and specificity to standard filter paper preparations for *P. falciparum* rna extraction. Using these techniques can simplify PCR-based case confirmation and rna extraction under field conditions.

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MOLECULAR TEST FOR *VIVAX* MALARIA WITH LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHOD IN CENTRAL CHINA

Feng Lu[1], Qi Gao[2], Junhu Chen[1], Yue Wang[1], Huayun Zhou[2], Jun Cao[2], Weimin Wang[2], **Eun-Taek Han**[1]

[1]Kangwon National University College of Medicine, Chuncheon, Gangwon-do, Republic of Korea, [2]Jiangsu Institute of Parasitic Diseases, Wuxi, China

In central China, vivax malaria was considerably serious with the highest incidence and most malaria cases in the country. High-throughput and cost-effective testing methods are essential for vivax malaria diagnosis in this area. This study was performed to evaluate the loop-mediated isothermal amplification (LAMP) method to diagnosis vivax malaria in comparison with the results of nested PCR with blood filter paper based on the microscopic examination results. LAMP detected vivax malaria parasites in 140 of 140 microscopically positive samples (sensitivity, 100%), whereas nested PCR detected *P. vivax* in 139 of 140 samples. No false-positive results were obtained by LAMP or nested PCR among 120 fever positive samples as a negative by microscopic examination. These two molecular detection tests yielded high sensitivities and specificities. However, the LAMP method is simple to perform and less influenced by inhibitors than PCR method. In addition, LAMP reaction mixture can be prepared easily and kept stably. LAMP method is an accurate, rapid, simple and cost-effective method that may be useful for diagnosis in field.

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DETECTION OF *P. FALCIPARUM* IN SALIVA USING RAPID DIAGNOSTIC TEST AND POLYMARASE CHAIN REACTION (PCR) IN PATIENTS WITH ACUTE UNCOMPLICATED MALARIA IN SOUTHWEST NIGERIA

Grace O. Gbotosho[1], Christian T. Happi[1], Onikepe Folarin[1], **Keyamo A. Ochuko**[1], A. Sijuade[1], A. Sowunmi[1], A. Oduola[2]

[1]Malaria Research Laboratories, College of Medicine, University of Ibadan, Ibadan, Nigeria, [2]Malaria Research Laboratories, College of Medicine, University of Ibadan, Ibadan, Nigeria

The gold standard method for diagnosis of malaria is microscopic examination of Giemsa stained blood films which necessitates drawing blood by finger prick. Diagnosis of malaria from other biological specimen using non-invasive techniques provides an alternative strategy in the management of malaria. This study describes the potential diagnosis of malaria using human saliva by employing rapid diagnostic test based on detection of parasite lactate dehydrogenase, polymerase chain reaction (PCR) and genotyping of *Plasmodium falciparum*. Thirty-eight patients age ranging from 3 to 17 years were recruited into the study. Eighty-nine percent (34/38) of the patients were microscopically positive for falciparum malaria with parasitemia ranging from 2040 to 422,040 parasites/µl blood. Eleven percent (4/38) of the patients were microscopically negative malaria. Using OptiMAL-IT® rapid diagnostic kit, 71% (27/38) and 29% (11/38) of samples from the patients were positive for the presence of *P. falciparum* in capillary blood and saliva samples respectively. Nested PCR was successful in 82% and 89% of filter paper blood samples and saliva samples respectively. Matching polymorphic GLURP genotypes were often found between the corresponding saliva and peripheral blood amplicons of each individual that was matched, with varying inter-individual genotypes. The sensitivity and specificity for detection of *P. falciparum* in saliva using the OptiMAL-IT® rapid diagnostic kits compared to microscopy were 32% (95% CI= 17.3-47.5%) and 100% (95% CI= 45.0-100%) respectively with an agreement of 0.086. While the sensitivity and specificity for detection of *P. falciparum* using PCR compared to microscopy were 94% (95%CI= 79.7-99.2%) and 50% (95% CI= 15.4-84.6%) respectively with an agreement of 0.44. Overall, this study illustrates the principle of detecting *P. falciparum* infection by rapid diagnostic kit and PCR in human saliva samples. There is however a need for further studies in a larger population for better understanding of the clinical implication of these findings.

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HIGH-THROUGHPUT QUANTITATIVE MULTIPLEX REVERSE TRANSCRIPTASE 5′ NUCLEASE PCR ASSAY FOR QUANTITATION OF *PLASMODIUM FALCIPARUM* GAMETOCYTEMIA

Emily G. Clemens[1], Megan E. Reller[1], Karen C. Carroll[1], Tamaki Kobayashi[2], William J. Moss[2], J. Stephen Dumler[1]

[1]The Johns Hopkins University School of Medicine, Baltimore, MD, United States, [2]The Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, United States

Plasmodium falciparum gametocytemia detection is critical to malaria transmission and control studies that are now limited by low microscopic sensitivity and cumbersome molecular assays amenable only to limited subject numbers. We designed a high-throughput, quantitative assay for *P. falciparum* (*Pf*) gametocytemia using a 96 or 384 well one-step reverse transcriptase PCR assay, *pfs25* (25 kDa ookinete surface antigen precursor gene transcribed only in gametocytes) primers and a 5′ nuclease fluorescent probe. To assess analytical and clinical sensitivity, we tested 0.8 to 2 µL RNA samples obtained from *P. falciparum* gametocytes grown *in vitro*, known numbers of cultured gametocytes spotted on filter papers, and blood spots from returning U.S. travelers with *Pf* malaria (n=7) and endemic-area residents (n=26 Zambians). To assess specificity, we used blood spots from 3 patients with *P. vivax* and RNA from *Trypanosoma brucei rhodesiense* and *Anaplasma phagocytophilum* cultures. Analytical sensitivity was linear from 10^4 through 10^0 gametocytes, or 1.4×10^6 to 17 *pfs25* transcripts. No *pfs25* transcripts were detected in the *P. vivax*, *T. b. rhodesiense*, or *A. phagocytophilum* controls. Gametocyte RNA was detected in blood of 6/7 U.S. travelers with *Pf* malaria and in blood of 2/26 Zambian residents, both with microscopic gametocytemia. Of *Pf* malaria patients, parasitemia varied from <0.25-50% and transcript levels from 6 to 1.8×10^3 (median 44)/µL blood. There was no correlation between parasitemia and gametocytemia (R=0.183), although microscopic and molecular gametocytemia were highly correlated (R=0.995). Molecular parasitemia did not differ between travelers and residents (p=0.056). Multiplex reverse-transcriptase PCR for *Pf* gametocytemia is rapid, highly sensitive and specific, and quantitative. It could support high-throughput studies, can use RNA in dried blood spots, and should be useful for population studies where exquisite sensitivity and quantitation of gametocytes is required to assess transmission of *Pf*.

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FIELD PERFORMANCE OF THE RAPID DIAGNOSTIC TESTS PARACHECK AND FALCIVAX IN TSUNAMI-AFFECTED DISTRICTS OF ACEH AND NIAS, INDONESIA

Sigrid Hahn[1], David Muriuki[2], Braden Hexom[1], Richard Allan[2]

[1]Mount Sinai School of Medicine, New York, NY, United States, [2]The MENTOR Initiative, Skipton, United Kingdom

After the 2004 Indian Ocean tsunami, the MENTOR Initiative helped implement a malaria prevention and control program in affected areas. MENTOR introduced the use of laboratory confirmation for all suspected cases of malaria, using microscopy as well as the rapid diagnostic tests (RDTs) Paracheck and the newer FalciVax. This is the first publication about RDTs in tsunami-affected areas of Indonesia, and one of only one evaluations of FalciVax's performance under field conditions. In 2005 and 2006, two clinic-based surveys were conducted in tsunami-affected districts. Patients reporting fever within the past 48 hours were included. Using microscopy as a gold standard, we assessed the accuracy of

Paracheck (in 2005) and FalciVax (in 2006). 569 patients were included in 2005. Overall, 12.5% of slides were positive for *Plasmodium*. The sensitivity of Paracheck for *P. falciparum* was 74% and the specificity was 97%, yielding a positive predictive value (PPV) of 78% and a negative predictive value (NPV) of 96%. 618 patients were included in the 2006. The overall slide positivity rate was 15%. The sensitivity of FalciVax, which can detect both *P. falciparum* and *P. vivax*, for *P. falciparum* was 81% and the specificity was 91%, with a PPV of 49% and an NPV of 98%. Sensitivity and specificity for *P. vivax* were 64% and 96%, respectively. In conclusion, the performance of Paracheck was similar to previously published reports. FalciVax showed good sensitivity and high specificity for *P. falciparum*, but a lower sensitivity for detecting *P. vivax*.

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REFERENCE LABORATORY VERIFICATION, VALIDATION, AND QUALITY ASSURANCE OF REAL-TIME PCR AND ICT FOR THE DIAGNOSIS OF MALARIA

Krishna Khairnar, Rachel Lau, Donald S. Martin, **Dylan R. Pillai**

OAHPP –University of Toronto, Toronto, ON, Canada

Malaria is a mosquito-borne disease caused by the parasite of genus Plasmodia spp. There are at least 4 species that infect humans of which *Plasmodium falciparum* can be fatal if not treated immediately. It is endemic in countries in Africa, South America, India and South Asia. In this study, we validated RTPCR for the detection of all 4 species from n = 425 clinical specimens from travelers and new immigrants to Ontario, Canada from May 2006 - January 2009. RTPCR was compared to microscopy and immunochromatographic (ICT) detection of Plasmodia species. A total of n = 425 whole blood isolates (containing positives for each of the 4 species and negatives) from returning travelers to Ontario in the period May 2006 - January 2009 were analyzed by a novel RTPCR method (Dr. Stephanie Yanow, personal communication). DNA was extracted from whole blood using QIAmp DNA kit (Qiagen). ICT was performed according to manufacturer's (Binax, Carestart) instructions. Microscopy was performed at the reference lab level. RTPCR is able to specifically detect all 4 species of Plasmodia with excellent analytical sensitivity and specificity compared to reference microscopy and ICT results. RTPCR is also able to detect Plasmodia parasites in a small percentage of microscopy-negative or inconclusive samples. The analytical sensitivity, specificity, limit of detection, cost, precision, workflow, inter-reader variability for each method were evaluated. Confounders such as gametocyte only specimens were analyzed. External quality assurance of RTPCR was assessed by sharing specimens with another jurisdiction (Provlab, AB, Canada). In conclusion, we describe a large, multi-centre study on species-specific RTPCR detection of Plasmodium parasites in human whole blood samples when compared to ICT and microscopy. RTPCR is a fast, reproducible, and potentially quantitative procedure with high analytical sensitivity and specificity. RTPCR can be used as a supplementary diagnostic tool to detect malaria in patient blood samples where microscopy is inconclusive. RTPCR has the potential to be quantitative and therefore guide clinical therapy. Both ICT and RTPCR were valuable adjuncts to standard microscopy.

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USE OF THE INTEGRATED MANAGEMENT OF CHILDHOOD ILLNESS CRITERIA AND SYNDROMIC DIAGNOSIS OF MALARIA IN RURAL SIERRA LEONE

Obinna N. Nnedu

University of Washington, Seattle, WA, United States

Increasing resistance to older anti-malarial drugs has prompted many African countries, including Sierra Leone, to adopt artemisinin-based combination therapy as first-line therapy. Since laboratory testing is often unavailable in rural areas, the cost-benefit and viability of this approach may depend on accurately diagnosing malaria using clinical criteria. We assessed the accuracy of syndromic diagnosis for malaria made by community health officers in 3 peripheral health units in rural Sierra Leone, with particular emphasis on the Integrated Management of Childhood Illness (IMCI) criteria. Of 175 children diagnosed with malaria on syndromic grounds, 143 (82%) were confirmed by the Paracheck-Pf test. In a multivariate analysis, splenomegaly (p =.04) was the only clinical sign significantly associated with laboratory-confirmed malaria, while sleeping under a bed net was protective (p =.05). IMCI criteria for malaria may not be particularly useful in Sierra Leone, while other clinical and epidemiological indicators may help guide diagnosis.

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NEXT GENERATION QUINOLINE METHANOLS FOR MALARIA CHEMOPROPHYLAXIS AND IPTX

Erin E. Milner[1], Sean R. Gardner[1], Jayendra B. Bhonsle[1], Diana P. Caridha[1], Dustin Carroll[1], Lucia Gerena[1], Montip Gettayacamin[2], Charlotte A. Lanteri[1], Qiqui Li[1], ThuLan Luong[1], William F. McCalmont[1], Victor Melendez[1], Jay K. Moon[1], Norma E. Roncal[1], Jason C. Sousa[1], Anchalee Tangteung[2], Qiang Zeng[1], Jing Zhang[1], Peter Wipf[3], Lisa Xie[1], Geoffrey S. Dow[1]

[1]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, [3]University of Pittsburgh, Pittsburgh, PA, United States

Utilizing mefloquine as a scaffold, a next generation quinoline methanol (NGQM) library was constructed to identify a candidate that exhibits the key minimum aspects of the common target product profile for IPTx. These include the normal safety profile common to all drug candidates in addition to low cost of goods, excellent potency, a long half-life and duration of action, and an improved central nervous system tolerability profile relative to mefloquine. Medicinal chemistry efforts were focused upon modifying the scaffold, which has yielded interesting chemotypes around which to begin a rational synthetic program. The library of over two hundred analogs yielded compounds that inhibited drug sensitive and resistant strains of *Plasmodium falciparum* (W2, D6, C235 and C2A) and demonstrated superior therapeutic and cross-susceptibility indices relative to mefloquine. Herein we report selected chemotypes and the emerging structure activity relationship for this library of quinoline methanols.

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IDENTIFICATION AND DEVELOPMENT OF A NOVEL CHEMICAL SERIES WITH ACTIVITY AGAINST BOTH BLOOD- AND LIVER-STAGES OF *PLASMODIUM FALCIPARUM*

Clare E. Gutteridge[1], Brett W. Sadowski[1], Michael C. Baxter[1], Michael T. O'Neil[2], William F. McCalmont[2], Lucia Gerena[2]

[1]United States Naval Academy, Annapolis, MD, United States, [2]Walter Reed Army Institute of Research, Silver Spring, MD, United States

Several hundred compounds with significant *in vitro* efficacy against *Plasmodium falciparum* were assessed for their potential as a new lead against malaria. Using criteria including properties disclosed in the literature, synthesizability, "drugability" and potential for toxicity, a lead with an *in vitro* IC_{50} of 75ng/mL against blood-stage *P. falciparum* was selected for development. By synthesizing a variety of analogs of this lead, some of which included additional substituents, we have gained an understanding of structure-activity relationships for the compound series and have prepared analogs with increased *in vitro* efficacy. Additionally we have established that the series has promising activity *in vitro* against liver-stage *P. falciparum*. Results from *in vitro* toxicity testing and *in vivo* efficacy testing in *P. berghei*-infected mice will also be presented.

