are prioritized for sleeping under the net.

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### DO INSECTICIDE TREATED NETS PROTECT AGAINST MALARIA INFECTION IF THEY HAVE HOLES?

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Pyrethroid insecticide treated nets (ITN) are one of the most effective and widely used means of malaria prevention. In areas where *Anopheles* mosquitoes are no longer susceptible to pyrethroid insecticide the effectiveness of ITNs may be compromised. Mass distribution of ITNs was carried out in Equatorial Guinea in 2007 as part of comprehensive malaria control activities. High frequencies of the resistance associated *kdr* gene in *An.gambiae* populations have been observed in both the continental and island regions of Equatorial Guinea. The ownership, use and condition of ITNs, and the prevalence of infection with malarial parasites in children were monitored through annual malaria indicator surveys. The condition of nets was classified with respect to whether they were long lasting, treated or untreated, and whether they were intact, with small holes or with large holes. Infection in children was analysed in relation to whether the child slept under a net and the condition of the net. Results show that prevalence of infection is associated with net condition, with children who slept under treated nets with holes having a higher risk of infection than those who slept under treated nets that were intact. If confirmed, this finding may be an indication of the epidemiological impact of insecticide resistance on the effectiveness of pyrethroid based vector control.

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### PLACENTAL MALARIA IN PREGNANT WOMEN USING ITN/LLIN AND IPT AS CONTROL MEASURES IN THREE SELECTED TOWNS OF SOUTHEAST NIGERIA

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In recent years ITN/LLIN and IPT have been considered appropriate measures to help reduce or prevent Malaria infections in pregnant women. This study was carried out on 844 pregnant women in Afikpo, Okigwe and Umuahia towns of Southeast Nigeria to evaluate the role of ITN/LLIN and IPT in Malaria control. The Placentas of consenting women were obtained post delivery (following Ethical clearance by relevant authorities) and histological sections were prepared, stained and observed under the microscope for *Plasmodium* parasites. Of the 844 women examined, 225 (26.7%) used ITN/LLIN, 276 (32.7%) used IPT while 343 (40.6%) used other measures. The ITN/LLIN group had 36.9% infection with 83 of 225 infected. The IPT group had 39.1% infection with 108 of 276 infected while those who used other measures had 216 of 343 (63%) infected. The difference between the ITN/IPT group and the other measures group was statistically significant ( $P < 0.05$ ). There was also variations between the towns with Okigwe having the lowest infection of 27.3% among those using ITN/LLIN and IPT while Umuahia had the highest, 48.3% with ITN/LLIN and 70.8% with IPT. The significance of these results was discussed in relation to Malaria in pregnancy.



## EFFECT OF INCENTIVES ON INSECTICIDE-TREATED BED NET USE IN SUB-SAHARAN AFRICA: A CLUSTER RANDOMIZED TRIAL IN MADAGASCAR

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Insecticide-treated bed nets (ITNs) have been shown to reduce morbidity and mortality due to malaria in sub-Saharan Africa. Traditional ITN distribution campaigns have focused on education as a means of driving demand for ITNs, but behavioral incentives for ITN use could be more efficient. To date, behavioral incentives have been studied mostly in developed countries, and no study has yet looked at the effect of incentives on the use of ITNs. Reported here are the results of a cluster randomized controlled trial testing household-level incentives for ITN use following a free ITN distribution campaign in Madagascar. The study took place from July 2007 until February 2008. Twenty-one villages were randomized to either intervention or control clusters. Households in both clusters received a coupon redeemable for one ITN. After one month, intervention households received a bonus for ITN use, determined by visual confirmation of a mounted ITN. Data were collected at baseline, one month and six months. Both unadjusted and adjusted results, using cluster specific methods, are presented. At baseline, 8.5% of households owned an ITN and 6% were observed to have a net mounted over a bed in the household. At one month, there were no differences in ownership between the intervention and control groups (99.5% vs. 99.4%), but net use was substantially higher in the intervention group (99% vs. 78%), with an adjusted risk ratio of 1.24 (95% CI: 1.10 to 1.40;  $p < 0.001$ ). After six months, net ownership had decreased in the intervention compared to the control group (96.7% vs. 99.7%), with an adjusted risk ratio of 0.97 ( $p < 0.01$ ). There was no difference between the groups in terms of ITN use at six months; however, intervention households were more likely to use a net that they owned (96% vs. 90%;  $p < 0.001$ ). In conclusion, household-level incentives have the potential to significantly increase the use of ITNs in households in the short-term, but, over time, the use of ITNs is similar to households that did not receive incentives. Using incentives to target vulnerable populations may be even more cost-effective. Providing incentives for behavior change is a promising tool that can complement traditional ITN distribution programs and improve the effectiveness of ITN programs in protecting vulnerable populations in the short-term. Further study of the cost-effectiveness of these incentives and their longer term effects is warranted.

## IDENTIFICATION OF NOVEL COLOR VARIANT IN THE ANOPHELES ARABIENSIS PATTON (DIPTERA: CULICIDAE)

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Sufficient malaria vector control has proved difficult over the past years. The traditional first line of attack on malaria, killing the mosquitoes, initially proved effective, but was soon hampered by development of insecticide resistance. To circumvent this, a new genetic strategy is being developed which involves vector replacement with genetically modified Vectors (GMV) incapable of supporting development of malaria parasites. Transgenic technologies make use of markers for genetic mapping and manipulation. We have isolated color variants in *Anopheles arabiensis* Patton. The aberration is apparently sex-limited to females. To determine inheritance pattern, we attempted to purify a stock homozygous for black spot (Bs) by selection of spotted females and crossing them to sibling males but the attempt was unsuccessful. When we crossed Bs females to sibling males, serendipitously, a surge of 10% lethal mutation resulted from these crosses. Mutant larvae were characterized by black pigmentation of entire body and larval development progressed normally but died at larval-pupal ecdysis. Among the F1 progeny resulting from

the crosses between Bs females and wild-type males, melanotic lethal mutants were completely absent and penetrance of 44% was observed. Our preliminary data from the above crosses superficially indicates that Bs is incomplete dominant and the aberration is lethal when homozygous. Further investigation involving genetic crosses to describe genotypes of individuals and inheritance pattern are in progress.

## SITE-SPECIFIC INTEGRATION AND EXPRESSION OF A PLASMODIUM FALCIPARUM RESISTANCE TRANSGENE IN ANOPHELES STEPHENSI

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We used the *phi C31* site-specific integration system to produce transgenic *Anopheles stephensi* lines that express two effector molecules designed to target the human malaria parasite *Plasmodium falciparum*. These effector molecules are composed of an antimicrobial peptide, *An. gambiae* Cecropin A, joined to a single-chain antibody (scFv) derived from a *P. falciparum*-specific monoclonal antibody. The M4B7 immunotoxin contains an scFv designed to recognize Pfs25, a surface protein expressed by ookinetes, while the M2A10 immunotoxin contains an scFv designed to recognize circumsporozoite protein, a protein expressed on the surface of sporozoites. Previously characterized *Anopheles cis*-acting DNA regulatory elements were included in the transgene to coordinate immunotoxin production with parasite development. While the *An. gambiae* carboxypeptidase gene regulatory elements stimulate M4B7 expression in females within the first 12 hours post blood meal (hPBM), the *An. stephensi* vitellogenin gene regulatory elements direct expression of M2A10 in females ~12-24 hPBM. Through Southern blot, fluorescent hybridization *in situ*, RT-PCR, and western blot analyses, we confirmed transgene integration and expression. Having produced four transgenic lines that each contain a single copy of the M4B7/M2A10 transgene integrated into a different genomic location, we were able to observe the affect of flanking genomic DNA upon expression of these two immunotoxin genes.

## CLONING OF THE BREAKPOINTS OF FIXED 2RO AND 2RP INVERSIONS IN THE ANOPHELES GAMBIAE COMPLEX

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*An. gambiae* is a major vector of malaria and it belongs to a complex of seven sibling species which are morphologically indistinguishable. However, their behavior, ecological adaptation, vectorial capacity and geographical distribution differ. Studying the phylogenetic relationships and comparative genomics among the members of the complex is crucial to understand the genetic changes of evolving traits. This can help us identify the evolutionary changes that can be related to the gain or loss of human blood choice during the evolution. It has been confirmed that the structure of breakpoints can clarify the direction of evolution. *Anopheles gambiae* and *An. merus* can be distinguished based on two overlapping inversion, 2Ro and 2Rp. In this study, the inversion breakpoints of 2Ro and 2Rp in *An. merus* and their homologous sequence in the outgroup species *An. stephensi* have been analyzed. Genes adjacent to inversion breakpoints had been identified. Four genes from the *An. gambiae* 2Ro inversion breakpoints, and four genes from *An. gambiae* 2Rp inversion breakpoints were labeled and used as probes to screen the *An. merus* phage library. The same genes were also used to screen the BAC library of the outgroup species *An. stephensi*. Positive phages and BAC clones were obtained from the proximal 2Ro and 2Rp breakpoints. Twelve phages and BAC clones have been isolated and sequenced. A phage

clone from the proximal 2Ro breakpoint was used for Fluorescent *In Situ* Hybridization (FISH) with *An. gambiae*, *An. merus* and *An. stephensi* polytene chromosomes. Our results from FISH analysis of the 2Ro breakpoint showed that the phage DNA hybridizes to both breakpoints in *An. gambiae* and to one breakpoint in *An. merus* and an outgroup species *An. stephensi*. The results demonstrated the common organization of the 2Ro breakpoint in *An. merus* and *An. stephensi*. Since the gene order is the same in the inversion breakpoint within outgroup species, we can conclude that the 2Ro inversion can be considered closest to ancestral in *An. merus* or the inversion have originated independently in *An. merus* and *An. stephensi*

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#### ALLELIC GENE STRUCTURE VARIATIONS IN HUMAN MALARIA VECTOR *ANOPHELES GAMBIAE*

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Malaria, transmitted by anopheline mosquitoes, kills more than one million people annually. *Anopheles gambiae* is the major malaria vector. Vector control is an efficient approach for malaria control. Malaria resistant or insecticide resistant mosquitoes have been observed in nature, and genetic variations underlie these phenotypes. This study focuses on allelic gene structure variations that change protein sequences, functions or regulation. By analyzing 235,971 *A. gambiae* ESTs, we found about 2,340 transcript structure variation events in 1,490 genes. About 78% of transcript structure variations were located within the coding sequence (CDS) regions, and >65% of variations at the CDS regions have the same open-reading-frame, which indicated that most transcript structure variations just insert or delete some amino acids or functional motifs without changing the whole protein structure. From the same set of ESTs, we detected 113,367 single nucleotide polymorphisms (SNPs) that were present in more than one EST. Using these multi-hit SNPs as tags, we discovered that more than 28% of transcript structure variation events were contributed by different gene alleles in *A. gambiae*. Furthermore, genome sequences from two dozen individual wild *A. gambiae* mosquitoes from Kenya confirmed that allelic gene structure variation plays a major role in transcript diversity in this important human malaria vector. The genes with allelic gene structure variations will be novel genetic markers for genome-wide direct-association studies of malaria resistance and insecticide resistance.

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#### GENOME-BASED MICROSATELLITE DEVELOPMENT IN *CULEX QUINQUEFASCIATUS* WITH BROAD APPLICATION TO THE *CX. PIPPIENS* COMPLEX

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Mosquitoes in the *Culex pipiens* complex are among the most medically important vectors for human disease worldwide and include major vectors for lymphatic filariasis and West Nile virus transmission. However, detailed genetic studies in the complex are limited by the number of genetic markers available. Here we describe methods for the rapid and efficient identification and development of single locus, highly polymorphic microsatellite markers for *Cx. pipiens* complex mosquitoes via *in silico* screening of the *Cx. quinquefasciatus* genome sequence. Six laboratory colonies representing four *Cx. pipiens* and two *Cx. quinquefasciatus* populations were utilized in the preliminary assessment of 35 putative loci identified within 16 *Cx. quinquefasciatus* supercontig assemblies (CpipJ1) containing previously mapped restriction fragment length polymorphism (RFLP) genetic marker sequences. We identified and validated 12 new microsatellite markers distributed across all three linkage groups that

amplify consistently in *Cx. pipiens* strains from Japan, Johannesburg, Mozambique, and North America. To increase genotyping efficiency we developed groups of 3-5 microsatellite loci each for multiplex-ready PCR. Field collections from three cities in Indiana were used to assess ten microsatellite loci for their application to natural populations. All were highly polymorphic with 7 to 24 alleles (Mean 13.4) per locus and polymorphism information content (PIC) ranging from 0.654 to 0.882 (Mean = 0.765). Results of AMOVA indicated that most of the genetic variation was within individuals (89.20%) and within populations (10.57%) while only 0.23% was among populations. Pairwise FST values were low (0.0003-0.0043) among all three cities suggesting little population structuring at distances ranging from 110 to 260 km.

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#### GENE-OF-INTEREST EXPRESSION IN TRANSGENIC *AEDES AEGYPTI* AFTER TRANSFORMATION WITH A TRANSPOSABLE ELEMENT OR THE PHIC31 SITE-SPECIFIC RECOMBINATION SYSTEM

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During the last few years germline transformation of mosquitoes has become more widely applied to study gene function or to express anti-pathogen effector genes. In most cases germline transformation has been achieved by using a non-autonomous transposable element (TE) such as mariner *Mos1*, *piggyBac* or *Minos* as an insertion vector for the transgene. The gene-of-interest is inserted into the plasmid DNA of the TE, which is then co-injected into the mosquito embryo along with a helper plasmid expressing the TE transposase. One major caveat when using a TE is the fact that the integration site of the transgene into the host genome is unpredictable and uncontrollable. This often leads to position effects causing poor gene-of-interest expression. The PhiC31 system has been described as an elegant alternative to avoid such position effects. The basic components of the system derived from bacterio-phage PhiC31 are a 'phage' attachment site (attP), a 'bacterium' attachment site (attB) and the integrase, which catalyzes recombination between the two sites. The attP site is inserted into the host genome via a TE. In a subsequent experiment the resulting 'docking strain' is then 'super-transformed' with a donor plasmid encoding the corresponding attB site and the gene-of-interest. We transformed *Aedes aegypti* with plasmid DNA encoding EGFP under control of the bloodmeal-inducible, midgut-specific carboxypeptidase A promoter using the *Mariner Mos1* TE or the PhiC31 system. Here we compare gene-of-interest expression patterns between TE and PhiC31 generated mosquitoes to validate the efficacy of either approach. We also describe integration patterns and integration loci of the transgenes in both systems.

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#### FUNCTIONAL ANALYSIS OF ELMO AND F-BOX/LRR IN THE MOSQUITO, *AEDES AEGYPTI*

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It has been widely accepted that invertebrates harbor only innate immunity. The innate immunity includes phagocytosis, encapsulation, melanization and secretion of antimicrobial peptides (AMPs). Five AMPs, named Attacin, Cecropin, Defencin, Dipterican and Gambicin have been identified in the mosquito *Aedes aegypti*. ELMO was shown to be involved in *D. melanogaster* development and cytoskeleton stability. It was demonstrated to affect phagocytosis in mammals. Previous research showed that F-box/LRR play important role in protein-protein interaction and in ubiquitylation. Therefore, we ought to explore the functions of ELMO and F-box/LRR in the mosquito *A. aegypti*. We made use of RNA

interference (RNAi) technique to silence the mRNA expression of ELMO and F-box/LRR in *A. aegypti*, followed by the challenge of *Staphylococcus aureus* or *Escherichia coli*. The survival assay was performed to analyze the mosquito resistance to these bacteria. Our results revealed that *Aedes aegypti* showed resistance to *S. aureus* in the absence of ELMO and F-Box. Therefore, we speculated that ELMO and F-box may serve as negative regulators in Toll pathway. Next, the expression of Cecropin A, a downstream target of Toll pathway, was examined. The results showed that silencing of ELMO resulted in the over-expression of Cecropin A upon *S. aureus* challenge, suggesting that ELMO negatively regulate the expression of Cecropin A. Finally we made use of FITC-labeled bacteria to observe the effect of phagocytosis in *A. aegypti*. The result showed that silencing of ELMO can increase the phagocytic ability to *S. aureus* in the mosquito. It's suggested that ELMO played a negative role in phagocytosis to Gram positive bacteria. Interestingly, silencing of F-box/LRR in the early pupal stage revealed a significant reduction of emerging adults. Our findings showed novel role of ELMO and F-box/LRR in the mosquito *Aedes aegypti*.

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### THE WEST-SIDE STORY OF ANOPHELES GAMBIAE MOLECULAR FORM SPECIATION

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Throughout west and central Africa, *Anopheles gambiae* M and S molecular forms are characterised by largely overlapping geographical/temporal distributions, high levels of gene-flow restriction, low degree of inter-form genetic differentiation. Floating paracentric inversions on chromosome-2, probably involved in ecological adaptation to marginal sub-niches, are shared by the 2 forms, although with different frequencies of alternative inverted arrangements. In fact, while in forested/humid areas of west and west-central Africa M and S are both characterised by a standard homokaryotype, in northern savannah areas they show a very high level of chromosomal differentiation. We here report the first data on M and S population structure in the area along the Gambia river, where a frequency of 3-7% M/S hybrid has been observed. The results show that in the western part of the study area the M-form presents a very unusual chromosomal constitution, undistinguishable from that of sympatric S-form (i.e. high frequencies of 2Rb, 2Rd and 2La arrangements). The resulting karyotypes - never observed before at high frequencies in M-form - are also found in M-populations from the rice-cultivated central area of the transect, suggesting that M-form is able to adapt to this peculiar environment even in the absence of high frequencies of inversions 2Rbc and 2Ru, usually associated to comparable ecosystems in Mali and Burkina Faso. On the other hand, the 2Rbc and 2Ru inverted arrangements are observed in the few M-specimens found at the eastern part of the transect, where sympatric S-populations are prevailing and are characterized by increased chromosomal complexity, based on 2Rj, 2Rbk and 2Rcu arrangements typical of S-form in eastward geographic areas. These observations, coupled with the results from the analysis of 20 microsatellite loci on chromosome-X and -3, allow to speculate on the peculiar status of M and S forms at the western extreme of their range and on the multiplicity of genetic adaptive mechanisms allowing the great ecological flexibility of *A. gambiae* along its range.

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### GLOBAL CROSS-TALK OF GENES IN RESPONSE TO DENGUE VIRUS INFECTION IN THE MOSQUITO AEADES AEGYPTI

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The mosquito *Aedes aegypti* is the primary global vector of dengue virus (DENV). The early time periods after infection with DENV define critical cellular processes that determine success or failure of the virus to establish infection in the mosquito. To identify genes involved in these processes, we performed genome-wide transcriptome profiling between susceptible and refractory *Ae. aegypti* mosquitoes at two early periods after challenging them with DENV. Our data reveal extensive transcriptional networks of mosquito genes that are expressed in modular components in response to DENV infection. The genes that responded coordinately to DENV infection in the susceptible strain were clustered in one specific expression module whereas in the refractory strain they were distributed in four distinct modules. The susceptible- and refractory-specific differential expression of five randomly chosen genes from the array data were validated by qRT-PCR. The specificity of expression was also observed in two additional *Ae. aegypti* strains, DS3 and Moyo-In-Dry, in response to DENV infection. The susceptible response module in the global transcriptional network showed enrichment with genes related to energy metabolism and DNA replication whereas the refractory response modules showed enrichment with different metabolism pathway genes including cytochrome P450 and DDT degradation genes, and also genes associated with cell division and apoptosis. Two additional modules were identified that represented a common core set of coordinately expressed genes in both the susceptible and refractory mosquitoes. These core response modules are enriched mostly with genes related to different signal transduction pathways including the wnt, MAPK, mTOR and JAK-STAT pathways. These pathways may have important roles in the global-cross talk among the host factors during the early infection period that could trigger the appropriate host action in susceptible and refractory mosquitoes.

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### AEDES AEGYPTI: AN EMERGING MODEL FOR THE STUDY OF DEVELOPMENTAL BIOLOGY IN VECTOR MOSQUITOES

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Mosquito genome projects have stimulated increased interest in the potential for arthropod-borne disease control by genetic manipulation of vector insects. Although targets of particular interest include developmental regulatory genes, extremely little is known about the genetic regulation of vector mosquito development. We have recently developed methodology for analysis of gene and protein expression during embryonic, larval, and pupal development of the vector mosquito *Aedes aegypti*. We have also achieved knockdown of developmental genes through microinjection of siRNAs into *Aedes aegypti* embryos. This methodology is permitting detailed analyses of the functions of developmental regulatory genes and the selective inhibition of such genes during *Aedes aegypti* development. We are presently using these techniques to characterize the *Aedes aegypti* *semaphorin* and *plexin* homologs, genes which are known to regulate numerous developmental processes in *Drosophila melanogaster*, including development of the olfactory system. Expression data support the hypothesis that the roles of the *sema1a* and *plexin* genes are generally conserved between fruit fly and mosquito development. This hypothesis is currently being tested by siRNA-mediated knockdown of *sema1a*. Our investigation, in combination



with our ongoing functional analyses of additional developmental genes of vector importance, is helping to establish *Aedes aegypti* as an emerging model for vector mosquito development.

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#### MICROGEOGRAPHIC GENETIC DIVERSITY OF *ANOPHELES NUNEZTOVARI* S.L. FROM CORDOBA AND ANTIOQUIA, COLOMBIA

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*Anopheles nuneztovari* s.l. has a wide distribution in northern South America and is an important vector of malaria in Colombia and Venezuela. To test genetic diversity of *An. nuneztovari* s.l. at a microgeographic scale (approximately 150 km), mtDNA *COI* gene sequences were analyzed from 145 specimens collected in four Colombian localities: Montelíbano and Puerto Libertador in Córdoba department/state, and El Bagre and San Pedro de Urabá in Antioquia department, July 2007- February 2010. Nucleotide and haplotype diversity values were higher in the populations of Antioquia. There were 20 unique haplotypes, 4 shared among all the localities and a few (13) from both states were tip alleles, suggesting high demographic stability in the populations. A statistical parsimony *COI* gene network showed the most common interior haplotype (38% of all sequences analyzed) was represented in all collection sites. Overall, different analyses indicated low to moderate genetic differentiation and high gene flow among all populations tested from Córdoba and Antioquia; neutrality tests also supported demographic equilibrium. Despite the fact that *An. nuneztovari* s.l. is a species complex, the four populations in this study comprise a single mtDNA evolutionary unit. Continuation of this study with the analyses of additional markers will further elucidate the genetic structure of this important Colombian malaria vector and the understanding of its population genetics will contribute to the improvement of local malaria control strategies.

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#### SUPPRESSION OF DENGUE VIRUS REPLICATION IN THE SALIVARY GLANDS OF TRANSGENIC *Aedes aegypti*

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Genetic control strategies for vector-borne diseases based on population replacement require development of genetically-modified mosquitoes that provide resistance against the target pathogen. In order to achieve this, regulated expression of anti-pathogen effector molecules in a sex- and tissue-specific manner by using *cis*-regulatory DNA sequences is essential. Salivary glands of the female *Aedes aegypti* play an important role in the transmission of the dengue viruses and therefore are ideal sites for the expression of effector molecules. The *Aedes aegypti* *30K a* and *30K b* genes are expressed exclusively in the distal-lateral lobes of the female salivary glands and are separated by a 263 bp intergenic region. The *cis*-regulatory sequences of the *30K a* and *30K b* genes were used to express EGFP reporter and an anti-dengue effector gene in the salivary glands of female mosquitoes. The anti-dengue molecule, *Mnp*, consists of an inverted repeat sequence derived from the coding region for the membrane precursor region of the DENV-2 genome. Transgenic mosquitoes expressing *Mnp* fed on blood infected with DENV-2 showed reduced prevalence and mean intensities of infection of the virus in the salivary glands compared to control mosquitoes. The DENV-2 transmission potential also was reduced significantly in the mosquitoes carrying the

*Mnp* transgene compared to the controls. Work is in progress to achieve complete resistance against the virus by expressing the anti-effector gene in multiple tissues simultaneously.

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#### PREVALENCE OF INTESTINAL PARASITES, ANAEMIA AND ANTHROPOMETRIC STATUS AMONG CHILDREN UNDER FIVE YEARS OF AGE IN LAMARAME (SENEGAL)

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In order to target interventions against malaria and other tropical diseases, we conducted a baseline study at the new Health Demographic Surveillance System (HDSS) of Lamarame (Senegal) where malaria is seasonal. Our aim was to assess the prevalence of intestinal parasite (IP), anaemia and malnutrition among children under 5 years. A cross sectional survey was carried in January 2010. A two level random cluster sampling technique was used. A total of 30 clusters (villages) covered by Lamarame health post were randomly selected based on probability proportional to population size. Children were examined by a study physician after parents had given informed consent. For each child, anthropometric measures (weight, height, age) were taken. Height for age and weight for age z-scores were calculated by Epi info software using the NCHS/WHO international reference values. Haemoglobin rate was measured with HemoCue®; stool samples were collected and examined using the Ritchie technique. The study was approved by the Senegalese national ethical committee. For 722 examined children the average prevalence of IP was 26.2% [CI95% 22.9-29.5]. *Giardia intestinalis* was found in 15.6% [CI95% 13-18.5], *Entamoeba coli*: 10.9% [CI95% 8.7-13.4], *Hymenolepis nana*: 1.8% [CI95% 0.9-3], *Ascaris lumbricoides*: 0.42% [CI95% 0.08-1.2] and *Enterobius vermicularis*: 0.28% [CI95% 0.03-0.9]. IP prevalence was significantly higher in villages located at a distance  $\geq 1$  km from the health post (73.9% versus 26% RR: 1.7 [CI95% 1.2 - 2.6]). Prevalence of anaemia (Hb <11 g/dl) was 66.4%. Severe anaemia (Hb < 8g/dl) and moderate anaemia (8<Hb<11 g/dl) was found in 12.7% [CI95% 10.4-15.4], and 53.7% [CI95% 49.9-57.3], respectively. Stunting (HAZ<-2SD) was found in 21.6% [CI95% 18.6-24.8] and underweight (WAZ<-2SD) in 16.5% [CI95% 13.8-19.4]. In a logistic regression model, stunting was significantly associated with: severe anaemia (aOR 3.65 [2.01-6.64]) moderate anaemia (aOR 2.03 [1.27-3.26]), living at a distance > 1 Km from the health post (aOR: 3.74 [1.62-8.64] and number of children in the household (aOR1.5 [1.15-1.95]). Anaemia and stunting constitute a public health problem in Lamarame despite periodical mass administration of vitamin A and mebendazole treatment for STH. Protozoan infections such *Giardia* and *E. coli* are frequent in the area. Additional interventions are needed to target these parasitic diseases.

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#### RECURRENT AMEBIC LIVER ABSCESS

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A 44 year old man of Iraqi origin presented to our institution with a 5 day history of fever, chills and vomiting. He was treated for amebic liver abscess (proven by aspiration) in 2001 and 2003. The patient was treated with a combination of aspiration of the pus and metronidazole. Since his arrival from Iraq in 2000, the patient had not traveled outside of Michigan. On examination, he was febrile (39°C), with tachycardia (120/min); the rest of the exam was normal. Pertinent laboratory findings were an

elevated white blood cell count (15,500/ $\mu$ l), serum alkaline phosphatase (684 IU/ml) with negative hepatitis serologies. Ultrasound of abdomen showed an 8x7x7 cm abscess in the right lobe of the liver. Aspiration showed "anchovy paste", consistent with amoebic liver abscess. Antibody to Entamoeba was elevated. The patient was successfully treated with aspiration of the pus and metronidazole, along with oral iodoquinol as a luminal cysticidal agent. It is likely that the lack of use of a cysticidal drug for the previous episodes resulted in persistent colonization of the colon, with subsequent reactivation causing invasive liver disease. There have been few reports of recurrent amoebic liver abscess; it occurs in about 0.04% of cases. Most recurrent abscesses are seen within a year of the first episode. Our patient represents the first documented instance of such a late recurrence. Available data show that metronidazole is a poor luminal cysticidal agent. The case history of our patient highlights the risk of recurrence of liver abscess if intestinal amoebic cysts are not eradicated during treatment.

### 314

#### PREVALENCE OF INTESTINAL PARASITIC PATHOGENS IN DIARRHEAL AND NON-DIARRHEAL HUMAN STOOL SAMPLES IN TURKEY, 2001-2010

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Intestinal parasites are two major groups including protozoa and helminths that inhabit the gastro-intestinal tract in humans. They are one of the major health problems of especially poor and under-developed countries. This study was undertaken to determine the prevalence of intestinal parasitic infection in patients with diarrhea and non-diarrhea at the Gulhane Military Medical Academy, Military Hospital in Turkey. This retrospective study reviewed the hospital records of 26842 patients admitted over a ten-year period from 2001 to 2010. We used a standardized data collection form to obtain data for sociodemographic characteristics and laboratory diagnosis. Stool samples were collected from 26842 patients who applied to Gulhane Military Medical Academy Parasitology Laboratory between 2001 and 2010. During the study period, 2.2% (1314/57375) of stool samples were tested positive for sixteen species of intestinal parasites, by using standard parasitological techniques. Multiple infections with 2-4 parasitic species constituted 0.1% of 26842 infected cases. *Giardia intestinalis* (2% of the 26842 cases) was the most common parasitic cause of diarrhea among the patients. Its prevalence appears to be decreasing in recent years. Fifteen other species of intestinal parasites were identified. *Entamoeba coli* (0.8%) and *Blastocystis hominis* (0.3%) ranked second and third in prevalence, respectively. *Enterobius vermicularis* (0.2%) was more common in non-diarrheal samples. Prevalence of intestinal parasitic infection was lowest (14%) in winter, gradually increased during the spring, reached peaks of 56% between July and October, and gradually decreased to 2% in December. These data will help provide accurate estimates of the prevalence of intestinal parasites, which are crucial for the development of policies and strategies to enhance their effective control. The present study has demonstrated that *G. intestinalis*, *E. coli* and *B. hominis* are common parasitic causes of diarrhea in Turkey.

### 315

#### SUCCESSFUL TREATMENT AN IMMUNE-COMPETENT PATIENT WITH REFRACTORY GIARDIASIS USING NITAZOXANIDE AND GENETIC CHARACTERIZATION OF THE *GIARDIA INTESTINALIS* ISOLATE

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*Giardia intestinalis* is a most common protozoan infection in the world. It is the cause of both epidemic and endemic diarrhea and upset of the gastrointestinal system. There are a number of drugs whose efficacies are well studied and accepted for the treatment of patients with this infection. However, some individuals experience treatment failure, despite having received successive courses of treatment that have been documented to result in a cure for most patients. In humans, nitazoxanide has been reported to be effective against a broad range of parasites, including *G. intestinalis*. We report the case of a 21 year old male immuno-competent patient admitted to Gulhane Military Medicine Academy Hospital in February 2008. He had nausea, vomiting and fever. The results of an extensive evaluation including fibrinogen, immunoglobulin G, A, and M tests were initially negative. He had a pain in region of epigastric and upper gastrointestinal endoscopic examination was performed on this patient. Chronic gastritis was diagnosed through endoscopic examination and a plenty of *G. intestinalis* trophozoites was detected in samples obtained from stomach and bulbous. And he received oral metronidazole, 500 mg given t.i.d. for five days. After the patient received the treatment, *Giardia* cysts were still present in stool samples. He complained of watery diarrhea with 7-8 times a day. Subsequently, the patient underwent two sequential treatment regimens that consisted of secnidazole, 2 g given once and albendazole, 400 mg given b.i.d., for 5 days. Stool samples tested negative for *Giardia* during therapy but revert to positive after the treatments. Successful treatment with nitazoxanide, 1 g given b.i.d. for 15 days, resulted in stool samples that tested negative by microscopy. He reported no side effects and the results of microscopic evaluation of his stool samples have remained repeatedly negative for *Giardia*. Genetic analyses of *Giardia* cysts isolated from patient revealed that they were from Assemblage B. In this report, nitazoxanide was found to be active against a metronidazole and albendazole-resistant *G. intestinalis* isolate from an immune-competent patient. If its anti-giardial efficacy is confirmed in additional studies, nitazoxanide may be considered as an alternative therapy for resistant giardiasis.

### 316

#### PREVALENCE OF SPECIES AND SUB-TYPES OF *CRYPTOSPORIDIUM SPP.* AND *GIARDIA DUODENALIS* IN FOUR COMMUNITIES IN THE PERUVIAN AMAZON BASIN

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Childhood diarrhea is an important cause of morbidity and mortality in children less than 5-years of age. The prevalence and genetic diversity of *Cryptosporidium spp.* and *Giardia duodenalis*, pathogens associated with childhood diarrhea, were assessed in microscopy-confirmed samples from children  $\leq$ 5 years in four riverside communities located in the Peruvian Amazon basin. Stool samples (one per child) and data on diarrhea in the previous 2 weeks were collected in 2009 from

453 children by the U.S. Naval Medical Research Center Detachment (Naval Medical Research Center Detachment) in collaboration with the Peruvian Ministry of Health. Participants' stool samples were first examined microscopically for parasites. Nineteen samples were positive by microscopy for *Cryptosporidium* and 7 samples were further genotyped by SSU rRNA PCR-RFLP. Five children had *C. hominis* and two had *C. canis*. A fragment of the gp60 locus was sequenced and sub-types for 4/5 of the *C. hominis* samples were obtained: Ib (2 samples), Ie, and a novel Ig sub-type. Eighty samples were microscopy positive for *Giardia*; 72 of those were confirmed positive by SSU rRNA-based real-time PCR. Forty-three samples were successfully genotyped by sequence analysis of the TPI locus. Two genotypes were detected: assemblage A in three samples, and assemblage B in 40. Within assemblage A, we detected two different sub-types. Subtype analysis of samples with assemblage B revealed 11 distinct subtypes, and the presence of mixed infections with B subtypes in 7 samples. There were no mixed infections with assemblages A and B. The genetic diversity of *Cryptosporidium* and *Giardia* in these communities Amazon basin is similar to that found in other endemic settings, where transmission may occur through direct contact, food- or waterborne routes. Although the geno- and sub-types of *Cryptosporidium* and *Giardia* detected in this study are associated with anthroponotic transmission, the presence of large number of samples with assemblage B of *G. duodenalis*, and *Cryptosporidium canis* in two children also suggest potential zoonotic transmission.

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#### ENTAMOEBA HISTOLYTICA AND ENTAMOEBA DISPAR IN CYST-POSITIVE FECAL SAMPLES FROM SMALL COMMUNITIES IN THE PERUVIAN AMAZON

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Cysts of pathogenic *Entamoeba histolytica* and non-pathogenic *E. dispar* have similar morphologic characteristics. The differentiation of these species is important because it leads to different therapeutic courses or additional diagnostic testing. We determined the prevalence of *E. histolytica* and *Entamoeba dispar* in children <5 years-old. Four-hundred fifty-six samples as well as data on diarrhea and fever were collected in 2009 from four riverside communities located in the Peruvian Amazon basin. Ova and parasite examination identified cysts with morphology of *Entamoeba histolytica/dispar* in 34/456 samples (7.5%). These samples were further analyzed by species-specific PCRs to identify *E. histolytica* and *E. dispar*, using two tests for each: conventional PCR and Real-Time TaqMan-PCR. These methods amplify fragments of the small-subunit rRNA gene. Conventional PCR detected 11 samples positive for *E. dispar*, while the TaqMan assay detected 7 additional positives (total = 18). In contrast, *Entamoeba histolytica* was not detected in any sample by either assay. The absence of *E. histolytica* in the samples microscopically diagnosed with *E. histolytica/dispar*-like cysts, and the detection of *E. dispar* in 18/34 samples suggest that non-pathogenic amoeba are quite frequent in these communities. These findings highlight the importance of species-specific tests for *E. histolytica* and further differential diagnosis among people with bloody dysentery, particularly those whose samples had microscopy positive results for *E. histolytica/dispar*.

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#### EPIDEMIOLOGY OF ENTAMOEBA HISTOLYTICA INFECTIONS AMONG ABORIGINAL CHILDREN IN PERIPHERAL VILLAGES IN SELANGOR, MALAYSIA

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*Entamoeba histolytica* infection is one of the most common parasitic infections in the world particularly in developing countries. A community-based study to determine the prevalence and predictors of this infection was carried out among Aboriginal children aged between 2 and 15 years in selected peripheral villages in Selangor, Malaysia. Socioeconomic data were collected using pre-tested questionnaires. Of 281 trichrome stained fecal smears examined, 25(8.9%) children were positive for *E. histolytica*. The prevalence of *E. histolytica* infections increased with age and associated with large family size and significant wasting ( $P < 0.05$ ). Binary logistic regression confirmed that large family size ( $\geq 8$  members) was a significant predictor of *E. histolytica* infections (OR=3.34; 95%CI=1.31, 8.52). The subjects were asymptomatic or presented non-specific symptoms that could be attributed to amoebiasis. In conclusion, the predictors found in this study are known to be important determinants of *E. histolytica* infections. Further studies are needed and molecular approaches to distinguish the invasive *E. histolytica* from the non-invasive parasites such as *E. dispar* and *E. moshkovskii* may lead to a better understanding of the burden of this infection in Malaysia.