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RANDOMIZED CLINICAL TRIAL (RCT) WITH A CROSSOVER STUDY DESIGN TO EXAMINE THE SAFETY AND PHARMACOKINETICS OF A 2100 MG DOSE OF AQ-13 AND THE EFFECTS OF A STANDARD FATTY MEAL ON ITS BIOAVAILABILITY

Fawaz Mzayek[1], Haiyan Deng[2], M. Azam Hadi[2], Vidya Mave[2], Frances J. Mather[2], Christopher Goodenough[2], David M. Mushatt[2], Juan J. Lertora[3], **Donald Krogstad**[2]

[1]University of Memphis, Memphis, TN, United States, [2]Tulane University Health Sciences Center, New Orleans, LA, United States, [3]Clinical Center, National Institutes of Health, Bethesda, MD, United States

Our previous studies have shown that AQ-13 is active against chloroquine(CQ)- and multi-resistant strains of *Plasmodium falciparum*. They have also shown that oral doses of 1400 and 1750 mg AQ-13 are as safe as equivalent oral doses of CQ, and have similar pharmacokinetics. These studies were performed to address two questions: 1] Is a 2100 mg dose of AQ-13 (700 mg per day x 3 days) safe to include as a third arm in the initial dose-finding Phase 2 (efficacy) studies of AQ-13 in Mali?, and 2] What are the effects of food (the standardized FDA fatty meal) on the bioavailability and pharmacokinetics of AQ-13? To address these questions, we performed a randomized clinical trial (RCT) with a crossover study design in which all subjects were admitted twice (stage 1 and stage 2) to receive 700 mg AQ-13 orally on days 1, 2 and 3 and then followed for adverse events and drug and metabolite levels during the 5 day inpatient stay and a 5 week outpatient follow-up (total of 6 weeks). The purpose of randomization was to allocate individual volunteers to receive the 700 mg dose on day 1 after an overnight fast or a fatty meal in stage 1. After completing the 6 week follow-up for stage 1, volunteers were readmitted for the crossover. Those who received the day 1 dose of AQ-13 after a fatty meal in stage 1, received it after an overnight fast in stage 2, and vice versa. By using each subject as their own control, we examined the effect of the fatty meal on bioavailavility. In comparison to the 1400 and 1750 mg doses, pharmacokinetics (based on drug and metabolite levels) after the 2100 mg dose demonstrate similar times to peak blood concentration and similar terminal half-lives. In contrast, the peak blood levels and overall drug exposure (based on the area under the curve) were higher after the 2100 mg dose. Based on Holter monitoring, there is no evidence for adverse events such as drug-induced arrhythmias. Note that tachyarrhythmias similar to those reported with CQ poisoning have not occurred after either the 1400, 1750 or 2100 mg doses. Based on these results, we propose to compare the 1400, 1700 and 2100 mg doses of AQ-13 to each other and to Coartem in the initial dose-finding efficacy (Phase 2) studies of AQ-13 in Mali.

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INITIATING PRECLINICAL EVALUATIONS OF REVERSED CHLOROQUINES

Steven J. Burgess[1], Bornface Gunsaru[2], Jane X. Kelly[1], Shawheen Shomloo[1], Westin Morrill[1], Martin J. Smilkstein[3], **David H. Peyton**[2]

[1]DesignMedix, Portland, OR, United States, [2]Portland State University, Portland, OR, United States, [3]Oregon Translational Research and Drug Development Institute, Portland, OR, United States

Reversed Chloroquines (RCQs) are a novel class of antimalarial compounds which are made from a chloroquine (CQ)-like moiety and a portion which has activity as a chemosensitizer or reversal agent (RA) against chloroquine-resistant (CQR) *Plasmodium falciparum*. Here we disclose various RCQs which have *in vitro* IC50 values in the low-nanomole to picomole range against both CQS and CQR *P. falciparum*. We also present an SAR study, exploring the structural features that give these exceptionally high activities against CQR *P. falciparum*. In addition, we present *in vitro* toxicity and other preclinical work for a set that represents a range of physical chemical properties of these drug candidates. Several of the best RCQs have been tested in rodents for oral availability, *in vivo* efficacy and toxicity.

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LOW DOSE CYSTEAMINE POTENTIATES THE ANTI-MALARIAL ACTIVITY OF ARTEMISININ DERIVATIVES *IN VIVO*

Gundula Min-Oo[1], Kodjo Ayi[2], Silayuv E. Bongfen[1], Anny Fortin[3], Mifong Tam[1], Mary M. Stevenson[1], Kevin C. Kain[2], Philippe Gros[1]

[1]McGill University, Montreal, QC, Canada, [2]University of Toronto, Toronto, ON, Canada, [3]Dafra Pharma, Turnhout, Belgium

We have used a mouse model of infection to identify a role for the *Vanin*-encoded pantetheinase enzyme in host response to blood-stage malaria. The pantetheinase reaction produces a potent aminothiol, cysteamine; administration of this small molecule was shown to limit *Plamodium chabaudi* replication and increase survival in susceptible, pantetheinase-deficient, A/J mice. Cysteamine shows significant inhibitory effects on early parasite replication *in vivo*, using prophylactic, therapeutic and standard 4-day drug treatment regimens. Continuous exposure to cysteamine also blocks replication of chloroquine-sensitive and resistant isolates of the human *P. falciparum* parasite *in vitro*; however, the effect of cysteamine is specific to *Plasmodium* with no detectable effect on *T. cruzi* or *Candida albicans* replication in mice. Moreover, cysteamine shows additive effects *in vivo* when administered in combination with sub-optimal doses of chloroquine and, more importantly, shows strong synergistic effects when administered with low dose artemisinin derivatives (artesunate or dihydroartemisinin). Low-dose cysteamine can significantly potentiate the activity of artesunate; additionally, at doses of artesunate that show no effect on parasite replication (0.05-0.1mg/kg), the addition of cysteamine results in 60-70% inhibition of parasitemia. Cysteamine already has a safe history of clinical use for the treatment of nephropathic cystinosis in children. Taken together, these results indicate that cysteamine has strong potential for use in monotherapy or combination therapies against malaria.

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TETRACYCLIC BENZOTHIAZEPINES: A NOVEL CLASS OF *PLASMODIUM FALCIPARUM* CYTOCHROME BC1 INHIBITORS

Carolyn Dong[1], Sameer Urgaonkar[2], Joe Cortese[2], Javier Francisco[3], Jose F. Garcia-Bustos[3], Frederico M. Gomez-de-las-Heras[3], Vishal Patel[4], Leila Ross[5], Jeffrey D. Dvorin[6], Manoj T. Duraisingh[1], Dyann Wirth[1], Ralph Mazitschek[7], Jon Clardy[5]

[1]Harvard School of Public Health, Department of Immunology and Infectious Disease, Boston, MA, United States, [2]Broad Institute Infectious Diseases Initiative, Boston, MA, United States, [3]Drug Discovery Biology Group, Diseases of the Developing World Center, GlaxoSmithKline R&D, Tres Cantos, Madrid, Spain, [4]Harvard School of Public Health, Department of Immunology and Infectious Disease and Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology, Boston, MA, United States, [5]Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology, Boston, MA, United States, [6]Harvard School of Public Health and Children's Hospital Boston, Division of Infectious Diseases, Boston, MA, United States, [7]Broad Institute Infectious Diseases Initiative and Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology and Massachusetts General Hospital, Center for Systems Biology, Boston, MA, United States

Within the scope of our efforts to identify novel inhibitors of the electron transport chain (ETC), a whole-parasite screen was performed using a *Plasmodium falciparum* transgenic strain expressing cytosolic *Saccharomyces cerevisiae* dihydroorotate dehydrogenase (scDHOD), which renders the parasite insensitive to inhibitors of the mitochondrial ETC. Small molecule inhibitors of multi-drug resistant parasite growth that had previously been identified by our group were tested for a growth rescue phenotype in the scDHOD transgenic strain. Out of this whole-parasite screen, tetracyclic benzothiazepines (BTZs), a novel, highly potent anti-

malarial chemotype, showed remarkable activity and selectivity against mitochondrial ETC activity. A new synthetic methodology was developed and an exploratory library of analogs was synthesized yielding compounds with increased potency (IC_{50} = 16 nM). Further examination of the mode of action revealed the BTZ compounds to be specific inhibitors of the atovaquone target, cytochrome bc_1, likely via binding to the Q_0 site of cytochrome *b*, while only marginal (>100 fold) inhibitors of the human homolog. Atovaquone resistance in the human malarial parasite, *P. falciparum*, rapidly arises in the absence of co-treatment with proguanil due to mutations in the cytochrome bc_1 complex of the ETC. A lack of cross-resistance with atovaquone-resistant parasite strains suggests that the BTZs inhibit cytochrome bc_1 activity in a different manner than atovaquone, making the tetracyclic BTZ chemotype a promising alternative to existing mitochondrial inhibitors of *P. falciparum* viability.

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PLASMODIUM FALCIPARUM GAMETOCYTOCIDAL ACTIVITY OF PROTEASOME INHIBITOR EPOXOMICIN

Beata Czesny, Samrawit Goshu, **Kim C. Williamson**

Loyola University Chicago, Chicago, IL, United States

Malaria transmission is mediated by mature gametocytes, which are resistant to the anti-malarials commonly used clinically to kill asexual parasites. Consequently, transmission can continue even after effective treatment. In *Plasmodium falciparum* complete sexual differentiation requires 10-12 days and the mature gametocytes produced are viable and can be transmitted for over a week. New anti-malarial compounds with gametocytocidial activity should help block the spread of malaria. The work reported here demonstrates that nanomolar concentrations of the proteasome inhibitor epoxomicin effectively kills all stages of intraerythrocytic parasites. Twenty four hours after treatment with 100 nM epoxomicin the total parasitemia decreased by 78%, asexuals decreased by 86% and gametocytes by 77%. Forty eight hours later, 72 hours after treatment, no viable parasites remained in the 100 nM or 10 nM treatment groups. Epoxomicin also blocked oocyst production in the mosquito midgut. In contrast, cysteine protease inhibitors only block hemoglobin digestion in early gametocytes and have no effect on later stages. Moreover, once the cysteine protease inhibitor is removed sexual differentiation resumes. These findings provide strong support for the further development of proteasome inhibitors as anti-malarial agents that are effective against asexual, sexual, and mosquito midgut stages of *P. falciparum.*

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METABOLIC PROFILING OF PRIMAQUINE USING *IN VITRO* AND *IN VIVO* APPROACHES

Xiannu Jin, Dustin Carroll, Thu-Lan Luong, Gavin Black, Tanya Jenkins, Raul Olmeda, Necole Reese, Qigui Li, Lisa Xie, Jing Zhang, Michael T. O'Neil, Michael P. Kozar, Ai J. Lin, Colin Ohrt, Jason C. Sousa, Victor Melendez

Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD, United States

The metabolism of primaquine (PQ), an 8-aminoquinoline with broad spectrum antimalarial activity yet incomplete metabolic profile, is being evaluated using *in vitro* and *in vivo* approaches. The drug was studied *in vitro* by incubations in the presence of pooled human hepatocytes, pooled human liver microsomes, and recombinant cytochrome P-450 isoenzymes, followed by LC-MS analyses. The results indicate that primaquine was dominantly metabolized by CYP450 3A4, followed by CYPs 1A2 and 2D6, as confirmed in microsomal and hepatocyte studies with the inclusion of specific P450 inhibitors. Both matrices yielded quantifiable levels of carboxy-primaquine but no detectable levels of any other metabolite. However, we observed no significant metabolism of primaquine by recombinant P450s after one hour incubations. Current *in vitro* efforts are aimed at further characterizing the P450 isoenzymes while identifying other enzymes (FMOs, MAOs) potentially influencing primaquine metabolism. *In vivo* work showed that mice treated with a single oral dose of primaquine at 50 mg/kg yielded levels of carboxy-primaquine greater than 3µg/ml at T=60 minutes, with metabolite's levels falling to less than 50% after three hours. Two relatively stable minor putative metabolites (M- 422 and M-452, respectively) were observed for up to four hours. Interestingly, analysis of plasma samples from a pig treated with a 30 mg IV dose yielded six minor putative metabolites other than carboxy-primaquine. Two of these (M-238 and M-262) appear stable (8 and 1 hrs respectively). Based on our findings a metabolic profile for primaquine is proposed. The relative contribution of the putative metabolites to the efficacy and/or toxicity of primaquine is yet to be characterized.

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IN VITRO AND *IN VIVO* METABOLISM AND DISPOSITION STUDIES OF IMIDAZOLIDINEDIONE ANALOGS

Jason C. Sousa, Raul Olmeda, Dustin Carroll, Gavin Black, Xiannu Jin, Necole Reese, Tanya Jenkins, Xihong Wang, Ramadas Sathunuru, Liang Zhang, Ai J. Lin, Qigui Li, Lisa Xie, Jing Zhang, Michael P. Kozar, Victor Melendez

Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD, United States

A series of imidazolidinedione analogs are currently under evaluation by the U.S. Army for their potency against liver-stage malaria. In this study, a subset of these analogs have been subjected to a series of *in vitro* metabolism and *in vivo* pharmacokinetic studies as a means of selecting the most appropriate compound(s) for *in vivo* efficacy studies. Metabolic stability was measured via incubation in both human and mouse liver microsomes. Half-lives ranged from 13 to >60 minutes and from 6 to >60 minutes in human and mouse microsomes, respectively, with median values of >60 for both species. The potential for drug interaction was measured for the principal P450 metabolic enzymes (1A2, 2C9, 2C19, 2D6, 3A4) using expressed isoenzymes and fluorescent markers of enzymatic activity specific for the given enzyme. Most compounds displayed low potential (IC50s >10µM) for interactions with CYPs 1A2, 2C9, 2D6 or 3A4, while several compounds showed a moderate interaction potential (IC50s between 1-10µM) for CYP 2C9. Determinations of apparent permeability coefficients using Caco-2 cells showed a significant range (2 orders of magnitude, from low to high intestinal permeability) within this subset of compounds. For the pharmacokinetic studies, mice were orally dosed and drug plasma and liver levels were measured over 48 hours. C_{max} and AUC values, like Caco-2 permeability, showed a range in values spanning 2 orders of magnitude. Elimination half-lives and liver/plasma ratios for these compounds are also presented. The data obtained served to systematically evaluate the compounds for further development.

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IN VITRO AND *IN VIVO* METABOLIC PROFILE OF TWO DEOXO-IMIDAZOLIDINEDIONE ANALOGS

Thu-Lan Luong, Xianjun Liu, Ai J. Lin, Qigui Li, Lisa Xie, Jing Zhang, Raul Olmeda, Michael P. Kozar, Jason C. Sousa, Victor Melendez

Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD, United States

A series of newly synthesized Deoxo-Imidazolidenedione analogs are being evaluated by the U.S. Army for development as candidate antimalarials. This study provides *in vitro* and *in vivo* metabolic profile and possible pathways for two selected analogs, namely Analog 1 and Analog 2. 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 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 the extraction. *In vitro* microsomal incubations of Analog 1 yielded six putative metabolites. Hydroxylation (+16) was the major metabolite followed by methylation (+14), regardless of the specie evaluated. Analog 2 yielded five putative metabolites. As for Analog 1, hydroxylation was the major metabolite followed by methylation. *In vivo,* both analogs yielded more metabolites than the *in vitro* microsomal assays. Analog 1 had 16 metabolites in plasma and 15 metabolites in liver. Meanwhile, Analog 2 had 12 metabolites in plasma and 14 metabolites in liver. The *in vivo* metabolites of both analogs 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 both analogs under consideration. The relative contribution of the putative metabolites to efficacy and/or toxicity is yet to be characterized. The findings will contribute to the development of new analogs with desired attributes.

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IN VITRO METABOLISM AND DISPOSITION EVALUATIONS OF QUINOLINE METHANOL ANALOGS OF MEFLOQUINE MODIFIED AT THE 4-POSITION

Dustin Carroll, Geoffrey S. Dow, Erin Milner, William F. McCalmont, Charlotte A. Lanteri, Norma E. Roncal, Lucia Gerena, Xiannu Jin, Raul Olmeda, Necole Reese, Lalaine Anova, Constance Asher, Jason C. Sousa, Victor Melendez

Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD, United States

A series of newly synthesized quinoline methanol analogs of the antimalarial mefloquine are being evaluated by the U.S. Army for development as a prophylactic drug(s). The overall objective is to identify efficacious, orally active compounds with relatively long half-life and no CNS toxicity compared to mefloquine. This work provides *in vitro* metabolism and disposition data for selected analogs with relatively high antiparasitic potency when tested *in vitro*. The IC50s against four strains of *P. falciparum* (W2, D6, C235, C2A) ranged from <0.48 to 80 ng/ml or <1 to 236 nM. Metabolic stability was measured following incubations with liver microsomes in a high throughput mode. LCMS analysis of the extracted microsomal samples yielded median half-life values of >60 and 20 minutes for human and mouse, respectively. The potential for drug interaction was measured for the principal P450 metabolic enzymes (1A2, 2D6, 2C9, 2C19, 3A4) using expressed isoenzymes and fluorescent markers of enzymatic activity specific for the given enzyme. Most compounds displayed low potential (IC50s>10μM) for CYPs 2C9 or 3A4 interaction and moderate potential (IC50s between 1-10μM) for CYPs 1A2, 2C19 or 2D6 interaction. Metabolite identification data by LC-Trap/MS following a slow gradient show masses consistent with hydroxylated and dealkylated products as the must abundant metabolites in microsomal preparations for all four species (human, monkey, rat, mouse) evaluated. Determination of apparent permeability coefficients using Caco-2 cells suggests several analogs have reduced permeability, presumably due to an efflux mechanism. The *in vitro* data generated identify key attributes of candidate compounds and provide a systematic approach that contributes to the down selection of compounds more likely to succeed *in vivo*, while identifying those compounds in need of structural modifications.

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DEVELOPMENT OF A NETWORK OF INVESTIGATORS TO STUDY ANTIMALARIAL PROPHYLAXIS IN THE ASIA-PACIFIC REGION

David Saunders[1], Gregory Deye[2], Kent Bennett[2], Colin Ohrt[2]

[1]Armed Forces Research Institute of the Medical Sciences, Bangkok, Thailand, [2]Walter Reed Army Institute of Research, Silver Spring, MD, United States

Advanced clinical development of new drugs for antimalarial chemoprophylaxis has been hindered by ethical and scientific hurdles to conducting adequate and well controlled pivotal studies to permit product licensure by the US Food and Drug Administration (FDA). Despite new guidance issued by FDA on antimalarial drug development, no new chemoprophylaxis drugs have been studied or submitted for NDA in at least 10 years, since the licensure of atovaquone-proguanil. Antimalarial chemoprophylaxis studies have historically been conducted in semi-immune populations in endemic areas using a superiority design with placebo controls. The ethical issues surrounding this approach include the use of placebo, and access by the population studied to the product being developed. The use of personal protective measures which may or may not be known to investigators, and the ability to conduct directly observed therapy are important confounders to correct interpretation of results. An alternative approach is to study non-immune populations. However, the risk of use of placebo in these populations is higher, and may or may not be in accordance with the accepted standard of care in the population being studied. In this setting, a non-inferiority design comparing the drug of interest to an active comparator is preferable. Unfortunately, interpretation of results is confounded by the lack of a well-validated surrogate endpoint of malaria infection. The Walter Reed Army Institute of Research has conducted studies of several well characterized biomarkers, but to date, a validated surrogate endpoint remains elusive. A combined approach to licensure which includes the study of semi-immune and non-immune populations will be discussed, while recognizing the limitations of both approaches. Because of the relatively large sample sizes likely to be involved, and the reduction in malaria burden in the Asia-Pacific region in recent years, a multi-center approach to conducting pivotal clinical studies is most likely to be successful. The Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand, is developing plans to conduct antimalarial prophylaxis studies, and seeks interested collaborating investigators in the region with suitable access to both semi and non-immune patients at risk for contracting malaria. Desired characteristics of collaborating centers will be discussed.

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RAPID INCREASES IN THE PREVALENCE OF PFDHFR AND PFDHPS MUTANT HAPLOTYPES DURING THE PERIOD OF SP USE AS THE FIRST-LINE ANTIMALARIAL IN KISUMU, KENYA

Maroya D. Spalding[1], Fredrick Eyase[2], Hoseah Akala[2], Sheryl Bedno[2], Rodney L. Coldren[2], Norman C. Waters[3], William J. Moss[1]

[1]Johns Hopkins School of Public Health, Baltimore, MD, United States, [2]United States Army Research Unit-Kenya, Nairobi, Kenya, [3]Walter Reed Army Institute of Research, Silver Spring, MD, United States

Widespread antimalarial resistance in Kenya resulted in two drug policy changes within a decade: sulfadoxine-pyrimethamine (SP) replaced chloroquine as the first-line antimalarial in 1998 and artemether-lumefantrine (AL) replaced SP in 2004. In 1999, shortly after SP became the first-line antimalarial, we conducted a baseline study of the prevalence of molecular markers of chloroquine and SP resistance in Kisumu, Kenya, a malaria endemic area near Lake Victoria. A subsequent study was performed from 2003-2005, spanning the period in which AL replaced SP. Blood spots were collected from patients who presented at New Nyanza Hospital outpatient clinics, provided informed consent, and were malaria smear-positive. Samples were genotyped to determine the prevalence of *pfdhfr*, *pfdhps*, and *pfcrt* mutations associated with drug resistance

in each study period. The prevalence of mutations at *pfdhfr* codons 51, 59, and 108 was greater than 85% at baseline and was similar at follow-up, except for a significant increase in the prevalence of the S108N mutation from 87.2% to 99.6% (p<0.001). Substantial increases were observed in the prevalence of mutations at *pfdhps* codons 436, 437, and 540, increasing from 44.3% to 68.6% (p<0.001), 35.9% to 98.8% (p<0.001), and 59.0% to 95.9% (p<0.001), respectively. The *pfdhps* mutations A581G and A613S/T, which were not detected in 1999, were present in 84.8% (p<0.001) and 60.5% (p<0.001), respectively, of isolates from the second study period. The quintuple mutant, formed from the *pfdhfr* N51I/C59R/S108N triple mutant and the *pfdhps* A437G/K540E double mutant, is a molecular marker for SP treatment failure in Africa and increased in prevalence from 22.9% to 53.3% (p<0.001). Notably, the highly drug resistant mutation *pfdhfr* I164L was absent during both study periods. Although our studies spanned a period when chloroquine pressure was expected to decrease, the prevalence of the *pfcrt* mutation K76T associated with *in vivo* treatment failure increased from 81.6% to 97.1% (p<0.001). Thus, during the period in which SP was used as the first-line antimalarial in Kisumu, highly SP resistant parasites became the predominant haplotypes in the parasite population, mutations at *pfdhps* codons 581 and 613 emerged, and the prevalence of the chloroquine resistant haplotype did not abate.

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COLOMBIAN NETWORK FOR SURVEILLANCE OF *PLASMODIUM FALCIPARUM IN VITRO* SUSCEPTIBILITY TO ANTIMALARIAL DRUGS: PARASITE'S PHENOTYPE AFTER THE ARTEMISININ-BASED COMBINATION THERAPIES (ACT) IMPLEMENTATION

Gustavo Diaz[1], Zuleima Pava[1], Javier Martínez[2], Lidia Montenegro[2], Diego Echeverry[1], Pedro Gil[3], **Claribel Murillo**[1]

[1]Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM), Cali, Colombia, [2]Universidad del Valle, Facultad de Salud. Escuela de Bacteriología y Laboratorio Clínico, Cali, Colombia, [3]Karolinska Institute, Stockholm, Sweden

Two ACTs (artemether-lumefantrine and artesunate-mefloquine) were implemented in late 2006 in Colombia as first line treatment for falciparum-malaria, replacing failing amodiaquine based therapies. Taking into account the observed capacity of Colombian origin *Plasmodium falciparum* to evade previous chemotherapy regimens, the emergence of resistant strains to artemisinin derivatives is likely to occur in the next years. Our aim was to determine the spatial/temporal changes of *P. falciparum in vitro* susceptibility to relevant antimalarials drugs. During 2007 and 2008, 99 *P. falciparum* fresh isolates from Colombian Pacific towns of Tumaco (Southern Coast) and Quibdó (Northern region) were analyzed. The parasites were tested *in loco* for their *in vitro* susceptibility to 6 antimalarials drugs: chloroquine (CQ), amodiaquine (AQ), desethylamodiaquine (DAQ), mefloquine (MQ), lumefantrine (LUM) and dihydroartemisinin (DHA, the common active metabolite of both artesunate and artemether). Using two techniques in parallel, ELISA-Histidine rich protein II and the WHO reference microtest of schizont maturation, the 50% inhibitory concentrations (IC_{50}) were determined. The reference strains W2 and D6 were used as controls. The IC_{50} determination was performed with HN nonlin software. The isolates showed high susceptibility to DHA and LUM with IC_{50} geometric mean (GM) <10nM, along the two years in both regions. The IC50 to MQ from Quibdó were significantly higher than those from Tumaco. Additionally low susceptibility to CQ and DAQ (IC_{50} GM>100nM and >60nM, respectively), was prevalent and stable in both regions. This study provides baseline information about the local parasite population's response against the previous chemotherapy regimens after the ACT implementation and the initial patterns of *in vitro* susceptibility to the compounds of ACT. This information has been obtained thanks to ongoing implementation of a sentinel network for surveillance of *P. falciparum*-resistance using *in vitro* methodologies.

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A RAPID PYROSEQUENCING METHOD FOR DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISM (SNPS) ASSOCIATED WITH ANTIMALARIAL RESISTANCE

Krishna Khairnar, **Dylan R. Pillai**

University of Toronto and Ontario Public Health Laborotories, Toronto, ON, Canada

The emergence of drug-resistant *Plasmodium falciparum* is a serious public health threat. Provided that the genetic basis for the drug resistance is known, genetic markers can be exploited for monitoring the drug resistance pattern in a population. We have developed a rapid screening method for molecular markers of drug resistance in *P. falciparum* malaria that may inform clinicians on patterns of drug resistance. A pyrosequencing protocol was developed in a 24 well format as a rapid (~2 hrs) and reliable method to identify approximately 30 single nucleotide polymorphisms (SNPs) in ATPase6, chloroquine resistance transporter (pfcrt), cytochrome b (cyt b), multidrug resistance protein (pfmdr1), dihydrofolate reductase (dhfr) and dihydropteroate synthetase (dhps) genes of *P. falciparum* that are associated with artemisinin, chloroquine, atovaquone-proguanil, mefloquine, and pyrimethamine-sulfadoxine resistance respectively. The pyrosequencing protocol developed in our laboratory was able to screen the SNPs in all the six gene targets of *P. falciparum* associated with drug resistance. The pyrosequencing was found to be a reliable method for genotyping, and it was also faster and less expensive than the commonly reported RFLP and direct sequencing methods. Molecular data for all resistance markers mentioned above will be presented for *P. falciparum* isolates obtained from returning travelers to Ontario during the period January 2007-2008. In conclusion, pyrosequencing was found to be a useful alternative method that can be used in a high throughput format for molecular surveillance of antimalarial-drug resistance. Molecular surveillance of imported *P. falciparum* malaria suggests that clinicians should be aware of emerging resistance.

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SCREENING AND GENETIC MAPPING TARGETS OF DIFFERENTIAL CHEMICAL-RESPONSE PHENOTYPES IN *PLASMODIUM FALCIPARUM*

Jing Yuan[1], Ronald Johnson[2], Ruiling Huang[2], Jennifer Wichterman[2], Hongying Jiang[1], Karen Hayton[1], David Fidock[3], Thomas Wellems[1], James Inglese[2], Christopher Austin[2], Xinzhuan Su[1]

[1]LMVR/NIAID/National Institutes of Health, Rockville, MD, United States, [2]NCGC/NHGRI/National Institutes of Health, Rockville, MD, United States, [3]Columbia University, New York, NY, United States

Studies of gene function and molecular mechanisms in *Plasmodium falciparum* are hampered by difficulties in characterizing and measuring phenotypic differences between individual parasites. We screened seven parasite lines, including parents of the three available genetic crosses, for differences in responses to 1,279 bioactive chemicals. Hundreds of compounds were active in inhibiting parasite growth; 650 differential chemical-response phenotypes (DCRPs), defined as pairwise IC_{50} differences of five fold or more between parasite lines, were cataloged. Major determinants for three DCRPs between the parents of a genetic cross were mapped, and candidate genes for three DCRPs, including one off-target gene, were identified by linkage analyses using progeny from a genetic cross and genetic transfection methods of allelic replacement. Differential sensitivity to dihydroergotamine methanesulfonate, a serotonin receptor antagonist, mapped to a malaria homolog of human P-glycoprotein (PfPgh-1), revealing an unexpected new drug target. These results show that differential responses of small molecules between parasite lines can be reliable phenotypes useful for exploring molecular mechanisms of pharmacologic interest. This study also provides an effective approach for investigating drug action and resistance

mechanisms in malaria parasite by utilizing a high-throughput chemical genomic strategy.