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#### GIARDIA LAMBLIA AND CRYPTOSPORIDIUM SPP. GENOTYPING IN CHILDREN UNDER FIVE YEARS OLD FROM PANAMA, CENTRAL AMERICA

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*Giardia lamblia* and *Cryptosporidium* spp are zoonotic parasites that can cause gastrointestinal disease and nutritional deficiency in children. In Panama, these two pathogenic protozoa are present with important prevalence in children, especially giardiasis. However, information on their genetic characteristics, distribution, and role in human disease is limited. We analyzed the genetic diversity and geographic distribution of both protozoa from infected children younger than five years. Stool samples were taken from 1,560 diarrheic and non-diarrheic children from eight different regions in Panama. Oocysts were microscopically detected using the formalin-acetate concentration procedure and Kinyoun stain. Of these samples, 201 presented *G. lamblia* and 79 presented *Cryptosporidium* spp. DNA was extracted from positive samples. Molecular diagnosis and characterization was possible in 120 *Giardia* and 24 *Cryptosporidium* positive samples. *G. lamblia* genotyping was performed using a PCR-RFLP analysis based on the polymorphisms of the *tpi* gene. For *Cryptosporidium* spp, the SSU rRNA gene was used as molecular marker. Genetic analysis revealed that 23.3% *Giardia* samples belonged to assemblage A, 69.0% belonged to assemblage B and 7.5% were mixed infections. Subtyping of assemblage A samples showed that type All is nine times more frequent than Al. *Cryptosporidium* genotyping showed that 62.5% were *C. hominis*, 21.0% *C. parvum*, 12.5% *C. meleagridis* and 0.04% *C. canis*. *Cryptosporidium* identification was confirmed by direct sequence analysis. Further genetic diversity within *C. parvum* samples was assessed by sequence analyses of the GP60 gene. This is the first report of *G. lamblia* and *Cryptosporidium* spp genotype in human isolates from Panama. *G. lamblia* organisms belonging to assemblage B and *C. hominis* are the



predominant genotypes in different regions of Panama. Further studies are required to evaluate the molecular epidemiology and to develop prevention and control measures of both protozoa parasites in Panama.

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### ANTHROPOGENIC DISTURBANCE INCREASES PREVALENCE OF PATHOGENIC PROTOZOA IN WILD RODENT RESERVOIRS IN UGANDA

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*Giardia* and *Cryptosporidium* are zoonotic pathogens that cause widespread morbidity in the developing world. Despite the impact of these pathogens, little is known about their reservoirs in nature or how anthropogenic disturbance affects their patterns of infection. To better understand this interplay, we sampled rodents for these pathogens along a disturbance gradient traversing primary forest, logging concessions, small-scale and commercial agricultural plots and villages in Kabarole District, Uganda. Trapping webs were utilized to create accurate density and diversity estimates of the rodent community along each transect of this grid. Rodent fecal samples were screened for the presence of both protozoa via immunofluorescent antibody testing (IFA). Pathogen prevalence was calculated as number of individuals of a species expressing positive results divided by total number of individuals of that species sampled. Effects of rodent species and geographic location on pathogen prevalence were examined using parametric statistics. The brush-furred rat (*Lophuromys* sp.) was found to have significantly higher prevalence of *Giardia* compared to other wild rodent species examined ( $P < 0.01$ ). In addition, *Cryptosporidium* was only observed in *Lophuromys* sp. Neither *Cryptosporidium* nor *Giardia* were observed in the invasive black rat (*Rattus rattus*). Every rodent sample collected from the undisturbed forest was negative for both pathogens and rodent samples from the most disturbed site had significantly higher prevalence of both *Cryptosporidium* and *Giardia* compared to all other sites ( $P < 0.01$ ). These results suggest that *Lophuromys* sp. may serve as a viable reservoir for these neglected tropical pathogens and that anthropogenic disturbance may be associated with their prevalence in rural, forested Africa. These results highlight the need for future research into the epidemiology, cross-species transmission ecology, and clinical consequences of *Giardia* and *Cryptosporidium* not only in humans and livestock, but also in the wild animals that share their environments.

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### COMMON DIAGNOSTIC METHODS FOR *ENTAMOEBIA* SPECIES LEAD TO OVER-DIAGNOSIS OF THE PATHOGENIC *ENTAMOEBIA HISTOLYTICA*, IN POPULATIONS OF EASTERN VENEZUELA

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In Venezuela, since 1990, infection frequencies are reported from 6.8 to 42% throughout the country. Between 2005 and 2008, 124,142 diarrhea cases were reported in children, 7,366 of these were reported as amebiasis, with Capital District, Zulia, Anzoátegui and Sucre states showing highest prevalences. Even though WHO since 1997 have recommended to differentiate the pathogenic species *Entamoeba histolytica* from the other non pathogenic species of the genus, in order to rationalize the treatment, few places carry out such differentiation. In Venezuela, the main diagnostic method is based on the microscopic observation of the trophozoites and cysts, which has a proven sensitivity of about 60% and cannot differentiate among *E. histolytica*, *E. dispar*

and *E. moshkovskii* because of their identical morphology, except in cases where hematophagous trophozoites are seen. To study the real prevalence of these species in the states of Sucre and Anzoátegui, we have diagnosed 1,045 fecal samples from symptomatic and asymptomatic individuals using nested-multiplex PCR for the detection of *E. histolytica*, *E. dispar* and *E. moshkovskii*. These samples were previously diagnosed by clinical laboratories using microscopic observation in saline physiologic solution and Lugol. The results show variations in the prevalence from 11 to 20% for the complex *E. histolytica/E. dispar* using microscopy, while PCR detected infection in symptomatic and asymptomatic individuals with 3.3% of cases being diagnosed as *E. histolytica*, 3.8% as *E. dispar* and 0.6% of mixed infections. *E. moshkovskii* has not been detected so far in these populations. These results make evident the over-diagnosis of amebiasis by conventional diagnostic methods and the lack of differentiation among the species of the genus, indicating the treatment in many more cases than really needed, which can lead to parasite resistance to common drugs to treat amebiasis.

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### CHANGES IN PROFILE OF EXPRESSED PROTEINS IN *TRYPANOSOMA BRUCEI BRUCEI* PRIOR TO BRAIN INVASION DURING EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

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*Trypanosoma brucei* the causative agents of African Trypanosomiasis is known to invade the brain during the encephalitic stage of the disease. However mechanisms by which trypanosomes invade the brain are not well understood. During infections, the onset of the encephalitic stage is preceded by several waves of parasitemia, suggesting that some changes may occur in trypanosomes enabling them to traverse the blood brain barrier (BBB). We compared the traversal efficiency of trypanosomes harvested from experimentally infected mice during the early stage of the disease (early stage trypanosomes) and trypanosomes harvested during the late stage of the disease (late stage trypanosomes) *in vitro* using Mardin Darby Canine Kidney (MDCK) monolayer. Late stage trypanosomes were found to traverse the biological barrier more effectively than early stage trypanosomes. When analyzed by two dimensional gel electrophoresis, later stage trypanosomes were found to express proteins that were not expressed by early stage trypanosomes which points to possible differential transcriptional and translational events that enable an active crossing of the BBB. Identification of these proteins by mass spectrometry is underway.

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### SURVEILLANCE FOR CYCLOSPORIASIS IN THE UNITED STATES, 1997-2008

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Cyclosporiasis is a food- and waterborne enteric disease caused by the parasite *Cyclospora cayatanensis*. Outbreaks have been investigated by CDC since the mid-1990s; however, U.S. data on sporadic cyclosporiasis cases have not been summarized previously. Health departments report cyclosporiasis cases via the National Notifiable Diseases Surveillance System. Of the 1,092 sporadic cases reported to CDC for the period 1997-2008, 338 (31%) had a history of international travel during the 14 days before illness onset, 382 (35%) did not (i.e., "domestic" cases), and the travel history was not reported for the other 372 (34%). The destination was known for 287 (85%) of the 338 travel-associated cases. The most frequent destinations were Mexico (59/338; 18%), Guatemala (40; 12%), and Peru (37; 11%). Domestic cases were reported largely from Florida (124; 32%) and the Northeastern United States (New York City [44; 12%], Massachusetts [31; 8%] and Connecticut [27; 7%]). Most

reported domestic cases (265; 69%) occurred or were diagnosed from April through August. Both travel-associated and domestic cases occurred equally among males and females ( $p=0.80$ ). The median age of travel-associated and domestic case-patients was 41 years and 47 years. At least some food history was available for 52% of domestic cases, but varied widely due to state-specific differences in interview instruments, and case-patient response rates. While a large percentage of travel-associated cases visited known *Cyclospora*-endemic countries, our data suggest that *Cyclospora* could be transmitted more frequently than previously thought in some countries such as Mexico. Most domestic cases were reported by several eastern states during the spring and summer months. Vehicles of infection for sporadic cases are not usually identified because food histories are sparse or incomplete, a known limitation of sporadic case surveillance. Furthermore, because there is no *Cyclospora* molecular subtyping capability to link individual cases, some outbreak-associated cases were likely misclassified as sporadic.

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#### FOLLOW-UP OF THE 1977 TOXOPLASMOSIS OUTBREAK FOR OCULAR DISEASE

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In 1977 an outbreak of *Toxoplasma gondii* infection occurred in Georgia associated with exposure in an indoor horse arena. Cat feces containing the organism were most likely stirred-up when horses ran on the dirt floor, and were inhaled or ingested by riders and observers. Thirty-seven persons were found to be infected with *T. gondii* (by clinical exam and/or serologically [IgM and IgG IFA]). Twenty-five persons received a follow-up eye examination 4 years later, and 1 person was identified with an ocular lesion diagnosed as toxoplasmosis. Starting in 2004, we again attempted to locate persons from the 1977 toxoplasmosis outbreak and offer them an ocular examination by a retinal specialist with ocular photographs if lesions were found. Of the 37 persons infected with *T. gondii* in the outbreak, 14 (38%) were located and agreed to an ocular examination. Of these 14, 13 (93%) were female, the median age was 16 years (range 10-47 years) in 1977, and the median age at time of examination was 42.5 years (range 35-72 years). Of the 23 persons not examined, 19 (83%) were female and the median age was 27 years (range 17-38 years) in 1977. Among the 14 persons examined, 3 (21%) were diagnosed with ocular lesions typical of toxoplasmosis. The person identified with an ocular lesion at four years had ocular symptoms at that time; the other 2 did not have ocular symptoms associated with their lesions. If these 3 persons were the only ones with ocular disease out of the 37 persons in the outbreak, the ocular disease rate would still be 8%. As a result of exposure to *T. gondii* during this outbreak, a relatively high percentage of persons developed ocular disease over time.

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#### CRYPTOSPORIDIUM PARVUM PROTEINS RECOGNIZED BY PATIENTS WITH ACTIVE CRYPTOSPORIDIOSIS

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The protozoan parasite *Cryptosporidium* has been implicated in foodborne and waterborne outbreaks. The active infection is not necessarily detected with the current serological methods available using *Cryptosporidium* proteins. The present study was designed to determine *Cryptosporidium* proteins recognized by human sera from *Cryptosporidium*-infected individuals using one-dimensional and two-dimensional western blot analyses, and their identification by mass spectrometry in order to determine antigenic proteins for use in diagnosis of cryptosporidiosis

during outbreak investigations. *C. parvum* antigens of 14, 16, 21 or 26 kDa detected in one-dimensional analysis, or 17.5 detected in two-dimensional analysis, reported previously in the literature, did not distinguish recent infections ( $p < 0.05$ ). In one-dimensional analysis a *C. parvum* protein of 57 kDa reacted more strongly with acute human sera ( $p < 0.05$ ) whereas the results of two-dimensional analysis suggest that *C. parvum* antigens of 43.4, 50.3, 50.3, 47.6, 64.7, and 50.3 kDa, with pIs of 5.4, 7.0, 7.2, 5.3, 6.6, and 6.7, respectively, may be used as markers of early *Cryptosporidium* infection. A *Cryptosporidium* serine/threonine phosphatase, actin protein, a dynein heavy chain, phosphoglycerate kinase, a chaperone-related protein, and three *Cryptosporidium* hypothetical proteins were identified by tandem mass spectrometry. Antigenic peptides were predicted from these proteins and *Cryptosporidium*-specific peptides are suggested. These proteins and peptides might be useful to detect early *Cryptosporidium* infections and to determine human immune response associated to a specific *Cryptosporidium* species.

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#### INDUCTION OF INFLAMMATORY AND ANTI-APOPTOTIC RESPONSES BY DIFFERENT STRAINS OF TOXOPLASMA GONDII IN MACROPHAGES AND MICROGLIAL CELLS

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*Toxoplasma gondii* is a highly successful intracellular parasite capable of causing severe disease among immunocompromised and newborn populations. The majority of strains of *T. gondii* belong to three distinct clonal lines known as types I, II, and III. The outcome of the immune response to infection is influenced by the parasite strain type. This study examined the kinetics of gene expression in macrophages and microglial cells infected with types I, II, or III of *T. gondii*. Emphasis was placed in pro-inflammatory and anti-apoptotic factors as their expression have been reported to change significantly in parasite-infected cells. The kinetics of host gene expression was examined by RT-PCR in response to infection. In addition, the protective effects of parasite infection against apoptosis were assessed by inhibition of caspase-3. A type II strain of *T. gondii* (ME49) elicited a greater expression of pro-inflammatory cytokines in macrophages and microglial cells compared to types I (GT-1) and III (CTG). These differences were minimal early in infection (2-4 h), but became pronounced at middle (8-12 h) and late stages of infection (24 h). Contrary to this, the induction of anti-apoptotic genes was equivalent among the different type strains throughout infection. Of note, induction of the pro-inflammatory response occurred early in infection while the anti-apoptotic response exhibited a delayed profile regardless of the strain. Lastly, experiments with cells lacking the Toll-like receptor adaptor molecule Myd88 (Myd88<sup>-/-</sup>) showed a dependency on this factor for the pro-inflammatory response but not the anti-apoptotic response. The results suggest that the outcome of host gene expression in response to *T. gondii* is determined by the parasite type in a time-dependent manner and is selective to particular subsets of genes. The capability of *T. gondii* to induce an anti-apoptotic response in the absence of a critical immune regulator reflects a complex level of modulation of host functions that might extend to other intracellular parasites.

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### CPG-ODN, ARGININE OR ALANYL-GLUTAMINE REDUCE CRYPTOSPORIDIAL INFECTION IN MALNOURISHED HUMAN INTESTINAL EPITHELIAL CELLS

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*Cryptosporidium parvum* is a parasite that invades intestinal epithelial cells and is a leading cause of childhood diarrhea worldwide, and intensified by malnutrition, has substantial impact on immune, cognitive, and physical development. Oligodeoxynucleotides with unmethylated CpG motifs (CpG-ODN) act as immune adjuvant for vaccines by binding to Toll-Like receptor 9. Arginine (ARG) and alanyl-glutamine (AQ) are amino acids necessary for regulation of cell cycle, polyamines production, cell migration and proliferation. ARG also increases levels of nitric oxide. Previous studies showed that CpG-ODN, ARG and AQ reduce *C. parvum* infection in neonatal and malnourished mice. We now study the effect of these molecules on HCT8 cells (human intestinal epithelial cells) infected with the parasite. HCT8 cells were grown in culture plates (n=3-4 wells/group) and subjected to either 1% ("malnourished cells") or 10% ("nourished cells") Fetal Bovine Serum in glutamine-free media. Cells were then pre-treated with ARG (1mM), AQ (10mM), CpG-ODN (100µg/mL), or combinations of the two amino acids alone or together with CpG-ODN. 24 hours later, cells were infected with 105 *C. parvum* oocysts/well. At 6 hours after infection (time point=0 hours), cells were washed and received fresh media with the treatments. Cells were harvested for DNA extraction at 0 and 24 hours. A ratio of parasites per 106 cells was calculated using Real-Time qPCR for specific parasite and HCT8 gene. CpG-ODN (p=0.0109), ARG (p=0.0117) and AQ (p=0.0127) decreased the *C. parvum* infection mainly in the groups with 1%FBS but not in 10%FBS. These findings demonstrate the potential use of a combination of CpG-ODN, ARG and AQ as a novel approach to control *C. parvum* infection, especially during malnutrition, to enhance the host immune responses and repair the intestinal injury.

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### AN APICOMPLEXAN LINEAGE-SPECIFIC POLYTROPIC MEMBRANE PROTEIN AS A POTENTIAL DIAGNOSTIC AND DRUG TARGET FOR HUMAN CRYPTOSPORIDIOSIS

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Human cryptosporidiosis, a waterborne gastroenteritis, is an important cause of morbidity amongst infants in many tropical countries, and is a potentially life-threatening complication in HIV-infected and other immunocompromised individuals. Human cryptosporidiosis is caused by two major species of intestinal epithelial cell parasites, including *Cryptosporidium parvum* which infects both humans and farm animals and *C. hominis*, a primarily human parasite. We have recently identified a novel family of apicomplexan lineage-specific polytopic membrane proteins in *C. parvum* and *C. hominis*. One of the members of this unique family of proteins, CpAlp854, is abundantly expressed on the oocyst wall and in apical organelles of sporozoites. Immunofluorescence assays revealed that this protein is surface-accessible to antibodies. Interestingly, CpAlp854 is also abundantly expressed on merozoites of parasites cultured *in vitro* in HCT-8 cells. This is the first polytopic membrane protein that has been partially localized to the apical organelles in *Cryptosporidium*. Polyclonal anti-serum raised against an antigenic peptide of CpAlp854

blocks the excystation of the sporozoites from oocysts. We and others have recently shown that the orthologs of these groups of proteins in other apicomplexan parasites, including *Plasmodium falciparum* and *Toxoplasma gondii*, are associated with the cytoskeleton and may be involved in zoite invasion into host epithelial cells. The abundant expression and surface localization of CpAlp854 provide an attractive target as a diagnostic marker and potential target for the design of anti-cryptosporidial compounds or vaccine to combat human cryptosporidiosis.

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### MALNUTRITION IMPAIRS HOST DEFENSES AGAINST CRYPTOSPORIDIAL INFECTION AND VACCINE RESPONSES IN WEANED MICE

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*Cryptosporidium parvum* is a protozoan that leads chronic diarrhea worldwide mainly in developing areas, where intensified by malnutrition, has devastating impacts on immune, cognitive, and physical development. The availability of the gene sequence for *C. hominis* surface antigen (CP15) opens opportunities to examine novel vaccine candidates. We developed a malnourished weaned mouse model with malnutrition induced by 2% versus 20% (malnourished and nourished respectively) protein diet for 5-7 days to study infection with *C. parvum* and immunization with CP15 vaccine. Malnourished and nourished groups included: vaccine alone, infection alone, vaccine plus infection, and uninfected. Vaccinated mice received the antigen as recombinant with cytolysinA in *Salmonella* serovar typhi CVD908-htrA given intranasally, followed by 2 intraperitoneal boosts (10 days between each) with synthetic CP15 and Freund's adjuvant. Infected mice received 5x10<sup>7</sup> oocysts/mouse via gavage. Mice were weighed daily and stools collected over 15 days post-infection. Parasite shedding determined by Real-Time qPCR in DNA extracted from stools. Mice were sacrificed 20-40 days after immunization; spleen, mesenteric nodes and serum were collected. Cytokines and antibodies were quantified by ELISA. qPCR results showed malnourished animals have heavier infection and more weight loss. In the group that was vaccinated and infected, the vaccine did not show significant protection against *C. parvum* infection. Immunologic results showed that among mice with vaccine alone, malnourished mice have increased levels of IFN-γ and IL-10, but reduced IL-6 and IL-2 and antigen-specific antibody responses compared with nourished mice. Among infection alone, malnourished mice have increased IFN-γ but decreased antigen-specific antibody responses compared with vaccine alone group. These findings demonstrate that malnutrition impairs immune responses to immunization and parasite challenge.

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### GENETIC CHARACTERIZATION, METRONIDAZOLE SUSCEPTIBILITY TESTING AND SECRETED PROTEASE ACTIVITY OF HISTORICAL AND CLINICAL TRICHOMONAS VAGINALIS ISOLATES

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*Trichomonas vaginalis*, a protozoan parasite that infects the human urogenital tract, causes, the most common non-viral, sexually transmitted disease in the world. Over 170 million cases of trichomoniasis occur worldwide annually. Trichomoniasis is associated with vaginitis, cervicitis,



chronic prostatitis, and non-gonococcal urethritis, and is a risk factor for HSV and HIV transmission. *Trichomonas* infection increases the incidence of premature rupture of placental membranes, low birth weight infants, and can be transmitted to neonates during passage through the birth canal. Frontline treatment is metronidazole, which is usually very effective and well tolerated. However, an estimated 2.5-10% of cases of trichomoniasis display some degree of resistance to initial treatment. We have characterized 23 historic and 170 clinical isolates of *T. vaginalis* based on genetic profiles and susceptibility to metronidazole. Restriction fragment length polymorphism (RFLP) analysis using a cytoplasmic heat-shock protein 70 (Hsp70) hybridization probe with digested genomic DNA was used in molecular typing of *T. vaginalis* isolates. RFLP results illustrate the substantial genomic diversity present in *T. vaginalis* and indicate that a large number of genetically distinct *Trichomonas* isolates of clonal lineage may be responsible for human trichomoniasis.

We have also developed a multi-locus sequencing typing (MLST) scheme for *T. vaginalis* for use with these strains. We expect that this method will provide a more rapid, easily reproducible technique for typing. Our initial scheme utilizes 12 house-keeping genes to determine the relatedness of individual *T. vaginalis* isolates. This should provide a sufficient level of discrimination power for typing this organism. We have successfully generated 400-500 bp PCR products for 8 *T. vaginalis* isolates. The number of single nucleotide polymorphisms (SNPs) observed at a single locus ranges from 1-7 SNPs. We compare the two typing techniques in terms of reproducibility, precision, and rapidity. The Alamar Blue™ colorimetric assay was used to test isolate susceptibility to metronidazole. Susceptibility testing shows the prevalence of high drug resistance found in this study to be of 3-5%, similar to the national average. Secreted Cysteine protease activity was assessed via a fluorometric analysis. The variation in activity among *T. vaginalis* isolates is greater than threefold.

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#### CRYPTOSPORIDIOSIS - AN INNER CITY HOSPITAL EXPERIENCE

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*Cryptosporidium* spp. is a known cause of protracted diarrhea in immunocompromised patients. It is also reported with higher prevalence among children in underdeveloped countries. With HAART therapy the epidemiology of cryptosporidiosis may be changing and it is worth describing a recent experience from an inner city hospital in the Bronx with a large immigrant and HIV population. A retrospective chart review identified patients diagnosed with *Cryptosporidium* spp. from 2003 to 2009. Individuals that were HIV positive (HIV+) were compared to those that were HIV negative (HIV-). 47 patients with Cryptosporidiosis were identified. Of the 47, 29(62%) were HIV-, the mean age was 21±18 years and 16(33%) were female. Forty-one (87%) patients had diarrhea and 30(64%) were hospitalized at diagnosis. Twenty-three (49%) patients had traveled within one month to an underdeveloped country and all were visiting friends and relatives. Travel destinations in order of decreasing frequency were Latin America, Africa, Middle East, Eastern Europe and Asia. Fifteen of 47(32%) were treated with nitazoxanide. HIV+ patients had a median CD4=37/mm<sup>3</sup> (Min 1/mm<sup>3</sup> and Max 952/mm<sup>3</sup>) with 29% having a CD4>100/mm<sup>3</sup>. There was no difference with respect to sex or presence of diarrhea in those that were HIV+ and HIV-. HIV+ patients were more likely to be hospitalized, have a longer length of stay and be admitted to an ICU (p<.001, p=.047 and p<.001, respectively). HIV- patients were younger than the HIV+ patients with a mean age of 10.9 ± 12 years (p<.001) and were more likely to have traveled (p<.001). Three of 18 (17%) with CD4<10/mm<sup>3</sup> died within 6 months and no HIV+ patients had biliary involvement. Ten patients with a mean CD4/

mm<sup>3</sup> of 54±1.2 had persistent diarrhea documented. In conclusion, *Cryptosporidium* spp. is an important cause of travelers' diarrhea in children undertaking high risk travel to endemic regions. Cryptosporidiosis continues to occur in HIV+ patients with CD4<100/mm<sup>3</sup>, causing persistent diarrhea, but is also seen in those with higher CD4 counts.

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#### IMMUNOPHENOTYPIC LYMPHOCYTE ANALYSIS OF MEMORY CELL RESPONSES IN CRYPTOSPORIDIOSIS

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*Cryptosporidium parvum* is a protozoan parasite that infects the epithelial cells of the small intestine causing diarrheal illness in humans. While T cells are known to be important in resistance and recovery from infection, little has been characterized as to the phenotypic expression of surface effector and memory markers after infection. Using a IL-12 KO model of acute infection, we depleted mice of CD4+ and CD8+ cell populations after either primary or secondary infection. Depletion of either CD4+ or CD8+ prior to primary infection significantly amplified infection in both groups. However, CD8+ depleted mice eventually recovered while CD4+ depleted mice were not able to recover from a primary infection. Mice depleted of either CD8+ or CD4+ cells after recovering from an acute infection and challenged demonstrated resistance to re-infection. We then used flow cytometry to characterize expression of different effector and memory cell markers 30 days after infection. Subpopulations with varying effector and memory cell potentials were defined based on the expression of specific cell surface molecules and activation markers. We found that infected mice had a higher percentage of activated CD4+ levels (CD69 and CD71) between days 3 and 7 post infection but that these markers rapidly declined over the course of infection. Increases in the percentage of effector markers (CD62Llow) on CD4+ and CD8+ cells were found in the spleen and mesenteric lymph node by day 7. Memory cell phenotype (CD62Lhi, CD44hi) increased and were the predominant cell population by day 30 post infection. Adoptive transfer of CD44hi cells of either CD4+ or CD8+ populations from immune mice into naïve mice provided protection from infection, suggesting that both subpopulations play a role in memory responses.

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#### INFLUENZA SURVEILLANCE AMONG CHILDREN AND PREGNANT WOMEN PRESENTING TO HEALTH CARE FACILITIES IN BAMAKO, MALI

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Virtually all epidemiologic data demonstrating an increased risk of complicated and fatal influenza illness among pregnant women and young infants derives from studies in industrialized countries in temperate or sub-tropical zones. To better define the burden of influenza in developing countries, we conducted surveillance among pregnant women and children < 36 months of age presenting to the Emergency Department (ED) of Hôpital Gabriel Touré and health centers in Banconi in Bamako, Mali. Women in the third trimester of pregnancy and children aged 0 to 35 months presenting with influenza-like illness (ILI) were identified. Per week of surveillance, up to 48 cases whose illness had lasted fewer than 4 days were sampled; this included up to 6 infants < 2 months of age, 24 children 2- 35 months of age and 18 pregnant women. After obtaining consent, nasal and throat swabs were obtained for analysis by real-time PCR for influenza virus. From November 2009 to February

2010, we recorded 1260 consultations for ILI including 1146 children and 114 pregnant women. Among these, samples were collected from 275 (24%) children and 40 (35%) women. Of the children, 24 (8.7%, 24/275) tested positive for 2009 H1N1 and 16 (5.8%, 16/275) had influenza B. Pregnant women tested positive for 2009 H1N1 in 10% (4/40) of cases and influenza B virus in 10% of cases (4/40). We found one case of co-infection (2009 H1N1 and B) in a pregnant woman. All cases were treated as outpatients and resolved without complications. In conclusion, ILI in Mali is associated with influenza A (2009 H1N1) and B infection. This preliminary surveillance suggests that broadening activities and conducting them throughout the year will provide invaluable information regarding the utility of vaccination in this setting.

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#### HEPATITIS B IN IRAQ: CRISIS AND RESCUE OPTIONS

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Hepatitis B is still of a global importance with more than 300 million people infected with hepatitis B virus (HBV) majority are resident in developing countries. In early eighties Iraq was considered as an area with intermediated endemicity of HBV infection and was pioneer among the Middle East countries in introducing HBV vaccination and incorporating it into Expanded Program of Immunization (EPI). The crisis escort the country from early eighties, inform of wars and sanctions, have created crisis which is continues smolder. The country suffered a humanitarian crisis from the massive degradation of the country's infrastructure and disability of health care systems and shortages of medicine and vaccines all over the country. Subsequences of the crisis, that many children who were born in eighties or early nineties either were non-vaccinated or have escaped one or two doses of HBV vaccine. This susceptible pool of children grows into adulthood and has been added to the preexisting reservoir of adult carriers of hepatitis B. Therefore, HBV carrier cases accumulate, especially HBeAg positive cases with increased tendency for transmission to others This paper will describe the breakdown of health care systems and its impact on HBV infection rates during crisis and the challenges facing the country and the rescue options needed for control of this infection.

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#### THE ROLE OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS NONSTRUCTURAL AND STRUCTURAL PROTEINS DURING INFECTION OF THE ENZOOTIC MOSQUITO VECTOR, *CULEX TAENIOPUS*

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Venezuelan equine encephalitis generally exists in two ecological cycles: enzootic and epizootic. Viral strains that circulate in the enzootic cycle (ID and IE) are maintained in rodents and mosquito vectors (*Culex taeniopus*) and are typically unable to cause disease in equids. Epizootic viruses (IAB and IC) are able to infect equids and persist in a replication cycle between equids and epizootic mosquito vectors (*Aedes taeniorhynchus*). Previous studies indicate that the genotypic determinate for the epizootic phenotype is primarily found in the E2 glycoprotein of the structural regions of the virus. Interestingly, recent studies comparing the efficiency of epizootic, enzootic, and intermediate phenotypic strains suggest that there might be regions outside of the structural regions that play a role in determining the viral phenotype. We hypothesize that while the structural regions clearly play a role in establishing the epizootic phenotype, that regions outside of the structural regions may be important for fitness in the enzootic mosquito, *Cx. taeniopus*. To evaluate our hypothesis, we generated various chimeric VEEV viruses between epizootic (IAB Trinidad Donkey) and enzootic (IE 68U201) viruses to evaluate infection and dissemination within the enzootic mosquito vector. As expected, the IAB wild type was unable to infect or disseminate in *Cx. taeniopus*. The IE wild

type virus infected 81% and disseminated in 44% of exposed mosquitoes. Both chimeras IAB/IE/IAB and IE/AB/IE infected 53% of exposed mosquitoes, but only the chimera with IE derived structural proteins (IAB/IE/IAB) was able to disseminate in 16% of exposed mosquitoes. The fact that the IE/IAB/IE chimera was able to infect *Cx. taeniopus*, while the wild type IAB was not, indicates that the genetic determinates for the enzootic phenotype include regions outside of the structural proteins.

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#### INNATE IMMUNE PATTERN RECOGNITION RECEPTOR UTILIZATION BY RIFT VALLEY FEVER VIRUS

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Rift Valley fever virus (RVFV) is a zoonotic pathogen endemic to regions of Africa and the Arabian Peninsula. In the most severe cases, RVFV infection can cause retinitis, encephalitis, or hemorrhagic fever. The innate immune response to RVFV is suspected to be important in viral clearance but is still poorly defined. In animal models of RVFV infection, a strong protective role has been identified for type I interferon responses. In human infection, a delayed onset of interferon production is associated with the more severe forms of RVFV-induced clinical disease. Members of the Toll-like receptor (TLR) and RNA helicase families recognize viral patterns and stimulate type I interferon responses. In this study, human embryonic kidney (HEK) cells were used as a model for defining key innate recognition receptors during RVFV infection. HEK cells intrinsically express helicase RIGI and some basal levels of TLR3. HEK cells that overexpressed individual TLRs of interest were transiently transfected with luciferase reporter plasmids for NFκB and IFNβ signaling pathways. In addition, exogenous RIGI was transfected into cells to drive increased induction of the helicase pathway. Dominant negative constructs were utilized to block activity of adaptor molecules and subsequent signaling pathways. Results from *in vitro* studies were verified using primary bone marrow-derived dendritic cells and macrophages from wild-type and knockout mice. Recognition by endosomal TLRs drove NFκB mediated cytokine responses, whereas activation through helicases was the primary source for interferon production. The degree of dependence upon these receptors varied among cell types.

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#### HOST GENETIC FACTORS AND SUSCEPTIBILITY TO HTLV-1-ASSOCIATED MYELOPATHY/TROPICAL SPASTIC PARAPARESIS IN PERUVIAN HTLV-1 INFECTED

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HTLV-1 is a retrovirus associated to HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chronic neurodegenerative disease characterized by a paraparesis of the lower limbs. The causative factors that predispose to HAM/TSP development are not well known; high proviral load (PVL) has been associated to HAM/TSP in several populations. However PVL and viral factors *per se* do not explain fully HAM/TSP development. A multifactorial background is proposed for HAM/TSP, with host genetic, viral and environmental factors as contributors to disease susceptibility. A two stage study was performed to ascertain whether human genetic factors are associated to HAM/TSP disease in Peruvian HTLV-1-infected. Allelic distribution of 6 HLA, and 94 SNPs belonging to

45 genes were evaluated in 55 HAM/TSP patients and 114 asymptomatics (AC) HTLV-1 infected. SNPs with a trend of association ( $P < 0.1$ ) were evaluated in a second stage of 85 HAM/TSP and 146 AC. 36 Ancestry informative markers (AIMs) were analyzed to correct for population stratification. Logistic regression analysis was done in both stages to test for association between disease status and candidate genes. Age, gender, PVL and the first three principal components based on the AIMs were used as covariates. 12 SNPs from 9 genes showed a  $P < 0.1$  in the first stage: *IFN- $\gamma$ -874*, *MMP2-1306*, *NFKB1A*, *NKG2D*, *NKG7*, *PD1-1.9*, *RANTES-403*, *TGF- $\beta$ -509*, *TLR2*. In the second stage three of the twelve SNPs evaluated showed a  $P < 0.1$ , SNPs belongs to *NFKB1A* and *NKG2D* genes.  $P$ -values for the full data set were calculated to determine if the trend of association was in the same direction,  $P < 0.05$  were observed for SNPs from *NFKB1A* (2 SNPs) and *NKG2D* (3 SNPs) genes. In conclusion, although no correction for multiple testing was performed due to the exploratory nature of the study, the  $P$ -values observed suggest that *NFKB1A* and *NKG2D* genes might influence susceptibility to HAM/TSP. These findings need to be confirmed in a larger population and/or in a different population of HTLV-1 infected to assess the implication of these genes over HAM/TSP disease.

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#### LOW RATES OF HBV AND HIV CO-INFECTION IN TANZANIA

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Hepatitis B is a leading cause of liver disease in the developing world. Hepatitis B virus (HBV) shares infection routes with the human immunodeficiency virus (HIV). Therefore, areas with high endemicity of HIV, such as sub-Saharan Africa, tend to present high rates of infection with HBV. In the setting of HIV co-infection, both morbidity and mortality from HBV is increased compared to non co-infected populations. Moreover, with the use of antiretroviral therapy and its potential liver toxicity this relation becomes critically important. We conducted a prospective study to address the role of HBV co-infection in HIV-positive patients that were starting antiretroviral therapy in northern Tanzania. Appropriate approval was obtained from the research committee at Selian Lutheran Hospital in Arusha and the University of Minnesota in Minneapolis. Consent was obtained from participating subjects. Patients who started antiretroviral therapy were tested for hepatitis B s-antigen (HBsAg) and ALT in addition to routine laboratory tests, with the purpose of identifying those with chronic hepatitis B. Follow up with ALT and clinical symptom was intended to happen at 6 and 12 weeks when subjects returned for routine controls. Approximately 38% of our subjects were males and 62% females. Average age was 33 years. Surprisingly of 120 recruited patients, in two different periods of 3 months each, within 2 years, only 3 (2.5%) were positive for HBsAg. This rate of con-infection is much lower than previously reported in Tanzania for both general population and HIV-positive patients. The study was repeated in a smaller population using different reagents and laboratory settings and results were similar. Although occult hepatitis B (active hepatitis B virus with negative HBsAg) has been reported in HIV-positive patients, this is unlikely to account for such low numbers of HBV-infected individuals in an HIV-positive population. Further research to understand these results is warranted.

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#### ESTABLISHMENT OF A NATIONAL VIRAL HEMORRHAGIC FEVER SURVEILLANCE PROGRAM IN UGANDA

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Uganda has experienced many past outbreaks of viral hemorrhagic fevers (VHFs), including recent outbreaks due to Ebola (Bundibugyo) and Marburg virus, in 2007. It is also an endemic region for many other viral zoonotic diseases, including Rift Valley Fever (RVF) and Crimean

Congo Hemorrhagic Fever (CCHF), which have the potential to cause large outbreaks in animal and/or human populations. Because of this, the Special Pathogens Branch (SPB), Centers for Disease Control and Prevention, has collaborated with the Uganda Virus Research Institute (UVRI) to initiate a national VHF surveillance and laboratory network in Uganda. The overall goals of the VHF surveillance system are to enhance Uganda's capability to detect, diagnose, and respond to endemic VHFs in a timely manner. Through implementation, we will improve the collection and analysis of surveillance data, improve laboratory capacity for detecting viral pathogens and strengthen its role in epidemiologic surveillance and outbreak response. SPB has developed and implemented a standardized three tiered (suspect, probable, and confirmed) case definition for VHFs and a standardized case reporting form. The laboratory at UVRI has the capacity to perform diagnostic antigen-detection, IgM, and IgG enzyme-linked immunosorbent assays, as well as reverse-transcriptase polymerase reaction. These assays will be employed to confirm suspect VHF cases including Ebola hemorrhagic fever, Marburg hemorrhagic fever, RVF, CCHF, as well as arenaviruses and hantaviruses which may be endemic in Uganda. A comprehensive program, including training on collection of epidemiological data and clinical samples, has been initiated at multiple sites throughout Uganda. The sites were chosen based on proximity to areas with past VHF activity in human populations, or proximity to suspect natural zoonotic reservoirs. This network will help provide a baseline for VHF activity in Uganda and improve the early detection, diagnosis, and response to VHF outbreaks in Uganda.

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#### RESPIRATORY SYNCYTIAL VIRUS IN CENTRAL AND SOUTH AMERICA: GENETIC VARIABILITY IN STRAINS CIRCULATING FROM 2007 TO 2009

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Respiratory syncytial virus (RSV) is a major cause of viral lower respiratory tract infections among infants and young children. It has a negative-sense, nonsegmented, single-stranded RNA genome. The significance of subgroup differentiation has been suggested by some studies. Subgroup A RSV may be more virulent than subgroup B, and infection may result in greater disease severity among hospitalized infants. This virus expresses three transmembrane glycoproteins: the attachment glycoprotein (G), the fusion protein (F), and the small hydrophobic protein. The F and G proteins are important antigenically because they stimulate the production of protective immune responses. The G protein is of particular interest because variability in this protein is greater than that in the other proteins, both between and within the major antigenic groups of RSV. In this study, we evaluated the genetic diversity of both group A and B RSV strains by sequencing a variable region of the G protein gene of isolates collected during a three year period (2007-2009) in Central and South America. Nasopharyngeal throat swab specimens were collected at hospitals throughout Central and South America from patients who presented with a febrile, respiratory syndrome. Virus identification was accomplished using RT-PCR and this was followed by characterization and sequencing. From 7,198 samples collected, 185 (2.5%) were positive for RSV. Participants under 12 years of age accounted for 97% of the samples, clearly showing this virus is predominantly seen in children. We randomly selected 50 samples to be sequenced for genetic variability. Our results revealed the existence of two major antigenic groups of RSV, groups A and B, as two established strains circulating in Central and South



America during the last year. Two main branches were identified in group A: genotypes GA2 and GA5, with GA2 being predominant. The subgroup B that we observed was genetically similar to the virus isolated in Buenos Aires in 1999 (BA virus). These data provide a better understanding of the patterns of RSV strain circulation and the possible importance of strain differences to the consistency of yearly community outbreaks of RSV disease.

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#### EVIDENCE OF ARENAVIRUS INFECTION AMONG FEBRILE PATIENTS IN PERU

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Viruses from the family *Arenaviridae* are usually associated with hemorrhagic diseases that are transmitted to humans by distinct species of rodents. Arenaviruses are grouped into Old World and New World complexes based on their geographic distribution and serological cross-reactivity. Among the New World arenaviruses, Machupo, Junin, Guanarito, Chapare, and Sabia are known to cause severe hemorrhagic disease in humans, whereas Tacaribe virus has resulted in a single example of febrile disease with mild central nervous symptoms. In Peru, Allpahuayo virus (New World complex) has been the only arenavirus isolated from arboreal rice rats (*Oecomys bicolor* and *Oecomys paricola*) in Iquitos. However, human infections associated with Allpahuayo and other arenaviruses remain unknown. In 2000, the U.S. Naval Medical Research Center Detachment (Naval Medical Research Center Detachment), Lima, in collaboration with the Ministry of Health of Peru, initiated a passive surveillance study to investigate etiology of febrile illnesses. Patients presenting at health posts or clinics with fever  $\geq 38^{\circ}\text{C}$  of no more than seven days duration and headache, myalgia, or other nonspecific symptoms were enrolled in the study. Two paired-blood samples were collected, one during the acute phase of illness and the second sample 2-4 weeks after onset of symptoms. Samples were processed for virus isolation and serological evidence of fourfold or greater increase in antibody titer to a variety of vector-borne viruses. Serological testing for arenaviruses was done for evidence of anti-Allpahuayo and anti-Tacaribe IgM by enzyme immunoassay (EIA). A four-fold or greater increase in antibody titer (indicating seroconversion) to Tacaribe and Allpahuayo was found in six and three patients, respectively. A presumptive case was defined as having an acute sample with titers  $\geq 1:400$ . We found eight Tacaribe and one Allpahuayo case fulfilling this criteria. Virus isolation attempts were unsuccessful in all cases. The most common symptoms among Tacaribe and Allpahuayo patients with seroconversion included headache, chills, malaise, anorexia, myalgia, and arthralgia. Three of the Tacaribe cases had petechiae and one Allpahuayo case had bleeding gums. Additional studies are needed to confirm whether the patients were infected with Tacaribe and Allpahuayo virus or a related arenavirus. Results of this study suggest that arenavirus infection may be the cause of undifferentiated febrile illness in Peru.

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#### FIRST SEROLOGIC EVIDENCE OF HANTAVIRUS IN PERU

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Hantaviruses are rodent-borne virus of the family *Bunyaviridae* and have been identified as etiological agents of two human diseases: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome (HPS). HPS resulting from infection by hantavirus species such as Andes virus or Laguna Negra virus (LNV) has been reported in numerous countries of

South America including Chile, Bolivia, Paraguay, and Brazil. Surprisingly, there have been no reports of human hantavirus infection in nearby Peru, although a Rio Mamore-like virus (RMV) has been isolated from a rodent (*Oligoryzomys microtis*) in the Amazon basin city of Iquitos, Peru. The objective of this study was to provide serological evidence of human hantavirus infection in Peru. A cross-sectional serosurvey was conducted with serum samples obtained from 1,316 healthy volunteers residing in urban areas of Iquitos. Serum samples were tested for IgG reactive to LNV, Sin Nombre Virus (SNV), and RMV antigens using an enzyme immunoassay (EIA). From 1,316 serum samples, a total of 30 (2.3%) contained IgG reactive to one or more hantaviruses. Two (0.2%) were positive for anti-LNV-RMV-SNV IgG by EIA, nineteen (1.4%) were positive only for anti-SNV IgG, seven (0.5%) were positive only for anti-RMV IgG and two (0.2%) were positive only for anti-LNV IgG. There were no significant age or gender differences between the hantavirus-exposed and unexposed populations. In conclusion, the finding of this study indicates human exposure to one or more hantaviruses in Peru.

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#### PILOTING COMMUNITY INTERVENTION TO PREVENT NIPAH VIRUS TRANSMISSION USING BAMBOO SKIRTS

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Human Nipah virus (NiV) outbreaks have occurred regularly in central and north-western Bangladesh. Fruit bats are the natural reservoir of NiV. When people drink raw date palm sap, apparently contaminated with infected bats' saliva and urine, they occasionally contract NiV infection. In prior studies, gachhis (date palm sap collectors) expressed interest in placing a bamboo skirt over the sap flow and pot to prevent bat access to date palm sap. The aim of this study was to assess the acceptability and take-up of a promotion campaign to encourage the use of bamboo skirts by the date palm harvesting community. We conducted an intervention trial from December 2009- February 2010, in Boalmari sub-district in Faridpur district of Bangladesh. We facilitated 15 community meetings to introduce NiV infection, possible way of NiV transmission through date palm sap and how bamboo skirt interrupts bat access to sap. We identified gachhis and tree owners through a baseline survey. We randomly sampled 79 tree owners out of 1303 and took an equal sample of 79 gachhis out of 168. After one month, we assessed the early impact of the intervention by interviewing gachhis and tree owners, and observing the date palm trees. At baseline, no bamboo skirts were used in the community. One month after the intervention, 34% of gachhis (20/59, p-value <0.001) and 14% of tree owners (7/49, p-value <0.001), who drink raw sap, used bamboo skirts. In addition, 5% (3/59) of gachhis and 2% (1/49) of tree owners used jute skirts. Gachhis reported that jute stalk is locally available and required less time to make into a skirt compared to bamboo. Thirteen gachhis and five tree owners also reported covering their trees with cloth, mosquito nets and thorns. Observations confirmed the reported practices. In conclusion, many respondents adopted bamboo skirts implying the community's receptiveness to the intervention. Inclusion of locally available materials and increased duration of the intervention might further improve uptake.

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#### ASSESSMENT OF PLAQUE ASSAY METHODS FOR ALPHAVIRUSES

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Alphaviruses have been responsible for outbreaks involving thousands of human and equine cases of severe disease in the Americas. Confirmation

of alphavirus cases is based on viral isolation or a four-fold or greater increase in antibody titers between acute and convalescent samples. Specificity of antibodies to an alphavirus is usually confirmed by plaque reduction neutralization assays. However, the performance of two standard methods of PRNT (semisolid and solid) have not been compared for this group of viruses. In an attempt to identify the best method for alphavirus and neutralizing antibody recognition, we evaluated: 1) a semisolid method using a 0.6% carboxymethyl cellulose overlay, and 2) a solid method using a 0.4% agarose overlay. Initially, Mayaro virus (MAYV), UNA virus (UNAV) and Venezuelan equine encephalitis virus (VEEV) were titrated using both methods. Next, positive controls were evaluated to determine neutralizing antibody titers. To confirm results, we tested acute and convalescent sera from 19 patients who had MAYV isolated from their acute sample. The solid method consistently showed greater sensitivity than the semisolid method. The alphaviruses provided ~5-9 fold higher viral titers using the solid method and also gave a higher neutralization titer on positive controls. Specific MAYV neutralizing antibodies (40 or higher) were detected in 17 of 19 of the convalescent sera (and none of the acute) using the solid method while all samples were negative with the semisolid method. Neutralizing antibodies against VEEV and UNAV were not detected in these samples further confirming the specificity of the solid assay. In conclusion, our results provide evidence that the solid method is superior in detecting alphaviruses and alphavirus neutralizing antibodies and should be the method of choice when using PRNT as a confirmatory test.

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#### AVIAN FLU: PILOTING AN INTERVENTION TO REDUCE THE RISK OF TRANSMISSION TO BACKYARD POULTRY-RAISING FAMILIES IN RURAL BANGLADESH

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Available evidence suggests that most backyard poultry raisers in Bangladesh are not implementing recommended practices to reduce the risk of H5N1 transmission to humans. We developed and piloted preventive messages focused on slaughtering sick poultry, one of the riskier behaviors. We explored the acceptability and feasibility of the messages in the community. We implemented the intervention in two villages during June-August, 2009. We held five one-hour meetings to introduce bird flu, its clinical signs, transmission, and preventive messages through a pictorial flipchart and posters. Each participating villager attended one session. The messages encouraged covering one's nose and mouth with clothing while slaughtering, burying offal, hand washing, and cleaning slaughtering tools and site. We also suggested isolating sick poultry, burying carcasses, and avoiding buying, selling or consuming sick poultry. We documented community response using observation, in-depth interview, group discussion, and informal conversation. We observed both before and after intervention that villagers occasionally separated sick poultry, buried offal of sick and healthy poultry, and cleaned the slaughtering site. During interviews after the intervention, residents expressed willingness to slaughter their poultry that were sick with bird flu using the safer methods, but were unwilling to avoid slaughtering or selling sick poultry as they would lose household income. They reported they had never observed this disease in their area. Later, when poultry die-offs of unidentified cause occurred, their slaughtering practices were unchanged. In conclusion, the villagers already practiced some preventive behaviors, however, their normal practices risk transmission of influenza from poultry to people. More intensive communication and follow-up may increase these poultry raisers' risk perception, but financial loss is an important impediment to safer behavior. Encouraging safer slaughtering for all sick poultry or all poultry may be an achievable objective for behavior change.

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#### MOLECULAR EPIDEMIOLOGY AND GENETIC DIVERSITY OF RABIES VIRUS ASSOCIATED WITH BATS IN PERU

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During the last decade in South America, rabies perpetuated by the common vampire (*Desmodus rotundus*) increased considerably, especially in remote areas of the Amazon. Typically, domestic animals and humans are exposed by contact with infected wildlife. To determine current epidemiological links, viral diversity and disease spread, complete and partial sequences of the nucleoprotein (N) gene of viral isolates were studied from 2002 - 2007 in different mammalian species and distinct geographical areas. Phylogenetic analysis revealed that Peruvian rabies viruses could be grouped in at least seven distinct lineages. *D. rotundus* was incriminated as a principal reservoir and transmitter throughout Peru. Viruses isolated from vampire bats and livestock suggest long-term transmission pathways. Overlap of some lineages was found, as was evidence of spillover to other species, and a suggestion of geographic translocations. Two viral variants not previously identified in the Americas were found in a kinkajou (*Potos flavus*) and the insectivorous bat *Histiotus montanus*, from the south eastern part of the Amazon. One RV lineage was widely distributed from Colombia to Madre de Dios. This is the first comparative study on genetic diversity of rabies virus in Peru and its relationship with other variants in the Americas. Our results provide a better understanding of the molecular epidemiology of rabies associated with bats in Peru.

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#### DEVELOPMENT AND FIELD USE OF A SIMPLE, PORTABLE TEST TO QUANTIFY H<sub>2</sub>S-PRODUCING FECAL BACTERIA IN DRINKING WATER AS PREDICTORS OF DIARRHEAL DISEASE RISK

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Lack of access to safe water, improper sanitation and poor hygiene contribute to an ongoing global health and development crisis resulting in millions of deaths and infectious disease morbidity burdens affecting billions of persons annually. Inadequate water, sanitation and hygiene account for roughly 94 percent of the 4 billion cases of diarrhea that WHO estimates occur globally each year. In order to know if water is safe to drink and if WHO-recommended Water Safety Plans for hygienic water management are achieving microbially safe water, drinking water and its sources must be tested regularly. Given the lack of access to microbial testing of water in resource-limited settings and especially in most water supply settings in developing countries, there is a need for simple, low cost tests for fecal indicator microbes that can be performed by the water consumer at the point of use or by others on their behalf (water suppliers, community health workers, government agencies, etc.). This research focused on the development of a compartmentalized plastic bag for water quality testing for hydrogen sulphide (H<sub>2</sub>S)-producing fecal microbes in water to estimate their concentration as Most Probable Number (MPN). Analysis of lab spiked-sewage samples, natural water samples from North Carolina, and household drinking water samples from community water supplies in central Vietnam showed that there are significant relationships between H<sub>2</sub>S producing bacteria and *E. coli* (97% of samples were either both positive or both negative for *E. coli* and H<sub>2</sub>S producing bacteria in lab studies, as were 69% of samples from Vietnam). Furthermore, molecular analyses of the bacterial community structure of select Vietnamese water samples show that there is a strong relationship between positive H<sub>2</sub>S tests and water containing enteric pathogens and fecal indicator bacteria

of concern. Moreover, a household water and sanitation study in central Vietnam communities showed that there was a significant relationship between increasing levels of H<sub>2</sub>S-producing bacteria and diarrheal disease (Odds Ratio 1.28 (95% CI 1.051328-1.388585), p=0.008}. We conclude that the low-cost MPN compartment bag test for H<sub>2</sub>S-producing bacteria is simple and easy-to-use in field settings (as little or less time than both the IDEXX Colilert system and standard membrane filtration techniques), is low cost and is amenable to widespread commercial production and distribution.

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#### A QUALITATIVE EXPLORATION OF BARRIERS TO PROMOTE WATER TREATMENT TECHNOLOGY IN RURAL BANGLADESH

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Drinking contaminated water is major cause of morbidity and mortality among children <5 in Bangladesh. Point of use water treatment technologies could provide safe drinking water at the household level. To date, such interventions have failed to achieve high rates of regular use. This study was conducted to understand the barriers affecting the uptake of water treatment technology at the household level. We conducted this study in two villages where a local non government organization had promoted subsidized arsenic removal filters. We enrolled both users and non users who had a child < 5 years and a monthly income of < TK 5000 (US\$ 72). In-depth interviews identified practices of household water management, experience with water treatment technology, and perceptions of water quality and water born diseases. Water from tube-wells was the main source of drinking water. It was considered safest because it originated underground, despite being contaminated with iron and arsenic. Surface water was not preferred because it was turbid, and used for bathing and washing utensils. Safe drinking water was only judged by its appearance, not linked to germs or pollutants. Diarrheal diseases were not considered serious and were believed to be related to food, not water. Since arsenic poisoning is not immediately visible, informants did not recognize it as a serious health hazard. Barriers to use of water filters included collecting water directly from tube-wells for immediate consumption without storage. Users disliked the taste and smell of filtered water. The water flow was slow, maintenance was difficult and spare parts were not available. In conclusion, the idea of pathogen free water was not particularly meaningful to these village residents; rather they focused on its appearance, taste, and smell. Stressing the positive aspects of water treatment technology to the non-users, such as visible filtering of iron and improvement in color, along with ensuring access to affordable filters, and the necessary spare parts, could increase uptake.

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#### AN ASSESSMENT OF USE OF SIPHON FILTERS IN LOW INCOME COMMUNITIES OF URBAN DHAKA, BANGLADESH

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Siphon water filters are a low cost point of use water treatment technology providing safe drinking water at household level in low income populations. There is little evidence of why this group continues to use these filters or not. This study aimed to identify both motivators and barriers to sustained use. We conducted a 3 month follow up survey among a low income community in urban Dhaka, Bangladesh who received siphon filters at the end of a randomized control trial as part of an assessment process of willingness to pay, and who had recent

experience using the filters as part of this study. Some participants received this hardware free of cost while others had to pay up to 5 US\$. We interviewed household caregivers and tested water quality using the H<sub>2</sub>S method, a 24 hour color-coded system to confirm contamination. Among the 178 study participants, 82% (n=145) reported ever using the filters. Of these, 49% (n=71) reported using it within seven days of our interview. From household spot checks we observed that 37% (n=54) of households filters were used recently. There was no difference in usage between people who did or did not purchase the filters (28% vs. 30%, p=0.84). A higher proportion of observed recent users had better quality stored water compared to non users (30% vs. 18%, p=0.07), determined by bacterial growth from the H<sub>2</sub>S test. The most frequent reasons for not using the filter were using other treatment methods (11%, n=19), filter being broken or clogged (10%, n=18) and too troublesome (10%, n=17). People who perceived they are 'treating water like a modern person' were more likely regular filter users on multivariate regression analysis (OR: 7.4, 95% CI: 1.4, 38.4). Regular use was not strongly associated with perception of 'improving health' (OR: 2.4, 95% CI: 0.6, 10.2). In conclusion, although filter users had better water quality compared to nonusers, only one third of the people continued to use it after 3 months. Non health benefits were the primary determinant of regular filter usage.

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#### PERCEPTIONS, PRACTICES AND BARRIERS OF HANDWASHING IN RURAL BANGLADESH

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Handwashing with soap can substantially reduce the risk of diarrhea and respiratory disease, yet, observational studies in Bangladesh show that practice of hand washing with soap, soil, or ash is infrequent. We conducted a qualitative study in three rural villages of Bangladesh to explore perceptions, practices, and barriers to hand washing with different cleansing agents at different times. From the findings of structured observations from a previous study we knew that these villagers were using an agent for handwashing. To understand the perceptions and barriers of using an agent for handwashing a convenience sample of adult males and females was selected for in-depth interview (25). We used pocket voting (30), an interactive exercise with school children to assess the actual hand washing practices with different cleansing agents. Habitually, people rinse their hands with only water. They said, soap helps to get rid of stickiness, bad odor after defecation or to remove poison after spraying pesticides. Using soil after defecation was embedded in the context of religion, which is mainly practiced by the elderly. To avoid direct contact with stool, the anus is cleaned with three pieces of hard soil and then hands are rubbed with soil or ash before rinsing with only water. From pocket voting, we found girls practice better handwashing than boys. Most of the children indicated that they used a hand washing agent after defecation and the most common agent was soap followed by ash. Ash is only used after defecation, when soap is not available. After defecation, the absence of soap inside latrines was an important barrier to handwashing with soap. Respondents were reluctant to use the same soap for handwashing at other times which they used for washing hands after defecation. None of the informants washed their hands before cooking. According to most, hands should be washed when visibly dirty, while only a few perceived that hands should be cleansed to remove germs or to prevent diarrheal disease. In conclusion, these residents of rural Bangladesh wash their hands to remove visible dirt and not to reduce the risk of disease transmission. Messages to promote hand washing with soap may be more effective if they capitalize on people's desire for cleanliness.



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**BARRIERS TO CHILDREN ADOPTING A HANDWASHING HABIT IN URBAN BANGLADESH**

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Handwashing with soap can reduce diarrhea, a major cause of mortality in Bangladesh. Increasingly, hygiene promotion programs seek to inculcate handwashing habits among young children. We sought to understand physical and behavioral barriers to establishing handwashing habits in the home early in a child's life in low-income community in Dhaka. We conducted a household survey by visiting the 5<sup>th</sup> household in each compound of houses in the Dhaka study community. Through observations and interview, we recorded physical and behavioral barriers to use of soap for handwashing by children aged 2-7 years. Of 146 households enrolled, 143 (98%) had a handwashing station, defined as the place where hands are usually washed after using the toilet. The station was outside both toilet and cooking place in 82% of households and the mean distance from the latrine was 6 steps (SD=7.6). Water was available at 96% of the 143 handwashing stations on the interview day, although only 76% of respondents stated that water is always available. Soap was observed at the handwashing station in only 40 (28%) and was located at a mean height of 101 cm (40 inches). Among households that did not keep soap at the handwashing station, 75 % reported bringing soap from inside the house to wash hands. Respondents indicated that bar soap cannot be kept at the station because others might use it (42%), soap could be stolen (31%), and there is no convenient place to keep the soap at the station (13%). Of 54 respondents who had children 2-7 years old, 81% reported that the child knows where soap is kept at home. In 22 households that had children 2-7 years old and that had bar soap present at the handwashing station, 10 (45%) allowed those young children to access that bar soap. In conclusion, soap at the handwashing station is accessible to children aged nearly 4 years, according to international child growth standards. While many young children could reach soap at this height, soap is not actually kept at the handwashing station in a majority of households. Even when soap is kept at a handwashing station, a minority of children are allowed to access it, preventing many young children from independently forming a habit of handwashing after using the toilet. Community- and school-based handwashing programs seeking improvement of children's handwashing behavior in Bangladesh must aim for parental behavior change with respect to children's access to soap at home.

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**PILOT STUDY OF SERIAL SOAP WEIGHTS AS A NEW METHOD OF MEASURING HANDWASHING; DHAKA, BANGLADESH**

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Handwashing with soap reduces diarrhea and respiratory mortality for children < 5 in low-income countries. Accurate, inexpensive measures of handwashing are needed to evaluate handwashing interventions. Current measures are invalid or prohibitively resource-intensive. We tested the feasibility of serial soap weights to measure handwashing. Fieldworkers conducted 8 bi-weekly visits to 180 households with at least 1 child < 5 in an urban slum of Dhaka. At each visit, we interviewed participants and weighed soap products. Soap weights were included in analysis if there was no soap replacement in the interval. Soap weight differences

were compared to several measures of handwashing: visual inspections of respondent palms for cleanliness, presence of soap and water at the handwashing station, and handwashing with soap at critical times such as after defecation, as observed during 5hr structured observations (SO). Main handwashing products were bar soap (87%) and laundry soap (13%); all households also used their main handwashing product for either bathing or laundry. Soap weight differences were stable over the course of 8 visits (by regression on visit number, bar soap p-value 0.42, laundry soap p-value 0.86). Most (63%) respondents reported increased soap consumption on Fridays (a weekend day in Muslim Bangladesh). Mean bar soap use was 1.5g/day/person (95% CI 1.56- 1.62) & mean laundry soap use was 3.2g/day/person (95% CI 3.17 - 3.31). Compared to 3, 4, and 5-day intervals, the 2-day interval had the most soap weight data included in analysis (63%) due to fewer replacements between visits. Bar soap and laundry soap weight changes were not correlated with the SO measure of handwashing (Pearson's  $r = -.04$  and  $-.09$  respectively). Similarly, soap weight changes were not correlated with palm inspections or with presence of soap and water at the handwashing station. In conclusion, accurate soap consumption measurements may be possible with a few household visits 2 or 3 days apart, provided weekly spikes in soap use are accounted for. Soap weights had poor correlation with three different measures of handwashing, each with its own limitations. However, given the reliability of soap weight differences, the serial soap weight method merits further validation as a measure of handwashing behavior by testing its correlation with health outcomes.

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**ORAL REHYDRATION USE BY CAREGIVERS OF YOUNG CHILDREN WITH DIARRHEA IN A POOR PERI-URBAN DISTRICT IN THE DOMINICAN REPUBLIC**

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Oral rehydration is an effective strategy for decreasing morbidity and mortality from diarrhea. However, gaps have been reported in its use at the household level. This study examined the extent of use of oral rehydration and predictors of its use by caregivers of young children with diarrhea. Three samples of caregivers of young children from peri-urban Dominican Republic participated in a structured interview on child health which contained closed-ended questions about responses to childhood diarrhea. Of the 541 participating caregivers, 156 (29%) reported that the index child had had an episode of diarrhea within the last four weeks. Bivariate analysis was used to explore the relationship between hypothesized predictors and oral rehydration use. The following pattern of responses were reported for those whose child had had a recent diarrheal episode: went to the doctor (59%), used tea (49%), used package oral rehydration solution (40%), used coconut oil (33%), used an antibiotic (20%), used a homemade sugar/salt solution (4%). Those reporting having used oral rehydration were more likely to perceive their child to have been dehydrated, to have taken the child to a doctor, and to have also used an antibiotic. Children receiving oral rehydration were more likely to be younger and to have had more days of diarrhea. In conclusion, there are still gaps in the extent to which caregivers employ oral rehydration as a response to childhood diarrhea. Variables associated with oral rehydration use may provide a preliminary understanding of factors influencing its use.

### TRENDS IN DIARRHEAL DISEASE MORTALITY IN CHILDREN <5 YEARS OLD, ACCESS TO IMPROVED WATER SOURCES AND USE OF HOUSEHOLD WATER TREATMENT, CDC/KEMRI DEMOGRAPHIC SURVEILLANCE SYSTEM, NYANZA PROVINCE, KENYA, 2003-2008

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Diarrheal diseases are a leading cause of death in children <5 years old in Kenya. In 2003, a nationwide social marketing campaign was initiated in Kenya to reduce diarrhea risk through sale of chlorine solution for household water treatment; from 2003-2008, >7.1 million bottles were sold. To measure the impact of this program on diarrheal disease mortality, we examined data from the CDC/Kenya Medical Research Institute (KEMRI) Demographic Surveillance System (DSS), a longitudinal, population-based health and vital event registration system, designed to monitor health and demographic dynamics in Nyanza Province of rural western Kenya. We conducted an exploratory ecological analysis of DSS demographic and verbal autopsy data from 2003-2008 in two DSS sites. We calculated the diarrhea mortality rate per year in children <5 years old, and determined the proportion of households per year reporting access to improved water sources, and use of household water treatment. From 2003-2008, 999 (6%) of 17,232 deaths were determined by verbal autopsy to be diarrheal deaths; 628 (63%) were in children <5 years old. During this period, the diarrhea mortality rate decreased by 51%, from 73 to 36 per 10,000 children <5 years old, with the greatest decrease in 2005. During the same period, the proportion of households reporting access to improved water sources increased from 38% to 48%, with almost all of the increase occurring after 2006. The reported use of chlorine water treatment products steadily increased from 4% of households in 2003 to 32% of households in 2008, with 66% of the increase occurring after 2005. Although reported household chlorine water treatment and access to improved water sources increased during this period, most of these changes appeared after the largest decline in diarrheal mortality. Further study is warranted to determine other possible explanations for the decline in diarrheal mortality including trends in diarrhea treatment, association with other illnesses, such as malaria, HIV infection and malnutrition, and other environmental factors.

### EXAMINING THE SOCIAL AND ENVIRONMENTAL FACTORS THROUGH WHICH NEW ROAD DEVELOPMENT IMPACTS DIARRHEAL DISEASE INCIDENCE

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Road development has been linked to an increase in infectious disease incidence. A previous study found higher rates of diarrhea in villages that were less remote due to new road construction in northern coastal Ecuador. While remoteness is a distal factor that is associated with diarrhea, what remains to be examined are the proximal social and environmental factors through which decreasing remoteness impacts diarrhea incidence. In this study, we investigated the causal pathway between remoteness and diarrhea. We estimated specific indirect effects of remoteness through various proximal factors hypothesized to be associated with diarrhea, the direction of which were sometimes opposing. For example, decreasing remoteness was correlated with shorter residence time in the community, a proxy for increased movement of

people and pathogens, increasing risk of diarrhea. However, development also brought with it improved sanitation, decreasing risk for diarrhea. We used case-control data collected between July 2003 and February 2008 from 21 communities of varying remoteness in northern coastal Ecuador. Each community was visited seven times for a period of 15 days, during which all cases of diarrhea were identified through daily household visits. Both household and community controls were randomly selected upon case identification. Cases were defined as individuals with three or more loose or watery stools passed in a 24-hour period. Stool samples collected from cases and controls were used to estimate pathogen-specific diarrhea (*E.coli*, rotavirus and *Giardia*). For analysis, we employed a structural equations modeling approach, bootstrapping our 95% confidence intervals to determine relative effect sizes of the hypothesized specific indirect effects of remoteness on diarrhea. This study provides insight on the social and environmental changes that accompany development in rural areas as well as their impact on diarrheal disease burden.

### PERSPECTIVES ON DIARRHEA AND HOUSEHOLD WATER CHARACTERISTICS IN TRINIDAD, BOLIVIA

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Diarrhea is a primary cause of morbidity and mortality around the world. Improved water, sanitation, and hygiene are primary pathways of diarrhea prevention. The objective of this research was to identify household knowledge, attitudes, and practices surrounding household water use and diarrhea. We conducted 374 household interviews in 40 neighborhoods in peri-urban Trinidad, Bolivia during summer 2009. Median self-reported household income was \$2300 Bolivianos and an average of 5.9 people were reported living in each household. The majority of respondents considered the community to be healthy or very healthy (72%), and 94% considered diarrhea to be dangerous or very dangerous. At least half of respondents reported water and rotten food to be causes of diarrhea, while fewer than 15% identified poor hygiene behavior, such as lack of hand-washing at key moments, to be a cause of illness. Only half of households reported that they always or usually treating drinking water, and more than one third of households reported never treating water. Among households that reported always or usually treating water, most reported boiling (31%) or the use of a fabric, ceramic or sand water filter (23%). Notably, 22% of respondents report owning a ceramic filter, while only 7% report this type of filter as a method they use to treat water. The majority of households with ceramic filters obtained the devices from NGOs during widespread flooding (2007-2008). Approximately half of the households indicated that they would pay up to 200 Bolivianos (\$28 USD) for a ceramic water filter. These results indicate that improved hygiene education is needed in peri-urban Trinidad. Furthermore, improved education should accompany distribution of filters during natural disasters to improve uptake and consistent use of these devices for water treatment. Ultimately, these results will be utilized to design water, sanitation, and hygiene interventions for residents in Trinidad, Bolivia.

### THE IMPACT OF A WATER TREATMENT PROGRAM ON GASTROINTESTINAL PARASITE FREQUENCY

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Previously published results revealed a reduction in the proportion of individuals with parasites present in stool in Honduran villages with potable water and sanitation interventions. The communities received no intervention (Control), a water treatment intervention (Water) or water treatment and sanitary pit latrine with pour flush toilet (Toilet).

Any intervention was associated with a lower proportion of positive (+) tests, though the proportion + was higher in the Toilet group than with Water only. The purpose of this analysis is to generate a hypothesis for this discrepancy. The primary outcome is the magnitude of difference in proportion of stool + (*Giardia*, *Cryptosporidium*, *Entamoeba*) between groups with subgroup analysis for age and gender. There were 4 Control communities (N = 86), 4 Water (N=112) and 4 Toilet ( N=53). Magnitude is measured with relative risk (RR) with 95% confidence intervals (CI) and logistic regression. The relative risk of a + test for the Control group versus any treatment was 1.98(CI 1.28-3.08). The Toilet group had less + than Control (RR1.32,CI.77-2.25), but more than Water group alone (RR 1.97,CI 1.03-3.78). By gender a trend towards increased + in the female group is seen in the Toilet group only (RR 1.49,CI.58-3.9). By multivariate analysis controlling for age and gender, the odds (OR) of having a + test were 0.42 (CI.23-.79) for any treatment compared to no treatment. For the Toilet group alone there was a trend towards increased odds for female gender (OR1.6,CI.43-5.9) not present when all patients were analyzed (OR.98,CI.52-1.8). In conclusion, though underpowered for subgroup analysis, these results indicate that the reduced improvement associated with communities who received toilet and water interventions may be due to the greater proportion of females + for parasites. The fact that women clean the toilets may account for their suspected increased parasite burden. Future investigations will include adjustments to the Toilet intervention to further test this hypothesis.

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#### SEASONAL DIARRHEA INCIDENCE IN NICARAGUA, 2001-2002: IMPLICATIONS FOR THE EFFECTIVENESS OF UNIVERSAL ROTAVIRUS IMMUNIZATION

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Approximately 90% of diarrhea episodes are treated at home and are not captured by commonly-used hospital-based surveillance systems. In order to estimate the potential benefit of rotavirus immunization in Nicaragua, we measured the diarrhea incidence among a community-based sample of children during the dry and rainy seasons. Rotavirus, the most common cause of childhood diarrhea, is transmitted most intensely during the dry season in Central America, while bacterial pathogens are typically more common during the rainy season. The study was conducted using the Health and Demographic Surveillance System in León, Nicaragua. We randomly selected 414 households from the sampling frame of the Surveillance System. Field interviewers enrolled 726 children under age 5 from these households and returned every 2 weeks to record any diarrhea episodes. The children were followed for 13 weeks during the dry season and 20 weeks during the rainy season in 2001-2002. The diarrhea incidence rate was calculated by season and compared using Poisson regression analysis. Among a total of 726 enrolled children, 216 children experienced diarrhea; 108 occurred during the dry season and 194 occurred during the rainy season. Diarrhea incidence in all age groups increased during the rainy season. Overall, incidence was 0.072 (95% CI 0.058, 0.085) episodes per person-month during the dry season and 0.090 (95% CI 0.077, 0.102) episodes per person-month during the rainy season. In conclusion, we found a higher incidence of diarrhea during the rainy season, using a community-based sample of children. These findings have implications for the recent introduction of the rotavirus vaccine, which will likely reduce the burden of diarrhea during the dry season, but have less of an impact during the rainy season.

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#### AN EXAMINATION OF THE RELATIONSHIP BETWEEN LEVELS OF DRINKING WATER QUALITY INDICATORS AND THE OCCURRENCE OF SELF-REPORTED DIARRHEAL DISEASE: A PROSPECTIVE COHORT STUDY IN THE DOMINICAN REPUBLIC, 2005-2006

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Based on the 2010 report of the Joint Monitoring Program, 884 million people do not have access to clean water. Lack of access to clean water is a known contributor to the occurrence of diarrheal disease (JMP, 2010). The purpose of this study was to examine the potential associations between the occurrence of diarrheal disease and the levels of three water quality indicators, turbidity, total coliforms, and *Escherichia coli* (E. coli), in 185 households in Bonao, Dominican Republic in a four-month observational study of diarrheal disease in 2005-2006. Datasets included a biweekly water quality dataset and a weekly diarrheal disease occurrence dataset. These two datasets were merged using three different methods, which impacted the number of observations. T-tests and odds ratios were calculated for all three different datasets. Multivariate logistic regression was also conducted. P-values of <0.05 and 95% confidence intervals were used to determine statistical significance of water quality indicators in predicting diarrheal disease. There were 430 cases of diarrhea out of 14,245 observations. In the age-adjusted multivariate logistic regression, turbidity (OR = 1.36; p-value = .012) was the only water quality indicator found to be positively associated with the occurrence of diarrhea disease. In conclusion, this study strengthens the evidence supporting a positive association between turbidity and the occurrence of diarrhea as has been shown in two recent studies examining drinking water quality and diarrheal disease in the United States. Future studies are needed to further clarify which water quality variables are predictive of diarrheal disease.

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#### EXAMINING THE INFLUENCE OF ECONOMIC AND POLITICAL FACTORS UPON ACCESS TO IMPROVED WATER AND SANITATION IN SELECT AFRICAN NATIONS, 2005-2008

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Today, 884 million people worldwide lack access to safe drinking water and 2.6 billion are without access to improved sanitation facilities. While many nations are on track towards meeting the Millennium Development Goals of decreasing the proportion of those without improved water and sanitation, progress in many developing nations is lacking. The purpose of this study was to determine what influence political and economic factors have on the availability of improved water and sanitation in developing nations, focusing on sub-Saharan Africa. This study addressed the following research questions: 1) Do political factors, specifically political stability (PS) and government effectiveness (GE), have an impact upon the availability of improved water and sanitation resources in sub-Saharan Africa? 2) Is gross national income (GNI) associated with the availability of improved water and sanitation resources? Data from the Demographic and Health Surveys of 11 sub-Saharan African nations conducted from 2005-2008 and from the World Bank indicators on PS, GE and GNI were analyzed using logistic regression models to examine the association between political and financial indicators and access to water and sanitation. A total of 109,606 observations were included in this study. The majority had access to improved drinking water sources (65.9%) and travel times < 30 minutes (83.3%). Most used no form of household water treatment (81.1%) and did not have an improved sanitation facility (64.1%). Overall, the strength and direction of the



association between economic/political factors and access to water and sanitation varied. GE and GNI had the strongest positive associations with access to improved water source and household water treatment. GNI was positively associated with access to improved sanitation; political stability was inversely associated with travel time to water source. The results of this study indicate that GNI, PS, and GE are associated with water and sanitation access in sub-Saharan Africa. With this information, context-specific interventions to improve and expand water and sanitation services in the region can be developed, focusing on building stable, effective governments, and alleviating the burden of poverty.

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#### **SCHISTOSOMA MANSONI CERCARIAE DETECTION IN WATER SAMPLES USING DEAD-END ULTRAFILTRATION AND PCR**

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An estimated 207 million people worldwide are infected with *Schistosoma spp.*, a water-borne parasitic helminth, with an estimated 779 million people at risk in 76 endemic countries. Transmission of schistosomiasis is spatially and temporally restricted to water bodies inhabited by competent intermediate host snails. Because humans become infected through contact with cercariae-infested water, identification of active transmission sites can help maximize the effectiveness of control interventions. The standard cercariometric filtration method relies upon suction and requires technically skilled staff to perform the collection. Moreover, water turbidity can drastically limit the volume sampled. We applied a previously described dead-end ultrafiltration (DEUF) method (Smith, 2009) using commercially available hollow-fiber dialysis ultrafilters for concentrating *Schistosoma* cercariae from 100-liter surface water samples followed by detection using real-time PCR (Gomes, 2006). Seeded water samples were pumped through the ultrafilter; the filter was then backflushed with a surfactant solution. The backflush solution was further concentrated through two 150 µm screens using a 60cc syringe. Lysis buffer (1.5 mL) was then slowly pushed through the filter screens and cercarial DNA was extracted and analyzed by PCR. The DEUF method consistently detected 5 cercariae in 100 liters of surface water with turbidities of up to 90 NTU. The limit of detection with other cercariometric methods is 1 cercariae in 5-10 liters of 120 NTU water. DEUF is a simple and cost effective method that can be utilized by untrained field personnel for rapid sample collection (~1 hr to collect 100 L). PCR detection of cercariae in DEUF samples offers a reliable technique for detection of cercariae in natural waters, which could be of great value in mapping areas of schistosomiasis infection risk, estimating the force of transmission and assessing whether transmission has been reduced after an intervention.