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ACCUMULATION OF CHLOROQUINE (CQ) AND AN INVESTIGATIONAL AMINOQUINOLINE (AQ-13) BY CQ-SUSCEPTIBLE AND CQ-RESISTANT *PLASMODIUM FALCIPARUM*

Haiyan Deng, Simon J. Hocart, Donald J. Krogstad

Tulane University Health Sciences Center, New Orleans, LA, United States

In the studies reported here, we compared the accumulation of an investigational AQ active against both CQ-susceptible and -resistant parasites (AQ-13) by CQ-susceptible and -resistant *Plasmodium falciparum* to the accumulation of CQ (which is active only against CQ-susceptible parasites). Based on the peak blood levels achieved during recent pharmacokinetic (Phase 1) studies in human volunteers, these studies used 3 μM concentrations of AQ-13 and CQ in human plasma with 10% suspensions of red cells (10% Hct) containing 5% synchronous ring stage parasitemias or unparasitized red cells. After incubation for 2 hours at 37° C followed by centrifugation, AQ accumulation was measured by extracting the CQ or AQ-13 from the red cell pellet and plasma, and quantitating AQ accumulation using fluorescence HPLC. With CQ-susceptible parasites (Haiti 135/CDC strain), there was substantial accumulation of both CQ (mean accumulation of 976 fmols per 10^6 parasitized red blood cells [PRBCs]) and AQ-13 (5,172 fmols per 10^6 PRBCs). In contrast, with CQ-resistant parasites (Indochina I/CDC strain), there was readily measurable accumulation of AQ-13 (966 fmols per 10^6 PRBCs), but not of CQ (-200 fmols per 10^6 PRBCs). The accumulation of CQ and AQ-13 by the CQ-susceptible Haiti strain is consistent with the activity of both CQ and AQ-13 against CQ-susceptible parasites at low nanomolar concentrations (IC50s 3-5 nM). Conversely, the accumulation of AQ-13 but not CQ, by the CQ-resistant Indochina I strain is consistent with the activity of AQ-13, but not CQ, against those parasites (IC50s 5-10 and > 250 nM, respectively). These results suggest that there is no detectable accumulation of CQ by CQ-resistant *P. falciparum* parasites at the peak blood concentrations achieved with the standard 1500 mg therapeutic dose of CQ.

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THE GENETIC BACKGROUND OF *PLASMODIUM FALCIPARUM* DETERMINES THE EXTENT TO WHICH MUTANT PFCRT CONFERS RESISTANCE TO CHLOROQUINE

Stephanie G. Valderramos[1], Juan-Carlos Valderramos[2], Adele M. Lehane[3], Lise Musset[2], Lisa A. Purcell[2], Odile Mercereau-Puijalon[4], Eric Legrand[5], Kiaran Kirk[3], **David A. Fidock**[2]

[1]Albert Einstein College of Medicine, Bronx, NY, United States, [2]Columbia University, New York, NY, United States, [3]The Australian National University, Canberra, Australia, [4]Institut Pasteur, Paris, France, [5]Institut Pasteur de la Guyane, Cayenne, French Guiana

The emergence and spread of chloroquine resistance (CQR) in the human parasite *Plasmodium falciparum* has decimated the efficacy of CQ, once the antimalarial gold standard. Mutations in the parasite transporter PfCRT constitute the key determinant of *in vitro* CQR and are highly associated with CQ treatment failure. Nonetheless, clinical studies have shown that CQ can sometimes cure patients infected with mutant *pfcrt* parasites. While these observations can be partly attributed to variations in host immunity and/or drug pharmacokinetics, it is also possible that other genes are necessary to augment *pfcrt*-mediated CQR in some parasites. To test this hypothesis, we replaced the wild type *pfcrt* allele in the CQ-sensitive strains 3D7, D10, and GC03 with the mutant allele from the CQ-resistant 7G8 strain. Recombinant clones exhibited a spectrum of reduced CQ susceptibility as measured in 72 hr drug assays, ranging from high-level resistance to a modest shift in CQ response that did not meet the standard CQR criteria. Nevertheless, steady-state [^{3}H]-CQ uptake assays show that expression of mutant *pfcrt* produced similarly reduced rates of CQ accumulation in all three genetic backgrounds. Furthermore, in each background, mutant *pfcrt* enabled the clones to recrudesce *in vitro* after CQ treatment. Mutant *pfcrt* was also found to significantly impact parasite responses to other antimalarials used in artemisinin-based combination therapies, in a strain-dependent manner. We have also identified clinical isolates from French Guiana that harbor mutant *pfcrt* and manifest relatively low CQ IC_{50} values *in vitro.* These isolates nonetheless recrudesced after exposure to high CQ concentrations. One isolate, H209, harbored a novel C350R PfCRT mutation and demonstrated moderate quinine resistance. Taken together, these findings highlight a complex relationship between mutant *pfcrt,* drug accumulation and CQ response *in vitro,* and suggest that the extent to which mutant *pfcrt* confers CQ resistance is influenced by additional parasite factors.

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BALANCING FITNESS COSTS WITH CHLOROQUINE RESISTANCE: A "RAISON D'ÊTRE" FOR NOVEL PFCRT ALLELES IN SOUTHEAST ASIA?

Ines Petersen[1], Eugene Palatulan[1], Thierry Fandeur[2], Qin Cheng[3], Juan-Carlos Valderramos[1], **David A. Fidock**[1]

[1]Columbia University, New York, NY, United States, [2]Institut Pasteur, Paris, France, [3]Australian Army Malaria Institute, Enoggera, Australia

Chloroquine resistance (CQR) in *Plasmodium falciparum* has been attributed primarily to mutations in the chloroquine resistance transporter (PfCRT). These include the K76T mutation, a highly specific marker of CQR. K76T is always accompanied by geographically differing sets of additional mutations that are thought to restore normal function of the protein. Studies in Malawi recently found that the complete and prolonged discontinuance of CQ has resulted in the disappearance of parasites harboring mutant *pfcrt*, presumably as a result of these mutations encumbering a fitness cost in the absence of drug pressure. In contrast, in Cambodia CQ-resistant parasites are still predominant in the population, even though drug policies discontinued CQ use some 20 years ago. Both in Cambodia and in The Philippines, novel *pfcrt* alleles have recently been identified. In this study, we have examined the influence on CQ resistance and parasite fitness of unique *pfcrt* alleles from Cambodia and The Philippines. Genetic engineering of these alleles into the CQ-sensitive GC03 background identified notable differences in the extent to which mutant *pfcrt* can mediate CQ resistance and impact fitness. Using quantitative pyrosequencing, we performed competition experiments with co-cultured lines expressing wild type and mutant *pfcrt*. Our data show that the selected Cambodian *pfcrt* allele is able to mediate CQ resistance without causing a loss of parasite fitness. This allele is distinct from the Asian/African Dd2 allele that confers a high degree of resistance yet causes a loss of fitness. On the other hand, one of the two unique Philippine alleles was CQ-resistant but was less fit than the wild type control. Interestingly, we found a reduced degree of CQR in the other mutant Philippine allele, which manifested increased fitness as compared to the wild type. These data suggest that *pfcrt* alleles can continue to evolve to increase their fitness and establish a balance between fitness and resistance.

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MOLECULAR CHARACTERIZATION OF RESISTANCE TO ARTEMISININ DRUGS IN *PLASMODIUM FALCIPARUM*

Matthew S. Tucker[1], Lucia Gerena[2], Katherine Sorber[3], Michelle Dimon[3], Azliyati Azizan[1], Zhinning Wang[2], Qin Cheng[4], Dennis E. Kyle[1]

[1]University of South Florida, Tampa, FL, United States, [2]Walter Reed Army Institute of Research, Rockville, MD, United States, [3]University of California-San Francisco, San Francisco, CA, United States, [4]Australian Army Malaria Institute, Enoggera, Australia

Artemisinin (QHS) and its derivatives are effective against all stages of *Plasmodium* spp. and they provide faster clearance of parasitemia than any other drugs. These drugs are part of frontline combination therapies in areas where drug-resistant *P. falciparum* exists. Although clinical resistance to these drugs is usually not reported, recent studies suggest resistance to these drugs may be emerging. We have developed resistant parasites and are using these lines to determine the molecular basis of QHS resistance. Discontinuous exposure to artelinic acid (AL) or QHS *in vitro* produced AL and QHS resistant progeny of *P. falciparum* lines W2, D6, and TM91c235. Using this method, we produced parasites that could tolerate 340ng/ml of QHS (D6), 200ng/ml QHS (W2), and 280ng/ml of AL (TM91c235). After exposing D6 and D6.QHS340 to concentrations of QHS ranging from 28.2-2400ng/ml, we found D6 could tolerate up to 1500ng/ml QHS, and D6.QHS340 tolerated 2400ng/ml QHS. At each concentration level (less than or equal to 1500ng/ml), the resistant parasite grew back more quickly than D6. We are using a flow cytometry approach to further characterize recovery rates in these and other strains. Prior microarray and real-time PCR performed on drug selected progeny of W2 and TM91c235 identified differentially expressed genes and increases in *pfmdr1* copy number and/or expression. We are conducting these analyses on other drug selected lines, which includes new microarray studies of the most resistant parasites. Whole genome sequencing of D6 and D6.QHS2400 identified single nucleotide polymorphisms (SNPs) that may be involved in resistance. We are currently in the process of confirming these SNPs in resistant lines using pyrosequencing. Preliminary proteomic analyses found potential proteins that may be differentially expressed in D6 vs. D6.QHS2400 and W2 vs. W2.QHS200. Future research will focus on further dissecting whole genome sequence and proteomic data of parental and resistant parasites to further elucidate mechanisms of resistance to artemisinin drugs.

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ANTI-MALARIAL TREATMENT REGIMES FROM AN EVOLUTIONARY PERSPECTIVE

Silvie Huijben, Derek Sim, Brian Chan, Danielle Tomasello, Andrew Read

The Pennsylvania State University, University Park, PA, United States

Drug resistance is a serious problem in health care. In the case of malaria, resistance against most antimalarial drugs is widespread, except against the recently-deployed artemisinin derivatives. The effect of drug treatment regimens on the spread of resistance is largely unknown. Using the rodent model *Plasmodium chabaudi*, we compared the effects of a variety of 'patient' treatment regimes on infections consisting of resistant and sensitive parasites, testing the impact of each regime on host health, infectiousness and the transmission of resistant parasites. In untreated mixed infections, resistant parasites started at low frequencies in the initial inoculation produced gametocytes at densities that are barely detectable by PCR. However, drug treatment resulted in a rapid increase of resistant parasites, causing recurrent parasitaemia, increased anaemia, and a much increased transmission potential of resistant parasites. Shorter drug courses or lower drug dosages significantly reduced the fitness of the resistant parasites without compromising host health. Conventional drug treatment aimed at radical cure resulted in the greatest fitness gain for drug resistant parasites. These results demonstrate the need for more research on the role of drug treatment regimens on the spread of drug resistance in malaria. Currently recommended regimes inadvertently impose maximal selection for resistance when resistant parasites are present in an infection. There is a need to empirically evaluate the public health consequences of treating in excess of clinical need.

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VALIDATION OF PLATE COATING AND STORAGE TECHNIQUES FOR *IN VITRO* DRUG SENSITIVITY TESTING OF PIPERAQUINE, PYRONARIDINE, AND LUMIFANTRINE

Maneerat Rasameesoraj[1], Kuntida Tangthongchaiwiriya[1], David Saunders[1], Mark Fukuda[1], Harald Noedl[2], Pascal Ringwald[3], Paktiya Teja-Isavadharm[1]

[1]Armed Forces Research Institute of the Medical Sciences, Bangkok, Thailand, [2]Medical University of Vienna, Vienna, Austria, [3]WHO, Geneva, Switzerland

Recent data suggest that plate coating and storage conditions for *in vitro* drug sensitivity testing are more important for obtaining reproducible results than the technique used for assessing growth inhibition. This study validated the plate coating solvents and storage conditions for piperaquine (PIP), pyronaridine (PRN) and lumifantrine (LUM), drugs for the treatment of resistant *Plasmodium falciparum.* Seven solvents were evaluated: distilled water, 70% ethanol, 100% ethanol, 0.5% lactic acid in distilled water, linoleic acid and tween 80 (1:1), dimethyl sulfoxide (DMSO) and 100% ethanol+ linoleic acid + tween 80 (2:1:1). The 96-well culture plates were pre-coated with the best solvents for PIP, PRN, and LUM determined by the lowest IC50 using HRP2 ELISA method. Chloroquine (CQ) and dihydroartemisinin (DHA) in 70% ethanol were used as internal controls. Four storage conditions: freezer (-30ºC), refrigerator (4ºC), room temperature (25ºC), and outdoor (25-31ºC) were used and all the plates were stored at 1, 2 weeks, 1, 2, 3, and 6 months. The drug sensitivity was tested against cultured *P. falciparum* W2 (CQ resistant) and D6 (CQ sensitive) clones using an HRP2 assay. The IC_{50}'s at various condition**s** were considered reproducible/stable (variation was within ± 2 SD). The best solvent for PIP is 0.5% lactic acid in water for W2 and water for D6; for PRN it is 70% ethanol for W2 and D6; for LUM it is DMSO for W2 and linoleic acid and tween 80 (1:1) for D6. All the tested drugs PIP, PRN, and LUM coated plates tested against a W2 clone can be stored in all conditions for 6 months, except for PIP at 4°C which was stable for 2 months only. There were similar finding against the D6 clone; PIP can be stored in all condition for 6 months; and LUM for 3 months but at -30°C LUM was stable for 2 weeks only. In conclusion, different P.f. clones require different solvents and storage conditions for preparing drug coated plates. In general, all drugs tested can be stored in outdoor conditions up to 6 months, especially when used against a W2 clone.

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COMPARISON OF GENOTYPING USING CAPILLARY VS. GEL ELECTROPHORESIS FOR TWO ANTIMALARIAL DRUG EFFICACY TRIALS IN UGANDA

Vinay Gupta, Grant Dorsey, Philip Rosenthal, Bryan Greenhouse

University of California, San Francisco, San Francisco, CA, United States

In antimalarial drug efficacy trials in malaria endemic areas, molecular genotyping techniques are required to distinguish failures due to recrudescence from new infection after therapy. In 2007, the WHO recommended using capillary electrophoresis rather than gel electrophoresis for genotyping where feasible. This recommendation is based on increased discrimination of alleles with capillary electrophoresis, potentially reducing the risk of alleles matching by chance and being misclassified as a recrudescence. However, no direct evidence currently supports this recommendation. We compared efficacy results obtained with the two genotyping methods, using data and samples from two trials comparing artemether-lumefantrine (AL) and dihyroartemisinin-piperaperaquine (DP); one in Kunungu, Uganda, where transmission intensity is moderate (EIR=7), and the other in Apac, Uganda, where

transmission intensity is very high (EIR>1500). In both trials, samples from subjects with recurrent parasitemia were genotyped at the MSP2, GLURP, and MSP1 loci in a stepwise fashion to distinguish new infection from recrudescence. Laboratory personnel were blinded to the identity of samples. At present, we have completed analysis for MSP2 and GLURP. In Kunungu (n=408), of 20 pairs classified as recrudescences by gel, 11 (55%) were new infections by capillary; of 12 pairs classified as recrudescences by capillary, 3 (25%) were new infections by gel. The 42-day genotyping-adjusted failure rate was higher when using gel vs. capillary genotyping for both AL (7.5% vs. 5%) and DP (4% vs. 2.4%). In Apac (n=417), of 61 pairs classified as recrudescences by gel, 42 (69%) were new infections by capillary; of 28 pairs classified as recrudescences by capillary, 9 (32%) were new infections by gel. The 42-day failure rate was again higher when using gel vs. capillary genotyping for both AL (19% vs. 10%) and DP (15% vs. 6%). Our preliminary results, based on only the MSP2 and GLURP loci, show that using capillary vs. gel genotyping resulted in fewer classifications of clinical failures as recrudescences, especially in our high transmission site, likely due to improved allele discrimination of capillary electrophoresis.