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#### **USE OF THE BRISTOL STOOL CHART TO COMPLEMENT SELF-REPORTED DIARRHEA AS AN OUTCOME MEASURE FOR WASH RESEARCH**

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We discuss the use of the Bristol Stool Chart as a potential outcome measure for public health research on water, sanitation, and hygiene interventions. The literature on WASH interventions has relied on mothers' reports of whether or not their children suffered from diarrhea, since more objective measures such as stool samples are difficult to obtain and expensive to analyze. As an alternative health indicator, mothers could be asked to rank their child's most recent stool according to the Bristol Stool

Chart, a 7-point pictorial scale that ranges from (1) "separate hard lumps" to (4) "like a sausage or snake, smooth and soft" to (7) "watery, no solid pieces". The Bristol Stool Chart has been used for clinical purposes among adults in upper-income countries, but no previous studies have used the scale as an indicator of gastroenteritis among children in developing countries. We asked over 300 mothers in rural western Kenya to rank their child's most recent stool on repeat survey visits, with an average of 7 observations per child under age 5 for over 1100 unique children. We would not expect a perfect correlation between this measure and the clinical definition of diarrhea used in the same survey ("3 or more looser than normal stools in a 24 hour period over the past 7 days"), since the stool chart measure is only the previous stool compared to an absolute scale whereas the clinical definition is compared to the child's normal stool and references a 7 day period. Nonetheless, the prevalence rate of loose stools (ranking of "7") according to the chart (21%) is almost twice as high as for the clinical definition of diarrhea (11%), p-value<0.001. Use of the chart is limited in that mothers were unable to rank stools of children over age 3, as they did not know what older, more mobile children's stools looked like, and the chart did not adequately represent the stools of infants under 6 months, as evidenced by the fact that in over a third of such cases mothers selected the option "other" rather than one of the 7 categories on the scale.

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#### **AN EXAMINATION OF HOUSEHOLD DRINKING WATER STORAGE AND MANAGEMENT PRACTICES IN BONA0, DOMINICAN REPUBLIC FROM SEPTEMBER 2005-JANUARY 2006**

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More than 2.2 million people die each year from diarrheal disease. Most cases of diarrheal disease can be linked to a lack of access to clean water and sanitation. The proper usage of sanitation, hygiene and safe drinking water are all mechanisms by which to prevent or limit fecal contamination, and in turn, will reduce the risk of diarrheal disease. In an attempt to better understand the roles of drinking water storage, we analyzed data collected during a prospective cohort study performed in the Dominican Republic from September 2005 to January 2006. The purpose of this study was to determine if characteristics of household drinking water storage containers influenced the concentration of *E. coli* in the stored household drinking water in communities of Bona0, Dominican Republic. Drinking water samples were taken at approximately two week intervals and tested for turbidity, total coliforms and *E. coli*. In addition, information was collected about the storage container, drinking water source and use of dippers or other utensils for serving the water. After testing independent risk factors for *E. coli* contamination using t-tests and chi-squared tests, it was established that household storage practices have a significant impact on drinking water quality. Specifically, water samples collected from narrow-mouthed containers had significantly less *E. coli* (geometric mean of 10.5 MPN/100 ml) than those collected from wide-mouthed containers (geometric mean of 25.1 MPN/100mL water). In addition, household drinking water samples that were reported to be treated via boiling or chlorination had significantly lower concentrations of *E. coli* compared to samples that were reported to be untreated. The geometric average *E. coli* for all untreated samples was 19.5 *E. coli* MPN/100mL and for all treated samples was 7 *E. coli* MPN/100mL. The results of this study suggest an association between household storage practices and concentrations of *E. coli* in household drinking water. This highlights the importance of understanding the role of household water storage practices and the need to encourage hygienic practices that might prevent or reduce contamination of drinking water during storage in the home.

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**RELATIONSHIPS BETWEEN TURBIDITY, WATER QUALITY AND CHLORINATION IN RURAL COASTAL ECUADOR**

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Chlorination can provide an effective and low-cost method of treating drinking waters, but the specific efficacy and effectiveness of chlorination of turbid waters under real world conditions remains under-studied. We present the results of a study of the impacts of turbidity on water quality and chlorination of water in rural coastal Ecuador. This study follows up two previously published studies from the same region. In the first, we observed on average a more than half-log reduction of indicator organisms between the source of drinking water to its point-of-use. In the second, no significant differences were observed in log reductions between drinking water of households that reported chlorination of their water. This follow-up study explores the role of source water turbidity in explaining these previously observed results. We report on E.coli contamination levels from samples collected in four villages from source waters, under household storage conditions (with and without water chlorination), and under controlled conditions. We report on water sampled from households both before and after agitation of the container, to address whether our previously observed reductions in indicator organisms during storage in the household are due to settling of turbid source waters or die-off of organisms in the storage container. In addition we report water quality results from trials examining the efficacy of chlorine dosage regimes currently recommended by the CDC for waters of varying turbidity. The results address the following questions: (1) Is turbidity related to microbial contamination in source waters and/or stored water? (2) Are our previously observed reductions in microbial contamination during storage due to settling or die-off of organisms? (3) What is the efficacy of recommended chlorination dosage of turbid waters? (4) What is the effectiveness of chlorination of turbid waters in the household context? This research provides important new insight about the relationship between turbidity, water quality, and chlorination under village conditions.

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**CHARACTERIZATION OF AN *IN VITRO* MALARIA LUMINESCENCE-BASED LIVER STAGE DRUG SENSITIVITY SCREEN**

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*Plasmodium* liver stages represent an ideal target for antimalarial prophylaxis since elimination of liver stages prevents development of the disease-causing erythrocytic stages. Although rodent and non-human primate models are available for evaluating causal prophylactic activity, the amount of compound needed for *in vivo* testing is substantial, and thus, requires costly and time-consuming scale-up synthesis. Since *in vivo* models are not a viable option for high-throughput drug screening or developing a proper structure-activity relationship around lead molecules, the need exists for an *in vitro* liver stage screen to complement the existing antimalarial blood-stage assays. In this study, we report the development of a 96-well luciferase expressing *P. berghei*-HepG2 *in vitro* liver stage screen to identify potential antimalarials. Our assay protocol does not require post-drug washes, media changes, or cell lyses, resulting in high inter-experimental reproducibility, throughput, and automation amenability. Furthermore, our approach utilizes soluble, non-toxic

d-luciferin, which allows measurement of parasite growth over time. A panel of antimalarial drugs with known blood- and liver-stage activities were benchmarked in the system by determining their respective 50% inhibitory concentrations (IC50s). The results compare to other previously published *in vitro* liver-stage drug IC50 values. Counterscreening using MTT whole-cell toxicity assays was performed and selectivity indices calculated. Additionally, drug susceptibility time courses on select drugs were explored. Finally, activity profiles of novel compounds displaying liver-stage inhibition will be briefly highlighted. The results obtained indicate that the assay significantly increases our screening capabilities to discover potential antimalarials affecting liver-stage infection.

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**HEPATOCTE-BASED ASSAY TO ASSESS THE EFFECTS OF DRUGS ON THE PRE-ERYTHROCYTE STAGES OF *PLASMODIUM VIVAX***

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Recent data indicate that the impact of *Plasmodium vivax* (Pv) malaria on the health and economies of the developing world has been dramatically underestimated. Pv has a unique dormant stage in its hepatic cycle, the hypnozoite, which allows the blood stage infection to relapse in the absence of reinfection. Hypnozoites are characterized as persistent parasites of around 4 μm diameter. There have been no reports in the literature of assessment of drugs against the liver stages of Pv *in vitro*. We are developing a medium to high throughput assay to identify new drugs against Pv liver stages, including hypnozoites. As a first step we established a system for infecting human hepatoma (HepG2) cells with cryopreserved Pv sporozoites (PvSPZ). The cryopreserved PvSPZ invade HepG2 cells and develop into late liver stage schizonts or remain as dormant structures resembling hypnozoites. We next assessed the effects of primaquine (PQ), atovaquone (AQ) and chloroquine (CQ) on liver stage parasite development in a 3 day assay in a 96 well format. PQ showed 22%, 56%, 77% inhibition of liver stage parasite numbers at 1, 10, 100 μg/mL concentrations, AQ showed 20%, 66%, 86% inhibition at 1, 10, 100 ng/mL concentrations respectively and CQ did not show any significant inhibition. The high concentration of PQ needed to achieve inhibition similar to AQ could be due to an inability of the HepG2 cells to metabolize PQ. Dose dependent effects of PQ and AQ, but not by CQ indicate that our assay has the capacity to reproducibly evaluate drugs against the hepatic stages initiated from cryopreserved PvSPZ. These data provide the foundation for finalizing a medium to high throughput assay to identify new drugs for the elimination of Pv liver stages including hypnozoites.

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**EFFICACY OF ARTEMETHER-LUMEFANTRINE (AL) IN THE TREATMENT OF BLOOD STAGES OF *PLASMODIUM VIVAX***

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The significant burden of *Plasmodium vivax*, traditionally underappreciated, is currently in the spotlight, specially in southeast Asia, where chloroquine (CQ) resistant strains to this species led to the use of artemisinin-based combination therapies (ACTs) for its treatment. The effect of the currently recommended dosing regimen of AL on *P. vivax* was reviewed in the 4 Novartis sponsored studies which included patients

(pts) with mixed infection (*P. vivax* / *falciparum*) at baseline (n=13, 5, 16, 2). All pts were cleared from *P. vivax* by 48, 24, 42 and 24 hours, for each study, respectively. Reappearance of *P. vivax* was observed between day 18-49 in 6/13, 2/5, 3/16 and in 0/2 of pts, respectively (primaquine (PQ) was administered after day 7 in 1 pt). Literature review suggests that AL achieves a mean PCT of 1.2 days [0.9, 1.6] with 80% of pts having cleared parasites at D1, as reported previously. However, if not administered with PQ, the incidence of recrudescence, being re-infection or relapse, is around 50%, observed mainly after D28 (1;2). This is higher than observed with dihydroartemisinin-piperaquine (DP), probably due to the relatively shorter half life of lumefantrine compared to piperaquine. Moreover, AL combined with PQ (in pts not G6PD-deficient) (n= 38) is safe and provides faster PCT than the combination of CQ/PQ (mean 41.6 h vs 55.8 h, p<0.001), with a cure rate at D28 of 97.4%, as reported previously. AL is effective against blood stages of *P. vivax* and allows fast parasite clearance. If AL is administered alone, the risk of recrudescence, re-infection or relapse, remains however high and needs to be monitored. The combination of AL and PQ provides high rates of radical cure.

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#### EFFECTIVENESS AND SAFETY OF ARTEMETHER-LUMEFANTRINE IN THE USA: SURVEILLANCE IN PEDIATRIC AND ADULT PATIENTS WITH *PLASMODIUM FALCIPARUM* MALARIA

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Most studies of the safety and efficacy of artemether-lumefantrine (AL) have been conducted in malaria endemic regions, mainly in Southeast Asia and sub-Saharan Africa. However, fewer data are available in non-immune patients, as the largest clinical trial so far was performed in 165 non-immune patients from Europe and non-malarious areas of Colombia. This study showed that AL was effective (PCR corrected 28-day parasitological cure rate of 96.0%) and well tolerated, including in patients >70 kg of body weight. In April 2009, AL received marketing approval from the US Food and Drug Administration (FDA), who requested that a "descriptive study of the use of AL in non-immune travelers" be conducted as a post-marketing requirement. Therefore, the current evaluation, designed by Novartis in partnership with the US Centers for Disease Control and Prevention (CDC), aims to assess for a period of 5 years, pediatric and adult patients (US and foreign residents) diagnosed with malaria and treated with AL with regard to demographics, malaria immune status, treatment effectiveness, and adverse events (AEs). As malaria is a reportable disease in the US, the evaluation was designed to assess public health surveillance data reported to CDC using the 'Malaria Case Surveillance Report' form. This form was recently adapted to capture additional information including AEs and serious AEs. The evaluation will provide a unique opportunity to gather data in elderly patients as well as in patients with a BMI  $\geq 25$  kg/m<sup>2</sup>. Data collection and reporting methods will be described.

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#### PATHWAYS TO DISCOVERY OF NON-HEMOLYTIC 8-AMINOQUINOLINE ANTIMALARIALS

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8-Aminoquinolines (8AQ) are the only drug class that kills the key parasite stages necessary for the survival of malaria. For *Plasmodium falciparum* (Pf) malaria, this is the mature (stage 5) gametocytes (Pfg) which transmit the infection. For *P. vivax* (Pv) and *P. ovale* (Po), this is the sleeping liver stage or hypnozoite, which emerges weeks to months after the initial infection and causes relapse. Primaquine is the only approved drug that can do

this but it is not widely deployed because of toxicity concerns, especially hemolytic toxicity in population with glucose 6-phosphate dehydrogenase (G6PD) deficiency. G6PD deficiency is the most common enzymopathy and it is prevalent in malaria endemic regions. A Non-hemolytic 8AQ Consortium was formed to identify 8AQ or 8AQ combinations with an improved therapeutic index. The first objective of the consortium is to develop predictive models for hemolytic toxicity in G6PD-deficiency. Clear progress being made with one *in vitro*, two mouse and one Rhesus model. Once the models are fully qualified, they will be used to identify drug combinations with or isomers of existing drugs to mitigate the risk of existing drugs as high priority. Unique approaches are also being used to select new 8AQ from the 1800+ in the Walter Reed Army Institute of Research chemical information system and literature for assessment in the G6PD models, with priority to those with documented efficacy in humans or the Rhesus relapsing model. Investigations on mechanism of hemolytic toxicity are also being pursued. The current overall status of this project will be presented.

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#### INESS-INDEPTH EFFECTIVENESS AND SAFETY STUDIES FOR ANTIMALARIALS IN AFRICA: A PHASE IV STUDY PLATFORM

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Researcher-driven Phase I, II and III randomized, controlled, clinical trials have been well supported and establish the initial safety and efficacy of such products when delivered under ideal conditions. However, large scale Phase IV studies to determine the effectiveness and frequency of severe adverse events when the intervention is delivered in real-life systems is a missing piece of the drug development pipeline. In Africa, new drugs are offered for national policy decisions with as few as 6,000 patient exposures and no long term follow-up. Phase IV safety and effectiveness data, generated outside trial conditions, will be valuable for national malaria control programmes in Africa before the widespread use of such new treatments. INESS undertakes Phase IV effectiveness and safety studies of new combination therapies (and other drugs and vaccines) for malaria in at least 8 INDEPTH Demographic Surveillance System (DSS) sites in 4 countries of Africa over a four year period. The main product of the platform is a longitudinal evidence base to allow assessment of efficacious drugs in real life settings. The study employs several modules under two main modules of system effectiveness and safety. In the past one year, data has been collected from five DSS sites in Ghana and Tanzania on the current antimalarials. This year the study extends to include Burkina Faso and Mozambique and will begin data collection on newly registered ACTs. The experience of setting and carrying out a phase IV platform for investigation of not only antimalarials as well other health commodities in future is a rare experience. Experiences derived from the implementation of this platform are unique and tedious, particularly the set up of electronic data capture and biometric system for identification of patients and linking of health facility and DSS data. All these experiences are documented within INESS and is worth sharing with other researchers, academicians as well as policymakers.

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#### AN ATYPICAL PROTEIN PHOSPHATASE IS CRITICAL FOR THE INTRAERYTHROCYTIC DEVELOPMENT OF *PLASMODIUM FALCIPARUM*

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An insertion mutagenesis screen of *Plasmodium falciparum* identified PF13\_0027, annotated as a hypothetical protein, to be crucial for intraerythrocytic development. This protein with unknown function



consists of two distinct conserved eukaryotic domains: 1) an inactive rhodanese domain and 2) an atypical protein tyrosine phosphatase (PTP) domain. This tandem domain structure is similar to many eukaryotic dual specificity phosphatases, including the MAP kinase phosphatases. The atypical PTP putative catalytic domain of PF13\_0027 lacks the signature arginine residue and has an insertion adjacent its catalytic site. Most merozoites produced from the PF13\_0027 KO clone fail to initiate or complete invasion of new host erythrocytes resulting in a severely attenuated blood-stage growth phenotype. Although early asexual development appears normal, a defect in cell cycle regulation becomes evident in late trophozoites due to a significantly longer pre-S phase (52 hrs) versus wild-type parent (46 hrs). Another significant defect becomes noticeable in late schizont development as premature degradation of the parasitophorous vacuole membrane leaves merozoites free in the erythrocyte cytoplasm and the explosive rupture of egress is not usually observed. As a result of this defective development unusually large numbers of merozoites accumulate in the culture supernatants of this phosphatase null mutant, as merozoites fail to invade host erythrocytes. Microarray analysis revealed significant alterations of specific metabolic pathways suggesting a regulatory function for this phosphatase in asexual development and a key role in regulating progression into sexual stage development, consistent with its role in crucial signaling cascades.

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#### EVIDENCE FOR THE EFFLUX OF A RANGE OF ANTIMALARIAL DRUGS AND 'CHLOROQUINE RESISTANCE REVERSERS' FROM THE DIGESTIVE VACUOLE IN MALARIA PARASITES WITH MUTANT FORMS OF THE CHLOROQUINE RESISTANCE TRANSPORTER

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Chloroquine resistance in the malaria parasite *Plasmodium falciparum* is conferred by mutations in the *P. falciparum* Chloroquine Resistance Transporter (PfCRT). PfCRT localises to the membrane surrounding the digestive vacuole, an acidic organelle in which chloroquine accumulates to high concentrations and exerts its toxic effect. Previously, we have shown that chloroquine-resistant malaria parasites show an increased leak of H<sup>+</sup> ions from their digestive vacuole in the presence of chloroquine. This observation was attributed to the transport of chloroquine, together with H<sup>+</sup>, out of the digestive vacuole via mutant PfCRT. Here, we show that chloroquine:H<sup>+</sup> efflux from the digestive vacuole is present in transfectant parasites expressing mutant PfCRT in the context of different genetic backgrounds, including in parasites that are not rendered highly chloroquine resistant by the introduction of the mutant protein. Further, we show that a range of other antimalarial drugs, as well as various 'chloroquine resistance reversers' induce an increased leak of H<sup>+</sup> ions from the digestive vacuole in parasites expressing mutant forms of PfCRT, consistent with these compounds being substrates for mutant forms, but not the wild-type form, of PfCRT. The finding that chloroquine resistance reversers are substrates for mutant PfCRT has implications for the mechanism of action of this class of compound.

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#### THE MECHANISMS OF LATENCY OF MALARIA PARASITES IN THE MOSQUITO SALIVARY GLANDS

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Sporozoites, the invasive form of malaria parasites transmitted by mosquitoes, are quiescent while in the insect salivary glands. Sporozoites only differentiate inside of the hepatocytes of the mammalian host. We show that sporozoite latency is an active process controlled by a eukaryotic initiation factor-2 $\alpha$  (eIF2 $\alpha$ ) kinase (IK2) and a phosphatase. IK2 activity is dominant in salivary gland sporozoites leading to an inhibition of translation and accumulation of stalled mRNAs into granules. When sporozoites are injected into the mammalian host, an eIF2 $\alpha$  phosphatase removes the PO<sub>4</sub> from eIF2 $\alpha$ -P, and the repression of translation is alleviated to permit their transformation into liver stages. In IK2 knockout sporozoites eIF2 $\alpha$  is not phosphorylated and the parasites transform prematurely into liver stages and lose their infectivity. Thus, to complete their life cycle, *Plasmodium* sporozoites exploit the mechanism that regulates stress responses in eukaryotic cells.

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#### TARGETING PLASMODIUM SPOROZOITE-KUPFFER CELL INTERACTIONS WITH A PHAGE DISPLAY LIBRARY

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After inoculation by the bite of an infected mosquito, the *Plasmodium* sporozoite enters the blood stream and infects the liver with unique specificity. After capture by protruding glycosaminoglycans, sporozoites migrate along the sinusoidal wall until they find and invade a Kupffer cell, thus gaining access to the underlying hepatocytes. Previous evidence suggests that specific sporozoite-Kupffer cell interactions are required for invasion to occur. A screen of a phage display library for peptides that bind to rat Kupffer cells yielded three peptides. Notably, peptide binding to Kupffer cells strongly inhibited *Plasmodium berghei* sporozoite invasion. These observations support the hypothesis that the peptides bind to Kupffer cell receptor(s) for sporozoite invasion. In a separate set of experiments we found that antibodies against the candidate peptides recognize sporozoite protein(s) and importantly, inhibit invasion, suggesting that the candidate peptides structurally mimic sporozoite ligands that are required for invasion. These findings could provide the basis for the identification of novel protective antigens for use in pre-erythrocytic vaccines.

### LIMITED ABILITY OF *PLASMODIUM FALCIPARUM* *PFCT*, *PFMDR1*, AND *PFNHE-1* POLYMORPHISMS TO PREDICT QUININE *IN VITRO* SENSITIVITY OR CLINICAL EFFICACY IN UGANDA

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Quinine (QN) remains a standard drug for treating severe malaria in Africa, and it is increasingly used to treat uncomplicated malaria. However, failures of QN therapy are common. A recent effectiveness study in Uganda found that 23% of children treated with QN experienced recrudescence over 28 days of follow-up. Mechanisms of resistance to QN are uncertain, and it is unknown if QN treatment failures in Africa are due to drug resistance. Recent studies have identified associations between *in vitro* QN sensitivity and polymorphisms in the *pfct*, *pfmdr1*, and *pfnhe1* genes, all of which encode putative transporters. In particular, for *pfnhe1*, which encodes a putative Na<sup>+</sup>/H<sup>+</sup> exchanger, varied numbers of DNNND or DDNHNDNHND repeats in ms4760 have been associated with different levels of *in vitro* QN sensitivity. To better characterize mediators of QN sensitivity and treatment failure, we characterized associations between genetic polymorphisms, *in vitro* QN sensitivity, and QN treatment responses in Uganda. Among 172 fresh clinical isolates tested (IC50 range 15.4 - 760.9 nM, based on HRP-2 ELISA assays), a trend of decreasing sensitivity to QN was observed with accumulation of *pfmdr1* mutations at codons 86, 184 and 1246. All parasites had the *pfct* 76T mutation and wild type sequences at *pfmdr1* 1034 and 1042. Considering *pfnhe1*, sequence analysis showed that ms4760 is highly polymorphic, with 29 novel genotypes identified in addition to 19 previously reported. Two copies of either the DNNND or DDNHNDNHND repeat, compared to 1 or >=3 repeats, were weakly associated with decreased QN sensitivity, but differences were not significant. Considering samples from 66 subjects treated in the clinical trial, none of the polymorphisms noted above predicted QN treatment failure. Our data suggest that known polymorphisms in *pfct*, *pfmdr1*, and *pfnhe1*, while associated with QN sensitivity in some studies, are not robust markers for QN resistance, and each likely will be of limited value as a tool for the surveillance of QN resistance in Africa.

### NOVEL CHIMERIC VACCINES FOR CHIKUNGUNYA: IMMUNOGENICITY AND EFFICACY STUDIES IN A129 MICE

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Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that causes explosive outbreaks of febrile illness associated with rash, painful arthralgia and sometimes arthritis. There is currently no commercial vaccine for CHIKV. Therefore, the development of a new, safer vaccine is needed. We constructed a candidate CHIKV vaccine based on introducing RNA sequence elements to the wild-type CHIKV strain that prevent

efficient expression of the structural proteins in insect cells. To this end, we used the Encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), which is nonfunctional in insect cells (LR CHIKV/mutSG/IRES). Infection with this new virus construct was assessed in A129 homozygote mice, which are defective in interferon  $\alpha/\beta$  signaling, and compared it to the 181/25 live attenuated vaccine. Groups of adult A129 mice were injected intradermally with each of the CHIKV candidate vaccines. All mice injected with the 181/25 vaccine survived with no apparent signs of disease except a temporary ruffled appearance and body weight loss. The severity of this transient morbidity correlated inversely with the infecting dose of virus. In contrast, mice receiving the LR-CHIKV/mutSG/IRES construct remained healthy with no apparent signs of morbidity, weight loss or foot pad swelling. Mice immunized with either vaccine construct seroconverted with detectable neutralizing antibody responses measured on day 21 and 35 post immunization. When challenged intradermally with the 10<sup>2</sup> PFU wild-type CHIKV, all mice were protected with no clinical signs of disease (weight loss, fever or foot pad swelling). In contrast, control mice immunized with PBS succumbed to infection by day 3 post challenge. Immune sera collected from mice vaccinated with either candidate vaccine conferred full protection against lethal CHIKV challenge in passively immunized A129 mice. This result indicates the protective role of antibodies against CHIKV infection. Overall, these findings highlight that our new vaccine candidate is safe and efficacious and offers a promising strategy to prevent CHIKV epidemics.

### LONG-LASTING OVERMORBIDITY AND IMPAIRED QUALITY OF LIFE 30 MONTHS AFTER CHIKUNGUNYA INFECTION: COMPARATIVE COHORT OF FRENCH GENDARMES EXPOSED TO CHIKUNGUNYA IN 2006 IN REUNION ISLAND

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In June 2006, a survey studying the prevalence of Chikungunya (CHIKV) was settled among the French gendarmes exposed to the large 2005-2006 outbreak in Reunion Island (southwest Indian Ocean). The chikungunya prevalence in this cohort was 18.8% (126/671 participants). Our objective was to observe the long-lasting morbidity due to CHIKV infection within the same cohort by comparison of clinical symptoms and quality of life (QOL) between gendarmes infected with CHIKV (CHIK+) and non infected (CHIK-). Self-questionnaires collecting clinical symptoms, health care service consumption and QOL (SF-36) were sent by mail to all gendarmes who participate to the 2006 survey. Based on the two questions: "Do you think that you got chikungunya infection during your stay in Reunion Island?" and "Do you consider that you are healed?", patients were ordered in three groups: healed CHIK+, non healed CHIK+ and CHIK-. Among the 398 responders (92% male, median aged of 42.8 years), 101 (25.4%) were CHIK+. Between July 2006 and June 2008, CHIK+ subjects remained at least 5 times more symptomatic for rheumatic symptoms than CHIK- and their healthcare consumption was 1.7 time higher (9.5 consultations versus 5.5 consultations). In June 2008 (in median 30 months after infection), CHIK+ subjects still significantly more frequently complained for joints pain, swelling and stiffness than CHIK- with a gradient of severity between healed and not healed CHIK+ subjects. Moreover, at the same time, CHIK+ subjects considered that their pain moderately (52%) or highly (18%) reduced their activity while 81% of CHIK- declared no pain limitation. As well, all dimensions of SF36 and both physical (PCS) and mental component (MCS) summaries were impaired in CHIK+ subjects with a decreasing gradient from not healed (Mean PCS: 43.6; Mean MCS: 41.6) to healed CHIK+ (Mean PCS: 52.0; Mean MCS: 47.5), then to CHIK- (Mean PCS: 54.8; Mean MCS: 50.8, p<0.001). In conclusion, this comparative study among a young active male population concludes to a persistent high overmorbidity and impaired QOL due to CHIKV at 30 months of infection, even in patients considering themselves healed.

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### THE EFFECTS OF IMMUNE SUPPRESSION ON CHIKUNGUNYA VIRUS PATHOGENESIS IN MICE

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Chikungunya virus (CHIKV) is a mosquito-transmitted *Alphavirus* that causes an illness, characterized by fever, rash and incapacitating joint and muscle pain. CHIKV-induced arthralgia can be recurrent and persistent in some people for months and up to 5 years after infection. CHIKV is endemic and sporadically epidemic in Asia and Africa; the recent epidemic in Asia involved 2 million people. Despite its frequency, the pathogenesis of CHIKV in people is poorly understood. To help elucidate CHIKV pathogenesis, a mouse model of CHIKV infection was developed in our laboratory. Previous work has demonstrated that needle inoculation of CHIKV in young mice causes a self-limiting infection characterized by 3-4 days of viremia with persistence of virus in spleen and skeletal muscle for several more days as well as severe focal necrosis in the skeletal muscles. Recent published work has shown that CHIKV pathogenesis may be dependent on cells of the immune system. Suppression of the immune response of mice prior to infection with CHIKV is helping us to understand how immune-suppression changes CHIKV pathogenesis. The use of cyclophosphamide and glucocorticoid steroids decrease the inflammation associated with CHIKV infection in mice with no increase in mortality or sickness. Viremia levels are similar in treated and untreated mice. Results to be presented include histopathologic analysis of skeletal with and without immune suppression as well as the effect of CHIKV infection on blood chemistry and liver enzyme values. These results help us to better understand CHIKV infection in an at risk population as well as aiding in the development of therapeutic options in people.

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### INNATE IMMUNE RESPONSE TO RIFT VALLEY FEVER VIRUS INFECTION

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Rift Valley fever virus (RVFV), a member of the *Bunyaviridae* family, is an important pathogen in Africa and on the Arabian Peninsula. RVFV can cause severe disease; hemorrhagic fever or encephalitis, in humans and animals, particularly ruminants. Due to the rapid progression from symptoms to death and the high containment facilities needed to work on RVFV, there is little data on the effect of infection on the host innate immune response and how this correlates with pathogenesis. In an effort to characterize the host response to a vaccine strain (MP-12) and a wild-type strain (ZH501) in the mouse model, cytokine levels in infected C57BL/6 mice were measured in serum, liver, brain and spleen. These data have shown that ZH501 infection significantly increases chemokines (KC, MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP) while it decreases Th1 [IFN $\gamma$  and IL-12(p70)] associated cytokines when compared to MP12 infections in the organs examined. Interleukin-1 $\alpha$  and IL-1-responsive cytokines increase in ZH501 infection with peak concentration levels at 72 hpi. There is no change in type II interferon response between the three treatments except in the brain of ZH501 infected animals, where there appears to be an IFN $\gamma$  response late in infection. The most dramatic differences in cytokine concentration are seen between 48 and 84 hpi with 60 and 72 hpi appearing to be critical time points in mouse infection as these points represent the largest differences in cytokine response in this model. Blood chemistry analysis demonstrated an increase in liver enzymes and a decrease in liver and kidney function markers (glucose, bilirubin, and BUN) at 60-72 hpi in ZH501 infected animals while MP-12 and mock infected animals were largely unchanged. Given the recognized hepatotropism of RVFV, loss of liver function is not surprising. Data shown here demonstrate

that, unlike the vaccine strain, RVFV wild-type virus targets the brain and visceral organs and causes a significant inflammatory response. These significant differences in viral pathogenicity indicate that, despite inducing a productive infection in mice, the host is able to limit the effect of viral infection on the innate immune response.

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### PROTEOMIC CHARACTERIZATION OF THE RIFT VALLEY FEVER VIRUS NSS PROTEIN

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Rift Valley Fever Virus (RVFV), a member of the *Bunyaviridae* family, is a priority pathogen for both the CDC and USDA. While RVFV has a significant effect on livestock, it can also cause hemorrhagic fever in humans. Our research is aimed at determining critical RVFV host interactions to identify therapeutics targeted against the host. RVFV is a negative stranded RNA virus that encodes three segments: large (L), medium (M) and small (S). The S segment codes for the NSs protein which is nonstructural and is not required for viral replication, although it does play a major role in viral pathogenesis. NSs protein exists in both the nucleus and cytoplasm and forms distinct filamentous structures in the nucleus. It is known to suppress transcription of host mRNA through interactions with TFIIF subunit p44. In this study we used proteomic methods to identify novel host proteins that interact with NSs. Using mass spectrometry we identified multiple novel NSs interacting proteins, including Protein Arginine Methyltransferase 5 (PRMT5) and Heat Shock Protein 70 (HSP70). The association of NSs with PRMT5 led us to investigate methylation of NSs. Interestingly, Methylation Modification Prediction Server (MeMo) predicted multiple arginine methylation sites, but no lysine methylation sites. Treatment of MP-12 infected Vero cells with the general methyltransferase inhibitor, AdOx, resulted in decreased viral replication. Current studies are focused on identifying the site(s) of NSs methylation and effects of methylation on NSs function.

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### PATHOGENESIS OF MONKEYPOX VIRUS IN CYNOMOLGUS MACAQUES INFECTED BY THE INTRAVENOUS OR INTRABRONCHIAL ROUTE

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Monkeypox virus (MPXV), an orthopoxvirus, is a zoonotic disease of primarily Central Africa which causes outbreaks of disease associated with case fatality rates of up to 10%. In addition to variola virus, the causative agent of smallpox, MPXV is also a potential agent of bioterrorism. Infection of nonhuman primates (NHPs) with MPXV, primarily by the intravenous (IV) route, serves as a model of orthopoxvirus disease in humans although disease course is considerably accelerated. The objective of this study was to further characterize the pathogenesis of the IV model of MPXV infection and to determine the utility of intrabronchial (IB) inoculation of NHPs as a novel model that might more closely resemble the progression of MPXV disease in humans. Three NHPs inoculated IB with 5x10<sup>6</sup> PFU of MPXV (66% morbidity) were compared to NHPs inoculated IV with the standard 5x10<sup>7</sup> PFU dose (83% morbidity). Mean time to fever onset was 2.8 and 4.2 days for the IV and IB groups,



respectively. Lesions and infectious virus in oral and nasal swabs were detected 4 days post IV inoculation and 7 days post IB inoculation. In addition, mean day of moribundity for the IV route was 9.8 days and 20 days for the IB route. Virus distribution across 19 tissues was unaffected by route, although virus load was typically 10-fold higher after IV inoculation. Disease course in a subset of NHPs was also evaluated using PET/CT imaging. NHPs were imaged at several time points using  $^{18}\text{F}$ -FDG as a nonspecific indicator of inflammation. Inflammation and consolidation of lungs in NHPs infected by the IB route was visualized during the progression of disease and resolution was observed in one animal. Lymphadenopathy and suspected immune activation was also detected by PET/CT imaging in the axillary lymph nodes of NHPs infected by both routes. Taken together, these results indicate that the IB route results in severe lesional disease with a delay in the onset of disease symptoms and moribundity, but the severe viral pneumonia associated with this route may limit the application of this model.

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#### **PATHOPHYSIOLOGIC ASSESSMENT OF EBOLA VIRUS INFECTION WITH AND WITHOUT INTRAVENOUS FLUID TREATMENT IN A NONHUMAN PRIMATE MODEL USING A MULTI-SENSOR TELEMETRY SYSTEM**

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The pathophysiology of Ebola virus infection in humans and NHPs is not well characterized. We sought to characterize the pathophysiology of Ebola virus infection and impact of IV fluids in an NHP model. Nine adult rhesus macaques (6M/3F) were implanted with multi-sensor telemetry devices and internal jugular catheters and subsequently challenged with ~100 pfu IM *Zaire ebolavirus* (Kikwit, 1995). Five animals were controls and four animals received IV normal saline using a treatment algorithm. Physiologic signals were recorded continuously. Daily labs were collected for viremia, chemistries, hematology, and cytokines. All animals became ill. Pre- and post-challenge physiologic parameters were compared. 2/5 untreated and 1/4 treated animals survived beyond D10: 1 untreated animal was euthanized on D18 for an eye infection and two animals survived without sequelae beyond 5 months until euthanized. Viremia levels were lower in surviving animals. Pre-illness diurnal patterns for BP, pulse, temp, RR, and contractility index disappear after fever onset. Late-stage infection was associated with a progressive and steady decline in mean arterial blood pressure and systolic blood pressure that began 36 to 48 hours before ultimate obtundation and euthanasia. Lactic acidosis and renal failure developed in the final 24-48 hours in non-survivors. A decline in the cardiac contractility index was observed in animals that succumbed to infection. IV fluid boluses appeared to result in transient improvement in hemodynamic parameters. Early aggressive IV fluids in one animal appeared to stabilize BP and improve contractility index. A rise in respiratory rate and end-diastolic pressure was also observed in this animal. Graphs will be used to illustrate physiologic trends in individual animals and across animals. In conclusion, this data provides insight into physiologic changes in Ebola virus infection. Late-stage infection manifests as progressive hemodynamic compromise and may be responsive to fluids.

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#### **A MATRIGEL PLUG MODEL TO STUDY FILARIAL PATHOLOGY IN RATS**

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Lymphatic filariasis is caused by the parasitic nematodes *Wuchereria bancrofti* and *Brugia malayi* which infect over 120 million people worldwide. Most microfilaria-positive individuals are asymptomatic, but with subclinical lymphangiectasia. We hypothesized that the excretory-secretory products (ES) of the worms activate the lymphatic endothelium directly or indirectly through an accessory cell. Initial experiments suggested that the ES did not directly activate lymphatic endothelial cells (LEC). Monocytes have been shown to play a role in lymphangiogenesis by secreting soluble factors; so we analyzed the production of IL-8, IL-6 and VEGF-A by peripheral blood mononuclear cells (PBMC) from naïve donors following stimulation with filarial ES as these factors can support endothelial proliferation and function. ES-stimulated PBMCs produced significantly increased levels of IL-8, IL-6 and VEGF-A compared to cells cultured in media alone and CD14+ monocytes were the primary producers of IL-8 and VEGF-A. Furthermore, IL-8, IL-6 and VEGF-A induced *in vitro* tubule formation by LEC in Matrigel cultures. Therefore, we tested the ability of IL-8, IL-6 and VEGF-A to stimulate *in vivo* vessel formation in August rats using a Matrigel plug model to measure lymphangiogenesis. Haematoxylin and eosin staining as well as immunohistochemical analysis using antibodies specific for LEC markers such as LYVE-1, podoplanin and VEGFR-3 revealed that stimulation with rat recombinant IL-8, IL-6 and VEGF-A induced cellular infiltration into the Matrigel plugs and *in vivo* LEC tubule formation. In addition, supernatants from human naïve PBMCs stimulated with filarial ES products were also incorporated into the Matrigel plugs and induced *in vivo* vessel formation. Collectively, these data suggest that monocytes may support lymphatic function through the secretion of soluble factors to encourage vessel growth and contribute to the pathogenesis of filarial disease. Our Matrigel experiments establish a useful model to study the molecular mechanisms associated with filarial pathology *in vivo*.