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IN VITRO DRUG SENSITIVITY-PHARMACODYNAMIC CORRELATES IN A CLINICAL TRIAL OF VARYING DOSES OF ARTESUNATE IN CAMBODIAN ADULT PATIENTS WITH UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA

Stuart Tyner[1], Wiriya Rutvisuttinunt[1], Kritsanai Yingyuen[1], Panjaporn Chaichana[1], Siratchana Sundrakes[1], Piyaporn Saingam[1], Youry Se[1], Delia Bethell[1], Sabaithip Sriwichai[1], Chanthap Lon[1], Phisit Khemawoot[1], David Saunders[1], Sea Darapiseth[2], Sok Poeu[2], Jessica Lin[1], Kurt Schaecher[3], Paktiya Tejaisavadharm[1], Bryan Smith[1], Duong Socheat[2], **Mark M. Fukuda**[1]

[1]Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, [2]National Center for Parasitology, Entomology and Malaria Control, Pnom Penh, Cambodia, [3]9th Area Medical Laboratory, Aberdeen Proving Ground, Maryland

A growing body of evidence suggests that resistance of *Plasmodium falciparum* strains to the artemisinins is developing in Southeast Asia. This resistance phenomenon, characterized by elevated IC_{50}s, increased parasite clearance times, and clinical failures rates, is thought to be contained along a relatively limited area along the Thai-Cambodian border, but has not been fully characterized geospatially. This underscores the importance of both establishing correlates of IC_{50} values for dihydroartemisinin (DHA) to parasitological and clinical endpoints and expanding surveillance geographically to fully characterize the extent of resistant clones. We conducted an open label, randomized, dose ranging trial of artesunate monotherapy administered over 7 days to determine dose-response relationships in patients with uncomplicated *P. falciparum* malaria. Relationships between IC_{50} and clinical and parasitological outcomes were assessed. As of June 2009, over 100 subjects have completed study endpoints, with IC_{50}s for DHA ranging from 0.56ng/ml to 8.07ng/ml. *In vitro* drug sensitivity testing IC_{50} levels and their corresponding relationships to PCTs, PRRs and clinical outcomes will be presented.

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A NEW MALARIA EXPERIMENTAL CHALLENGE SYSTEM; INFECTION OF VOLUNTEERS BY THE BITE OF ASEPTIC *ANOPHELES STEPHENSI* MOSQUITOES INFECTED WITH *PLASMODIUM FALCIPARUM* (NF54) SPOROZOITES

Kirsten E. Lyke[1], Matthew Laurens[1], Matthew Adams[1], Peter Billingsley[2], Adam Richman[2], Mark Loyevsky[2], Sumana Chakravarty[2], Christopher V. Plowe[3], Kim Lee Sim[2], Robert Edelman[1], Stephen Hoffman[2]

[1]University of Maryland, Baltimore, MD, United States, [2]Sanaria, Inc., Rockville, MD, United States, [3]Howard Hughes Medical Institute, Baltimore, MD, United States

Experimental infection of malaria-naïve volunteers by the bite of *Plasmodium falciparum*-infected mosquitoes has provided critical information for malaria vaccine and drug development. The model relies on the bite of five infected mosquitoes to consistently induce malaria. To improve on this malaria challenge system, we examined the efficacy of malaria transmission using aseptically-raised mosquitoes. The aseptic rearing of mosquitoes may reduce the variability of sporozoite load among mosquito lots, decrease the already low risk to human volunteers of infection by concomitant microorganisms within the mosquito salivary glands, and improve reproducibility of the prepatent period and/or infection rate. In this randomized trial, we sought to determine the minimum number of *A. stephensi* bites required to safely achieve 100% volunteer infectivity. Eighteen adults aged 18-40 years (mean: 29 years) were randomized 1:1:1 to receive 1, 3 or 5 bites of *A. stephensi* mosquitoes infected with the chloroquine-sensitive NF54 strain of *P. falciparum*. Seventeen volunteers developed malaria, fourteen occurring on Day 11, with a mean prepatent period of 10.9 days (9-12 days). All volunteers were afebrile at diagnosis with a geometric mean parasitemia of 15.7 parasites/µl (range: 4-70) by microscopy. Polymerase chain reaction detected malaria in all smear-positive volunteers prior to microscopy (mean: 3.1 days, range: 0-4). The eighteenth volunteer (1 bite cohort) withdrew from the study on Day 13, and was PCR and smear negative on Day 13. The most common clinical solicited events included fatigue, fever and myalgia. Transient clinical laboratory abnormalities were occasionally noted. The geometric mean sporozoite count detected in challenge mosquitoes was 16,753 (range: 1,000-57,500). The use of aseptic *P. falciparum* sporozoite-infected mosquitoes is safe, is associated with an extremely precise prepatent period compared to the traditional model and, for the first time in humans, the bite of three *A. stephensi* mosquitoes has been shown to transmit *P. falciparum* malaria to 100% of recipients.

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ESTIMATING THE RATE OF ACQUIRING IMMUNITY TO SEVERE DISEASE DUE TO *PLASMODIUM FALCIPARUM* WITH AGE AND EXPOSURE

Deirdre Hollingsworth[1], Neil Ferguson[1], Jamie Griffin[1], Christl Donnelly[1], Chris Drakeley[2], Eleanor Riley[2], Hugh Reyburn[2], Azra Ghani[1]

[1]Imperial College London, London, United Kingdom, [2]London School of Hygiene and Tropical Medicine, London, United Kingdom

Immunity to severe disease is known to develop with repeat infections of *Plasmodium falciparum*. Previous analysis suggested that immunity to non-cerebral severe malaria is acquired after only a couple of infections. However, evidence from longitudinal studies shows that some young children experience multiple episodes of severe disease, suggesting that immunity may not be acquired so quickly. Methods: We developed a mathematical model for infection and episodes of severe disease by age and fitted it to the age-distribution of severe malaria cases in children, stratified by cerebral malaria, severe malarial anaemia and respiratory distress in northeastern Tanzania. Models were fitted to data across a range of settings, from very low to high transmission areas. The development of immunity to severe disease is investigated by evaluating

the fit of different model structures to the data. The estimated force of infection in each setting increased with the reported parasite rate, and maternal immunity was estimated to last approximately a year. Whilst slightly different model structures for the development of immunity to disease with exposure explained the data equally well, the best fitting models estimated that immunity to severe disease was not complete after two exposures, but had increased by 80-90% after four exposures. The models which best represent the patterns of severe disease from a range of transmission settings give plausible estimates both for the force of infection in each setting, for the duration of maternal immunity to disease and quantify the patterns of clinical manifestations with age. Our results suggest that immunity to severe disease increases with each exposure, but more gradually than previously suggested. Further longitudinal studies would be required to test these estimates and to investigate the effect of heterogeneities in exposure on disease

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ASYMPTOMATIC CARRIAGE OF *PLASMODIUM FALCIPARUM* PARASITES PROTECT AGAINST MALARIA ATTACKS DURING THE MALARIA HIGH TRANSMISSION SEASON IN CHILDREN UNDER FIVE YEARS LIVING IN A RURAL AREA OF BURKINA FASO

Tiono B. Alfred, Ouedraogo Alphonse, Diarra Amidou, Sanon Souleymane, Yaro Jean Baptist, Ouedraogo Espérance, Ouedarogo Amathe, Soulama Issiaka, Bougouma Edith, Konaté T. Amadou, Nébié Issa, Sirima B. Sodiomon

Centre National de Recherche et de Formation sur le Paludisme, Ouagadougou, Burkina Faso

Asymptomatic carriage of malaria parasites during the low transmission season in areas where malaria transmission is seasonal has been reported to play a potential role in protection against clinical attacks during the malaria high transmission season. The study was conducted in the rural district of Saponé where malaria is markedly seasonal with the high transmission occurring from June to October and the low transmission from November to May. A cohort of 555 children where screened during the low transmission season and asymptomatic carriers (AC) and Non Carriers (NC) of *P. falciparum* were enrolled for a one year longitudinal follow up; which consisted of home visits twice a week to detect malaria episodes. A health facility-based passive case detection system was also in place to complement the active follow up. A blood smear was obtained at any time the child present with fever (axillary Temperature ≥ 37.5) or history of fever within the last 24 h. At inclusion, the prevalence of AC was 51.7% with a geometric mean of *P. falciparum* parasite density of 3955.2 (95% ci [3124.6-4785.8]). At the end of the follow up, incidence of malaria attacks (any parasite density) was 6.8/1000 Child-Days at Risk (CDAR) in AC vs. 8.8/1000 CDAR yielding a reduction of 22.8%. A more important effect was observed when different threshold of parasite density was considered for the malaria case definition. The reduction was 32.9% (4.7/1000 CDAR in AC vs. 7.1/1000 CYAR in NC) and 36.3% (4.1/1000 CDAR in AC vs. 6.4/1000 CYAR) at the respective threshold of 2000 and 5000 trophozoites/µL. Geometric mean of parasites density was significantly higher during a malaria attack in NC as compared to the AC (44098.7 trophozoîtes/µL (95% ci [39787.5-48409.8] vs. 30801.9 (95%ci [26974.4-34629.4])). In conclusion, the study has confirmed the potential role of asymptomatic carriage of *P. falciparum* to protect individuals against clinical malaria attacks. In the era of malaria elimination goal, the host parasites interactions mechanisms underlying this protection need to be elucidate.

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BURDEN OF MALARIA IN PREGNANCY IN AREAS OF STABLE AND UNSTABLE MALARIA TRANSMISSION IN CHHATTISGARH, INDIA

Neeru Singh[1], Mrigendra P. Singh[2], Manmohan M. Shukla[2], Mohassir Hussain[2], Aditya P. Dash[3], Blair J. Wylie[4], Kojo Yeboah-Antwi[5], Lora Sabin[5], Venkatachalam Udhayakumar[6], Meghna Desai[6], **Davidson H. Hamer**[5]

[1]Regional Medical Research Centre (ICMR), Jabalpur, India, [2]National Institute for Malaria Research Field Station, Jabalpur, India, [3]National Institute for Malaria Research, Delhi, India, [4]Department of Obstetrics, Massachusetts General Hospital, Boston, MA, United States, [5]Center for International Health and Development, Boston, MA, United States, [6]Malaria Branch, Centers for Disease Control and Prevention, Atlanta, GA, United States

Malaria is endemic throughout India with 95% of the population at risk of infection. Both *Plasmodium falciparum* and *P. vivax* are present. Pregnant women are particularly vulnerable. The objective of this study was to assess the prevalence of peripheral parasitemia among pregnant women in two districts in Chhattisgarh, one with stable (Bastar) and another with unstable malaria transmission (Rajnandgaon) over a 12 month period (2007-2008). Two health facilities, one urban and one rural were selected in each district. After obtaining informed consent, 2,696 pregnant women presenting for antenatal care were enrolled, regardless of symptoms, and were screened for malaria by both Giemsa-stained blood smear and rapid diagnostic testing. In the low transmission district (Rajnandgaon), peripheral parasitemia was detected in only 1 of 1498 pregnant women (0.07%). In Bastar, where transmission is stable, the prevalence was 2.8% (34/1198) of which 14.7% was *P.vivax* and 85.3% *P. falciparum*. Prevalence was higher in the rural vs. urban sites (1.7% vs. 1.0, $p < 0.05$). Primigravidae were more susceptible to malaria as compared to others ($p<0.05$). Symptomatic women had a higher prevalence of malaria (9.6%, 23/239) as compared to subjects with no history of fever (0.5%, 13/2457; $p<0.0001$). Among women with parasitemia, anemia was common; 57% had moderate anemia and 28.6% severe anemia, while only 14.3% had normal hemoglobin values. Overall anemia was significantly more common in parasitemic than non-parasitemic pregnant women (mean Hb 8.2 ± 2.4 vs. 10.4 ± 3.4 g/dL, $p <0.0001$). In conclusion, the burden of malaria in pregnant women in the state of Chhattisgarh varies widely between high and low transmission districts. There is thus a need for improved control activities (e.g. ITN and improved case management) to reduce the burden of disease, with targeting of higher transmission areas.

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DISTRIBUTION OF HAPLOTYPES CARRIED BY PFCRT, PFDHFR AND PFDHPS IN TWO NEIGHBOURING GEOGRAPHICAL SITES FROM SOUTHERN COTE D'IVOIRE

Louis K. Penali[1], **Berenger A. Ako**[1], Offianan A. Toure[1], Marnie Briceno[2], Carol H. Sibley[2]

[1]Institut Pasteur de Cote d'Ivoire, Abidjan, Cote d'Ivoire, [2]Genome Sciences Department, Seattle, WA, United States

Years after the switch to SP and when ACTs were introduced as treatment policy, little was known about the geographical distribution of drug resistance associated haplotypes in Cote d'Ivoire. This work shows how distribution of haplotypes could be different in neighbouring sites. In 2005, the study was hold in Bonoua and Samo (less than 6km away from Bonoua). DNA extraction was performed from whole blood spotted filter paper according Qiagen protocol. Once extracted, DNA was amplified by PCR or Nested-PCR. Sequencing with Big Dye Terminator then followed. Analyzing software helped to screen all sequences at the following codon positions: Pfcrt C72C, V73V, M74I, N75E, K76T; Pfdhfr A16V, N51I, C59R, S108N/T, I164L; Pfdhps S436A, A437G, K540E, A581G, A613T/S. All samples from Bonoua (n=48) and Samo (n=49) were successfully amplified. For Pfcrt, Pfdhfr and Pfdhps respectively 48

(100%), 46 (95.8%) and 41 (85.4%) samples were successfully sequenced in Bonoua, and 45 (91.8%), 34 (70.8%) and 31 (64.6%) in the group of Samo. All 48 (100%) Pfcrt sequences carried the triple mutant CVIET in Bonoua, showing no variation. This haplotypes was also over-represented in Samo because carried by 39 (86.6%) strains. In addition, the latter site was much more heterogeneous with the six following haplotypes or combination of haplotypes represented in 1 (2.2%) sample each : MNK, MET, MET/MNK, MET/MEK, MNT/IEK, and MNT/NEK. Similar situation prevailed for Pfdhps in both sites with less haplotypes heterogeneity in Bonoua than in Samo. In the former, 100% of samples presented GKAA on position 437, 540, 581, 613 and variability resides only on the first position 436 for 3 (7.3%) samples carrying SGKAA, 13 (31.7%) AGKAA/SGKAA and 25 (61%) SGKAA/FGKAA. In the latter, mutations S436A, S436F, S436Y or A437G, A613S occured. Pfcrt and Pfdhps distributions of haplotypes from both sites were significantly different (p-values<0.05). For Pfdhfr, distributions in both sites were not significant (p-value>0.05) although NCSI and IRNI were dominant in Bonoua, with 23 (50.0%) and 15 (32.6%) samples respectively. In Samo, 13 (38.3%), 7 (20.6%), 4 (11.8%)and 10 (29.3%) samples carried NCSI, IRNI, NRNI and double infections respectively. In conclusion, inside regions of high endemicity, molecular epidemiology may vary even for neighbouring geographical sites depending either on drug pressure or movements of populations.