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#### **BASOPHILS ARE NOT NECESSARY FOR PROTECTION AGAINST CHALLENGE INFECTION IN A FILARIA VACCINE MODEL**

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Live, irradiated L3 larvae induce substantial protection against challenge infection with *Litomosoides sigmodontis*, the only murine model of filariasis in which the parasites develop to mature, microfilaria-releasing adult worms. Recently, other investigators have demonstrated that basophils play an important role in protective immunity against intestinal helminth infections. To establish the role of basophils during vaccine-induced protection against filariae, Balb/c mice were immunized with three weekly s.c. injections of 25 irradiated L3 larvae, depleted of basophils with weekly i.p. injections of anti-CD200R3 antibody, and subsequently challenged s.c. with 40 infective L3 larvae. Mice were sacrificed 4 weeks p.i. Vaccine-induced responses in mice were characterized by high levels of parasite-specific IgE and IgG1, eosinophilia, and elevated concentrations of circulating IL-4. In addition, splenic CD4+T cells of vaccinated mice produced IL-4 and proliferated in response to parasite-antigen. Vaccinated mice that were depleted of basophils exhibited an attenuated type 2 response characterized by reduced levels of parasite-specific IgE and IgG1, less eosinophilia, and lower concentrations of IL-4 in blood. Even

though type 2 immune responses induced by vaccination were lower in basophil-depleted mice, basophil depletion did not alter protective immunity induced by the vaccination regimen. Specifically, vaccinated and vaccinated+ basophil-depleted mice had average worm recoveries of only 3.5 and 3.2 worms per mouse, respectively, whereas infected mice had an average of 20 adult worms per mouse. These findings suggest that basophils amplify type 2 immune responses, but are not necessary for vaccine-mediated protection against this tissue-invasive nematode.

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#### EARLY EXPOSURE TO HELMINTH ANTIGENS INDUCES SPECIFIC TH1 AND TH2 CYTOKINES IN INFANTS WITH NO EFFECT ON SUBSEQUENT RESPONSE TO BCG

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Helminth infection during pregnancy have been reported to affect immune responses of the fetus and therefore may impact on how newborns respond to new antigens encountered early in life. Children and adults infected with helminths generally develop Th2-skewed immune responses as well as regulatory T cells which can compromise the immune response to Th1-producing vaccines such as BCG. We asked whether this is also the case in infants with developing immune maturity. Heparinized venous blood was collected from a total of 74 pairs of pregnant mothers and their infants at the age of 2 and 5 months, 1 year, 2 and 4 years of age. Whole blood assay was performed and samples were stimulated with crude *Brugia malayi* or *Ascaris lumbricoides* antigens. Measurement of Th1 (IFN- $\gamma$ ) and Th2 (IL-5) cytokines was done by luminex. Filial specific IgG4 from plasma was analyzed by ELISA. Maternal filarial status was determined by filarial antigen detection and intestinal helminths by direct examination of stool. While maternal cytokine responses to helminths showed more Th2 dominance, children showed much lower helminth-specific Th2 responses but these responses increased with increasing age. Th1 responses to helminth antigens were more pronounced in children at early age. Starting from 2 years of age, Th2 response to BmA was higher in children born to helminth positive mothers compared to those born to helminth-free mothers. When we examined responses to PPD after BCG vaccination, both children born to helminth-infected or helminth-free mothers showed no significant differences in the Th1 and Th2 productions against PPD for up to 4 years of age. The results indicate the priming of child immune responses to helminth antigen resulted in both Th1 and Th2 responses, with no effect on subsequent cytokine responses to PPD.

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#### CHRONIC HUMAN FILARIAL INFECTION LEADS TO ALTERED T CELL MEMORY AND A DEFECT IN EFFECTOR CELL TRANSITION

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Chronic filarial infection has been associated with defects in parasite-specific T cell responses. Whether this defect reflects altered T cell memory, as occurs in chronic viral infections, has not been examined. Using multiparameter flow cytometry to identify T memory cell subpopulations in well-characterized patient groups from the filarial-endemic Cook Islands, we examined the role of persistent infection in the maintenance of T cell memory. Compared to filarial-uninfected endemic normals (EN, n=6), phenotyping of CD45RA<sup>-</sup> cells demonstrated a much smaller CD4 central memory (CD27+CCR7+IL7R $\alpha$ +) compartment (Tcm) in mf+ infected patients (INF, n=5; GM% of CD45RA<sup>-</sup> cells=4.6 vs 8.4 in EN) as well as a smaller CD8+CD27-CCR7-IL7R $\alpha$ - effector compartment (Teff; GM%=32 vs 42). These contracted Tcm and Teff populations were still evident in patients previously mf+ who had cleared their infection (CL-Inf; n=6; CD4+Tcm=3.8%, CD8+Teff=22%). In contrast, the CD8+CD45RA+

effector memory compartment (Temra+), containing both Ag-experienced effector as well as anergic cells, was expanded in both the INF and CL-INF compared to the EN (GM% of CD45RA<sup>+</sup> cells=14 and 12 vs 9). Moreover, the density of IL7R $\alpha$ , necessary for T memory cell maintenance (but decreased in T effector cells), was significantly higher in the INF as well as the CL-INF for CD4+T effector memory cells (Tem; EN vs INF and CL-INF, p=0.030 and 0.009), CD8+Tem cells (EN vs CL-INF, p=0.026) and CD8+Temra+ cells (EN vs INF, p=0.017). The increased expression of IL7R $\alpha$  on these memory cell populations may indicate a defect in the ability of these cells to transition from memory to effector status. The fact that the Tem:Teff ratio was higher in both the INF and CL-INF compared to the EN group (CD4: EN vs INF and CL-INF, p=0.017 and 0.065; CD8: EN vs CL-INF, p=0.041) further supports this notion. Taken together, these data indicate that filarial-infected patients have contracted memory compartments and a defect in effector cell development compared to EN, which persists even following clearance of infection.

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#### CONCOMITANT INFECTIONS: REDUCED INFECTIVITY OF *PLASMODIUM GALLINACEUM* TO THE MOSQUITO, *ARMIGERES SUBALBATUS*, IS MEDIATED BY *BRUGIA MICROFILARIAE*

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Co-occurrence of multiple species of malaria parasites and co-occurrence of malaria and filarial worm parasites in a single human have been reported frequently in the tropics. But, little is known about the occurrence and prevalence of lymphatic filariasis and malaria transmitted by the same *Anopheles* vector and how these two parasites interact within this vector; therefore, it is important to gain an understanding of the interactions among parasites in a concomitantly infected vector to better design effective disease management programs. Herein, we present data evaluating the hypothesis that immune activation and/or development by filarial worms might negatively impact *Plasmodium* development in co-infected mosquitoes. To test this hypothesis in the laboratory, we conducted studies using the mosquito *Armigeres subalbatus* and the parasites *Brugia malayi*, *Brugia pahangi*, *Dirofilaria immitis*, and *Plasmodium gallinaceum*. Although they are a laboratory strain, *Ar. subalbatus* used in this study are natural vectors of *P. gallinaceum* and *B. pahangi* and they are naturally refractory to *B. malayi* (melanization-based refractoriness); therefore, using *Ar. subalbatus* as a model may provide a better depiction of the competition between filarial worm and malaria parasites within the same vector. Mosquitoes were dissected and oocyst mean intensities were analyzed six days after blood feeding on either *P. gallinaceum* alone or after taking a bloodmeal containing both *P. gallinaceum* and *B. malayi* or a bloodmeal containing both *P. gallinaceum* and *B. pahangi*. There was a significant reduction in oocyst mean intensity for all three biological replicates in mosquitoes that had a dual infection, regardless of *Brugia* species, as compared to those mosquitoes that were infected with *Plasmodium* alone; and this could have a significant impact on the measurement of vector infection and transmission dynamics, i.e., if filarial worm infection reduces the intensity of *Plasmodium* transmission, the elimination of filarial worms in a co-endemic locale could enhance malaria transmission.

### SECRETED FILARIAL PRODUCTS INDUCE HUMAN MONOCYTES TO HAVE THE FUNCTIONAL AND PHENOTYPIC CHARACTERISTICS OF ALTERNATIVE ACTIVATION

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A defect in antigen presenting cell (APC) function is among the many mechanisms proposed to mediate the profound filarial-specific T cell hyporesponsiveness seen in lymphatic filariasis. This concept is based on findings that monocytes from patients with patent filarial infections are studied with internalized filarial antigens and express markers associated with alternative activation of macrophages. To explore the role of the filarial-derived parasite antigens in the differentiation process of human monocytes, monocytes were exposed to microfilariae (mf) of *Brugia malayi* and their phenotypic and functional characteristics were compared to monocytes exposed to factors known to generate either alternatively activated (IL-4) or classically activated (MCSF) macrophages. Like IL-4, exposure to mf did not alter the mRNA expression of ARG-1 or iNOS, but did induce significant upregulation of CCL17, CCL18, CCL22 and down-regulation of monocyte HLA-DR surface expression as compared to mf-unexposed monocytes. Secreted products from mf also significantly downregulated the monocyte surface expression of PDL2 and CD86 and mRNA expression of TLR3, TLR5, TLR7 and TLR8 resulting in decreased production of IL-10 and TNF- $\alpha$  following TLR ligand stimulation. In contrast to MCSF-cultured monocytes, exposure of monocytes to mf resulted in significant inhibition of the phagocytic capacity of these cells similar to IL-4 driven monocytes. Despite a phenotype reminiscent of alternatively activated macrophages (AAM $\Phi$ ), mf failed to alter the ability to monocytes to mediate CD4+ and CD8+ T cell proliferation in response to anti-CD3. In summary, our data suggest that although, on balance, secreted filarial products skew monocytes toward an immunoregulatory phenotype suggestive of alternative activation, they also induce monocyte differentiation signals that involve innate immune pathways not typically found in AAM $\Phi$ . The role of these mf-altered monocytes in functioning to mediate the filarial-specific T cell hyporesponsiveness in human filarial infections is currently under study.

### HELMINTH-MEDIATED PROTECTION AGAINST AUTOIMMUNE DIABETES IN NOD MICE IS NOT DEPENDENT ON FOXP3+ REGULATORY T-CELLS

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Helminth infections exert beneficial effects on autoimmune diseases. Previously we showed that infection with the filarial nematode *Litomosoides sigmodontis* prevents the onset of diabetes in nonobese diabetic (NOD) mice and that the protective effect is independent of a Th2 immune response. In this study we tested the hypothesis that *L. sigmodontis*-induced regulatory immune responses prevent the onset of diabetes in NOD mice. Infection of NOD mice with the filarial nematode *L. sigmodontis* prevented the onset of diabetes and was associated with significantly increased frequencies of splenic and pancreatic lymph node CD4+CD25+FoxP3+ cells. Splenic FoxP3+ cells from infected NOD mice showed significantly increased proliferation as measured by Ki67 positivity and increased expression of CTLA-4. Transfer of spleen cells from 12-week old NOD mice into NOD.scid mice demonstrated that spleen cells from uninfected, but not *L. sigmodontis*-infected NOD mice, induced diabetes in NOD.scid mice. Lack of diabetes induction by splenocytes of

*L. sigmodontis*-infected mice did not appear due to active regulation of effector lymphocytes by FoxP3+ T-regulatory cells as splenocytes of *L. sigmodontis*-infected eGFP FoxP3 NOD mice depleted of FoxP3+ cells also did not induce diabetes in recipient mice. These results suggest that *L. sigmodontis* alters the potency of autoimmune-inducing cells in infected mice through a mechanism independent of active regulation by FoxP3+ cells. Similarly, continuous depletion of CD25+ regulatory T-cells with PC61 did not reduce the protective effect *L. sigmodontis* has on NOD mice. As *L. sigmodontis* infection in NOD mice increases splenic IL-10 and TGF $\beta$  production, we are currently treating helminth-infected and uninfected NOD mice with anti-IL-10R and anti-TGF $\beta$  to test whether helminth-induced protection is dependent on these immunoregulatory cytokines. These studies demonstrate that filarial worms protect against the onset of Type 1 diabetes in NOD mice by reducing the effector capability of autoimmune cells through a mechanism that is independent of Th2 responses or FoxP3+ T-regulatory cells.

### DENGUE OUTBREAK -- KEY WEST, FLORIDA, 2009

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In September 2009, three cases of autochthonous dengue were identified in Key West, Florida after a 70-year absence. An outbreak investigation to determine the incidence of and risk factors for recent dengue infection in Key West was conducted by the Florida Department of Health and the CDC's Dengue Branch. A stratified random sample of households within 1 kilometer radius of the index cases was selected. At each household, residents were asked to provide blood samples and medical and travel histories. Blood was tested for anti-dengue IgM and IgG antibodies. Antibody-positive samples were tested with a plaque-reduction neutralizing test (PRNT) to determine the infecting flavivirus serotype. For participants with fever in the past 7 days, samples were tested for dengue virus (DENV) by RT-PCR and non-structural protein-1 (NS-1) assay. Mosquitoes were collected from the area and tested for DENV by RT-PCR. Blood was collected from 240 persons in 175 households. Eight (3.3%) participants who had not recently traveled had evidence of recent dengue infection by IgM, RT-PCR, or NS1; 5 were identified as having DENV-1. Ninety-one (37.9%) participants were IgG positive, indicating possible past flavivirus infection. Of these, 5 (2.1%) persons with a dengue-like illness in the past 3 months were classified as having probable recent dengue infection by PRNT. Genotyping showed that mosquito and human isolates were closely related to each other and to a strain of dengue 1 isolated in Mexico. In multivariate analysis having a bird bath or wading pool in the yard or having ever lived outside the US were risk factors for dengue. Emptying water-filled containers or using prevention measures such as repellent were protective factors. Approximately 5% of Key West residents tested positive for recent dengue infection, making this the largest dengue outbreak in the continental United States outside the Texas-Mexico border region in over 60 years. With increasing international travel and ample *Aedes aegypti* mosquito populations, Key West and southern Florida may be at risk of future dengue outbreaks.



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### QUANTIFYING THE SPATIAL DIMENSION OF DENGUE VIRUS EPIDEMIC SPREAD WITHIN A TROPICAL URBAN ENVIRONMENT

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Dengue infection spread in quasi-naive populations occurs in an explosive and widespread fashion. Knowledge on the contribution different factors such as human movement, vector dispersal and the built environment to the propagation of dengue virus infection has been limited. We analyzed the spatio-temporal pattern a large dengue virus-2 (DENV-2) outbreak that affected the Australian city of Cairns (north Queensland) in 2003, quantified the relationship between dengue transmission and distance to the epidemic's index case (IC), evaluated the effects of indoor residual spraying (IRS) on the odds of dengue infection, and generated recommendations for city-wide dengue surveillance and control. We retrospectively analyzed data on the exact position of the most likely place of infection for 383 DENV-2 confirmed cases and on the location and timing of 1,163 IRS applications. Spatial and space-time analyses determined the intensity and directionality of clustering of dengue cases, whereas a Bayesian space-time regression assessed the impact of IRS in the odds of weekly dengue infection. About 63% of the cases clustered up to 800 m around the IC's house. Most cases were distributed in the NW-SE axis as a consequence of the arrangement of the built environment and, possibly, the prevailing winds. Infection spread rapidly, generating 18 clusters (comprising 65% of all cases); clusters varied in severity and extent as a function of their distance to the IC's house. Human movement contributed to ~40% of the observed transmission. IRS applications had a significant protective effect in the occurrence of dengue cases only when reached coverage of 60% or more of a house's neighboring premises. By applying sound statistical analysis, we described the spread of dengue virus with high detail and quantified the spatio-temporal dimension of dengue virus transmission within a complex urban environment. We foresee that some of the results and recommendations derived from our study may also be applicable to other areas currently affected or potentially subject to dengue epidemics.

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### CONTACT CLUSTER INVESTIGATIONS REVEAL A KEY ROLE OF HUMAN MOVEMENT PATTERNS IN THE TRANSMISSION OF DENGUE VIRUS

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*Aedes aegypti*, the principal vector of dengue virus (DENV), feeds during the day when people are active. Consequently, exposure is not limited to the individual's home, and the daily movement of individual humans will play a key role in defining the pattern and spatial extent of DENV transmission. This hypothesis predicts that there should be evidence of dengue infection in the locations visited recently by dengue infected individuals. To test this, we collected blood samples on day 0 and day 15 from febrile individuals (index) and contacts living in locations the index recently visited. Between August of 2008 and April of 2010, we conducted 66 contact cluster investigations initiated by DENV-positive

(30) and negative (36) febrile participants in Iquitos, Peru. We identified a total of 166 DENV infections by RT-PCR, IFA, and/or IgM ELISA (titer  $\geq 1:100$ ): 124 infections among participants within positive clusters and 42 among participants in negative clusters, for attack rates of 0.25 and 0.07 respectively ( $P < 0.001$ ). Overall, 56% of all infections occurred in sites outside the home. Within negative clusters, 74% of infections occurred outside of the home and attack rates were 0.06 for the home and 0.08 for other sites. Within positive clusters, 51% of infections occurred outside the home with attack rates of 0.32 for the home and 0.21 for sites outside the home ( $P = 0.02$ ). Patterns of increased dengue transmission in sites recently visited by dengue positive participants were especially marked in 2008 when DENV-4 first invaded Iquitos, but were maintained over the study period despite rapidly rising herd immunity. Our data indicate that 1) movement patterns of individuals play an important role in defining the pattern and spatial extent of transmission and 2) consideration of human behavior and movement patterns will increase the effectiveness of surveillance and control programs.

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### THE ROLE OF IMPORTED CASES AT DIFFERENT STAGES OF DENGUE EPIDEMICS

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Dengue/dengue hemorrhagic fever is the world's most widely spread mosquito-borne arboviral disease and threatens more than two thirds of the world's population. Cases are mainly distributed in tropical and subtropical areas in accordance with vector habitats for *Aedes aegypti* and *A. albopictus*. Rapid and frequent international travel also contributes to the geographical expansion of dengue epidemics. However, the role of imported cases in those countries/areas where dengue has not become endemic yet remains unclear. The specific aims of this study are to investigate the interplays between imported and indigenous dengue cases and climate conditions at different stages of epidemics. We analyzed bi-weekly, laboratory-confirmed dengue cases at their onset dates of illness from 1998 to 2007 to identify correlations between indigenous dengue and imported dengue cases in the context of local meteorological factors across different time lags. Our results revealed that imported cases have a role in igniting indigenous outbreaks in southern Taiwan (a non-endemic area) when favorable weather conditions are present and where *Aedes aegypti* mosquitoes are mainly distributed. In addition, the imported and indigenous dengue cases had a significant quantitative relationship only at the onset of local epidemics. However, this relationship became less significant once indigenous epidemics progressed past the initial stage. These findings imply that imported dengue cases are able to initiate indigenous epidemics when appropriate weather conditions are present. An early-warning surveillance system, that is able to integrate local meteorological data, is crucial to successful prevention and control of dengue, particularly in countries where dengue is non-endemic. The deployment of such an integrated system will be an invaluable tool for averting the global risk of dengue/dengue hemorrhagic fever epidemics in an era of climate change.

### A STOCHASTIC SIMULATION TOOL FOR SPATIALLY PRIORITIZING RISK IN THE TRANSMISSION OF DENGUE VIRUSES

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One of the unanswered challenges in providing cost effective allocation of integrated resources to prevent outbreaks of dengue viruses is that space-time virus transmission is reported to be erratic and unpredictable from place to place and year to year. Tools to provide improved understanding of transmission dynamics of a region in order to more effectively anticipate the location and timing of epidemics would be important contributions to prevention programs. We developed a Reversible Jump Markov Chain Monte Carlo Bayesian simulation tool to examine the space-time pattern of incidence in a large region over many years of transmission. The tool is used to identify geographic patterns in transmission that are innate to a region as well as spatial migrations in incidence behavior over time. It is also used to partition a large region into innate geographical structures with highly correlated incidence behavior over time. We applied this tool in evaluating the space-time transmission dynamics of dengue viruses throughout Thailand over 2+ decades (1983-2004). Our analysis revealed a definitive geographic pattern that subdivides the region into zones of synchronized risk and predicts geographically varying spatial extent in the spread of seasonal epidemics. We developed graphical representations of spatially correlated risk across the region at a range of spatial scales that are intrinsically defined by the data. We analyzed the spatial structure of risk in the context of space-time influences from the environment (geography and weather) and from human population dynamics and observed clear evidence of the interaction between both sets of forces (human and entomological) in regulating the spread of dengue viruses. This tool is also being applied to regions in other parts of the world. The simulation requires no initial assumptions about the spatial dynamics of the region in order to produce a space-time assessment of dynamics of risk. We will present an overview of the tool, the statistical model that drives the simulation, and the results of our analyses.

### A MULTI-HOST, MULTI-VECTOR SIR MODEL OF DENGUE 2 VIRUS IN SENEGAL

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Epidemics of Dengue 2 virus (DENV) have been observed in Senegal with interepidemic periods between 7 and 8 years. This cycle may be the result of persistence of DENV in one primate reservoir species with periodic spillover transmission to other primates including humans, or ongoing sustained transmission in multiple primate species. DENV infections have been detected in several primate species in Senegal, each with distinct demographic dynamics and potential differences in DENV transmission efficiencies. Here we report the results of a time series analysis of DENV incidence in multiple vector species in Senegal, and investigate the dynamics of models that incorporate multiple hosts and vectors, and determine which exhibit long interepidemic periods. We present a multi-host, multi-vector deterministic seasonally forced SIR model to investigate the impact of multiple host species on the incidence of DENV in Senegal.

We consider human, patas monkey, baboon, and green monkey as host species and *Aedes aegypti*, and *Ae. furcifer* as vector species. We first examine each primate-mosquito system as uncoupled, and then as coupled to the other primate-mosquito systems through non-zero cross-species biting rates. We find long-period multiannual cycles predominantly when transmission probabilities (primate to mosquito and mosquito to primate) are low. As we increase the coupling between systems we find that for low values of coupling (1/1000th of normal biting rates) we find large epidemics occur in all species at once with long interepidemic periods. We consider multiple formulations of scaling of biting rates of the two vectors by primate body size and determine the impact on long-term dynamics. In conclusion, little work has been done to model the impact of multiple host species on the incidence of DENV in sylvatic settings. The long interepidemic periods in Senegal have yet to be explained. Our work demonstrates that the inclusion of multiple host species has little impact on either length of period or the size of epidemic outbreaks in humans. We identify critical ranges of both coupled and uncoupled models that exhibit the long periods observed in the data.

### FIRST YEAR OF ENHANCED DENGUE SURVEILLANCE AT AN ACUTE CARE SETTING IN PUERTO RICO

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Dengue is an acute febrile mosquito borne illness caused by four dengue virus serotypes. In Puerto Rico, dengue is endemic and reportable by law. The Centers for Disease Control and Prevention (CDC) and the Puerto Rico Department of Health (PRDH) has operated a passive dengue surveillance system (PDSS) for over 30 years. In June 2009 CDC implemented an enhanced dengue surveillance system (EDSS) at a 161 bed, acute care hospital in Guayama, Puerto Rico. Upon implementation physicians and nurses were trained in dengue diagnosis, case management, and reporting. Case reporting was initiated by clinicians, and CDC staff oversaw day-to-day case reporting, reviewed case forms for accuracy and completeness, and prepared serum samples for delivery to the CDC laboratory. Clinical and epidemiological data and serum samples were collected from patients fitting dengue clinical criteria as defined by the World Health Organization. From June 1, 2009 to April 30, 2010, 837 suspected dengue cases from 12 municipalities across the Southeastern region were reported, 474 (57%) and 15 (2%) fit criteria for dengue fever (DF) and dengue hemorrhagic fever (DHF), respectively. Laboratory-positives (408, 49%) were identified in 7 municipalities; 351 (86%) were RT-PCR positive and 57 (14%) were dengue IgM positive. DENV-1, -2, and -4 were detected during in these municipalities, but DENV-1 made up the majority (94%) of infections. There were 155 (18%) laboratory-negative and 255 (30%) indeterminate cases. Laboratory-positives were equally distributed by gender, with a mean age of 23 years (median =17, sd=17). Most cases were between the ages of 10-14 (92, 23%) and 15-19 (65, 16%). Many laboratory-confirmed cases (46%) were hospitalized and no deaths occurred. Two hundred and fifty nine (64%) met the case definition for DF while 12 (3%) met the criteria for DHF. In its first year EDSS was instrumental in providing timely identification and characterization of dengue cases leading to a better description of dengue occurrence and severity in the Southeastern region of Puerto Rico.

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**EXPRESSION AND KINETIC CHARACTERISATION OF NON DISCRIMINATING *PLASMODIUM FALCIPARUM* APICOPLAST GLUTAMYL-TRNA SYNTHETASE (PFGLURS)**

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The genome sequences of malaria parasites have revealed many novel insights into parasite biochemistry and the identification of potential drug targets. We identified *Plasmodium falciparum* genes encoding glutamyl-tRNA synthetase (GluRS) and glutamyl-tRNA amidotransferase (Glu-AdT), enzymes that comprise an indirect aminoacylation pathway for the production of Gln-tRNA<sub>Gln</sub>, a key substrate for protein biosynthesis. In archaea, most bacteria, and (probably) plastids, this pathway is the sole route for the production of Gln-tRNA<sub>Gln</sub>, but it is not found in the eukaryotic cytosol. Bioinformatic analyses suggest that the *Plasmodium* GluRS and Glu-AdT orthologs are targeted to the apicoplast. Aminoacyl tRNA synthetases are potential drug targets because their catalytic activities determine the genetic code and therefore they are essential for protein synthesis and cell viability. We expressed the full-length as well as truncated forms (minus the apicoplast targeting sequence) of PfGluRS. Recombinant PfGluRS glutamylated the cognate substrate apicoplast tRNA<sub>Glu</sub> as well as the non-cognate substrate apicoplast tRNA<sub>Gln</sub>. The latter activity is diagnostic of GluRSs that participate in indirect aminoacylation, demonstrating that this pathway is functional in *Plasmodium*. Kinetic characterization was performed using conditions established to produce linear kinetics within a 3-minute reaction. Both forms of the enzyme exhibited a higher affinity towards the cognate tRNA<sub>Glu</sub> substrate as compared to the non-cognate tRNA<sub>Gln</sub> substrate. The truncated enzyme exhibited a marked lower preference towards tRNA<sub>Glu</sub> than the full-length enzyme while at the same time exhibiting a higher velocity (K<sub>cat</sub>) and catalytic efficiency (K<sub>cat</sub>/K<sub>m</sub>), suggesting that the removal of the apicoplast targeting sequence affects enzyme activity. Like many tRNA synthetases, PfGluRS is subject to product inhibition by PPI but upon addition of inorganic pyrophosphatase a 5-fold stimulation of the reaction rate was observed. We will describe the biochemical characterization of the apicoplast GluRS as well as progress towards expression and reconstitution of the PfGlu-AdT, the second enzyme in the pathway.

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**TRANSCRIPTIONAL ANALYSIS OF FATTY ACID STARVATION IN *PLASMODIUM FALCIPARUM***

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*Plasmodium falciparum* causes millions of infections worldwide and a high burden of mortality. As half the earth's population remains at risk, and drug-resistance remains a challenge, investigating parasite biology is essential. We previously described a novel *in vivo* transcriptional profile in *P. falciparum* obtained from blood samples of infected patients. The novel state appears to represent a starvation response, with induction of metabolic pathways and genes, including those for fatty acid biosynthesis. Parasites require exogenous fatty acids, and thus we test the hypothesis that this novel state may occur in response to limited availability of host fatty acids. We cultivated the 3D7 strain of *P. falciparum* in lipid-depleted media reconstituted with incremental concentrations of fatty acids known to support parasite growth *in vitro*. Samples were analyzed by gas chromatography coupled to a flame ionization detector. When cultures were subjected to the lowest concentration of fatty acids, we detected the induction of multiple fatty acid species, stearic acid being most abundant. We also performed metabolic labeling using <sup>14</sup>C-acetate, and confirmed

increasing synthesis of lipids as a result of decreasing fatty acid content in the culture media. Using parasites also cultivated under lipid-depleted conditions, we extracted RNA to analyze the gene transcripts for fatty acid synthesis. Using real-time PCR, we demonstrated a step-wise induction of FAS II gene transcripts as the fatty acid content of the culture media was decreased. We will present the correlating whole genome transcriptional profile in order to identify alterations in other GO functions associated with the fatty acid starvation state. Through the *in vitro* recapitulation of the starvation state we can characterize *P. falciparum*'s unique biology and its impact on the human host.

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**CORRELATION OF  $\alpha$ -TUBULIN AND PFG377 ORTHOLOG GENE EXPRESSIONS IN *PLASMODIUM VIVAX* GAMETOCYTES AND MOSQUITO INFECTION**

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*Plasmodium vivax* transmission from humans to mosquitoes requires the presence of infectious gametocytes in human peripheral blood. An index of infectiousness based solely on an analysis of gametocyte counts from blood films is unreliable. There is no correlation between gametocyte density, which is generally low, and mosquito infection. This may be due to the gametocyte examination by microscope is not sensitive and cannot identify the infective or mature gametocyte from the dead or immature ones. Gametocyte stages display a distinct pattern of gene expression than asexual stages. In *P. falciparum*,  $\alpha$ -tubulin and Pfg377 are gametocyte-specific genes essential for microgametocyte formation and the emergence of macrogametes from erythrocytes during the gametogenesis, respectively. The expression of these genes may be useful for predicting infectiousness of the gametocytes to the mosquito vectors. In this study, the transcripts of  $\alpha$ -tubulin and Pfg377 ortholog genes in *P. vivax* were determined by quantitative real-time PCR using 73 clinical blood samples. *Anopheles dirus* mosquitoes were fed on these blood samples to determine the infectiousness of the gametocytes. The parasites from those samples infective to mosquitoes expressed significantly higher levels of  $\alpha$ -tubulin and Pfg377 ortholog than those of in the non-infective group. However, there were the weak correlations between the expression of  $\alpha$ -tubulin and Pfg377 ortholog genes and the mean oocyst number in mosquitoes' midgut. The levels of expression of these genes may be useful to predict the *P. vivax* gametocyte infectiousness for malaria surveillance.

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***PLASMODIUM FALCIPARUM* QUANTITATIVE TRAIT LOCI DETERMINING INFECTIVITY TO *ANOPHELES GAMBIAE* MOSQUITOES**

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Malaria parasites differ in the prevalence (proportion infected) and intensity (number of oocysts per mosquito) of mosquito infections. Our main aim is to determine the number of parasite genetic loci that contribute to infectivity differences and locate them on a genetic map. We have previously identified two genetically distinct clones of *Plasmodium falciparum* (denoted 3D7 and HB3) that differ significantly ( $P < 0.0001$ ) in their ability to establish mature oocyst infections in *Anopheles gambiae* mosquitoes, natural vectors of human malaria. We have used a quantitative trait locus (QTL) approach to map the parasite loci contributing to differences in prevalence and intensity of infection. The prevalence and intensity of infection in *A. gambiae* mosquitoes were measured for 20 progeny clones from the 3D7 X HB3 genetic cross. The



progeny clones were genotyped using a custom-built Affymetrix molecular inversion probe 10K malaria panel array with a coverage of ~1 SNP per 3 kb to generate a genetic map. Here we present data for a major locus on chromosome 12 of the parasite which contributes both to prevalence and to infection intensity. This locus is responsible for 94% of the observed phenotype for infection intensity and 44% of the observed prevalence phenotype. The locus contains ~27 open reading frames. Additional loci on other chromosomes make minor contributions to the traits. This study demonstrates for the first time a single parasite QTL having a major effect on mosquito infection.

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### PERUVIAN *PLASMODIUM FALCIPARUM*: HISTORICAL BOTTLENECKS OR RECENT INTRODUCTIONS?

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Population genetics principles have previously been used to explain the impact of natural selection on the spread, maintenance, and decline of drug-resistant alleles and global population structure of *Plasmodium falciparum*. In South America, this population structure was demonstrated to be the least diverse in the world yet highly differentiated. In Peru, malaria control efforts reduced the incidence of malaria after the 1950s, which could have induced bottlenecks. During the 1990s, multiple epidemics of malaria occurred in Peru. We tested the hypothesis that Peruvian *P. falciparum* populations rapidly expanded from locally bottlenecked populations or founder migrants from neighboring areas. We investigated the genetic relatedness of *P. falciparum* parasites (n=220) using samples from the Peruvian Pacific Coast (Bellavista, La Arena, Zarumilla) and the western (Pampa Hermosa, Ullpayacu), central (Padre Cocha), and eastern (Caballococha) Peruvian Amazon collected in 1999 and 2000. We sequenced *dhfr*, *dhps*, *pfcr1*, and *pfmdr1* (genes linked to sulfadoxine-pyrimethamine and chloroquine resistance), 54 proximal microsatellite markers, and 12 neutral markers. We tested our data with pairwise Fst, AMOVA, median joining network diagrams, pairwise linkage disequilibrium, and the Bottleneck application. Our findings include the first description of genotypes collected in coastal Peru. Across all sites, parasite lineages demonstrated limited genetic diversity and multilocus linkage disequilibrium (LD) across all 4 resistance genes and proximal microsatellites, and neutral markers. Our results indicate there were 5 clonal lineages that rapidly expanded, with some representing bottlenecked local populations and others recent introductions. In addition, population structure exhibited admixture rather than isolation by distance. *P. falciparum* population structure should be carefully considered when planning and interpreting molecular epidemiology-based surveillance data.

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### GENOME-WIDE ASSOCIATION STUDIES FOR ANTIMALARIAL RESISTANCE UNCOVERS NOVEL TARGETS IN *PLASMODIUM FALCIPARUM*

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*Plasmodium falciparum* malaria's rapid adaptation to new drugs allows it to remain one of the most devastating infectious diseases of humans. Understanding the genetic basis of these adaptations is critical to successful intervention. Genome-wide association studies (GWAS) are a promising approach to directly identifying genetic variation that may be contributing to both established and emerging drug resistance. We developed a high-density genotyping array and applied it to 57 culture-adapted parasites, characterizing population structure and applying long-haplotype tests to identify signatures of selection. Coupled with drug-sensitivity phenotyping, we performed association studies with 13 antimalarials. Using recently developed GWAS tools such as the EMMA and HLR tests, we were able to control for population structure and detect known and novel resistance loci at genome-wide significance. Functional analysis of one of the novel halofantrine hits revealed that PF10\_0355 overexpression decreases sensitivity to halofantrine, mefloquine and lumefantrine but not to structurally unrelated antimalarials, and that resistance is mediated by increased gene copy number. This demonstrates the effective application of these GWAS methods, and shows the usefulness of GWAS more generally for understanding the genetic basis for antimalarial drug resistance in the wild, potentially identifying important biomarkers for surveillance as elimination and eradication efforts are pursued.

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### NEXT-GENERATION SEQUENCING FOR BASIC BIOLOGICAL AND TRANSLATIONAL STUDIES OF THE MALARIA PARASITE

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We report on advances in adapting next-generation Illumina sequencing into a 'post-genomic' research tool useful for investigating pertinent biological questions associated with malaria parasites. We have devised a hybrid selection protocol to significantly enrich parasite DNA in samples containing large amounts of host DNA, and demonstrated the viability of this enrichment approach on whole-genome-amplified samples. These strategies will not only allow for far more efficient sequencing of non culture adapted parasite samples, but will enable sequencing of samples previously considered unsuitable, such as DNA derived from blood spots on filter papers. We have also developed a method to estimate the multiplicity of infection (MOI) with unprecedented accuracy in clinical

samples that leverages the enormous power of Illumina sequencing in an economical way using a multiplexed approach to sequence PCR amplicons of highly polymorphic loci. We report on the multiplicity as well as the proportional representation of parasite strains within a collection of complex infections from Senegal, and will discuss the biological relevance of accurate MOI estimation for future drug, vaccine, and epidemiological studies. Finally, we will report on methodologies developed for field assessment of key genetic variants in epidemiological and surveillance studies using high resolution melting technologies. Collectively, we will discuss methodological and technical advances to identify genetic loci of biological interest and survey for these loci directly from patient-derived materials as well to assess parasite numbers and types as intervention strategies are applied.

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#### **RANDOMIZED STUDY COMPARING ARTESUNATE PLUS AMODIAQUINE TO ARTHEMETER PLUS LUMEFANTRINE FOR THE REPEATED TREATMENT OF RECURRENT *PLASMODIUM FALCIPARUM* UNCOMPLICATED MALARIA OCCURRING IN COHORT FOLLOWED DURING TWO YEARS IN SENEGAL**

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The use of artemisinin combination therapy (ACT) is currently recommended for treating uncomplicated malaria. Our objective was to assess the efficacy and safety of repeated administrations of two fixed-dose combination ACT: artesunate + amodiaquine (ASAQ) and artemether-lumefantrine (AL) in consecutive episodes of *Plasmodium falciparum* malaria. A randomized, investigator-blinded, comparative study was conducted in a rural community of central Senegal from August 2007 to January 2009. Children and adult patients with uncomplicated *P. falciparum* malaria were randomized to receive ASAQ once daily, or AL twice daily for three days. Drug doses were given according to body weight range. Treatments for first episodes were supervised and unsupervised for subsequent ones. ECG and audiograms were performed in patients > 12 years of age. Primary outcome was adequate parasitological and clinical response rate after PCR correction on Day 28 for the first episode. A total of 840 patients were screened, 366 patients were enrolled in the two groups (184 for ASAQ and 182 for AL) and followed-up during 2 malaria transmission seasons. In the ITT population, ACPR after PCR correction at D28 for the 1st episode was 98.4% vs 96.2% respectively in the ASAQ and AL groups. A 100% ACPR rate was also obtained at D28 in the 60 and 4 patients who experienced respectively a 2nd and a 3rd episode. Treatment-related AEs were reported in 11.7% of the patients without significant differences between the 2 groups. A better improvement of hemoglobin rate at D28 was noted in the ASAQ group. No sign of ototoxicity was demonstrated. A widening of the QTc interval was observed in both groups during treatment with no clinical consequence. Study results confirmed the satisfactory efficacy and safety profile of ASAQ and AL. Moreover, in patients who were treated at least twice, repeated administration of ASAQ or AL did not result in any significant safety issue.

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#### **COGNITIVE FUNCTIONING AFTER CEREBRAL MALARIA IN UGANDAN CHILDREN BELOW FIVE YEARS: A PROSPECTIVE STUDY**

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Earlier studies in African children aged 5 to 12 years have shown cognitive deficits mainly in attention and memory after an episode of cerebral malaria (CM). We present preliminary results of cognitive function after CM in children less than 5 years of age. Sixty nine Ugandan children aged 18 months to 4.9 years admitted with CM at Mulago Hospital were assessed for fine and gross motor skills, receptive and expressive language skills, memory and visual spatial skills a week after discharge and six months later. Test scores were compared to 67 community controls recruited from the families of the CM group or other similar families. Children with CM more frequently had impairment in one or more of the areas tested as compared to community children at baseline (17% vs 9%;  $p=0.15$ ) and at 6 months (14% vs 8%;  $p=0.48$ ), but these differences were not statistically significant. Children in the CM group having cognitive impairment at 6 months had a lower WAZ score than those not impaired (-3.28 vs -1.29;  $p=0.001$ ). No other factor was associated with impairment at 6 months. In conclusion, in children < 5 years of age with CM, malnutrition at admission may play a role in the persistent cognitive impairment. Analysis of tests of attention, the area found most impaired in studies of children over 5 years of age, is currently ongoing. Final analysis to assess risk of cognitive impairment in children <5 years of age with CM will be performed when study cohort enrollment is complete.

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#### **SELECTION AGAINST THE EL TOR ALLELE OF *CTXB* IN *VIBRIO CHOLERAE* O139 AND O1 REVEALS THEY HAVE DISTINCT NICHES**

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*Vibrio cholerae* causes cholera, a severe diarrheal disease causing an estimated 3 to 5 million cases and c. 120,000 deaths per year worldwide. *V. cholerae* has two epidemic serogroups: O1 and O139, the latter arose from the former by lateral gene transfer of a novel serogroup encoding region. Our working paradigm is that the two serogroups are a single disease entity. Recently new variants have arisen that are clinically more severe and have the 'classical' allele at *ctxB* (cholera toxin-coding) locus. Ninety isolates of O139 collected systematically from patients with cholera admitted in to the Infectious Diseases Hospital in Kolkata between 1992 and 2000 were genotyped at the *ctxB* locus and at the five loci used in MLVA (multilocus variable tandem repeat analysis). Our MLVA produced a network of genetic relatedness consistent with the genotypes evolving over time. The *ctxB* genotypes correlated with the MLVA genotypes. The 'El Tor' allele of *ctxB* was replaced selectively by the 'classical' allele. The selection coefficient was estimated to be greater than 10<sup>-6</sup>. A similar selection coefficient was estimated among O1 *V. cholerae* where the 'El Tor' allele of *ctxB* was replaced selectively by the 'classical' allele. The selective sweeps occurred at different times in *V. cholerae* O139 and O1 serogroups. Our findings are consistent with the idea that the two serogroups have two distinct niches and should be thought of not as one disease, but rather as two distinct disease entities. Like different pathogroups of diarrheagenic *Escherichia coli* (EPEC, EAEC, etc), we should think of *V. cholerae* O1 and O139 as independently evolving distinct diseases.

### NORTH AMERICAN PARAGONIMIASIS FOLLOWING INGESTION OF RAW CRAYFISH IN THE MISSOURI OZARKS

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Seven autochthonous cases of North American paragonimiasis due to *Paragonimus kellycotti* were published between 1969 and 2007. This presentation will describe 6 cases seen at a single medical center in Missouri over the past 3 years. The patients (5 M, 1F) ranged in age from 12 to 32 years. All patients reported having ingested raw crayfish during camping or canoe trips along float streams in SE Missouri. Patients presented with fever and cough associated with eosinophilia 3 weeks to 3 months after crayfish ingestion. The diagnosis of paragonimiasis was made weeks to many months after the onset of symptoms, generally after several failed therapeutic trials of antibiotics and/or steroids. All patients had pulmonary infiltrates and/or pleural effusions with eosinophilia (>10% in peripheral blood). Other clinical manifestations included pericardial effusion (2 patients), migratory subcutaneous nodules (2 patients), and visual changes with an occipital lobe lesion present on MRI (1 patient). Numerous procedures were performed on these patients including thoracentesis, pleural biopsy, bronchoscopy with lavage and lung biopsy, pericardiocentesis, and laparoscopic cholecystectomy. *Paragonimus* ova were not identified in stool or sputum in these patients, and only 3 of 6 patients had positive serology tests for antibodies to *P. westermani*. All patients promptly responded to treatment with praziquantel (75 mg/kg/d in three divided doses for 2 days). It was distressing for us to see patients who had suffered so much from this easily preventable and treatable infectious disease. Therefore, we worked with the Missouri Department of Health and Senior Services to develop a health advisory letter for physicians (to shorten the time to diagnosis and treatment) and a warning poster for campgrounds and canoe rental businesses (to warn people not to eat raw crayfish). Physicians should consider the diagnosis of paragonimiasis in patients with pulmonary symptoms, fever and eosinophilia.

### THE EFFECT OF GENITAL SCHISTOSOMA HAEMATOBIIUM INFECTION ON FEMALE FERTILITY

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A cross-sectional study in an *Schistosoma haematobium* endemic area of rural Zimbabwe was done in order to examine the association between genital schistosomiasis and fertility. In a community-based study all resident women of Mupfure Ward, between the ages of 20 and 49, not pregnant, not virgins, and not passed menopause, who had lived in the schistosomiasis endemic area for more than 3 years were invited into the study. Four hundred and eighty three women were interviewed about reproductive health issues and underwent a gynecological investigation. Genital, urinary and, faecal specimens were examined for parasite ova, analyses were done for sexually transmitted diseases. Logistic regression was used to control for the influence of sexually transmitted diseases and HIV on the association between schistosomal infection and fertility. Women with genital schistosomiasis had fewer children. The presence of *S. haematobium* ova in genital tissue was found to be significantly associated with infertility (Adj. OR 3.6, 95% CI 1.05-12, p=0.034). We have previously published that *S. haematobium* was not associated with abnormal menstruation. Furthermore, the uterus rarely harbours *S. haematobium* ova. However, many case reports have shown partial or fully

blocked Fallopian tubes where ova are deposited. Previous reports indicate that anti-schistosomal treatment may reverse infertility, however this study could not confirm this. Larger studies are needed to determine the mechanism of infertility and if these women are more prone to abortion.

### TOWARDS ENHANCED SURVEILLANCE FOR MONKEYPOX: APPLICATION OF A ROBUST CLINICAL CASE DEFINITION

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Monkeypox virus is endemic in central and western Africa. Surveillance data from January through May 2009 in Tshuapa District, Democratic Republic of the Congo, yield an estimated annual incidence of 3 per 10,000; a five fold increase from the 1988 reported disease incidence. Misdiagnosis and confusion with varicella infection occurs. Adequate detection of cases and valid contemporary incidence measures of monkeypox will help to understand whether the disease is reemerging in the Congo Basin and to inform vaccine utilization policy. A surveillance case definition was developed based on published clinical symptoms of human monkeypox infection. Using this case definition, 33 suspect monkeypox cases from multiple African countries submitted to the US Centers for Disease Control and Prevention's (CDC) Poxvirus Team for consultation were evaluated by an independent observer using the data collected by healthcare workers, including photographs for each case. Using descriptive clinical information from six cases where there was laboratory testing for Orthopoxvirus or monkeypox virus, the specificity of the surveillance case definition was 0.8 and the sensitivity was 1.0. Using photographic evidence only, the specificity and sensitivity of the case definition were both 1.0. Photographic evidence classified 14 of 33 suspect cases (42.4%) as monkeypox, 4 (12.1%) as possible monkeypox or indeterminate, and 15 (45.5%) as not monkeypox. Healthcare workers misdiagnosed as many as 57% of those examined visually and 90% by clinical description as monkeypox. The concise surveillance case definition performed well and is expected to be useful in a clinical setting. Diagnosis of human monkeypox is easily confused with other vesiculopapular rash illnesses, particularly with varicella infections. Application of rigorous enhanced surveillance methods including a case definition, adequate case form and data collection, photographs of the patient, and laboratory testing of samples, and continuing training in their use, will help define the incidence and epidemiology of human monkeypox.

### PROSPECTIVE COHORT STUDY ON THE BURDEN OF LEPTOSPIROSIS AND ITS TRANSMISSION IN THE URBAN SLUM ENVIRONMENT

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Leptospirosis has emerged as an urban slum health problem worldwide. However prospective studies have not been performed to evaluate its disease burden and obtain information on transmission factors which is needed to design effective interventions. We enrolled 9,862 inhabitants from a slum community in Salvador, Brazil in 2004 and followed the



cohort for a three-year period by: 1] active hospital-based surveillance to identify leptospirosis cases and 2] annual serosurveys of a sample of 2,003 subjects to identify *Leptospira* infection, defined as seroconversion in the microscopic agglutination test. Generalized estimation equation model was used to account for the time dependence of repeated measure data in risk factor analysis. The mean annual incidence for severe leptospirosis and *Leptospira* infection was 24 (95% CI, 3-73) cases and 2,690 (1,870-,3739) infections per 100,000 inhabitants, respectively. The infection-to-severe disease ratio was 114 (53-290). The annual incidence of secondary infections during the follow-up period was 8,475 (4,139-15,033) per 100,000 inhabitants. Independent risk factors for *Leptospira* infection were increasing age, male gender (RR 1.99; 95% CI 1.32-3.02), low per capita daily household income (0.83; 0.71-0.97 per US\$1) and elevation of residence above the lowest point in the valley (0.98; 0.97-1.00 per m;  $P=0.02$ ), a proxy for flooding risk. In conclusion, our findings showed that 2.7% of the urban slum residents were infected each year with *Leptospira*. Severe disease occurred in a small proportion (<1%) of infections, indicating that the disease burden for leptospirosis may be larger than previously believed. Re-infection was a frequent phenomenon, suggesting the presence of high-risk groups within slum communities who are repeatedly exposed to the pathogen. Furthermore, our findings emphasize the need for public health responses which address the social factors that produce unequal health outcomes among slum residents, as well as the infrastructure deficiencies, such as lack of closed drainage and sanitation systems, which serve as source for exposure to contaminated floodwater.

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##### IMMUNOMODULATION BY *SCHISTOSOMA MANSONI* BY IMPAIRMENT OF HOST PURINERGIC SIGNALING PATHWAYS

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Schistosomes are human parasitic flatworms that constitute an important public health problem globally. The parasites live for years, sometimes decades, in what is putatively a very hostile environment - the blood of vertebrates - yet they seem to elicit little if any protective reaction from two of the host's major defensive systems: the hemostatic system and the immune system. We hypothesize that this is because schistosome nucleotide metabolizing ecto-enzymes (NMEEs, alkaline phosphatase (SmAP), ecto-phosphodiesterase (SmPDE) and ecto-ATP-diphosphohydrolase (SmATPDase)), among a small subset of proteins expressed on the parasite surface membranes, dampen host pro-inflammatory and pro-thrombotic purinergic signaling mechanisms. In this way, these surface enzymes attenuate the host's ability to focus damaging thrombotic and immunological mediators in the parasite's vicinity, as reported previously. In this work, we show that the expression of all 3 NMEE genes is upregulated following vertebrate host invasion and that all are located in the tegument, by immunofluorescence and immuneEM. RNAi treatment targeting each NMEE gene results in potent suppression of gene expression, as determined by quantitative real-time PCR and by western analysis. The viability of suppressed versus control parasites is similar in culture but is significantly diminished *in vivo*. We show that, unlike parasites whose SmAP and SmPDE genes are suppressed, parasites whose SmATPDase gene is suppressed are significantly impaired in their ability to catabolize the potent pro-inflammatory molecule, ATP. We also show that parasites whose SmAP gene is suppressed, unlike parasites whose SmPDE and SmATPDase genes are suppressed, generate the potent anti-inflammatory molecule adenosine by catabolizing AMP. These data are consistent with the idea that some NMEEs provide an important immunomodulatory role for schistosomes within their hosts by impairing host purinergic signaling pathways.

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##### AN INVESTIGATION OF POLYMORPHISM IN THE TETRASPANIN-2 GENE OF *SCHISTOSOMA MANSONI* FIELD ISOLATES

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Tetraspanin-2 (tsp-2) is a four transmembrane-domain protein located in the tegument of *Schistosoma mansoni*. As yet, its function is unknown though mammalian homologues are thought to mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility. In addition to the four transmembrane domains, the protein is characterized by two extracellular loops. A recombinant version of the large extracellular loop 2 (LEL2) has been used by Loukas and colleagues, as reported previously, to vaccinate mice that were subsequently challenged with *S. mansoni* resulting in reductions of 57 % and 64 % in the mean adult worm burden and liver egg burden over two independent trials. Clearly, tsp-2 has the potential to be an effective vaccine against *S. mansoni*, however, this potential may only be realized if tsp-2 proves to have a limited level of non-consequential polymorphisms.

Using *S. mansoni* field isolates obtained from 5 people who live and/or work close to infected water sources in Kisumu, Western Kenya we are studying the nucleotide and inferred amino acid sequences of the gene and transcript encoding tsp-2 in at least 8 worms from each individual. Our preliminary data suggests the presence of an indel prior to the sequence encoding LEL2 that results in a frame shift and premature stop codon in a significant proportion of the transcript sequences. As this indel appears after a short polyA tract we are trying to determine if it is a result of an amplification or sequencing error. Several other polymorphisms have also been noted. We also hope to present data that sheds further light on the issue of tsp-2 polymorphism in *S. mansoni* obtained from a wider population of African and South American field isolates.

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##### INNATE IMMUNE PRIMING OF ADAPTIVE RESPONSES TO SCHISTOSOME INFECTION

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Schistosomes are intravascular helminths affecting approximately 200 million people throughout the tropics and subtropics. Upon infection, previous models proposed that an early Th1 response to schistosomes is replaced at roughly 6 weeks post infection by a Th2 response initiated by egg deposition. However, our data show that, in addition to IFN- $\gamma$ , CD4<sup>+</sup> T cells produce IL-4 and IL-10 in response to worm antigens during early infection. We hypothesize that production of IL-10, a regulatory cytokine, creates an immunomodulatory milieu permissive for parasite establishment and development. To test this hypothesis, we attempted to establish the identity of the IL-10-producing CD4<sup>+</sup> T cells and elucidate how this response is induced. To determine whether CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> natural T regulatory (nTreg) cells are an important source of IL-10, wild type mice were treated with monoclonal antibodies that deplete nTreg cells by inhibiting IL-2 signaling. This approach demonstrated that depletion of nTreg cells did not significantly reduce IL-10 production, suggesting an inducible CD4<sup>+</sup> T cell population, rather than nTreg cells, are the predominant source of IL-10. Our analysis of the innate APC response to schistosome infection supports a model whereby schistosome worms induce a population of myeloid suppressor cells, which subsequently interfere with primary activation of naive T cells. The precise identity of this myeloid suppressor population and their effects on T cell priming are currently under investigation.

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### TRANSCRIPTIONAL PROFILING OF *SCHISTOSOMA JAPONICUM*-STIMULATED ALTERNATIVELY ACTIVATED MACROPHAGES

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Alternatively activated macrophages (AAMφs) play important roles in a number of Th2 driven pathologies including asthma and allergy and a number of parasite infections. In *Schistosoma mansoni* infections AAMφs have been associated with profibrotic and immunomodulatory roles. However, the molecular mechanisms involved in the activation of AAMφs and their function are not well understood. Our own studies and those of others investigating *Schistosoma japonicum* infection strongly suggest the presence of AAMφs in *S. japonicum*-infected tissues. However, the effect of *S. japonicum* antigens on macrophage activation and the role of AAMφs in *S. japonicum* infection have not been investigated. In the present study we demonstrate, for the first time, that *S. japonicum*-secreted egg antigens are able to induce the alternative activation of macrophages as characterised by the significant induction of *Chi3l3* and *Arg1* expression. *Retnla* was not significantly induced in these macrophages suggesting that the specific function of these cells may differ to those induced by *S. mansoni* and other parasites. Closer examination of the gene expression profile of these cells identified other pathways that may confer immunomodulatory activity, independent of *Retnla* expression, including modulated expression of T-cell co-stimulatory molecules and chemokines. *S. japonicum*-stimulated alternative activation of macrophages was additionally associated with deactivation of classical activation pathways and altered expression of cell surface receptors and complement components that may alter phagocytic activity. There was no evidence of direct profibrotic activity. Together these data significantly enhance our understanding of the mechanisms associated with alternative activation of macrophages, highlight the importance of the context of activation in directing AAMφ phenotype and function, and provide significant insight into the role of these cells in schistosomiasis japonica.

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### DEVELOPMENT AND IMMUNOSCREENING OF AN IMMUNOMICS PROTEIN MICROARRAY TO INVESTIGATE SCHISTOSOMIASIS

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Schistosomiasis causes significant human morbidity and mortality and, despite mass chemotherapy programs, reinfection rates remain high and long-term transmission is undiminished. Mathematical modelling has demonstrated that an anti-schistosome vaccine could assist in the elimination of this disease. For this purpose we have developed a 289 feature protein microarray for *Schistosoma japonicum* and *S. mansoni* as a vaccine antigen discovery tool. Proteins were selected on the basis of known or predicted surface localisation, secretion/excretion, life-stage specificity, and potential antigenicity. This study represents the first use of an immunomics protein microarray in schistosome research. The schistosomiasis-resistant brown rat, an excellent model of resistant human immunity, has demonstrated consistent antibody recognition of ~10 antigens including calcium-binding proteins, a CutA-related precursor, a protease inhibitor and *S. mansoni* homologs. These serum antibodies

will be compared with tissue and life-stage specific responses in draining lymph nodes, spleen and BAL fluid, particularly after repeat infections. We will complement this animal model data with the screening of human sera from patients in schistosomiasis-endemic regions of China. In excess of 100 samples have been collected from putative-resistant and susceptible individuals as well as acute, severe and chronic disease states. These individuals were identified from large-scale field surveys and during treatment in established clinics. Using this data we will identify a subset of proteins for future animal vaccine trials in mice (susceptible model). By simultaneously examining two species of human schistosomes we hope to discover cross-reactive antigens that may lead to vaccine candidates and diagnostic/susceptibility markers for disease. This project will further our understanding of resistant host immune responses and parasite immune evasion and potentially identify future vaccine candidates.

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### IMBALANCE OF REGULATORY AND ACTIVATED T CELLS IN HUMAN *SCHISTOSOMA HAEMATOBIIUM* INFECTIONS

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Acquired immunity against helminths is characterised by a complex interplay between Th1 and Th2 immune responses, and is often manifest only with increasing age. Data from experimental models suggest that immunity is also influenced by regulatory T cells (Treg), but as yet studies on Treg in human schistosome infections are limited. We therefore characterized regulatory and activated T cell (Tact) populations in Zimbabweans (aged 8-60 years) exposed to *Schistosoma haematobium* parasites. Activated T cells were classified as CD4+CD25+FOXP3- while Treg were defined as CD4+(dim)CD25+(high)FOXP3+CD127low. The participants were partitioned into two age groups, young children (8-13 years) in whom schistosome infection levels were rising to peak and older people (14+ years) with declining infection levels. Treg proportions rose significantly with increasing infection in the younger age group resulting in an increase of the Treg:Tact ratio with level of infection. In contrast Treg were negatively correlated to infection intensity in the older age group. The balance between regulatory and effector responses differ significantly between young individuals in whom high infection is associated with an enhanced regulatory phenotype and older infected patients in whom the regulatory response is attenuated. This may reflect different stages of the development of protective schistosome acquired immunity.

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### WHOLE BLOOD CYTOKINE RESPONSES IN URINARY SCHISTOSOMIASIS: THE EFFECT OF PRAZIQUANTEL TREATMENT ON NATURAL IMMUNE RESPONSES

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Urinary schistosomiasis is a debilitating disease caused by chronic *Schistosoma haematobium* infection. 1/3 of sub-Saharan Africans are currently infected with the most severe cases aggregated in school-age children. Praziquantel is the drug of choice to clear infection, but fails to prevent re-infection in endemic communities. Our study investigates the changes to the natural human cytokine response following praziquantel treatment. Whole blood was collected from 255 Zimbabweans (aged 3-84 years) inhabiting an *S. haematobium*-endemic region. Blood was cultured with crude parasite (cercariae, egg and adult worm homogenates) and purified vaccine candidate (GST and Sh13) antigen preparations for 48 hours. Culture supernatants were harvested and a panel of 13 cytokines (IFN $\gamma$ , TNF $\alpha$ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17A, IL-21 and IL-23) was measured via Enzyme-linked Immunosorbent Assay (ELISA). *S. haematobium* infection was quantified by urine filtration.

*Schistosoma mansoni*, soil-transmitted helminth and malaria positive cases were excluded. Following initial sampling, participants were treated with a single dose of praziquantel. Serology and parasitology was repeated 6 weeks and 6 months post treatment. Multivariate data was analysed by ANOVA and uncorrelated variables were grouped using principal components analysis (PCA). Prior to treatment *S. haematobium* prevalence was 51.44% and mean infection intensity was 28.5 eggs/10ml urine and both peaked at age 11-14 years. Both infection intensity and prevalence were significantly reduced at 6 weeks post-treatment. Pre and post-treatment cytokines showed distinct patterns according to antigen stimulation. The age distribution of cytokine production was altered by treatment, suggesting long-term effects of treatment on host immunity to *S. haematobium*.

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##### SERO-PREVALENCE AND DISTRIBUTION OF KALA-AZAR IN POKOT COUNTY, AMUDAT DISTRICT, EASTERN UGANDA

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Visceral Leishmaniasis (Kala Azar) is the only clinical manifestation of leishmaniasis so far reported in Uganda and has been largely confined in Pokot County. Here, it is caused by *Leishmania donovani* and transmitted by the sand fly *Phlebotomus martini*. Records of 2006 from Amudat hospital indicates that KA accounted for about 17% of hospital in-patients, but actual prevalence of this disease is not known in Pokot County. This study sought to determine KA prevalence using Direct Agglutination Test (DAT) and describe its distribution in Pokot County in order to inform control measures in Amudat district, Karamoja region, Uganda. A cross-sectional study was conducted in February to March 2010. The study participants were children aged  $\geq 5$  years and adults  $\geq 18$  years randomly selected from the various strata in the selected clusters (Manyattas) obtained using Bennett's formula. A structured questionnaire was used to elicit the demographic profile and other characteristics of the participants. Standard operating procedures were performed for DAT using blood samples collected from participants on blotting papers at Amudat Hospital laboratory. Data was entered in EPIINFO and exported to STATA, used to produce frequencies and cross tabulation of DAT outcome and independent variables. Multivariable logistic regression was used to find association between the DAT outcome and potential selected risk factors. A total of 285 respondents were interviewed, response rate, 100%. The prevalence of Kala azar in Pokot County was 17.2% (49/285). The prevalence of clinical KA cases in the community was 2.5%. KA occurs commonly in children 10 years and below and is more common in males than females (1.33:1). DAT positive participants were more in sub counties with more clinical cases. Loroo Sub County had the highest number of DAT positive participants followed by Karita and Amudat at 30, 14 and 5 of the 49 positive participants respectively. In conclusion, Kala Azar is common in Pokot County and is most likely to spread to other parts of the county unless the ministry of health makes deliberate efforts and spearheads making available critical resources necessary to control and/or eliminate Kala Azar in Pokot.

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##### IS SUDANESE VISCERAL LEISHMANIASIS DIFFERENT FROM VL ON THE INDIAN SUBCONTINENT?

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Globally, there are two major foci of visceral leishmaniasis (VL): on the Indian subcontinent, in particular Bihar state in India, and eastern and southern Sudan. In Sudan VL occurs both endemic and epidemic. Since the 1990s important observations have been made that indicate

that Sudanese VL has unique characteristics in epidemiology, clinical presentation, diagnosis and treatment that contrast with reports from the Indian subcontinent. Transmission in Sudan is believed to be both anthroponotic and zoonotic with dogs and rodents as potential reservoirs, while in India VL is thought to be exclusively anthroponotic. More than 50% of VL patients in Sudan develop Post Kala-azar Dermal Leishmaniasis (PKDL) after treatment for VL compared with 5% in India, although higher PKDL rates are now reported from Nepal and Bangladesh, probably the result of improved reporting. The interval between VL and PKDL in Sudan is 0-6 months, while this is longer (often several years) in the Indian subcontinent. While PKDL causes considerable additional morbidity, it may have important implications for transmission. In addition, other post-kala-azar manifestations such as mucosal and ophthalmic leishmaniasis have been reported that have not been described elsewhere. Serological tests have different performance; while the rK39 strip test in India has sensitivity of nearly 100%, in Sudan this is less than 75%. Response to treatment is also different; on average the response to stibogluconate is good, while in India important resistance exists probably the result of inadequate dosing and compliance. In contrast, in a recent trial, the efficacy of paromomycin was considerably less in Sudan compared with what was found in India. Parasites circulating in Sudan are genetically more diverse (heterogeneous) through recombination that may explain at least some of these differences mentioned. Understanding the basis of these differences is important as this may lead to a different approach in clinical management of VL.

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##### EQUITY IN CUTANEOUS LEISHMANIASIS TREATMENT ACCESS: CHALLENGES AND OPPORTUNITIES FROM KABUL, AFGHANISTAN

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This presentation analyzes some of the determinants at the primary healthcare level in Kabul (Afghanistan) affecting the access to treatment among cutaneous leishmaniasis (CL) patients. The analyzed determinants include two major levels of assessment. First, the socio-economic status of the patients seeking for treatment at the primary healthcare level, addressing both refugee and civilian population in Kabul. Second, the features of the leishmaniasis service delivery system: the availability, access and use of the anti-leishmaniasis drugs; the operational status of the centres providing healthcare management; the healthcare workers policies applied to medical and no-medical national staff providing treatment to cutaneous leishmaniasis patients; the coordination and governance dynamics among different strategic and implementing partners operating in Kabul in the CL control services provision (UN, national program for control of leishmaniasis and NGOs). The analysis will address these elements among the national centres in Kabul, comprising the period 2009-2010 and illustrating how the differently combined determinants to access ultimately impact on the capacity of the public health sector to ensure equity among patients. The analysis aims at recognizing the operational and strategic challenges posed to the access to leishmaniasis control activities in Kabul among patients, with the ultimate goal to provide a way forward to share with decision and policy makers.



## HOW CAN TSETSE POPULATION GENETICS CONTRIBUTE TO AFRICAN TRYPANOSOMIASIS CONTROL?

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In sub-Saharan Africa, tsetse transmitted Trypanosomiasis have an enormous impact on both human health and economic development. Both the World Health Organisation and African countries through the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) have recently asserted their determination to rid the sub-continent of these diseases, and it is increasingly recognised that vector control should play an important role. This review mainly focuses on population genetics of tsetse of the palpalis group, the main vectors of sleeping sickness, and reports recent results on tsetse population structure and on measures of gene flow between populations in different countries (Burkina Faso, Senegal, Guinea, Ivory Coast). Implications of these studies for large-scale tsetse control programmes being undertaken in West Africa are important, particularly regarding the definition of control strategies (suppression or eradication).

## EVIDENCE THAT THE TYPE IIA STRAIN OF *TRYPANOSOMA CRUZI* IS ADAPTED TO CONGENITAL TRANSFER

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It is well known that *Trypanosoma cruzi* represents a genotypically diverse family of organisms. Although some studies have suggested that the pathological outcome to infection may be associated with specific isolates, no correlation between strain and modification in transmission strategy has been identified. We have previously demonstrated in mice that the Type Ila strain of *T. cruzi* found in the southeastern United States is transferred congenitally at a significantly higher rate than the Type I strain from the same region. Using an *in vitro* cell culture model for human placental syncytial trophoblasts, we have tested whether the Type Ila strain has an enhanced ability to invade and replicate in these cells. Cultures of BeWo cells were exposed to either a Type I or Type Ila isolate of *T. cruzi* and assessed microscopically at 48, 72, and 96 hours for the percentage of cells infected and the average number of intracellular amastigotes. Cultures exposed to Type Ila isolate had significantly higher percentages of infected cells, as well as increased average numbers of intracellular amastigotes. Control infections carried out in DH-82 canine macrophage cells found that the Type I isolate was at least equal to the Type Ila strain in the ability to invade and replicate under these non-placental cells. Our results confirm that significant differences exist in the ability of these two isolates to invade syncytial trophoblast cells, suggesting adaptations in the Type Ila strain toward congenital transmission. This study not only provides the first *in vitro* evidence of strain-associated tissue tropism for *T. cruzi*, but also supports previous hypotheses for the evolution of the Type II strain in placental animals.

## A NEW APPROACH TO IDENTIFYING DRUG LEADS FOR CHAGAS' DISEASE: HIGH THROUGHPUT SCREEN AGAINST AN INTRACELLULAR PATHOGEN

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*Trypanosoma cruzi* is the parasitic agent of American trypanosomiasis or Chagas' disease, a neglected infectious disease affecting around 10 million people and an overwhelming human and economic burden throughout Latin America. A surge of patients identified in developed countries in recent years has highlighted its importance in global health. Discovery of new chemotherapy without the severe side effects associated with nifurtimox or benznidazole is essential. It is becoming evident that multi-drug therapy can prevent or significantly delay the onset of Chagas' disease pathology. To facilitate the rapid screening of large drug-like libraries, we have recently developed and validated an image-based high throughput screening assay for the pathogenic amastigote stage of *T. cruzi*. Our assay can be used with a variety of *T. cruzi* isolates and host cells and simultaneously measure trypanocidal efficacy and drug cytotoxicity to mammalian host cells. We can use various parasite strains with different biological characteristics (e.g. *T. cruzi* resistant to nifurtimox and benznidazole, clinical strains) and a range of host cells from primary human cell cultures to established cell lines (e.g. muscle cells, macrophages, hepatocytes). Our high content assay can be easily adapted to screen drugs against other intracellular pathogens such as *Leishmania* and *Toxoplasma gondii*. We are currently exploring large libraries of compounds by high throughput screening to identify hits with trypanocidal efficacy and drug-like properties.

## MOLECULAR GENETIC STUDIES OF *GLOSSINA FUSCIPES FUSCIPES* AND *TRYPANOSOMA BRUCEI RHODESIENSE* IN EASTERN UGANDA

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Tsetse flies (Diptera: Glossinidae) are vectors of several species of pathogenic trypanosomes in sub-Saharan Africa causing Human African Trypanosomiasis (HAT) and African Animal Trypanosomiasis. Uganda has two forms of parasites, *Trypanosoma brucei rhodesiense* and *T. b. gambiense* causing HAT. Tsetse flies infest two thirds of Uganda with *Glossina fuscipes fuscipes*, predominating followed by *G. pallidipes* and *G. morsitans*. Genetic studies indicate genetic differentiation of *G. f. fuscipes* into Southern and Northern as separated by Lake Kyoga with co-occurrence of the two populations in central Uganda. Studies have indicated high dispersal rates in *G. f. fuscipes*. Such dispersal rates need monitoring local patterns and stability of genetic homogeneity over time at spatial scales to provide useful information for designing effective control programs. Little is known about the genetic stability of *G. f. fuscipes* populations and the genetic changes associated with temporal changes. These regions also span the historical disease foci caused by *T. b. rhodesiense* parasites. No information is available on the fine scale differentiation of parasite populations resident in distinct flies, animal reservoirs and humans. This project is to analyze: (1) The spatial and temporal stability of the genetic structure of *G. f. fuscipes* spanning southern and northern tsetse populations and (2) Parasite genotypes in infected flies, animal reservoirs, and in humans along the same transect. We are using nuclear and mitochondrial DNA markers to assess *G. f.*

*fuscipes* populations and *T. b. rhodesiense* isolates from the same regions. Data are being collected on nine populations with 600 tsetse flies and 200 cryo-preserved *Trypanosoma* isolates from infected tsetse, vertebrates and humans. We will discuss the results of these analyses in light of the previous population level genetic data and their potential impact in providing insights on control measures. In addition, we will discuss the genetic differentiation observed among lineages of trypanosomes collected in the same region.

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### ROSIGLITAZONE ADJUNCTIVE THERAPY IMPROVES THE OUTCOME OF EXPERIMENTAL CEREBRAL MALARIA IN *PLASMODIUM BERGHEI*-INFECTED MICE TREATED WITH ARTESUNATE

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Evidence is accumulating for the emergence of artemisinin resistant parasites. Treatments that modulate the host response to malaria may be useful adjunctive therapies that could potentiate clinical outcomes for artemisinin-based therapies. We have previously shown that rosiglitazone, an FDA approved PPAR $\gamma$  agonist, improved survival in an experimental model of cerebral malaria, and given as adjunctive therapy, improved parasite clearance times in Thai adults with uncomplicated malaria. Here we investigated whether rosiglitazone given in combination with artesunate would improve disease outcome in a model of *Plasmodium berghei* experimental cerebral malaria. Mice infected with *P. berghei* were given a sub-curative dose (10mg/kg) of artesunate for 4 days starting on day 3 post infection, with or without rosiglitazone (2.5mg/kg). Mice receiving artesunate in combination with rosiglitazone had a significant improvement in survival over mice receiving artesunate only (100% vs 50% respectively;  $P < 0.0001$ ), and were completely protected from cerebral malaria. Although both artesunate and combination-treated mice had similar levels of sequestered parasites in their brains, combination-treated mice had significantly less blood brain barrier permeability, as determined by Evan's blue staining, than artesunate-treated mice. Further, combination-treated mice had higher plasma levels of angiopoietin 1 and lower levels of soluble ICAM-1 throughout infection, indicating less endothelium activation compared to artesunate-treated mice. In summary, we have shown that rosiglitazone, a compound that modulates the host response to infection, improved the outcome of experimental cerebral malaria when administered in combination with artesunate.

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### MOLECULAR DETERMINANTS OF EXPERIMENTAL CEREBRAL MALARIA IN THE BRAIN AND CIRCULATION

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Cerebral malaria (CM) is a primary cause of deaths caused by *Plasmodium falciparum* with the majority of cases occurring in young children living in sub-Saharan Africa. Improved methods for early prognosis and differential diagnosis will help in reducing the high mortality rate of CM. To better understand the host molecules that mediate the pathogenesis of CM, we identified over 200 host biomarkers of experimental cerebral malaria (ECM) caused by infection with *P. berghei* ANKA parasites by performing microarray analyses in the brain tissue of moribund, non-moribund, and three type of resistant mice infected with *P. berghei* ANKA parasites. We next assessed the biological relevance of CD14 and galectin-3, two biomarkers significantly over expressed in brain tissue of mice with ECM, and found that both CD14 and galectin-3 deficient mice were significantly protected from ECM. Next, we identified over 300 potential prognostic/diagnostic indicators of ECM in the circulation by comparing the whole

blood transcriptional profiles of resistant (BALB/c) mice to two susceptible strains (C57BL/6 and CBA/CaJ) of mice during ECM. A panel of ECM associated genes detectable in the peripheral blood has been selected to create a diagnostic signature of ECM by real time PCR. Bioinformatics analysis of this dataset has indicated that during ECM, erythropoiesis is dysfunctional, platelet and blood clotting related genes are down-regulated, and cell surface glycosylation is modified. Furthermore, computational analysis of immunity related genes suggests that distinct mechanisms of immunopathogenesis may operate in susceptible C57BL/6 and CBA/CaJ mice. The biological relevance of a few selected circulatory biomarkers of ECM is currently being assessed in biochemical and immunological studies in mice. Finally, circulatory biomarkers of ECM will be tested in human studies to identify prognostic/diagnostic markers of CM in African children.

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### TOWARD A RHESUS G-6PD DEFICIENT MODEL

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Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most prevalent and well characterized enzymopathies found in about 400 million people worldwide. Presently, there is no validated model to predict G6PD deficiency related hemolytic potential for drugs, which limits development of antimalarial drugs in the 8-aminoquinolone class. We present results of preliminary steps in the development of a rhesus model of G6PDD that could be used to evaluate hemolytic potential of drugs. Healthy rhesus monkeys were phlebotomized. Glutathione (GSH) was depleted from erythrocytes by incubation with diethylmaleate (DEM) and buthionine sulfoximine (BSO) *ex vivo*. After labeling treated and untreated cells with separate fluorescent dyes they were transfused back to the donor animals. Animals then received primaquine 4mg/kg (n=2) or vehicle 2ml/kg (n=2) daily for 9 days. Daily flow cytometry was used to measure cell life-span. Concentrations of primaquine and its major metabolite, carboxyprimaquine were measured by chiral selective LC-MS. Methemoglobin and complete blood cell counts were measured. Comparison of % cells remaining, and of the ratio of treated to untreated cells showed a trend toward faster clearance of GSH depleted cells when exposed to primaquine than either untreated cells exposed to primaquine or depleted cells exposed to vehicle control. The greatest clearance of these cells is within two days after drug exposure. After racemic primaquine treatment, there is no difference in parent drug absorption, distribution and elimination between primaquine enantiomers. However, plasma concentration of the metabolite (-)carboxyprimaquine is 10 times higher than either the parent compound or (+)carboxyprimaquine. In conclusion, preliminary results show promise in the ability of a rhesus GSH depletion model to detect G6PDD-related hemotoxicity. Further validation of the model is required and is on-going.

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### ISOLATION OF VIABLE *PLASMODIUM FALCIPARUM* MEROZOITES TO DEFINE ERYTHROCYTE INVASION EVENTS AND ADVANCE VACCINE AND DRUG DEVELOPMENT

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During blood-stage infection by *Plasmodium falciparum*, merozoites invade red blood cells (RBCs). Currently there is limited knowledge of

cellular and molecular invasion events, the kinetics of invasion and no established assays to readily measure and quantify invasion-inhibitory antibodies or compounds for vaccine and drug studies. This is in part due to the technical limitations of isolating viable merozoites from parasite cultures *in vitro* due to their short half-life. We have developed novel methods to isolate merozoites, at high yield and purity that retain their invasive potential and viability. Using these methods we have made important insights into the biology of invasion, defining the kinetics of and requirements for merozoite invasion of RBCs. Using purified merozoites, we have developed and optimized an assay to measure the invasion-inhibitory activity of antibodies and compounds distinct from other mechanisms of growth inhibition of asexual stage parasites. Interestingly, the assay was more sensitive for detecting inhibitory activity than established growth-inhibition assays. Furthermore, it was possible to fix merozoites at different stages of invasion for visualization by immunofluorescence microscopy and electron microscopy. Using this we demonstrate that processing of the major merozoite antigen MSP1 occurs at the point of RBC invasion. These findings have important implications for defining invasion events and molecular interactions, understanding immune interactions, and for the identification and evaluation of inhibitors to advance vaccine and drug development.