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GENETIC DIVERSITY IN THE *PLASMODIUM FALCIPARUM* AND *P. VIVAX* MALARIA *CELTOS* GENE FROM SOUTHEAST ASIA, AFRICA AND SOUTH AMERICA REVEALS A HIGHLY CONSERVED GENE WITH FOCAL REGIONS OF NON-SYNONOMOUS MUTATIONS UNDER IMMUNE SELECTIVE PRESSURE CONFIRMED BY GENE SEQUENCING, 3D STRUCTURE PREDICTION AND PEPTIDE MAPPING

Eric B. Ockenhouse[1], Elke Bergmann-Leitner[2], Matthew Riley[2], Jonathan D'Ambrozio[3], M. Lee[4], X. Hu[4], Anders Wallqvist[4], Paul Graf[5], John Waitumbi[6], Evelina Angov[2], Christian F. Ockenhouse[2]

[1]Bethesda-Chevy Chase High School-Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]USMMVP-Walter Reed Army Institute of Research, Silver Spring, MD, United States, [3]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [4]TATRC, MRMC, Frederick, MD, United States, [5]NMRCD-Peru, Lima, Peru, [6]USAMRU-Kenya, Kisumu, Kenya

The *Plasmodium* protein Cell-traversal protein for ookinetes and sporozoites (CelTOS) also known as Ag2, plays a critical role in cell traversal of host cells in both the mosquito and the vertebrate and is required for the successful establishment of infection. The CelTOS protein has been identified as a promising pre-erythrocytic vaccine candidate against both *P. falciparum* and *P. vivax* malaria infections. The protein is highly conserved among rodent, non-human primate, and human malaria species and induces cross-species protection when used as a vaccine immunogen. Here we describe the extent of genetic diversity and the presence of single nucleotide polymorphisms (SNPs) in the *celtos* gene from both *P. falciparum* and *P. vivax* parasites obtained from three continents. There is a high degree of sequence homology among all *P. vivax* (98% homology) and *P. falciparum* (91% homology) malaria parasites. The specific SNPs are not randomly distributed but occur in two distinct regions of the gene, suggesting a strong selective pressure on the parasite resulting from host immunity. Phylogenetic tree analysis of the PfCelTOS protein sequence demonstrates geographic similarity and clustering between Asian and African isolates. Two B-cell epitopes within the PfCelTOS protein were predicted based upon 3D structural models using high performance computational algorithms that were not predicted using sequence-based prediction methods. We compared one such B-cell epitope in the carboxy-terminal region of the PfCelTOS protein to the cluster of SNPs which encoded non-synonomous mutations. Peptide mapping using overlapping synthetic peptides in ELISA assays with anti-Pf CelTOS hyperimmune sera confirmed that this region undergoes selective pressure. Future investigations will focus on regions of the molecule that may be selected by cell mediated immune pressure and should be evaluated in the context of eventual clinical trials of a CelTOS-based vaccine candidate.

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ASSESSMENT OF MALARIA MORTALITY IN AFRICAN CHILDREN LIVING IN A MALARIA STABLE TRANSMISSION AREA IN BURKINA FASO: A PART OF MALARIA VACCINE TRIALS SITE CHARACTERISATION STUDY

Konate A. None, Lankoande Malik, Ouedraogo Alphonse, Yaro Jean Baptiste, Ouedraogo Esperance, Tiono Alfred, Sirima Sodiomon Bienvenu

CNRFP, Ouagadougou, Burkina Faso

Reliable mortality data are an important prerequisite for planning malaria vaccine trials, yet such data are often not available in sub-Saharan Africa (SSA). Demographic surveillance systems (DSS) implementing the verbal autopsy (VA) method are the only possibility to observe cause-specific mortality of a population on a longitudinal basis in many countries. This study was carried out in the Saponé Health District. The Saponé district is a rural area in South-West of Ouagadougou, the capital city of Burkina Faso with stable endemic malaria and 86 000 individuals. Children below five years were enrolled. The DSS was used as an approach to assess the mortality. It consisted of a general census of the entire population of the health district. Regular updates of the database were done through a systematic quarterly home visit to collect vital events. We present all-cause, malaria-specific and seasonal mortality rates in children under 5 years from 2006 to 2007. Cause of death was ascertained by verbal autopsy. A total of 268 deaths were analysed. From which malaria was the most prevalent (48.5%) followed by diarrhoea and malnutrition (16.4%), respiratory infections (6.7%) and meningitis (5.2%). All cause mortality rates of children < 5 years were higher in the dry than the rainy season (11.9 vs 9 per 1,000 person-years). Malaria specific mortality was 42.3% during the low transmission season (dry season) and 57.7% during the high transmission season (rainy season). From the malaria specific mortality 0 (0%), 11 (8.5%), 41 (31.5%) and 78 (60%) occurred respectively in children < 1 month, 1-5 months, 6-11 months and 1-4 years. In conclusion, malaria is the most important cause of death in this remote area of SSA, even considering the low specificity of malaria diagnosis in young children. Developing malaria vaccine to control its mortality is of prime importance to reduce the high childhood mortality in the endemic areas of SSA.

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THE DYNAMICS OF MIXED AND ALTERNATING SPECIES INFECTIONS OF *PLASMODIUM VIVAX* AND *P. FALCIPARUM* IN LOW TRANSMISSION AREAS

Rosalynn L. Ord[1], Jean Hernandez[2], Connie Leeper[3], Praveen Bharti[4], Neeru Sing[4], OraLee Branch[1]

[1]New York University, New York, NY, United States, [2]Universidad Nacional de la Amazonia Peruana, Iquitos, Peru, [3]University of Alabama at Birmingham, Birmingham, AL, United States, [4]Regional Medical Research Centre for Tribals, Jabalpur, India

Regions where both *Plasmodium vivax* and *P. falciparum* are endemic, mixed species infections are observed less frequently than expected, and the parasite levels are likely to be controlled in a density-dependant manner. It is unclear whether host-mediated immune factors, parasite-parasite interactions, or a combination of both contribute to these observations. It is also possible that that the sensitivity of the diagnostic methods used is insufficient to detect mixtures, particularly if the densities of each species are very different. In low transmission settings, infections are spaced in time by months. This provides the opportunity to study single inocula infections and the dynamics of individual and mixed infections, as well as the alternation of species over a short time (cryptic infections) and the associated clinical manifestations. Data from Peru

(2003 - 2005) shows that mixed infections show a significantly reduced haematicrit and an increase in fever are reported compared to either single or cryptic infections. Our hypothesis is that mixed infections cause greater pathology. Further, presenting as a cryptic or alternating mixture, infection is a method of maintaining an environment most protective to both parasite species and the host. We have used quantitative PCR methods to assess how the parasite densities of both *P. vivax* and *P. falciparum* vary within single and mixed infections over time. We observe the presence of single- and mixed-species in samples assumed negative by standard PCR techniques, suggesting that either species is often overtly suppressed, rather than being entirely eliminated from peripheral circulation. Markedly, overtime there is an alternation of species suggesting interaction even more density interaction limiting co-existence. Clinical and parasite correlations with the single species and mixed/cryptic infections will be discussed.

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THE LONGITUDINAL PROROGATION OF COMPLEX *PLASMODIUM FALCIPARUM* INFECTIONS CULTIVATES GENETICALLY DISTINCT CLONAL POPULATIONS

Patrick L. Sutton[1], Lindsay Prado Torres[2], Claudia Silva[2], OraLee Branch[1]

[1]New York University, New York, NY, United States, [2]Universidad Nacional de la Amazonia Peruana, Iquitos, Peru

Sexual recombination in malaria parasites occurs within the mosquito midgut. Here, out-crossing between different parasite genotypes may occur if they exist together at this phase in the life cycle. The probability of recombination within a blood meal from a complex infection is a factor of the transmission rate and the genetic diversity within the parasite population. In high transmission regions, the frequency of infective mosquito bites may predict the formation of complex infections by super-infection. The high parasite diversity in such populations makes these super-infections almost guaranteed to contain different parasite genotypes. In the Peruvian Amazon, after the outbreak and 1995-1998 malaria epidemic there have been less than 0.5 *Plasmodium falciparum* infections/person/year reported. From prior investigation, we found considerable population-level diversity: with many different *P. falciparum* genotypes when testing by antigen-encoding genes. However, there was a low frequency of super-infection as shown genotyping weekly-taken blood samples in our cohort study in Iquitos. We hypothesized that complex infections are propagated since when the transmission rate was higher (1995-1998 epidemic). To test this, we utilized 19 microsatellite markers scattered throughout the *P. falciparum* genome. In 303 infections occurring in Zungarococha, Iquitos, from 2003 until 2007 determined the loci-by-loci polymorphisms and parasite haplotype(s) of all loci combined. We defined clonal populations on 2 levels: (1) by an ancestral (phylogeny) approach using the program STRUCTURE 2.2 and a pair-wise comparison (cladistic) approach. The complex infections were genetically dissimilar from their counterparts propagated in single-clone infections. We compare the genetic diversity over time and space: considering both microsatellite markers and an antigen encoding gene. We find evidence of extensive recombination between parasites in complex infections. Moreover, there is a sharing of new polymorphisms in the complex infections that persist over time. The results support the hypothesis that these complex infections were first mixed years earlier and have been propagated to form distinct *P. falciparum* populations. The implications on evolution and the pathology and epidemiology of the malaria infections will be discussed.

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GENETIC DIVERSITY OF *PLASMODIUM FALCIPARUM* FROM WESTERN KENYA HIGHLAND AREAS PRONE MALARIA EPIDEMICS

David M. Menge[1], Kacey C. Ernst[2], John M. Vulule[3], Chandy John[1]

[1]University of Minnesota, Minneapolis, MN, United States, [2]University of Arizona, Tucson, AZ, United States, [3]Kenya Medical Research Insitute, Kisumu, Kenya

Characterizing the genetic diversity and population structure of *Plasmodium falciparum* from highland areas will increase understanding of the emergence of malaria epidemics and the origin and spread of parasite phenotypes in populations. *P. falciparum* isolates from two adjoining highland areas in western Kenya were genotyped and analyzed for genetic structure and diversity at two antigen loci; merozoite surface protein 1 and 2 (msp-1 and 2) and a panel of 12 microsatellite loci. In comparison to other studies in low malaria transmission areas, we observed high genetic diversity with mean expected heterozygosity (He) <0.7 in both areas and a high level of multi-clonal infections in both areas (79% and 78% for microsatellite loci, and 61% and 72% for antigen loci for Kipsamoite and Kapsisiywa, respectively). There was significant linkage disequilibrium of parasite populations from both areas but no population structures except for the microsatellite loci Poly-α and TA81, which showed marginal genetic differentiation index of 0.021 (P = 0.03) and 0.019 (P = 0.04), respectively. This data imply non-isolation from endemic low land areas and can useful in unraveling parasite reproductive strategy and local transmission in relation to malaria control strategies.

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SEVERE MALARIA IN BATTAMBANG REFERRAL HOSPITAL, CAMBODIA FROM 2006 TO 2008

David Saunders[1], **Chanthap Lon**[2], Nillawan Buathong[1], Ngo Sithy[3], Youry Se[2], Ans Timmermans[1], Jessica Lin[1], Mark Fukuda[1], Duong Socheat[4], Delia Bethel[1]

[1]Armed Forces Research Institute of the Medical Sciences, Bangkok, Thailand, [2]Armed Forces Research Institute of the Medical Sciences, Phnom Penh, Cambodia, [3]Battambang Referral Hospital, Battambang, Cambodia, [4]CNM, Phnom Penh, Cambodia

Patients with severe malaria are in critical condition and require emergency therapy with parenteral drug therapy. Even when treated with appropriate antimalarial drugs, severe malaria in austere or resource-limited settings in the developing world may be associated with high mortality rates because of complications for which treatment may not be available, such as acute renal failure and acute respiratory distress syndrome. Little has been reported in the peer reviewed literature about the burden of severe malaria in the government referral hospital in Battambang (BRH), Western Cambodia's second largest city. However, data from the Cambodian National Malaria Center (CNM) in 2007 indicates that Battambang Province had the second highest mortality rate for probable and confirmed malaria in Cambodia. All cases of malaria admitted to the BRH from January 2006 to December 2008 with a discharge diagnosis of severe malaria were reviewed for demography, mortality, and referral patterns. There were 2,648 reported cases of severe malaria among 59,848 confirmed malaria cases in 2007 nationally, with a case fatality rate of 8.3%. There were 132 cases and 23 deaths (17.4% mortality) from severe malaria in 2007 reported from BRH, out of 4,105 confirmed malaria cases reported in Battambang province that year. Detailed data on mortality, demographics and referral patterns from 2006-2008 will be presented. Factors contributing to mortality and severity will be discussed. In conclusion, Western Cambodia continues to have a significant number of severe malaria cases, suggesting that interventions to improve access to early diagnosis and treatment of malaria remain priorities for this region.

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VALIDATING SEVERE ANEMIA AS A PROXY FOR MALARIA: EVIDENCE FROM A NATIONAL SURVEY

Jasbir K. Sangha, Kiersten B. Johnson

ICF Macro, Calverton, MD, United States

This study uses data from the Tanzania HIV/AIDS and Malaria Indicator Survey 2007-08 to discern if there is correlation between severity of anemia and malaria. As part of the survey all children 6-59 months residing in the selected households were tested for hemoglobin and malaria using the HemoCue and Paracheck Pf™ rapid diagnostic test (RDT) respectively. The RDT test detected parasites in 17.6% of children. The difference in the prevalence of malaria by place of residence was statistically significant, large cities including Dar-es-Salaam had low prevalence of malaria (1.1%) compared to the country side (19.8%). Among those children who tested positive for malaria, 37.9% were severely anemic (Hb <7 gm/dl) and 24.7% and 14.5% had moderate and mild anemia respectively. Children with malaria were three times more likely to be severely anemic than children who did not have detectable levels of parasites. As expected, significant inverse relationship was observed between prevalence of malaria and socio-economic variables such as wealth and mother's education. Findings remained statistically significant even when we restricted the analysis to only those children who slept under a mosquito net a night before the survey. Risk of severe anemia doubles in households where no children sleep under a mosquito net compared to those households where some children sleep under a mosquito net (36.5% compared to 18.3%). By restricting the analysis to only those children who slept under a mosquito net a night before the survey, we found even a stronger association between malaria and severe anemia. Children who tested positive for malaria were four times more likely to be severely anemic than children who did not have detectable levels of malarial parasites. These findings confirm that severe anemia can be used a proxy for malaria in low-resource settings.