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### PLASMODIUM FALCIPARUM FIELD ISOLATES USE COMPLEMENT RECEPTOR 1 (CR1) AS A RECEPTOR FOR INVASION OF ERYTHROCYTES IN A COMPLEMENT-INDEPENDENT MANNER

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The malaria parasite *Plasmodium falciparum* invades erythrocytes using complex and incompletely understood mechanisms. A major invasion pathway relies on sialic acid (SA) residues of glycoporphin present on the erythrocyte surface. However, some *P. falciparum* strains have the ability to invade neuraminidase-treated erythrocytes which lack SA. We recently reported that complement receptor 1 (CR1, CD35) is a SA-independent invasion receptor for many laboratory strains of *P. falciparum*. To determine the role of CR1 in the invasion of erythrocytes by *P. falciparum* field isolates, we tested eight isolates obtained from Western Kenya. In addition, we determined whether C3 plays a role in the CR1-dependent invasion of erythrocytes by laboratory and field isolates. All the parasites examined demonstrated an ability to invade erythrocytes in a SA-independent manner, although invasion rates varied among different isolates. Anti-CR1 and soluble CR1 (sCR1) partially inhibited invasion of intact erythrocytes in a majority of isolates tested. In addition, invasion of neuraminidase-treated erythrocytes was nearly completely blocked in the presence of sCR1 and anti-CR1, confirming that CR1 is the major erythrocyte receptor that mediates sialic acid-independent invasion in these field isolates. Sequence analysis of the hypervariable region of the *P. falciparum* AMA-1 gene showed considerable diversity among the isolates tested, suggesting that the use of CR1 as a receptor is likely widespread in field parasites. Although CR1 is a receptor for C3b, CR1-dependent invasion was not affected by heat-inactivation or by C3 depletion of plasma, suggesting that parasite ligands may be interacting directly with CR1. Taken together, the data demonstrate that CR1 is an important mediator of both SA-dependent and SA-independent erythrocyte invasion by *P. falciparum* field isolates. The identification of this receptor should facilitate the search for parasite ligands that interact with it and the formulation of an effective blood stage vaccine.

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### MULTIPLE ROUTES TO HOSTS - COMPARATIVE ANALYSIS OF PROTEIN TRAFFICKING IN APICOMPLEXAN PARASITES

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Apicomplexan parasites such as Haemosporaea and Piroplasmae cause blood diseases world wide. Amongst these, *Plasmodium* is the causal agent of the devastating diseases of malaria. Apicomplexa parasitize their host intracellularly, many are enclosed within a membranous parasitophorous vacuole (PV). To colonize host cells across the membrane barrier, Apicomplexa secrete an estimated 10-20% of their total proteins. These proteins are deployed on the parasite surface, host cellular environment and host cellular surface. We have identified several trafficking signals that can be responsible for targeting different groups of proteins at hosts. We have also analyzed the trafficking machineries that recognize the trafficking signal and deliver the proteins. Trafficking into host is likely to have evolved multiple times within the Apicomplexa parasites. These delivered proteins are presumably involved in manipulation of host cell metabolism and evasion of host immune responses.

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### MOLECULAR ANALYSES OF PLASMODIUM FALCIPARUM AND PLASMODIUM VIVAX PARASITES INVOLVED IN HEPATIC DYSFUNCTION

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*Plasmodium falciparum* and *P. vivax* contributes to the majority of the human malaria cases throughout the world. In India due to the geo ecological diversity both the parasites are found with variable distribution in different geographical areas. Severe manifestations due to *P. falciparum* has been known for a long time, but the recent reports of severe manifestations due to *P. vivax* like cerebral malaria, hepatic dysfunction, acute renal failure, ARDS, circulatory collapse, severe anemia, hemoglobinuria, abnormal bleeding due to *P. vivax* mono-infections from Bikaner, as reported previously, and other parts of the world has substantiated the need to look into this parasite with a new perspective. The pathogenesis of severe *Plasmodium* malaria is not clear, but is believed to be multi-factorial, due to its diverse clinical nature. We have analyzed the expression profile of the Indian *P. falciparum* and *P. vivax* isolates showing hepatic dysfunction alone as well as in combination with other manifestations like cerebral malaria, acute renal failure, severe anemia etc. Global Expression analysis of few *P. falciparum* and *P. vivax* hepatic dysfunction cases has shown appreciable differences when compared with non severe parasite from the same region. A substantial differential expression in the parasite encoded surface antigens like PfEMP-1, rifins, stevora and surfins (in *P. falciparum*) and vir genes, Duffy Binding Protein, Reticulocyte Binding Protein etc. (in *P. vivax*) has been observed.



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### COMPLEMENT RECEPTOR 1 IS THE "X" RECEPTOR (SIALIC ACID-INDEPENDENT) FOR *PLASMODIUM FALCIPARUM* IN THE HUMAN ERYTHROCYTE

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*Plasmodium falciparum* is a highly lethal malaria parasite of human red blood cells. The molecular mechanisms of erythrocyte invasion are incompletely understood. *P. falciparum* depends heavily on sialic acid (SA) present on glycoproteins to invade erythrocytes. However, a significant proportion of laboratory and field isolates are also able to invade erythrocytes in a SA-independent manner. The identity of the erythrocyte SA-independent receptor has been a mystery for decades. We report that the complement receptor 1 (CR1) is the major SA-independent receptor (X receptor) for the invasion of erythrocytes by *P. falciparum*. Soluble CR1 (sCR1) as well as polyclonal and monoclonal antibodies against CR1 inhibited SA-independent invasion in a variety of laboratory strains and wild isolates. Merozoites were observed interacting directly with CR1 on the erythrocyte surface by immunofluorescent microscopy. Also, the invasion of neuraminidase-treated erythrocytes correlated with the level of CR1 expression. Finally, both sialic acid-independent and dependent strains invaded CR1 transgenic mouse erythrocytes preferentially over wild-type erythrocytes but invasion by the latter was more sensitive to neuraminidase. This suggests that in the normal red cell both SA-dependent and independent strains interact with CR1 in the invasion process. However, only SA-independent strains can do so without the presence of glycoprotein sialic acid. Our results close a longstanding and important gap in the understanding of the mechanism of erythrocyte invasion by *P. falciparum* necessary for the development of an effective blood stage vaccine.

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### MODULATION OF INTERFERON REGULATORY FACTORS (IRFS) UNDERLIES THE SUPPRESSION OF MALARIA-SPECIFIC IMMUNE RESPONSES IN HUMAN PATENT FILARIAL INFECTION

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Having demonstrated previously that patent filarial infection suppresses the production of malaria-specific IL-12p70, IFN- $\gamma$  and CXCL-10 (mediated through IL-10) in a malaria/filarial co-endemic region of Mali, we sought to elucidate the mechanisms underlying this suppression. Using reverse transcriptase quantitative PCR to assess the expression levels of malaria antigen-specific IL-12R $\beta$ 1, IL-12R $\beta$ 2 and IRF-1, molecules known to regulate the IL-12/IFN- $\gamma$  pathway, in blood obtained from 18 filaria-infected (Fil+) and 17 filaria-uninfected (Fil-) individuals from a malaria-endemic region of Mali, we found that Fil+ individuals had lower

expression of IRF-1 ( $p = 0.04$ ) but not IL-12R $\beta$ 1, IL-12R $\beta$ 2 than did Fil- subjects; this diminished IRF-1 expression could be reversed by neutralizing anti-IL-10 antibody. Because IL-12 in humans is produced primarily by dendritic cells (DCs), we used flow cytometry to assess the frequency of DCs (mDCs and pDCs) producing IL-12 or IFN- $\beta$  respectively from Fil+ and Fil- subjects. We found that Fil+ subjects had lower frequencies of IL-12+ mDCs ( $p = 0.0037$ ) after malaria antigen stimulation than did the Fil- subjects; there were no differences in the frequencies of IFN- $\beta$ -producing pDCs between the two groups. Using an *in vitro* model of DC filaria/malaria co-infection, we found that mDCs pre-exposed to *Brugia malayi* microfilariae produced lower levels of CXCL-9, CXCL-10, IL-12p35, IL-12p40, IL-12p19 and CXCL-11 ( $p = 0.0025$ ,  $p < 0.0001$ ,  $p = 0.0002$ ,  $p = 0.0006$ ,  $p = 0.0034$  and  $p < 0.0001$ , respectively) following malaria antigen stimulation and had markedly downregulated expression of IRF-1, IRF-2 and IRF-3 compared to mf-unexposed mDC ( $p = 0.0031$ ,  $p = 0.0001$  and  $p = 0.0039$ , respectively). Other cytokines (TNF- $\alpha$ , IL-10, IL-1 $\alpha$ , IL-1 $\beta$  and IL-6) were upregulated in the context of co-infection.

Thus, our data demonstrate that the suppression of malaria-specific IL-12/IFN- $\gamma$ /CXCL10 appears to be mediated by the modulation of IRFs (IRF-1 particularly) that play key roles in Th1 differentiation.

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### INDUCTION OF AN ENVIRONMENTAL STRESS RESPONSE IN *PLASMODIUM FALCIPARUM* USING HUMAN INNATE IMMUNE CELLS

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Infection with *Plasmodium falciparum* accounts for 1-3 million deaths annually, primarily among children in sub-Saharan Africa. The advent of artemisinin-combined therapies has led to a decrease in malaria-associated deaths however, the recent emergence of resistant strains calls for not only the development of more effective vaccines but also a better understanding of the parasite's biology in the human host. Through *in vivo* expression profiling we have previously shown that during the erythrocytic cycle *P. falciparum* has three novel transcriptional states; one of which resembles an environmental stress response (ESR) that has not been observed *in vitro*. Our goal was to elicit this *in-vivo* parasite ESR *in-vitro* by using human immune cells that are likely involved in the immunologic response during the erythrocytic cycle. We incubated *P. falciparum* 3D7 with physiologic levels of human PBMCs and PMNs and then isolated parasite RNA at different life stages in order to evaluate their gene expression. Our results show that we were able to elicit the *in-vivo* parasite stress state *in-vitro*, and that certain gene families involved in both stress and virulence were differentially expressed. Our *in-vitro* stress state yielded changes in the expression of a number of heat-shock proteins; an effect that was significantly correlated with the *in-vivo* ESR. Moreover, we also found induction of stress-related genes such as spermidine synthase. Our results also demonstrate that the parasite can alter genes encoding surface targeted proteins that function in immune evasion/virulence specifically proteins of the *var* and *rif* gene families. The differential regulation of the *rifin* proteins in our *in-vitro* stress state may help to shed light on the role of these proteins in the host response. Overall, these results illustrate for the first time an *in-vitro* parasite stress state induced by host immune cells and provide evidence of specific gene usage that may accurately reflect what occurs physiologically in the circulation of an infected host and play a role in pathogenesis.

### RESVERATROL, A COMPONENT OF RED WINE, IMPAIRS THE CYTOADHERENCE OF *PLASMODIUM FALCIPARUM*-INFECTED RED BLOOD CELLS BY REDUCING THE EXPRESSION OF PFEMP-1

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Sequestration of *Plasmodium falciparum*-infected red blood cells (IRBCs) is critical to parasite survival and is centrally involved in the pathogenesis of malaria. Adherence of IRBCs to microvascular endothelial cells (MVECs) enables parasites to avoid clearance from the bloodstream by the spleen. Cytoadherence is also implicated in microvascular inflammation and endothelial dysfunction. Rosetting is also believed to contribute to obstruction and ischemia-induced inflammation in microvessels. Both cytoadherence and rosetting are associated with severe and fatal *falciparum* malaria. *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), a family of parasite-encoded antigenically-variant proteins, mediates cytoadherence and rosetting and is encoded by *var* genes. The expression of *var* genes is regulated by parasite-encoded sirtuin 2 (PfSir2), a histone deacetylase. The polyphenol resveratrol (RV) activates PfSir2 and was recently shown to transcriptionally repress, in a differential manner, all three major sub-families of *var* genes. We thus hypothesized that RV impairs the cytoadherence and rosetting of IRBCs. To test this, we infected RBCs with the HB3 and FCR-3 *P. falciparum* lines in the presence of increasing concentrations of RV. After one cycle of parasite invasion and development to the trophozoite stage expressing PfEMP-1, we found that RV impaired (up to 57%) adherence to MVECs in a dose-dependent manner. Using the rosetting *P. falciparum* line 'varO', we found that RV also reduced (up to 40%) rosette frequencies in a dose-dependent manner. These findings were associated with moderate reductions in the levels of PfEMP-1 on the surface of IRBCs detected by flow cytometry. These reductions in cytoadherence, rosetting, and PfEMP-1 levels were not associated with decreased parasite viability. These data suggest the possibility that commercially-available RV - a component of red wine - may attenuate the virulence of *P. falciparum* by impairing cytoadherence and rosetting *in vivo*. Our findings thus provide a rationale investigating whether RV, in combination with antimalarial chemotherapy, could improve the survival of patients with severe malaria.

### PLASMODIUM FALCIPARUM-INFECTED ERYTHROCYTES CONTAIN URIC ACID PRECIPITATES THAT ARE HIGHLY INFLAMMATORY

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Malaria life-threatening pathology is caused or exacerbated by excessive inflammatory responses in the host. Further understanding of the mechanisms involved in this process is needed to develop more effective therapies against malaria-induced pathology. We previously defined uric acid (UA) as a mediator of malaria-induced inflammation in mouse and human cells as reported previously. We have now discovered that *Plasmodium falciparum*-infected erythrocytes contain UA precipitates. Using both immunofluorescence with specific antibodies and lysate fractionation, we have detected UA precipitates in *P. falciparum*-infected erythrocytes in all cycle stages. UA precipitates are localized in the Plasmodium cytosol and are released into the medium upon schizont rupture. The inflammatory properties of UA precipitates (also named

crystals) are well known because they are the causative agent of gout and are also considered a danger signal for the immune system. Direct release of UA precipitates in the blood upon schizont rupture may cause strong inflammatory responses during malaria infection. We found that addition of UA inhibitory drugs, allopurinol and uricase, reduced secretion of inflammatory cytokines (TNF, IL-1 $\beta$  and IL-6) from human peripheral blood mononuclear cells in response to Plasmodium-infected erythrocytes, suggesting that a decrease in UA levels *in vivo* may reduce the host inflammatory response and pathology. We obtained intracellular UA precipitates derived from Plasmodium-infected erythrocytes. These precipitates caused increased expression of the dendritic cell activation markers, CD40, CD80 and CD86 *in vitro*. This inflammatory effect was sensitive to uricase treatment, confirming their identity. This suggests that Plasmodium-derived UA activates the host inflammatory response and may contribute towards malaria pathology. Inhibiting UA formation may therefore decrease malaria-induced pathology, and this will establish the basis for developing specific therapies against this devastating disease.

### VAR2CSA ELICITS BROAD REACTIVE ANTI-ADHESIVE ANTIBODIES

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Pregnancy Associated Malaria (PAM) has harmful consequences for both the mother and foetus, primarily due to the accumulation of infected erythrocytes (iE) in the placenta. A member of the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) family, called VAR2CSA, is a variant surface antigen (VSA) which mediates adherence of iE to a placental receptor - chondroitin sulphate A (CSA). Women with PAM-related placental infection develop VSAPAM-specific anti-CSA adhesive antibodies after successive pregnancies that protect them from the severe consequences of PAM. Identifying which part of the VAR2CSA protein elicits broad reactive anti-CSA adhesive antibodies will provide a breakthrough for developing an anti-PAM vaccine. A cohort of 1000 pregnant women, recruited before 24 weeks of pregnancy, was followed until delivery with the aim of accurate quantification of the effects of PAM on foetal and maternal health in Korogwe, North-eastern Tanzania. The overall aim of this longitudinal study is to optimize strategies for preventive intermittent treatment and facilitate development of a vaccine against PAM. Parasite isolates collected from pregnant women were cultured to late trophozoite and schizont stages and then tested for their ability to transcribe and express VAR2CSA by using qRT-PCR and flow cytometry, respectively. Antibodies raised in rats against different VAR2CSA Duffy binding like (DBL) domains of the FCR3 strain were assessed for their ability to inhibit adhesion of the PAM-derived *P. falciparum* iE to CSA *in vitro* using a static inhibition of binding assay (IBA). Based on qRT-PCR and flow cytometric analyses, we show that parasite isolates from pregnant women transcribe and express VAR2CSA on the surface of iE. The IBA results show that antibodies targeting particular VAR2CSA DBL domains inhibit the adhesion of most clinical isolates tested. In conclusion, immunization of rats with particular recombinant VAR2CSA protein domains based on the FCR3 sequence elicits broad reactive anti-adhesive

antibodies. Our findings bring us closer to identifying which part of the VAR2CSA protein may be used as a basis for developing an anti-PAM vaccine candidate.

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#### ANTIBODIES AGAINST VAR2CSA OF PFEMP1 DBL2X AND DBL3X DOMAINS INHIBITED ADHESION OF IE TO CHONDROITIN SULFATE A

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Over 500 million cases of clinical malaria occur annually. Malaria is the leading cause of infant mortality in under-developed countries. During pregnancy, *Plasmodium falciparum* infected erythrocytes (IE) bearing a preferentially expressed VAR2CSA surface protein sequester on placental syncytiotrophoblast by binding to Chondroitin Sulfate A (CSA). This phenomenon occurs mostly in primigravidae resulting in maternal anemia, low birth weight and in severe cases death of the fetus. However, after multiple pregnancies, multigravidae women develop blocking antibodies against VAR2CSA protein. VAR2CSA is a member of PfEMP1; a family of structurally related proteins with its extracellular portion made up of six Duffy-Binding-Like (DBL) domains and four Cysteine-rich Inter-Domain Regions. We refolded and purified recombinant DBL2X, DBL3X, and CSA binding sub-domain 3 of DBL3X (DBL3X-S3) and sub-domain 3 of DBL2X (DBL2X-S3) from *E. coli* inclusion bodies. DBL2X-S3 and DBL3X-S3 bind with higher specificity and lower affinity to CSA expressed on CHO-K1 cells compared to DBL2X and DBL3X which bind with lower specificity and higher affinity, respectively. Rat and rabbit antibodies raised against the DBL domains recognized homologous parasite IE and some heterologous parasite IE expressing alternative alleles of VAR2CSA. Preliminary results obtained with combinations of antibodies against these DBL domains suggest additive inhibition of IE binding to CSA expressed on CHO-K1 cells. Several of the rat and rabbit antibodies raised against these DBL domains showed limited inhibition of maternal field isolate binding to CSA, however, further studies are required. Taken together, these individual DBL domains of approximately 25-30 kDa can be produced in large quantities and scale in *E. coli*, hence favoring them as viable vaccine candidates for PAM.

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#### DISPARITIES IN ACCESS TO SANITATION IN BOLIVIA

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Bolivia is the only country in Latin America that is falling short of Millennium Development Goal #7 target for sanitation. Understanding where access to sanitation is the lowest, and the socio-economic factors associated with lack of access to sanitation, aid in identification of the populations most in need. Bolivia's population is estimated to be up to two-thirds indigenous Amerindian, and these groups dominate the rural population. Among the rural population, 57% (≈1,894,000 people) do not have access to a toilet or latrine. Previous studies have demonstrated that children in rural Bolivia are at greater risk of morbidity, malnutrition and impaired development associated with diseases linked to inadequate water, sanitation and hygiene. This analysis provides an in-depth assessment of disparities in access to sanitation by comparing

the relative influence of location, socioeconomic factors (household construction materials, number of household members), educational status and gender for major ethnic groups in Bolivia using the most recent data from the nationally representative Demographic and Health Survey (DHS). The language that the head of household reported as learning to speak first was selected to indicate ethno-linguistic group. Across the 3 major indigenous ethno-linguistic groups of Bolivia, the primary correlates with access to sanitation differ: among the Aymara people (20% of total population, 46% household sanitation coverage within group), rural location is the strongest correlate with low sanitation coverage; among the Quechua (27% of total population, 48% household sanitation coverage within group), rudimentary household construction materials are most strongly associated with lack of household sanitation; and among the Guaraní and other Llano region groups (1% of total population, 53% household sanitation coverage within group), larger household size is associated with less access to sanitation. These differences in the primary correlates with lack of household sanitation across the ethno-linguistic groups of Bolivia can inform regional sanitation programs by identifying the populations with the greatest need and helping implementers to better target population selection and sanitation intervention strategies to be more effective for the geographic and social context of their programs.

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#### AN EVENT-BASED MODEL FOR ENVIRONMENTAL TRANSMISSION OF GIARDIA INFECTION

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Diarrheal illness is a major burden in the developing world, with a median of ~3.2 episodes per year in children under 5 years. *Giardia* is a major contributor to this burden. Where resources are insufficient for distribution of clean drinking water and removal of human waste, many less expensive anti-diarrheal interventions have been investigated. These include latrine construction, handwashing with soap, and various methods for household water treatment (HWT). Published trials of these interventions often claim reductions in diarrheal illness of 30% or more. However, most trials are short-term, and nearly all are subject to bias. Furthermore, characteristics of interventions and communities vary greatly, and interventions are seldom maintained after the trial is over. Since long-term trials require much time and money, a simulation approach may be helpful for assessing the effectiveness of interventions in various contexts. A simulation model describing *Giardia* transmission in an isolated, underdeveloped community was programmed using Octave 3.0. It uses the Gillespie event-based algorithm to stochastically track susceptible, exposed, and infectious individuals and the number of cysts in the water source. Dose-response modeling determines exposure outcomes from ingestion of contaminated water. The model also includes an HWT intervention that reduces the number of cysts in drinking water for community members who use it.

Results (preliminary): If there is no intervention, the model equilibrates at a hyperendemic state, with ~90% of the population infected. If the entire community uses an HWT intervention that reduces cysts in drinking water by 99.0%, the prevalence gradually declines to ~8% after 1 year. If only 75% of the population uses the intervention, ~36% of the population is infected after 1 year. If everyone in the community uses the intervention on 95% of their drinking water, but continues to drink untreated water 5% of the time, the model equilibrates at ~67% infected after ~150 days. Highly consistent use of HWT may be necessary to control giardiasis in hyperendemic communities. Further refinements of the model may alter these conclusions. Household structure and additional transmission routes (e.g., contaminated hands) will be included in future versions of the model, allowing simulation of additional interventions (e.g., handwashing and sanitation).



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**CRYPTOSPORIDIUM CONTAMINATION OF SURFACE AND WATER SUPPLIES IN HAITI**

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Cryptosporidiosis is one of the most frequent causes of diarrhoea in Haiti. Transmission in children less than five years-old, HIV-infected individuals, and people living in low socio-economic conditions is frequently due to consumption of water or food contaminated by *Cryptosporidium* oocysts. This study examined the circulation of *Cryptosporidium* oocysts in surface waters and in public water supplies in the district of Port-au-Prince and in the surface water and groundwater used by the population of Les Cayes (Haiti). Data were gathered in 37 sample sites in Port-au-Prince and in 15 sites in les Cayes and in surroundings of the city (bathing water, household waste water, spring water, boreholes, water supply, domestic wells). Each sample of 100 litres of water was collected and immediately filtered using a polyethersulfone capsule. Oocysts were isolated using an immuno-magnetic method and counted under fluorescence microscopy after labelling with a monoclonal antibody. In the district of Port-au-Prince, 24/37 (65%) of water samples collected were contaminated by *Cryptosporidium* oocysts and the number of oocysts per 100L ranged from 4 to 1,274. In the reservoirs used by people living in peripheral areas, 10/11 (91%) of samples collected were contaminated with a mean number of 140 oocysts per 100L. In water samples from public standpipes provided by Camep, the public company of water distribution in Port-au-Prince, 7/13 (54%) were contaminated. All surface water 4/4 collected in Port-au-Prince or in peripheral areas was highly contaminated. In Les Cayes 8/15 (53%) samples contained *Cryptosporidium* oocysts and the number detected varied from 5 to 100 (mean 29) / 100 L of water filtered. In conclusion, a commitment to environmental improvement in Port-au-Prince and in Les Cayes is required to improve the quality of drinking water and to limit the risk of human transmission of cryptosporidiosis.

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**DETERMINANTS OF HOUSEHOLD WATER QUALITY IN PERI-URBAN SETTINGS**

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Public standpipes providing access to treated drinking water from municipal distribution systems are an increasingly common approach in urban environments; however, little data is available on the quality of drinking water at the household-level in urban and peri-urban environments. In February 2009, we assessed the determinants of household water contamination in a peri-urban settlement in Kisumu, Kenya. Data collection included: water quality measures at all drinking water sources; a population-based survey of 1,000 households, water source selection, and water handling practices; and water quality measures (fecal coliform and *E.coli* concentrations) of all household stored drinking water. Socio-economic position was assessed through an inventory of household goods and respondents divided into wealth quintiles. Logistic regression models were developed to determine the association between drinking water contamination and household behaviors and socio-economic characteristics. A total of 88 potential drinking water sources were identified, including 25 municipal standpipes and 63 shallow wells. Three of the municipal taps tested positive for *E.coli* contamination. Over 91% of respondents reported collecting drinking water from a municipal tap; and 47.9% of household stored drinking water samples tested positive for *E.coli* contamination. Significant predictors of *E. coli* contamination included: ever using a well as a drinking

water source (OR=2.6), having a water treatment product in the house (OR=0.74), storing water in a narrow-mouthed container (OR=0.68). There was a marginally significant reduction in the odds of contamination among wealthier households when compared to poorer households. In conclusion, findings suggest that efforts to provide clean drinking water through public standpipes are not sufficient to guarantee clean drinking water at the household level. Even when clean drinking water is provided from municipal distribution systems, household water contamination is mediated by a variety of household-level behavioral factors.

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**EVALUATION OF POT-CHLORINATION OF WELLS DURING A CHOLERA OUTBREAK, BISSAU, GUINEA-BISSAU, 2008**

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Waterborne cholera epidemics are a major public health problem in sub-Saharan Africa. Guinea-Bissau has experienced five cholera epidemics since 1994. The most recent epidemic occurred in 2008, causing >14,000 cases and 225 deaths. In the capital city, Bissau, UNICEF-designed pot-chlorinators were used to disinfect shallow wells, a common source of drinking water. We evaluated the ability of pot-chlorinators to achieve free residual chlorine (FRC) levels in well water adequate to inactivate *Vibrio cholerae*. Thirty wells were randomly selected from six neighborhoods. Pot-chlorinators - bottles filled with gravel, sand, and calcium hypochlorite granules - were placed in each well. FRC was measured before and 24, 48, and 72 hours after placement and compared with WHO-recommended levels of  $\geq 1.5$  mg/L during cholera outbreaks and 0.2-5mg/L in non-outbreak settings. Water turbidity, presence of well covers, distance from wells to latrines, and rainfall were noted and pH was measured at each well 24, 48, and 72 hours post-chlorination. Complete post-chlorination data were collected from 26 wells; 15 (58%) were <2 meters deep, with well volumes of 0.6-8.0 m<sup>3</sup>. Twenty-four (92%) wells were <30 meters from a latrine. Four (15%) wells were covered on all observation days; rain fell on the second night at all wells. Four to 15% of wells had turbid water over the observation period; rainfall and presence of a lid did not appear to affect water turbidity. All wells had a pH <8 at baseline, 24, and 48 hours post-chlorination; one well had a pH >8 at 72 hours. At baseline, no wells had FRC >0.09 mg/L. Four (15%), one (4%), and no wells had FRC  $\geq 1$  mg/L and 16 (62%), 4 (15%), and 1 (4%) wells had FRC between 0.2-5 mg/L at 24, 48, and 72 hours post-chlorination, respectively. Pot-chlorinators failed to achieve WHO-recommended FRC levels in wells during a cholera outbreak, and may convey a false sense of security to local residents. Pot-chlorination should be discouraged and alternative approaches to well-water disinfection promoted.

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**CERAMIC WATER FILTERS REDUCE DAYS OF DIARRHEAL ILLNESS IN HIV-INFECTED INDIVIDUALS IN LIMPOPO PROVINCE, SOUTH AFRICA**

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Individuals infected with HIV frequently suffer from diarrheal illness transmitted by water-borne pathogens. Locally produced ceramic water filters impregnated with colloidal silver can be a sustainable solution to purify water in resource-limited settings. This work investigates if these

filters can reduce the rates of diarrheal illness in individuals being treated for HIV in rural South Africa. This randomized, controlled trial recruited HIV-infected individuals receiving anti-retroviral therapy (ART) from a private clinic. After randomization, individuals either received a ceramic water filter along with training on its use (intervention) or received routine clinical care which included recommendations about drinking treated water (control). Participants in both groups completed daily diarrhea diaries and submitted the diaries weekly for 40 weeks. Influent and effluent water samples were tested using the membrane filtration method to evaluate the number of colony-forming units of fecal coliforms. Stool samples were collected at enrollment and evaluated for *Cryptosporidia* sp. and enteroaggregative *E. coli* (EAEC) by PCR. 65 participants completed the study with 35 in the intervention arm and 30 in the control arm. 90% of participants were female. Average age was 41.5 years. All participants were receiving chronic ART. 18/71 (25%) participants reported diarrhea within the month prior to enrollment in the study. At baseline, 27/76 (35.5%) had *Cryptosporidia* sp. and 16/76 (21%) had EAEC in their stool samples. Influent water samples yielded an average of 6,863 CFU/100 mL. Following filtration, the effluent samples showed 0 CFU/100 mL. After 40 weeks of follow-up, the participants in the control arm have reported a total of 176 days of diarrhea, and those in the intervention arm have reported 68 days. These findings represent rates per person-year of 7.6 days and 2.6 days respectively. ( $p=0.011$ ). In conclusion, silver-impregnated ceramic water filters significantly reduce the number of days of diarrhea in HIV-infected patients taking ART in rural South Africa who have high levels of fecal contamination in their drinking water and high prevalence of enteric pathogens.

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### POST-IMPLEMENTATION ASSESSMENT OF CERAMIC WATER FILTERS DISTRIBUTED TO TSUNAMI-AFFECTED HOUSEHOLDS IN SRI LANKA

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This study was a post-implementation assessment of tsunami-affected households in Sri Lanka that received ceramic water filters (CWF) during a distribution program in 2008. The research design was a cross-sectional study to determine the number of households still using the filter, factors associated with filter use and disuse, and the microbiological effectiveness of the filters. Data was collected by in-person oral interview from September to December 2009. Based on self-reported results, 76% of recipient households were still using the filter at the time of survey. At the time of survey, filters in user households had been in use from 6 months to >2 years, depending on the time of distribution. The data suggest that the main drivers of filter use and disuse are household water source, filter breakage, filter flow rate, and perception of water quality. Breakage was the most frequently cited reason for stopping filter use; this includes both filter breakage and storage container breakage. Logistic regression modeling showed that the variables with the greatest effect on continued filter use were having tap or well water, perceiving water as dirty, and perceiving water as unsafe. Households that had tap water were more likely to discontinue filter use, while households that had wells were significantly less likely to discontinue filter use. Source water quality in many survey households was fairly good; ~50% of filter households had <1 *E. coli*/100 mL in their water, as did ~70% of non-filter households. Analysis of *E. coli* levels in untreated and filtered water indicates that the microbial quality of water is improved by filters. These results suggest that filters improve water quality and have high levels of user satisfaction; sustained filter use needs to be maintained by the establishment of supply chains for replacement filters and user education about water quality.

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### DEVELOPMENT OF A MULTIPLEX PCR-BASED SUSPENSION ARRAY ASSAY FOR THE SIMULTANEOUS IDENTIFICATION OF FIVE *ENTAMOEBEA* SPP. COMMONLY FOUND IN HUMAN STOOLS

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Six species of the genus *Entamoeba*, i.e., *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. polecki*, *E. coli*, and *E. hartmanni* can be found in human stools. Of these only *E. histolytica* is considered pathogenic causing intestinal and extra-intestinal disease. *E. histolytica*, *E. dispar* and *E. moshkovskii* are morphologically identical. *E. polecki*, *E. coli*, and *E. hartmanni* can be differentiated morphologically from *E. histolytica*, but some of their diagnostic morphologic features may overlap creating issues for the differential diagnosis. Nevertheless, all these species can be differentiated using DNA-based approaches. The objective of this study was to develop a rapid, high-throughput screening method using a suspension array technique for the simultaneous detection and differentiation of *Entamoeba* species. PCR amplification was performed with biotinylated *Entamoeba* sp 18 S rRNA gene primers JVF and EntaRev, designed to amplify a fragment of approximately 360 bp of the *Entamoeba* spp studied. Regions of this fragment that had could differentiate among *E. histolytica*, *E. moshkovskii*, *E. dispar*, *E. hartmanni* and *E. coli* were selected to design hybridization probes to link to Luminex beads. The assay was standardized with cloned DNA and evaluated with ten DNA extracts from samples obtained from individuals that had these amebas in their stools. Using these approach we were able to correctly identify *E. histolytica*, *E. dispar*, *E. hartmanni*, *E. coli* and *E. moshkovskii* in all studied. These results show that this method could be used in the future for diagnostic detection of *Entamoeba* spp in fecal samples.

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### HUMAN PRIMARY EPITHELIAL CELL MODEL FOR *CRYPTOSPORIDIUM* INFECTION

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Cryptosporidiosis is caused by protozoan parasites that infect human intestinal epithelial cells. Studies of human infection have been limited by *In vitro* and *in vivo* models (no untransformed cells). In previous studies we have used human explants to study *Cryptosporidium* infection; however the model has been limited by the time because the cells go quickly to apoptosis. We hypothesized that by using anti apoptotic proteins and specific environments is possible to culture intestinal primary cells. Therefore the objective of this work was to develop a human primary epithelial cell model for studying *Cryptosporidium* infection. We obtained epithelial cells from human small intestine that would otherwise have been discarded after small intestinal surgeries. The cells were cultured in matrigel using specific enterocyte medium supplemented with growth and anti-apoptotic molecules. After 1 week, the cells formed organoids-like structures. The organoids were obtained, dissociated and seeded in culture chambers. The cells were characterized by IF, EM, ELISA and PCR. Cells remained viable and growing for >1 month. The cell population included intestinal stem cells, but also matures cells (with microvilli, tight junctions, and surface alkaline phosphatase). Cultured cells were infected with *Cryptosporidium* sporozoites and different stages of *Cryptosporidium* including oocysts were observed. Real time-PCR studies were performed to show an increase in the number of parasite during the time of

infection. Those results suggest the parasite is completing the life cycle in our system. This should provide an improved tool to study host-parasite interactions in intestinal infections as cryptosporidiosis.

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## CRYPTOSPORIDIUM IN NIGERIA

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Epidemiological study to determine the association of *Cryptosporidium* and other enteric protozoan parasites with diarrhea in Owerri and its environs of Imo- State, Nigeria was carried out between September 2002 and May 2005. Further determined was the relationship between these enteric parasites, especially *Cryptosporidium*, with HIV/AIDS. A total of 3054 stool samples from patients attending various health institutions in the study area was examined. Of these, 1204 (39.4%) were diarrheic while 1850(60.6%) were non-diarrheic. Enteric parasites were detected in 572 (47.5%) of the diarrheic stool samples. Enteric parasites identified in diarrheic stool samples include, protozoans (28.7 %), helminths (4.9%), and mixed infections (1.8%). The enteric protozoans identified include; *Entamoeba histolytica* (10.1%), *Giardia duodenalis* (7.9%), *E. coli* (5.8%) and *Cryptosporidium* species (4.9%). *Cryptosporidium* infection was higher in children aged <1-5 years and higher in those of 21-60. Age related protozoan infections showed significant variations ( $p<0.05$ ) between children aged <1-20 years and subjects aged 21 years and above. Of the 3054 stool samples examined, 356 were from HIV positive patients. 52 (14.6%) had *Cryptosporidium* oocysts in their stools while 14 (0.5%) of 2698 stool samples from non-HIV patients had *Cryptosporidium* oocysts. *Cryptosporidium* associated diarrhea showed significant difference ( $p<0.05$ ) among HIV positive (17.8%) and HIV negative (1.4%) diarrhea patients. Protozoan infections of HIV positive diarrhea patients showed significant difference ( $p<0.05$ ) from similar infections of HIV negative diarrhea patients. Cryptosporidiosis had a high pathogenicity and was found in association with diarrhea and only rarely in non-diarrhea samples. From this study *Cryptosporidium* is associated with diarrhea as much as *Giardia lamblia* and *E. histolytica* especially in HIV patients and children. Since diagnosis of *Cryptosporidium* species is possible with simple staining technique, it is suggested that routine examination for *Cryptosporidium* be part of the parasitological routine especially with AIDS patients. The epidemiological significance of these results is discussed, especially in the context of controlled measures.

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## THE EFFECT OF MATERNAL BREAST FEEDING ON TIME TO FIRST CRYPTOSPORIDIAL INFECTION AMONG CHILDREN IN A SEMI-URBAN SLUM IN SOUTH INDIA

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*Cryptosporidium* spp. are a major cause of parasitic diarrhea in children worldwide. Although breast feeding protects children from gastrointestinal illnesses, there is no conclusive evidence on the degree of protection conferred by exclusive breast feeding on acquisition of cryptosporidiosis. As part of an ongoing cohort study on cryptosporidial transmission among children in south India we ascertained the effect of exclusive breast feeding on time to first cryptosporidial infection. Over a 9-month period we recruited 150 children from households using packaged or municipal drinking water (75 children in each cohort) for weekly follow-up from the time of introduction of supplementary feeding until the first identified cryptosporidial infection. Surveillance stool samples were examined monthly and during diarrheal episodes for the presence of

*Cryptosporidium* spp. by PCR PFLP at the 18S rRNA locus. The mean (SD) age at introduction of supplementary feeding was 19.8 (6.1) weeks. Over 4195 child-weeks of observation, 46 children developed cryptosporidial infection 21.9 (9.2) weeks after stopping exclusive breast feeding. The first symptomatic infection occurred earlier than asymptomatic infection (16.4 vs. 24.1 weeks post-weaning,  $P=0.01$ ; for 33 symptomatic and 13 asymptomatic cases, respectively). The duration of exclusive breast feeding was inversely related to the time to first infection (Spearman's  $\rho=-0.47$ ,  $P=0.02$ ) in the municipal water cohort, even after adjusting for symptomatic infections ( $P=0.01$ ). Such an association was not observed in the packaged water cohort (Spearman's  $\rho=-0.08$ ,  $P=0.73$ ). In conclusion, these preliminary data suggest a complex relationship between duration of exclusive breast feeding and the time to first cryptosporidial infection. Immune responses, household hygiene & water handling practices might also play an important role and will be further examined.

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POPULATION GENOMICS OF THE SEXUALLY TRANSMITTED HUMAN PATHOGEN *TRICHOMONAS VAGINALIS*

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*Trichomonas vaginalis*, the causative agent of human trichomoniasis, is the most prevalent non-viral sexually transmitted infection with over 174 million new global cases occurring every year. Historically it has been considered a "self-clearing female nuisance infection", but more recently it has been associated with increased risk of HIV transmission, making detection of the parasite and treatment of the disease an important part in the fight against AIDS. Currently little is known about the genetic diversity and population structure of the species, but with the publication of the *T. vaginalis* genome in 2007, new molecular tools are becoming available. We have developed and validated a panel of 21 microsatellite and six single copy gene markers to evaluate the population genomics of new clinical isolates collected from female patients attending New York City STD clinics, as well as extant isolates collected from around the world. Using an array of population genomic tools, we have detected significant genetic diversity within the species and have found that it is maintained across global regions. We have also found evidence of a two-clade population structure that may be correlated with parasite virulence. These findings will be important in understanding the spread of drug-resistance, in determining virulence factors, and in understanding why many individuals remain asymptomatic while others have severe manifestations of disease.



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### DIFFERENTIAL ABILITIES OF *TOXOPLASMA GONDII* AND *NEOSPORA CANINUM* TO DEVELOP RESISTANCE AGAINST THE ACTION OF PHENYLATED PENTAMIDINE-DERIVATIVES (ARYLIMIDAMIDES) *IN VITRO*

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*Toxoplasma gondii* causes a variety of clinical diseases in man, is one of the most common opportunistic infections in immuno-compromised patients, and also represents a major veterinary medical and economic burden. *Neospora caninum* is a major cause of abortion in cattle and neuromuscular disease in dogs, and is closely related to *T. gondii*. We have earlier shown that a phenylated pentamidine derivative, DB750, inhibited the proliferation of *T. gondii* and *N. caninum* tachyzoites *in vitro*, with IC50 values of 0.16µM and 0.24µM, respectively. By *in vitro* culture and step-wise increase of drug concentration, *T. gondii* (ME-49) tachyzoites were able to adapt to DB750 treatment, up to a max. conc. of 1.2µM, and the adapted strain was named *T. gondii*\_DB750. In contrast, *N. caninum* (Nc-1 isolate) failed to adapt to the drug. Screening of a range of other pentamidine derivatives against *T. gondii* led to the identification of DB745, a compound that is structurally related to DB750, with highly improved IC50 (0.03µM). DB745 did not notably affect host cell (human foreskin fibroblast, HFF) proliferation nor host cell integrity at concentrations up to 3µM. Short term pretreatment of HFF with 1µM DB745 prior to infection and subsequent removal of the drug severely reduced the capacity of infecting *T. gondii* to proliferate intracellularly. In contrast to DB750, DB745 also inhibited invasion of tachyzoites into HFF. The DB750-adapted strain showed reduced susceptibility towards DB745, since *T. gondii*\_DB750 exhibited an IC50 for DB745 of 0.07µM, which is more than 2 times higher than the one originally reported for DB745 in the non-adapted parasites, but still well below the IC50 values originally reported for DB750. Since *N. caninum* was unable to adapt to increasing DB750 concentrations, DB750 was applied in a mouse model for acute cerebral neosporosis, and showed promising results and reduced cerebral parasite burden and viability. Thus, arylimidamides might represent useful treatment options against neosporosis, but, due to ability of *Toxoplasma* to readily adapt to drug pressure, probably less against toxoplasmosis.

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### DEVELOPMENT AND EVALUATION OF CHIMERIC ANTIGENS FOR THE VACCINATION AGAINST NEOSPOROSIS

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*Neospora caninum* (Apicomplexa: Eimeriina: Sarcocystidae) is reported as the leading cause of bovine abortion, thus the disease represents an important veterinary health problem and is of high economical significance, as reported previously. Currently, only one vaccine against bovine neosporosis is available on the market (Bovilis Neoguard®, Intervet). This vaccine is based on tachyzoite protein extract but confers only partial protection against the disease, as reported previously. Basically, an efficient vaccine against *N. caninum* infection in cattle (or other animals) should prevent tachyzoite proliferation and dissemination in pregnant dams to avoid transplacental transmission to the fetus, and prevent tissue cyst formation in animals that have been infected with oocysts or tissue cysts. This could be achieved by a vaccine that stimulates protective cellular immune responses as well as antibody responses at both mucosal sites and systemically. It is very unlikely that this could be achieved by a single antigen, but most likely by a mixture of parasite antigens or a vaccine that contains a number of relevant antigenic domains of different proteins. The overall goal of our investigations on

*N. caninum* is to develop a vaccine that limits both the cerebral infection and the transplacental transmission. Since promising results were obtained with a combination of the recombinant forms of three secreted proteins, NcMIC1, NcMIC3 and NcROP2 in the reduction of cerebral infection and vertical transmission in infected mice (as reported previously), we focused on the use of these proteins for further vaccination strategies. In order to increase the immunogenic potential of these antigens, the production of different chimeric proteins based on their putative antigenic domains was investigated. Antibodies against these proteins were raised in mice and tested for their inhibitory effect on the host cell invasion by *N. caninum in vitro*. Their capability to recognize the native proteins was also assessed by Western blot and immunofluorescence. A vaccination trial in mice is currently under investigation and the survival rate and health of the challenged mice as well as an assessment of the parasite burden in brain will be performed. Moreover, the cellular and humoral immune responses will be assessed. A summary of the results achieved so far will be presented.

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### DEVELOPMENTAL EXPRESSION OF FUCOSYLATED CARBOHYDRATES IN MIRACIDIA AND PRIMARY SPOROCYSTS OF *SCHISTOSOMA MANSONI*: CHARACTERIZATION OF THE FUCOSYLATION MACHINERY

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Fucosylated carbohydrate epitopes (glycotopes) of the parasitic flatworm *Schistosoma mansoni* are key determinants in its development and immunobiology. Importantly, studies indicate that glycotope expression is developmentally and gender-specifically regulated, however the mechanism of differential expression is not well understood. Ongoing research seeks to identify and functionally characterize the enzymatic machinery that contributes to their production, specifically the enzymes involved in fucosylation, GDP-L-fucose synthesis, and GDP-L-fucose transport. A homology-based bioinformatics approach for gene discovery identified several schistosome genes that are putatively involved in fucosylation, including  $\alpha$ 3- and  $\alpha$ 6-fucosyltransferases, GDP-D-mannose-4,6-dehydratase (GMD), GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (GMER), and GDP-L-fucose transporter (GFT). At present, gene transcription has been confirmed and full-length transcript sequences have been determined via RT-PCR and 5'/3' RACE. Interestingly, most genes exhibit alternative splicing. Current analyses include Southern and northern hybridizations, antibody production for western blotting and immunolocalization, quantitative real-time PCR to assess relative gene transcription amongst developmental stages, and functional assays such as RNAi-mediated gene silencing in conjunction with phenotypic screening in snail-associated schistosome larvae. Additionally, the enzymatic function of heterologously expressed GMD, GMER, and GFT will be assessed using canonical bioassays, including the *in vitro* reconstitution of GDP-L-fucose synthesis and transport. Of significance, the identification and characterization of these genes may provide novel targets for drug discovery, which could be useful for the control of schistosomiasis in snail and mammalian hosts.

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### TRANSCRIPTIONAL PROFILING OF THREE DIFFERENT MODELS OF RESISTANCE TO TREMATODE INFECTION IN *BIOMPHALARIA GLABRATA*

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As the intermediate host for the trematode *Schistosoma mansoni*, the freshwater snail *Biomphalaria glabrata* plays a significant role in

the transmission of schistosomiasis to human populations. In the lab, *B. glabrata* has demonstrated its ability to defend against trematode infections by employing specific defense strategies to counteract parasite evasion or immuno-suppression. We here compare three different forms of resistance of *B. glabrata* to trematode infection: age-based, strain-based, and acquired resistance. To make these comparisons, we used a *B. glabrata* oligo-based microarray (1152 features) emphasizing stress and immune-response factors. We monitored the transcriptional profiles of *B. glabrata* from 0.5 up to 32 days post-exposure. The age-based array compared susceptible juvenile M-line snails (4-8mm) to adult, resistant snails (10-14mm), both exposed to the trematode *Echinostoma paraensei*. The strain-based array compared the responses to *S. mansoni* of resistant BS-90 snails with those of susceptible M line snails. Finally, our acquired resistance array examined the response of M-line snails that were first exposed to irradiated miracidia of *E. paraensei* and then 8 days later, challenged with viable miracidia. The three treatments each revealed a unique transcriptional profile, with each highlighting potential resistance-associated transcripts. We discovered a common pattern in which susceptible snails, at 2 days post-exposure, displayed a significant down-regulation of certain immune-associated transcripts (FREP3, C1q-like lectin, Dermatopontin, and others). In contrast, resistant snails at the same time point up-regulated many of the same transcripts and lacked the marked overall pattern of down-regulation associated with susceptibility. We hypothesize that this up-regulation has a significant impact on the ability of the snail to resist infection, and we are now looking further into the individual, functional roles of these molecules in resistance.

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##### **SOMATIC DIVERSIFICATION OF FREP3, AN ANTI-PARASITE RESPONSE FACTOR IN HEMOCYTES OF THE SNAIL *BIOMPHALARIA GLABRATA***

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Trematode infection causes the snail *Biomphalaria glabrata* to respond with increased expression of fibrinogen-related proteins (FREPs), parasite-reactive lectins with N-terminal IgSF domains and a downstream FBG domain. Several mechanisms contribute to FREP (DNA and mRNA) sequence diversity in individual snails: presence of several FREP gene subfamilies each with a number of loci; retro/pseudogenes; alternative splicing; and a combination of point mutations and gene conversion drives somatic diversification. The underlying system for diversification of these innate-type immune factors in an invertebrate was explored by investigating the genomic architecture of FREP genes using BAC clones generated with DNA from *B. glabrata*. Medium throughput sequencing and SSCP was used to study diversity of genomic FREP sequences within subpopulations of bloodcells (hemocytes) of individual *B. glabrata*, in controls and following exposure to the digenetic trematode parasite *Echinostoma paraensei*. Two full-length FREP3 genes and several incomplete FREP3 gene subfamily-like sequences cluster within the 120kb genomic insert from *B. glabrata* in BAC clone 125N01. This tandem configuration is amenable to gene conversion. High fidelity PCR and DNA template derived from 20-40 hemocytes from single *B. glabrata* yielded diverse genomic sequences from exon 5 from FREP3. These results imply that *B. glabrata* hemocytes are functionally diverse.

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##### **ANTI-PATHOGEN RESPONSES IN *BIOMPHALARIA GLABRATA* SNAILS HARBORING LONG-TERM SCHISTOSOME INFECTIONS**

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Investigations of schistosome parasite- snail host interactions usually focus on early stages of infection. However, colonization by *Schistosoma mansoni* of *Biomphalaria glabrata* is merely the start of a long term,

intimate association in which the parasite modulates host immunity, physiology and reproduction to benefit parasite survival, growth and development. Some consider long term infected snails as an extended phenotype of the parasite. The survival of the immune-inhibited snail host is critical for continuation of the parasite's life cycle, and questions arise as to how immune function is organized in the "parasite/immuno-modulated host" entity to protect against other parasites and pathogens. Are snail defenses completely or selectively inhibited by *S. mansoni*, or does the parasite provide compensatory immuno-surveillance? Transcriptomic responses of *B. glabrata* to long term *S. mansoni* infection (up to and including patency) were recorded with an in-house developed *B. glabrata* oligo microarray (1152 features, emphasizing immune and stress factors). After initial upregulation, extensive downregulation of many (immune-relevant) transcripts was evident from infected snails starting at day 4. Of the immune genes, only FREP (fibrinogen-related protein)4 and galectin7 remained at increased levels, other features (including other FREPs) returned to control value or decreased.

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##### **CONTROLLING TRANSMISSION OF *SCHISTOSOMA JAPONICUM* IN SICHUAN PROVINCE, CHINA: CONTROL APPROACHES, EPIDEMIOLOGIC TRENDS, AND CHALLENGES**

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In Sichuan Province, schistosomiasis is endemic in 62 counties. With extensive efforts, schistosomiasis control in Sichuan achieved a milestone in 2008 - 39 counties achieved transmission interruption (i.e., elimination of transmission), while the remaining 23 counties achieved transmission control (i.e., human and cattle infection prevalences were below 1%, no infected snails were found in the past two years). Since then, an ambitious plan was instituted to eliminate the transmission of the disease throughout the province by 2015. Here, we present a systematic review of epidemiology and control of schistosomiasis in Sichuan, emphasizing epidemiologic trends, control experience, lessons learned, and challenges faced in moving towards elimination of the disease. Schistosomiasis control program in Sichuan started in the mid-1950s during which a snail control oriented strategy was implemented with modest success. In the mid-1980s, the introduction of praziquantel as a major chemotherapeutic agent for schistosome caused a major shift in strategy from snail to morbidity control, which resulted in a significant reduction in infected cases of humans and cattle. This progress was furthered through the support of the World Bank Loan Program (WBLP) during 1992-1996, over which time 47% and 62% reductions were observed in humans and cattle, respectively. However, an upsurge of 93.2% human cases was observed in 1999 compared to 1996 in the province and the transmission even re-emerged in some previously controlled area after the completion the WBLP Program. In 2004, an integrated control program that coupled extensive chemotherapy with snail control, and to a lesser extent, environmental modification was initiated. This integrated program yielded remarkable results - a 91% reduction in human cases observed in 2008 vs. 2004, bringing the overall human prevalence of infections below 1%. In spite of this achievement, many challenging questions emerged. To address these questions and to inform strategies moving forward, we are actively conducting epidemiologic studies and exploring disease modeling scenarios.

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### AN EVALUATION OF SURVEILLANCE METHODS FOR DETECTING SCHISTOSOMIASIS REEMERGENCE

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Schistosomiasis has reemerged in Sichuan Province, China, highlighting the challenges of sustaining reductions in this parasitic infection. Surveillance methods that rapidly identify areas where human infections have returned can direct the deployment of interventions to treat infections and prevent their further spread. We evaluated two surveillance methods commonly used in low prevalence and controlled areas, acute schistosomiasis case reports and surveys for *Schistosoma japonicum* infected snails, as well as alternative methods for detecting reemergence. Residents in 53 villages were tested for *S. japonicum* infection using the miracidial hatch test and the Kato-Katz thick smear procedure in a region where reemergence had been documented. We conducted surveys for *S. japonicum*-infected snails, tested cows and water buffalo for *S. japonicum* infection and examined county and provincial surveillance records for reports of acute schistosomiasis. The sensitivity and specificity of surveillance methods were estimated using the human infection surveys as the gold standard: villages were classified as positive if at least one human *S. japonicum* infection was detected. Human infections were detected in 35 villages. Acute schistosomiasis reporting and surveys for *S. japonicum* infected snails grossly underestimated the number of villages where human infections were present (sensitivity <10% for each method). Surveys for the presence of the snail host or *S. japonicum*-infected bovines had moderate sensitivity (69% and 59%, respectively) and specificity (44% and 67%, respectively). Limiting testing to adults age 30 to 49, the age group with the highest infection intensities, yielded higher sensitivity. Surveillance systems that rely on the detection of *S. japonicum* infected snails and reporting of acute schistosomiasis are ill-equipped to detect lapses in schistosomiasis control. While labor intensive, direct sampling of high-risk human populations defined by demographic characteristics or local environments should be considered.

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### CAUTIONING THE USE OF DEVELOPMENTAL MODELS FOR CLIMATE CHANGE PREDICTIONS: PREDICTING SCHISTOSOMA JAPONICUM INTERMEDIATE HOST DISTRIBUTION IN A FUTURE CLIMATE IN CHINA

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Research establishing quantitative relationships between climate and diseases carried by vectors or intermediate hosts often relies on degree-day functions, which incorporate temperature-dependence into development processes such as progression through an instar stage or reproductive maturation. These degree-day functions measure the vector or host developmental response to temperature in units of degree-days, which are accumulated only when the temperature exceeds a minimum threshold,  $T_{min}$ . Development is complete once the accumulated degree-days reach a certain threshold,  $D_{days}$ . These models are commonly used to predict the impact of future climate change on disease intensity, distribution, and timing. Though the simplicity of these models is appealing, little work has been done to analyze their ability to make long-term, regional, or global predictions of vector or intermediate host distributions given the influence of a changing climate. To assess the reliability of these models for such an application, we used a developmental model for *Oncomelania hupensis*, the intermediate snail host for the parasite *Schistosoma japonicum*, to investigate the sensitivity of host range predictions to degree-day model specification and parametric uncertainty. The model

included a temperature-dependent recruitment process, and used predicted snail densities as an estimate of population viability at each grid cell. Uncertainty in  $T_{min}$  and  $D_{days}$  strongly influenced *O. hupensis* range predictions, and significant bias was identified when degree-day models were misspecified or were applied to temperatures outside the range for which the model parameters were estimated. Range predictions based on degree-day models should be considered reliable only for the populations and temperature ranges used to estimate model parameters. This conclusion has important implications for predictions of the impact of global climate change on vector- and intermediate host-borne diseases.

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### THE EFFECT OF MASS DRUG ADMINISTRATION OF IVERMECTIN TO HUMANS ON WILD ANOPHELES GAMBIAE S.S. SURVIVORSHIP

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Ivermectin is an anthelmintic drug that is mass drug administered (MDA) to humans for the control of onchocerciasis and lymphatic filariasis in sub-Saharan Africa. We have shown in the laboratory that ivermectin reduces the survivorship of colonized *Anopheles gambiae* s.s. G3 strain, at concentrations that are present in human venous blood post-ingestion of 150µg/kg of ivermectin. Our hypothesis is that wild *An. gambiae* s.s. survivorship will be reduced post-ivermectin MDA of humans. In southeastern Senegal, there are abundant *An. gambiae* s.s. populations, high levels of malaria transmission, and ivermectin MDA. In 2008 and 2009 blood fed, *Anopheles* mosquitoes were aspirated from the insides of villagers' huts before and after ivermectin MDA in southeastern Senegal. Mosquitoes were held in an insectary for five days post collection and survivorship was monitored daily. Mosquitoes were also captured by CDC light traps hung next to bed nets in randomly selected huts before and after MDA. Mosquitoes were identified to species morphologically and molecularly if applicable, blood meals were identified, and *Plasmodium* spp. sporozoite infection was determined. *Anopheles gambiae* s.l., *An. funestus*, and *An. nili* were the primary malaria vectors captured in the area and other vectors such as *An. coustani* and *An. rufipes* were abundant. Statistical analysis of aspirated *An. gambiae* s.s. from treated villages demonstrates that there was a drop in survivorship post ivermectin MDA from up to six days post MDA. Data on the molecular species identification, sporozoite rates and blood meal identification is currently being analyzed. Given the effects of ivermectin on *Anopheles gambiae* s.s. survivorship, more frequent administration of ivermectin MDA may be used to interrupt malaria transmission.

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### THE AUTO-DISSEMINATION OF A POWERFUL MOSQUITO LARVICIDE AND CHEMOSTERILANT UNDER FIELD CONDITIONS: A NEW VECTOR CONTROL TOOL

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Recent proof-of-principal studies show that the natural behaviours of adult mosquitoes can be exploited for the highly efficient targeting of an insect juvenile hormone analogue (pyriproxyfen or PPF) to breeding sites. This is potentially far more efficient than conventional larviciding. The optimization and standardization of this method in the field would represent an exciting step in the development of a powerful new vector control tool. A commercial mosquito trap was adapted so that it could be used to 1) expose a natural population of *Aedes aegypti* mosquitoes to PPF and 2) release exposed individuals unharmed in order to facilitate the autodissemination of PPF. This "expose and release" tool was treated with a pulverised solid formulation of PPF and deployed in the field. Its effect



on the development of larvae and pupae in sentinel aquatic habitats was noted. Under experimental field conditions, 95% of larvae and pupae developing at sentinel aquatic sites failed to develop to adulthood. In comparison, less than 10% of larvae and pupae failed to develop during control periods (when no PPF was deployed). In a parallel set of field experiments, the exposure of adult females to PPF by these standardized tools was also seen to have a profound effect on the fecundity of the mosquito population. Only 48% of eggs laid at sentinel sites by the exposed population hatched. Almost 100% of eggs laid during control periods eclosed successfully. PPF is a known insect chemosterilant, but its effects on mosquitoes in the field have not previously been documented. The combined effects of autodissemination and auto-sterilization, demonstrated here using a standardized "expose and release" tool, a WHO-approved insecticide (PPF), and a naturally-occurring *Aedes* population, have enormous potential for mosquito control. Models show that both chemosterilant and auto-disseminative effects on this scale are likely to have profound impacts on mosquito abundance and, by implication, on disease transmission.

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### CLUSTER RANDOMIZED TRIALS OF INSECTICIDE TREATED MATERIALS (ITMS) FOR DENGUE VECTOR CONTROL IN LATIN AMERICA AND SOUTHEAST ASIA

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Prevention of dengue fever relies on control of its vectors, *Aedes aegypti* and *Ae. albopictus*, to prevent transmission. Prior studies in Latin America indicated that pyrethroid treated ITMs impact on dengue vector populations and potentially on dengue virus transmission, but this was the first large scale trial of ITMs for dengue control in SE Asia. The trials, in Venezuela (6000 households in 75 clusters) and Thailand (2000 households in 26 clusters), together offer the most comprehensive body of evidence to date on the potential and limitations of ITMs for dengue prevention. Novel aspects of the trials variously included: ITMs deployed as window curtains or container covers were tested alone or in combination; householders could choose their own ITMs; spill-over effects of the interventions into neighboring control clusters areas were monitored; coverage-dependent impact was assessed; the effect on both *Ae. aegypti* and *Ae. albopictus* (SE Asia) was analysed. Entomological indices were high at baseline in both trials and, although the types of ITMs differed between trials (Venezuela: window curtains and water storage container covers; Thailand: indoor door, wardrobe and window curtains), ITMs were adopted and maintained by the populations similarly (*i.e.* high initial acceptance, dropping to around 70% after 9 months). In Venezuela, results showed trends similar to those seen in previous trials, with an immediate drop in entomological indices post-intervention and an overspill effect in adjacent control clusters; impact on vector populations by each intervention was sustained throughout the trial but was most pronounced in the clusters which received both curtains and jar covers. In contrast, results from Thailand showed no measurable impact of ITMs on entomological indices. Reasons for this apparently dramatic difference in effect between both trials and the implications for the applicability of ITMs to dengue vector control initiatives, and the potential use of ITMs where pyrethroid resistant vector populations occur will be discussed.

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### WHOLE GENOME TRANSCRIPTIONAL PROFILING OF A HIGHLY INSECTICIDE RESISTANT POPULATION OF ANOPHELES GAMBIAE MOSQUITOES

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*Anopheles gambiae* populations from southern Ghana, Africa are known to be highly resistant to pyrethroid insecticides; the class used for bednets and increasingly for indoor residual spraying. The pyrethroid resistance phenotype was shown to be mediated by a combination of target site insensitivity (*kdr*) and metabolism via over expression of a cytochrome P450 (*Cyp6M3*). This resistance may, in due course, necessitate a switch to other insecticidal classes so in the present study we describe the patterns of resistance to two likely candidate compounds bendiocarb and DDT. Mosquitoes, collected from several sites in Accra, were exposed to either 0.1 % bendiocarb resulting in an LT50 of 1hr or 4 % DDT where a 6 hr exposure produced only 33% mortality; reflecting high levels of resistance to both compounds. Phenotyped specimens were subsequently screened for known target site insensitivity mechanisms and differentially expressed genes. To date studies of differential expression in mosquitoes have used a small candidate array approach. However this approach may not objectively screen all known transcripts and key resistance mediators could remain undetected. We describe the design and application of two whole-genome microarrays; a 4x44K and an 8x15K array were used to screen for genes differentially expressed in bendiocarb and DDT resistant mosquitoes respectively. Members of the three major enzyme families previously linked to resistance were represented in both experiments with candidates in the bendiocarb study including cytochrome P450s, GSTs and carboxylesterases, while P450s and GSTs were differentially expressed in the DDT experiments. However a number of novel candidates were also uncovered. Genes putatively linked to insecticide transport were up regulated in both the bendiocarb and DDT resistant mosquitoes. In addition a structural cuticular gene and a number of novel proteases were represented in the DDT result set. Real-time qPCR and recombinant protein expression systems have been employed to validate expression differences and confirm function *in vitro*.

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### EFFECTS OF CHEMICAL EXPOSURE ON Aedes Aegypti RECAPTURE RATES USING THE BG-SENTINEL™ TRAP UNDER SCREENHOUSE AND FIELD CONDITIONS

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As part of a larger research program focused on quantifying the effects of spatial repellents (SR) and contact irritants (CI) to reduce indoor densities of host-seeking *Aedes aegypti*, the BG-Sentinel™ trap is being evaluated as a tool for removing chemically repelled *Ae. aegypti* from the peridomestic environment and monitoring potential diversion of mosquitoes to untreated locations. This requires understanding the potential effects of chemical exposure on host-seeking behaviours of the female *Ae. aegypti* mosquito and subsequent trapping success. Screenhouse studies were performed to quantify trap recapture rates in the absence and presence of mosquito exposure to chemical. Effects of chemical were evaluated by exposing cohorts of female *Ae. aegypti* mosquitoes, positioned within sentinel cages, to candidate compounds inside treated and chemical-free experimental huts. Following exposure, cohorts were released inside the screenhouse and recapture rates monitored for two days. Further, BG-Sentinel™ traps were positioned at various locations near the treated and chemical-free experimental

huts and cohorts of *Ae. aegypti* females were released into the outdoor environment to quantify diversion based on recapture rates by trap and hut location. Results from these experiments indicate similar total numbers of *Ae. aegypti* recaptured under screenhouse conditions for both non-exposed and chemical-exposed mosquitoes. Further, there was no evidence of significant diversion from treated to control (chemical-free) huts in outdoor trials. This information will serve to better understand the role of a trapping device to augment a SR and CI vector control strategy and guide the optimization of the BG-Sentinel™ trap to serve as a complementary component of a Push-Pull vector control strategy currently in the proof-of-principle stage of development in Thailand.

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### PRINCIPAL VECTORS OF MALARIA AND FILARIASIS IN PAPUA NEW GUINEA (*ANOPHELES PUNCTULATUS* SIBLING SPECIES) ARE SUSCEPTIBLE TO STANDARD INSECTICIDES USED IN LONG-LASTING INSECTICIDE-TREATED NETS

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Pyrethroids and dichlorodiphenyltrichloroethane (DDT) affect insects by interfering with voltage-gated sodium channel proteins in neurons. In many parts of the world, mosquitoes have developed resistance to these insecticides. This has threatened to impede insecticide-based vector control programs. The primary mechanism of resistance is the knockdown resistance (*kdr*) allele, a mutation in the insects' voltage-gated sodium channel gene (*vgsc*) that inhibits binding of DDT and pyrethroids to the protein channel. Physiological resistance to DDT causes cross resistance to pyrethroids. Papua New Guinea (PNG) has a history of both DDT and pyrethroid use for the control of malaria vectors. The Global Fund is currently supporting the distribution of long-lasting pyrethroid-treated nets in the country for disease control. However, the status of pyrethroid resistance in the local vectors has never been determined. This study investigated the status of pyrethroid resistance in the major malaria and filariasis vectors, the *Anopheles punctulatus* group, in areas of PNG where DDT or pyrethroids have been used. The study employed World Health Organization standard susceptibility bioassays to detect *kdr* phenotypes in 2 to 5 day old female *Anopheles*. In the cone assay, mosquitoes were exposed to deltamethrin-treated netting (55mg/m<sup>2</sup>) for 3 minutes and the rate of knock-down was measured within 60 min post exposure. In the tube assay, mosquitoes were exposed to lambda-cyhalothrin-treated paper (18.35mg/m<sup>2</sup>) for 60 min during which time knock-down rate was measured. Mortality status was measured 24 hr post exposure for both assays. The *kdr* allele was diagnosed using a novel nested polymerase chain reaction amplification of a *vgsc* region that contains the mutation site. This was followed by a restriction digest using *DdeI* restriction enzyme. 100% knockdown and 100% mortality were observed in all populations. 100% mortality indicates a pyrethroid susceptible population according to the WHO percentage mortality index. All the mosquitoes that were genotyped were wild-type at the *kdr* locus.

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### RESISTANCE TO ORGANOPHOSPHORUS/CARBAMATES INSECTICIDES AND ACE-1 DUPLICATION IN *ANOPHELES GAMBIAE*: A CHALLENGE FOR MALARIA CONTROL

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Insecticide resistance is a rapid and recent evolutionary phenomenon with serious economic and public health implications. In the mosquito *Anopheles gambiae* s.s., main vector of malaria, organophosphates and carbamates resistance is mainly due to a single amino-acid substitution in acetylcholinesterase 1 (AChE1). This mutation entails a large fitness cost. However, a resistant duplicated haplotype (*ace.1D*) of the gene encoding AChE1 (*ace-1*) recently appeared in *A. gambiae*. In an upstream study, the duplicated haplotype was detected at molecular level in a framework of distribution study of *ace.1R* allele (resistant allele against carbamate and organophosphate) in natural populations of *A. gambiae* from West Africa. Using molecular phenotype data collected from natural populations from West Africa, we investigated the frequency of this duplicated haplotype by statistical inference. This inference is based on the departure from Hardy-Weinberg phenotypic frequency equilibrium caused by the presence of this new haplotype. The duplicated allele, *Ag-ace-1D*, reaches a frequency up to 0.65 in Ivory Coast and Burkina Faso, and is potentially present in Benin. This allele was recorded in both M and S molecular forms of *Anopheles gambiae* s.s. in different West Africa countries. It was generated by a single genetic event and present distribution suggests that this new allele is currently spreading. Unfortunately, the spread of this less costly resistance haplotype is potentially a major threat to public health, as it may impede *A. gambiae* control strategies, and thus increases the risk of malaria outbreaks.

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### ACUTE FEBRILE ILLNESS SURVEILLANCE IN A TERTIARY HOSPITAL EMERGENCY DEPARTMENT: COMPARISON OF INFLUENZA AND DENGUE INFECTIONS

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Dengue infections are often difficult to distinguish clinically from other acute febrile illnesses (AFI), including influenza. In 2009, an increased proportion of suspected dengue cases reported to the passive surveillance system in Puerto Rico were laboratory-negative in dengue-specific assays. As a result, enhanced AFI surveillance was initiated at the Emergency Department of a tertiary care hospital in southern Puerto Rico. From September to December 2009, 284 patients who presented with fever for 2-7 days and no identified source of infection were tested for influenza, leptospirosis, and enteroviruses, in addition to dengue. Thirty-one patients were confirmed as having dengue, 136 had influenza, 1 had leptospirosis, 3 had enterovirus, and 2 had dual infections; 111 had no infectious etiology identified. Median patient age was 17.9 years (range 0.5-82) and 55% were female. The majority were from Ponce (128, 45%) or neighboring Villalba (40, 14%) and Juana Diaz (38, 13%). Dengue patients were more likely than influenza patients to be residents of Villalba (58.1% versus 6.6%) and less likely to be from Ponce (3.2%

versus 54.4%). Nearly half (15, 48.4%) of all dengue patients met criteria for influenza (i.e., fever with cough or sore throat), and the majority (78.7%) of influenza patients met criteria for dengue fever. Dengue patients were more likely than influenza patients to have bleeding (80.6% vs. 26.5%), rash (38.7% vs. 8.8%), and positive tourniquet test (51.6% vs. 18.1%). Mean platelet count was  $74,484 \pm 58,000$  for dengue patients and  $189,639 \pm 57,400$  for influenza patients while mean white blood cell count was  $3,400 \pm 1,400$  and  $5,800 \pm 2,800$ , respectively. Clinical diagnosis can be especially difficult when outbreaks of other AFI occur during dengue season. Our findings highlight the focal nature of dengue outbreaks and suggest that physician notification to public health officials should be encouraged. With many dengue patients meeting the case definition for influenza and vice versa, complete blood count and tourniquet test may be useful to differentiate dengue from other AFIs.

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### SEVERE CO-INFECTIONS OF DENGUE AND PANDEMIC INFLUENZA A H1N1 VIRUSES

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Dengue and influenza are both acute-onset viral illnesses that can initially present with similar symptoms. Epidemics of influenza and dengue generally do not overlap in Nicaragua, and virus co-infections have not been documented. However, in September 2009, simultaneous high rates of transmission of pandemic influenza and dengue in Nicaragua resulted in co-infections. Here we report on four hospitalized patients with dengue-influenza virus co-infections. All patients were RT-PCR positive for dengue virus serotype 3 and for pandemic influenza A H1N1. Clinical findings at presentation ranged from influenza-like illness to severe dengue. The clinical progression of the infections varied by case, but all developed classic dengue symptoms and had interstitial and/or alveolar infiltrates. Three cases required intensive care including mechanical ventilation, and one was fatal. All of the cases requiring mechanical ventilation had asthma, and the fatal case was also obese. Thus, dengue-influenza virus co-infections may lead to severe disease and can be fatal. Due to the varied clinical presentation and difficulties differentiating dengue-influenza virus co-infections from single infections, especially early after symptom onset, it is advisable that testing for both viruses be performed when they are co-circulating.

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### DETERMINANTS OF RISK FOR CARDIOVASCULAR SHOCK AND MORTALITY IN HOSPITALIZED DENGUE PATIENTS IN HO CHI MINH CITY, VIETNAM

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Dengue represents a growing global public health challenge. Understanding trends in disease burden and epidemiology is important for vector control, allocation of health services and planning the introduction of vaccines and therapeutic drugs. We analysed clinical and demographic trends in the dengue case burden in Ho Chi Minh City, Vietnam, between 1996 and 2009, and assessed risk factors for dengue shock syndrome

(DSS) and mortality among 102,494 dengue patients admitted between 2000 - 2009. The dengue caseload across the three hospitals increased over the study period, to a peak in excess of 20,000 cases in 2008. Adults represented an increasing proportion of cases over time. The vast majority (13,595/14,079; 96.6%) of patients with DSS were children, with those aged 6 - 10 at higher risk of DSS than younger or older children. In contrast, the risk of mortality was highest in younger children and decreased with age (OR 0.52, 95% CI 0.36 - 0.75 in 6 - 10 year olds and OR 0.27, 95% CI 0.16 - 0.44 in 11 - 15 year olds, compared with 1 - 5 year olds). Overall mortality was low (0.20%) and progressively decreased during the study period (estimated change per year = -0.04%, 95% CI -0.06% - -0.02%). Males were overrepresented among dengue cases, suggesting a gender difference in healthcare seeking behaviour and/or susceptibility to disease. Strikingly however girls had a higher risk of DSS (OR 1.19, 95% CI 1.14 - 1.24) and death (OR 1.57, 95% CI 1.14 - 2.17) than boys. This hospital caseload indicates a startlingly high dengue disease burden in Ho Chi Minh City, with at least 1 in 400 people and 1 in 140 children admitted to one of the three study hospitals with dengue in 2008. In conclusion, the risk of DSS and death is highest in young female children. Young children are at greatest risk of death and this population should be targeted in clinical trials of dengue vaccines and therapeutics. The increased risk of severe outcomes in girls warrants further attention both in studies of dengue pathogenesis and of health-seeking behaviour, and in clinical care.

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### OLDER AGE IS A RISK FACTOR FOR SYMPTOMATIC DENGUE VIRUS INFECTION IN NICARAGUAN CHILDREN

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The Nicaraguan Pediatric Dengue Cohort Study is a prospective cohort study, established in August 2004, to examine the incidence and clinical manifestations of dengue virus (DENV) infection in children 2-14 years old in Managua, Nicaragua. Children were enrolled prospectively, with yearly participation of 3,693-3,795 children. Participants are encouraged to come to the study Health Center at first sign of illness and all medical care is provided free-of-charge. Participants with suspected dengue or undifferentiated fever are tested for dengue by RT-PCR, virus isolation, and serological assays. Additionally, yearly blood samples from all cohort members are collected to determine the incidence of inapparent DENV infection. Univariate and multivariable generalized estimating equations (GEE) with a Poisson model were used to examine risk factors for symptomatic disease given DENV infection. Variables included in the multivariate models were: year of study, immune status, sex, and age. In the first 4 years of the study, 159 acute dengue cases and 9 DHF/DSS cases were detected, yielding an incidence rate of 11.2 (95% CI 9.6, 13.1) acute dengue cases and 0.65 DHF/DSS cases per 1000 person-years. During the same period, 1,047 DENV infections (symptomatic and inapparent) were detected, yielding an incidence of 78.9 (95% CI 74.2, 83.8) DENV infections per 1000 person-years. The incidence of cases and infections as well as the ratio of cases to infections varied substantially year-to-year. Incidence of cases varied markedly by age, with the highest incidence rate of symptomatic dengue in 10 year-olds. In contrast, the incidence of DENV infection was more constant across ages, with the highest incidence observed in the youngest one-year age groups. In multivariable models, age group (9-12 years old) was a significant predictor of symptomatic disease given infection (incidence rate ratio (IRR) 1.9; 95% CI 1.2-2.9), but immune status was not (IRR 1.3; 95% CI 0.9-1.8). Stratifying by immune status revealed that age is an important risk factor for developing symptomatic infection among primary DENV infections (IRR 4.0; 95% CI