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LIMITED GLOBAL DIVERSITY OF ANTIBODY EPITOPES EXPRESSED BY PLACENTAL BINDING *PLASMODIUM FALCIPARUM* VARIANTS

Mirja Hommel[1], Salenna R. Elliott[1], Greg Kelly[2], Freya J. Fowkes[1], Michael F. Duffy[2], Joanne Chesson[1], Danielle I. Stanisic[1], Ivo Mueller[3], Marion Avril[4], Viju Soma[4], Joseph D. Smith[4], Stephen J. Rogerson[2], James G. Beeson[1]

[1]The Walter and Eliza Hall Institute, Parkville, Victoria, Australia, [2]Department of Medicine, University of Melbourne, Parkville, Victoria, Australia, [3]Papua New Guinea Institute of Medical Research, Magang, Papua New Guinea, [4]Seattle Biomedical Research Institute, Seattle, WA, United States

Malaria in pregnancy is characterised by the accumulation of *Plasmodium falciparum* infected erythrocytes (IEs) in the placenta. Isolates that cause malaria in pregnant women exploit gaps in the repertoire of variant-specific immunity and adhere in the placenta to chondroitin sulfate A and other molecules. Antibodies to surface antigens expressed by these isolates are acquired after exposure to placental malaria and appear to convey a substantial degree of protective immunity. Although the major antigens of placental-binding IEs are not fully defined, a specific variant of PfEMP1, known as var2csa appears to be an important target of antibodies and mediates IE adhesion in the placenta. Here we assessed the global diversity of placental-binding isolates to understand how immunity is mediated and the evolution of polymorphisms. Using a panel of placental-binding isolates from Africa, Asia, PNG, and Central America, we examined antibodies among pregnant women in Malawi and Papua New Guinea as representative populations of Africa and SE Asia. All isolates were recognised by antibodies in Malawi and PNG women. Antibody epitopes did not appear strictly conserved as many individuals demonstrated isolate-specific rather than pan-reactive or cross-reactive antibodies. There was a substantial degree of antigenic overlap and sharing of epitopes between isolates, including those from distant geographic locations. Furthermore, we identified polymorphic epitopes on var2csa domains that were recognised by antibodies among Malawi and PNG women, and var2csa sequence analysis suggests that these polymorphic epitopes have a wide geographic distribution. The limited diversity of placental-binding isolates and wide geographic distribution of epitopes may explain why immunity to malaria in pregnancy can be achieved after exposure during one pregnancy.

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ANTIBODIES AGAINST THE ERYTHROCYTE BINDING ANTIGENS OF *PLASMODIUM FALCIPARUM* ARE STRONGLY ASSOCIATED WITH PROTECTION AGAINST CLINICAL MALARIA AND HIGH PARASITEMIA

Jack S. Richards[1], Danielle I. Stanisic[2], Pascal Michon[2], Elijah Dabod[2], Chetan E. Chitnis[3], David E. Narum[4], Jennifer K. Thompson[1], Alan F. Cowman[1], Ivo Mueller[2], James G. Beeson[1]

[1]Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, [2]Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea, [3]International Centre for Genetic Engineering and Biotechnology, New Delhi, India, [4]National Institutes of Health, Rockville, MD, United States

Symptomatic malaria is caused by blood-stage parasitemia. Naturally acquired immunity can afford protection against symptomatic disease and high density parasitemia and is largely targeted towards blood-stage antigens. It is likely that antibodies against merozoite antigens make an important contribution to this protection. Antibodies may target merozoite surface proteins (MSP) or invasion ligands contained within the apical organelles. Although the erythrocyte binding antigens (EBAs; EBA175, EBA140, EBA181) have been identified as important ligands for erythrocyte invasion, there is little data on their potential significance as targets of protective antibodies, which may act by inhibiting merozoite invasion. We examined antibody responses in a cohort of 206 Papua New Guinean children who were all treated for parasitemia at enrolment and followed prospectively for 6 months to assess re-infection, episodes of high density parasitemia and symptomatic malaria. A number of *Plasmodium falciparum* merozoite proteins were examined as potential targets of protective antibodies, including MSP1, MSP2, apical membrane antigen 1 (AMA1), and the EBAs (covering different functional regions of the proteins). Of these, antibodies to the EBAs were strongly associated with protection from symptomatic malaria. Antibodies to regions III-V were more strongly associated with protection than antibodies to region II of each protein. The predominant antibody isotypes observed were IgG1 and IgG3. The isotype bias appeared to reflect the structural characteristics of the specific regions of the proteins and did not seem to be related to age, cumulative exposure, or factors specific to the individual. IgG3 was most strongly associated with protection, even in the setting of an IgG1 predominant response. These findings have significant implications for understanding human immunity to malaria and provide further support for the potential development of EBAs as vaccine candidates.

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ACQUISITION OF ANTIBODIES TO MEROZOITE SURFACE PROTEIN 3 AMONG RESIDENTS OF KOROGWE, NORTHEASTERN TANZANIA

Method D. Segeja, B.P. Mmbando, S.D. Misago, J.P. Lusingu, Martha.M. Lemnge

National Institute for Medical Research, Tanga Centre, Tanga, United Republic of Tanzania

Merozoite surface protein 3 (MSP3) is a polymorphic malaria parasite antigen which is among blood stage malaria vaccine candidates. It is believed to induce immunity through cytophilic antibodies that disrupt the process of erythrocytes invasion by merozoites. This study aimed at

assessing natural acquisition of antibodies to MSP3. This is the first study conducted on acquisition of antibodies to MSP3 in children and older individuals in Korogwe, northeastern Tanzania. Data from three strata was used in selection of one village for testing MSP3 malaria vaccine. Individuals aged 0-19 years living in lowland, intermediate and highland strata formed the study groups. Blood smears were prepared for malaria parasite identification and quantification. Enzyme linked immunosorbent assay was performed on 492 plasma samples. They were analyzed for reactivity of antibodies [immunoglobulins (IgM, total IgG, IgG1 and IgG3)] to MSP3. Highest malaria parasite prevalence (50.0%, 95%CI = 37.4, 62.6) was found in lowland stratum whilst the lowest prevalence was in highland stratum (9.8%, 95%CI=6.7, 13.7). The intermediate stratum had malaria parasite prevalence of 23.1% (95%CI=16.0, 31.7). Mean antibody levels of total IgG and IgM were higher in highlands stratum; and mean level of IgG1 and IgG3 were higher in individuals living in lowland stratum. All antibody levels were found to increase with increasing age. Rate of change of IgG3 antibody levels with age was 0.181(95%CI 0.154 - 0.208; p<0.001) and it was slightly higher than in IgG1. Higher levels of IgM and total IgG in highlands suggest recent malaria infection or presence of cross-reactive antigens. Higher levels of IgG1 and IgG3 in lowland stratum parallels that of parasite prevalence indicating these subclasses play a role in malaria protection. Individuals in intermediate stratum with lower parasite prevalence and lower levels of antibodies were selected for MSP3 phase 1b malaria vaccine trial.

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BIOMARKERS OF IMMUNO-HEMATOLOGICAL RELEVANCE ASSOCIATED WITH SEVERE MALARIA ANAEMIA IN GHANAIAN CHILDREN

Richard H. Asmah[1], Selorme Adupko[2], Micheal Ofori[2], Alexander Nyarko[2], Eugene Baah[1], Ben Gyan[2], Edwin Wiredu[1], John Tetteh[2], Patrick Ayeh-Kumi[1], Charles Brown[1]

[1]School of Allied Health Sciences, University of Ghana, Accra, Ghana, [2]Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana

Plasmodium falciparum related malaria infects about 500 million people every year, especially children, pregnant women and non-immune individuals visiting endemic regions. Presently, the global malaria problem is attributed to emergence of resistant parasites to most of the available drugs. Malaria infection results in inflammatory response marked by the production of cytokines such as IL-2, IL-4 and IL-10 amongst others. Reactive oxygen species generated during infection result in oxidative stress, affecting both parasite and host cells. Understanding the inter-relationship between hematological parameters, cytokines, reactive oxygen species and other agents influencing oxidative stress during the infection would help improve our perspective on the prognosis of *P. falciparum* malaria and the search for novel malaria control strategies. This work evaluated the inter-relationships between hematological factors, cytokines and free radical generation measured by superoxide dismutase (SOD) levels from pediatric malaria patients. Blood samples from 29 pediatric malaria patients and 12 healthy controls were used for the study. Hematological parameters, cytokines and SOD activity were measured using an auto analyzer, a commercial ELISA kit and a commercial SOD kit, respectively. Mean parasite density was 97.7/µl, mean hemoglobin level was 5.81g/dl, while RBC, WBC and platelet counts were $2.87x10^{12}$/l, $10.49x10^{9}$/l 112.14x109/l respectively, in the patients. There was positive correlation between WBC and platelets ($r = 0.636$, $p \leq 0.000361$), IL-2 and RBC ($r = 0.482$, $p \leq 0.01260$) while total erythrocyte SOD activity ranged from 25% to 71%. The SOD values correlated negatively with the levels of IL-4 ($r = -0.478$, $p \leq 0.0101$). Median levels of both IL-2 and IL-10 were significantly higher in patients than in controls. *Plasmodium falciparum* infection in Ghanaian children with severe malaria anemia led to correlating decreases in both platelets and WBCs counts with IL-4 levels having a significant influence on cellular reactive oxygen species generation.

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ANTIBODY RESPONSES TO EBA-175 REGION II IN ADULTS AND CHILDREN IN A MALARIA ENDEMIC AREA OF GHANA

Rafiq Okine, Dorothy Anum, Helena Lamptey, John Tetteh, Beverly Egyir, Ben Gyan, Kwadwo Koram, **Daniel Dodoo**

Noguchi Memorial Institute for Medical Research, Accra, Ghana

Antibody plays an important role in natural protection against malaria, but the antigenic targets of protection have not yet been conclusively identified. In malaria endemic regions, the risk of clinical malaria decreases with age and the strategy of vaccine development is to induce adult-like immunological responses in children who suffer disproportionately from the effects of malaria. The multiplication of blood stage parasites causes clinical malaria and involves the attachment of merozoite binding proteins to receptors on the surface of the RBC. Region 2 of EBA-175 has been shown to be important in erythrocyte invasion. In this study, IgG, IgM and IgG subclass levels were assesed in children and adults from a malaria endemic region of Ghana and in non-exposed individuals, using a standardized ELISA. Plasma samples for this study were obtained from children (1- 5 years) and adults (18-49 years) and 5 non-exposed individuals. The study showed differences in the quantity and quality of the antibody to EBA-175 region 2 between adults and children. IgG levels were higher in adults (GM Antibody Units = 21037) than in children (GM Antibody Units = 403), p<0.001. The predominant antibody in adults was IgG while that in children was IgM. In addition, IgG subtypes were different between adults (IgG1 and IgG3) and children (more of IgG2). Cytophilic to non-cytophhilic antibody ratio was significantly higher in adults (1.74) than in children (0.12), p<0.001. Levels of antibody in the 5 non-exposed individuals were around the lowest detection limits of the assay. This study has indicated that the quality and quantity of antibody responses in children (the ultimate target of anti-malaria vaccine) are very different from more exposed adults, whose type of immune responses one may wish to induce in vaccinated children to afford protection from disease. It may thus be important to include isotype and IgG subclass measurements in assessment of immunogenicity in the development of EBA-175 region 2 as a malaria vaccine.

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RELATIONSHIP BETWEEN CLINICAL MALARIA AND IGG RESPONSES TO A PANEL OF MALARIA SPECIFIC VACCINE CANDIDATES IN CHILDREN LIVING IN SEASONAL MALARIA TRANSMISSION AREA OF BURKINA FASO

Diarra Amidou[1], Ouedraogo Andre Lin[1], Tiono Alfred[1], Soulama Issiaka[1], Ouedraogo Alphonse[1], Charrif Mariama K.[1], Yaro Jean Baptist[1], Ouedraogo Espérance[1], Bougouma Edith C.[1], Sanon Souleymane[1], Konate Amadou T.[1], Gansane Adama[1], Corradin Gampietro[2], Dodoo Daniel[3], Sirima B. Sodiomon[1], Nébié Issa[1]

[1]CNRFP, Ouagadougou, Burkina Faso, [2]University of Lausane, Lausane, Switzerland, [3]Noguchi Memorial Institute, Accra, Ghana

Establishing a relationship between humoral responses to malaria specific antigens and relevant clinical outcome, applied to naturally acquired immunity, would significantly help to the clinical development of malaria vaccine. This study aimed to establish relationship between total IgG and malaria transmission seasons and relationship between IgG responses and clinical malaria episodes. Study was conducted in four villages of Saponé health district and included 529 children less than five years old. Two cross-sectional surveys were carried out: one at the beginning of low transmission season and the second at the peak of high transmission in 2007. During each survey, from each child, a blood smear was prepared for malaria diagnosis and 5 ml of blood taken for IgG measurement. Children were subsequently followed up for one year. Pre-erythrocytic (LSA1 and CSP) and blood stage (MR198 and MR48a) antigens were used for antibodies measurement. Strong relationship was found between IgG levels and age for MR48a, MR198 and for the CS peptides during both transmission season (P=0.000). Geometric means of IgG levels to the 4

peptides were higher at the peak of transmission compared to the lower transmission season. Surprisingly, the opposite was observed for the CSP 4146.7 (95%CI: 3826.1-4494.2) vs 2384.4 (95%CI: 2143.9-2652.0). Geometric mean of IgG levels at the lower and the peak of transmission was similar in children without malaria and those with at least one malaria episode for all four antigens except for CSP 3476.6 (95%CI: 2896.4-4056.8) vs 2551.7 (95%CI: 2245.0-2858.4) (P=0.004). In conclusion, these data suggest that these antigens may be affected by the level of malaria transmission and CSP antibody responses were associated with clinical protection. It is worthwhile investing in the development of these malaria vaccine candidates.

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CYTOKINE BIOMARKERS OF ASYMPTOMATIC MALARIA

Nana O. Wilson[1], Tammiki Bythwood[1], Wesley Solomon[1], Pauline Jolly[2], Nelly Yatich[2], Faisal Shuaib[2], Jonathan K. Stiles[1]

[1]Morehouse School of Medicine, Atlanta, GA, United States, [2]University of Alabama at Birmingham, Birmingham, AL, United States

In sub-Saharan Africa, approximately 30 million pregnant women are at risk for contracting malaria and suffering its adverse consequences annually. In a recent unpublished study conducted in Ghana, 10% of healthy pregnant women screened for *Plasmodium falciparum* HRP-II were HRP-II positive but asymptomatic for malaria. We tested the hypothesis that asymptomatic and healthy control pregnant women differ in their Th1/Th2 immunomodulator phenotype. Levels of cytokine, chemokine and growth factor in asymptomatic healthy pregnant women (positive or negative for *P. falciparum* HRP-II) were evaluated to determine their role in asymptomatic or symptomatic malaria. Plasma from healthy (n=15) and asymptomatic (n=25) pregnant women were evaluated for 27 cytokines, chemokines, and growth factors (IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, FGF-B, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1a, MIP-1b, PDGF-bb, RANTES, TNF-a, and VEGF) using a multiplex bead based immunoassay system. Angiogenic and pro-inflammatory factors did not show significant differences between the two groups. However, anti-inflammatory biomarkers (IL-10 and G-CSF) were significantly elevated among asymptomatic group compared to the healthy group (p= 0.031 and 0.041 respectively). Assessment of ratios of pro-inflammatory to anti-inflammatory factors (IL-1:IL-5, IL-1:IL-7, IL-1:G-CSF, IL-2:IL-13, IL-8:IL-10, Eotaxin:IL-13, IFN:G-CSF and MIPb:IL-10) revealed statistically significant differences among the two groups. Angiogenic to angiostatic biomarker ratios (IP-10:IL-10) were statistically significant (p =0.026) between the two groups. Thus, susceptibility to symptomatic malaria in pregnancy is associated with high levels of IL-10 and G-CSF and dysregulation of pro and anti-inflammatory biomarkers in pregnant women. Assessment of these biomarkers in conjunction with rapid detection of *P. falciparum* HRP-II (gene or protein) could be utilized in determining risk for asymptomatic malaria carriage.

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GENE CONTENT POLYMORPHISMS OF KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS (KIRS) IN THE SUSCEPTIBILITY TO AND PROTECTION FROM PLACENTAL MALARIA IN HIV-1 NEGATIVE AND HIV-1 POSITIVE PREGNANT WOMEN IN WESTERN KENYA

Yusuf O. Omosun[1], John Williamson[1], Caroline Othoro[2], Mary Carrington[3], Maureen P. Martin[3], John Ayisi[2], Anne Maria van Eijk[2], Juliana Otieno[4], Renu B. Lal[1], Richard Steketee[1], Bernard Nahlen[1], Feiko ter Kuile[5], Laurence Slutsker[1], Ya Ping Shi[1]

[1]Centers for Disease Control and Prevention, Atlanta, GA, United States, [2]Center for Vector Biology and Control Research, Kenyan Medical Research Institute, Kisumu, Kenya, [3]Cancer and Inflammation Program, Laboratory of Experimental Immunology, SAIC-Frederick, Frederick, MD, United States, [4]New Nyanza Provincial General Hospital, Ministry of Health, Kisumu, Kenya, [5]Liverpool School of Tropical Medicine, Liverpool, United Kingdom

Previous immunological studies have reported that high levels of IFN-γ producing Natural Killer (NK) cells are associated with protection against placental malaria (PM), and NK cell response is regulated by a family of killer cell immunoglobulin-like receptors (KIRs). Given the relevance of NK cells in protection against PM and the regulatory role of KIRs on NK cell functions, we examined the association between KIR gene content polymorphisms and PM in pregnant women of known human immunodeficiency virus (HIV) status. KIR genotypes were determined in 685 pregnant women who had participated in a birth cohort study to assess the effect of PM on vertical transmission of HIV-1. KIR specific polymerase chain reaction (PCR) and multiplex hybridization methods were used for genotyping of DNA from maternal blood cells. Gene content polymorphisms were determined as 1) presence of 16 genes in the KIR family including 8 inhibiting and 6 activating genes and 2 pseudogenes, and 2) homozygosity or heterozygosity of specific KIR genes. Overall results showed that KIR3DL2, KIR3DL3, KIR2DL4 and KIR3DP1 (3 inhibiting framework genes and 1 pseudogene respectively) were present in all pregnant women. The presence of the 12 remaining KIR genes in this population varied from 98% to 13% with lowest prevalence (13%) for activating gene KIR3DS1. Univariate analysis showed that among HIV-negative women the prevalence of two inhibiting genes, KIR2DL3 and KIR2DL1, was higher in women with PM than in those without PM (93% vs 81%, p=0.019; 100% vs 94%, p=0.03, respectively). In contrast, among HIV-positive women, prevalence of the inhibiting KIR2DL3 gene was lower in women with PM than those without PM (73% vs 87%, p= 0.012), whereas there was no difference for KIR2DL1. We further found that in HIV-negative women the prevalence of homozygosity of an inhibiting gene, KIR2DL2/KIR2DL2, was higher in women without PM than in those with PM (18%, vs 4%, p=0.005) while in HIV-positive women the prevalence of this KIR homozygosity was lower in women without PM than those with PM (14 vs 26%, p=0.031). These initial results suggest that 1) individual inhibiting genes, KIR2DL3 and KIR2DL1, and homozygosity of inhibiting KIR2DL2 gene may play a role in PM; and 2) HIV-1 co-infection could lead to changes in KIR repertoire which could either protect women from or render them susceptible to PM. Further multivariate analysis is ongoing to assess the relationship of KIR genes with PM and HIV.

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MSP-1_{19} HAPLOTYPE SPECIFICITY OF FUNCTIONAL ANTI-MSP-1_{19} ANTIBODIES IN PEDIATRIC ACUTE MALARIA INFECTION

Arlene E. Dent[1], Rhonda Kimmel[1], Christopher Yohn[1], Peter Sumba[2], John Vulule[2], Ann Moormann[1]

[1]Case University, Cleveland, OH, United States, [2]KEMRI, Kisumu, Kenya

Naturally acquired antibodies directed against *Plasmodium falciparum* Merozoite Surface Protein-1_{19} (MSP-1_{19}) have been associated with protection against malaria disease. Four predominant infecting MSP-1_{19}

haplotypes have been described in Kenya, but it is unclear if naturally acquired antibodies are haplotype-specific. To address this question, we examined MSP-1_{19} antibodies and infecting haplotypes in children living in a malaria endemic region of Kenya. Children (n=89, mean age 25 months (range 1 - 66 mo)) were recruited, provided a venous blood sample during an acute malaria infection, were treated with 6-doses of CoArtem™ (Artemether/Lumefantrin), and examined 4 weeks later when another blood sample was drawn. Samples were tested for MSP-1_{19} infecting haplotypes using an MSP-1-1_{19} specific PCR/Ligase Detection Reaction-Fluorescent Microsphere Assay (PCR/LDR-FMA); for antibodies against MSP-1_{42} (3D7--ETSR and FVO--QKNG) by ELISA; and for functional antibodies using an MSP-1_{19} specific invasion inhibition assay (MSP-1_{19} IIA) containing the ETSR haplotype. We found that during acute infection, the predominant MSP-1_{19} infecting hapotype was QKNG (42%) and EKNG (41%) followed by ETSR (13%) and QTSR (4%). Serologically measured antibodies to MSP-1_{42} (3D7_ETSR) were found to decrease slightly between acute and recovery time points whereas serologically measured antibodies to MSP-1_{42} (FVO_QKNG) did not change between the two time points. MSP-1_{19} IIA levels did not change between acute and convalescent time points. However, children who had infections with the ETSR infecting haplotype (n=19), MSP-1_{19} IIA levels were boosted at the time of infection compared with levels measured 4 weeks later (p=0.045). In contrast, overall antibodies titers against MSP-142 (3D7_ETSR) did not differ between the acute and recovery time points in individuals with ETSR containing infections. Thus, functional antibodies measured by MSP-1_{19} IIA may more accurately reflection MSP-1_{19} haplotype specific immunity.

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ENGINEERING APICAL MEMBRANE ANTIGEN-1 TO OVERCOME ANTIGENIC DIVERSITY IN THIS MALARIA VACCINE CANDIDATE

Michael Foley[1], Karen Harris[1], Madhavi Khore[1], Coley Andrew[1], Robin F. Anders[1], Sheetij Dutta[2]

[1]La Trobe University, Melbourne, Australia, [2]Walter Reed Army Institute of Research, Silver Spring, MD, United States

AMA1 is a leading malaria vaccine candidate but there is concern that vaccination with one form of AMA1 may not provide sufficient protection against diverse *Plasmodium falciparum* genotypes to be useful for controlling malaria. As there is a dominant strain-specific 'inhibitory' epitope in a domain I loop (loop Id) of AMA1 one strategy to overcome the problem of diversity would be to generate a form of AMA1 with loop Id mutated so as to enhance responses to epitopes that are common to all allelic forms of AMA1. Five of the most polymorphic residues in loop Id of the 3D7 and FVO forms of AMA1 have been mutated to either alanine, serine or glycine and the resulting AMA1 molecules displayed on the surface of bacteriophage. Reactivity with monoclonal and polyclonal antibodies indicated that the alanine and serine mutants of AMA1 have reduced binding to strain-specific antibodies, but retain binding to antibodies that recognize conserved conformational epitopes. In contrast the antigenic analyses indicated that the glycine mutants did not fold correctly when displayed on bacteriophage. The serine and alanine mutant 3D7 and FVO AMA1 proteins have been expressed in *E. coli,* refolded *in vitro,* and their immunogenicity in mice and rabbits is being examined. Antibodies raised to the mutant forms of AMA1 will be assessed for their ability to block parasite invasion by diverse strains of *P. falciparum.*

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EXAMINATION OF HUMORAL AND CELLULAR MEMORY RESPONSES TO *PLASMODIUM FALCIPARUM* IN THE PERUVIAN AMAZON

Eva Clark[1], Claudia Silva[2], Carlos Padilla[3], Oralee Branch[4]

[1]University of Alabama at Birmingham, Birmingham, AL, United States, [2]Universidad Nacional Amazonia Peruana, LIPNAA, Iquitos, Peru, [3]Instituto Nacional de Salud, Lima, Peru, [4]New York University, Department of Medical Parasitology, New York, NY, United States

The development of immunity to *Plasmodium falciparum* infections has been shown to require 2-10 years of persistent parasitemia, and this delay has been attributed to difficulties in development of protective antibody responses. However, in the low *P. falciparum* transmission region of Iquitos, Peru, we have previously shown that IgG responses to the 19kD region of Merozoite Surface Protien-1 (MSP119) seem to be relatively long-lived, and that there is a high percentage of asymptomatic malaria infections. To study more precisely the longevity of naturally acquired antibody responses to MSP119, serum samples from 186 Peruvian adults (≥14.5 years old) and 135 children (<14.5 years old) were chosen from our longitudinal study cohort. Associations among the longevity of antibody responses and age of the individual, parasite density/persistence, and amount of previous *P. falciparum* exposure were examined. We found that 25% of adults (>14.5 years of age) were able to maintain their responses higher than the negative cut-off value for 270 days after infection (median survival for adults = 128.5 days). Independent of age, individuals who have had at least two prior *P. falciparum* infections are capable of maintaining long-lived responses to MSP119 lasting for > five months. The evidence suggests that individuals living in this region form a unique cohort for the study of anti-malarial immunologic memory. Thus, the malaria antigen-specific memory B-cell population in our subjects was then evaluated by using plasmablast flow cytometry and B-cell enzyme-linked immunosorbent spot (ELISPOT) assays. An expansion of the plasmablast (CD19+, CD27+, CD38high cells) population was observed in certain individuals 5-10 days after a *P. falciparum* infection, which suggests a capacity for malaria-specific memory. Definitive evidence of MSP119-specific memory B-cells was found by ELISPOT analysis, showing B-cell memory develops in members of our Peruvian cohort, unlike prior studies showing a lack of functional immunologic memory to malaria in high transmission regions.

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MASSIVELY PARALLEL PYROSEQUENCING TO DEFINE IN HOST DIVERSITY OF *PLASMODIUM FALCIPARUM*: BLANTYRE, MALAWI

Jonathan J. Juliano[1], Victor Mwapasa[2], Steven R. Meshnick[1]

[1]University of North Carolina, Chapel Hill, NC, United States, [2]University of Malawi, Blantyre, Malawi

In sub-Saharan Africa, *Plasmodium falciparum* infections are typically polyclonal with individuals harboring as many as 13 different variants. The methods recommended to study diversity in malaria are over 20 years old and are clearly inadequate to define the true diversity of infections. The currently recommended nested PCR protocols detect only size polymorphisms. These methods fail to detect sequence polymorphisms and fail to detect minority populations in mixed infections. Newer methods for describing diversity are urgently needed. Massively parallel pyrosequencing (MPP) represents a new high throughput sequencing technique. The power of MPP and its utility for sequencing amplicons derives from its ability to sequence from single molecules within the mixture of molecules. When sequenced on this system, each amplicon molecule within the mixture is sequenced individually, allowing the identification of rare variants. This technique has been used extensively to study diversity of HIV. Using a Roche 454 sequencer, we evaluated the diversity of *P. falciparum* infections from pregnant women who took part in a trial of IPTp in Blantyre, Malawi. These samples had previously

been genotyped using a heteroduplex tracking assay for merozoite surface protein-1 (msp1) and represented a range of complexities (1-9 variants) by this method. We evaluated the diversity of msp1, merozoite surface protein-2 (msp2), circumsporozoite surface protein (csp) and the region of dihydrofolate reductace (dhfr) containing the dhfr-51,59 and 108 mutations. We found an increase in the described diversity when compared to the currently recommended nested PCR protocols.

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COMPARISON OF THREE DNA EXTRACTION METHODS FROM BLOOD SAMPLES COLLECTED IN MALARIA RAPID DIAGNOSTIC TESTS (MRDTS)

Luis Sanchez, Erik Mercado, Juan Contreras, Dionicia Gamboa

Instituto de Medicina Tropical "Alexander Von Humboldt", Universidad Peruana Cayetano Heredia, Lima, Peru

Malaria rapid diagnosis tests (MRDTs) are an alternative method for the malaria diagnosis, mainly used in remote areas where microscopy is not available. Nowadays, it is possible to recover DNA from the dried blood sample collected on a MRDTs, which can be used to carry out molecular epidemiology studies targeting markers like the ones related to drug resistance, immune response, among others, in order to understand the dynamic of the disease in areas where the accessibility is very difficult and no information is available. The aim of this study was to compare three DNA extraction protocols from blood samples collected in MRDTs in order to determine the best method that will allow obtaining good quality DNA from *Plasmodium sp.* that could be use in Polymerase Chain reaction (PCR). We randomly chose 9 MRDTs positive to *P. falciparum* from patients coming from communities in the area of San Lorenzo, Loreto (Peru) between February and September 2006. The DNA of each sample was extracted by three different extraction methods: QIAamp Mini Blood Kit®, Phenol-chloroform and Chelex-100. The quality of DNA was compared by PCR product. We carried out PCR tests on DNA extracted from the 9 MRDTs (OPTIMAL®) positive samples. 9/9 (100%) of samples extracted with QIAamp Mini Blood Kit®, 8/9 (89%) samples extracted with Phenol-chloroform and only 1/9 (11%) extracted with Chelex-100 were positive to *P. falciparum* by PCR. In conclusion, the PCR test determined that the best DNA quality was obtained from samples where the DNA was extracted using QIAamp Blood Mini Kit®. The samples extracted with Phenol-chloroform also amplified a PCR product; but the bands of the products showed lower quality in agarose gels. The Chelex-100 method was the least successful in isolate good quality DNA from *P. falciparum*.

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ASYMPTOMATIC MALARIA DETECTION BY PCR AMONG A COLLATERAL NATIVE POPULATION IN AN ENDEMIC REGION AT THE PERUVIAN-ECUADORIAN BORDER

Elizabeth Villasis[1], Jorge Cacho[1], Jorge Bendezu[1], Victor Neyra[1], Jaime M. Bernal[2], Dionicia Gamboa[1]

[1]Instituto de Medicina Tropical "Alexander Von Humboldt", Universidad Peruana Cayetano Heredia, Lima, Peru, [2]Laboratorio Referencial Santa Maria de Nieva, Red de Salud Condorcanqui, Amazonas, Amazonas, Peru

Asymptomatic malaria carriers are continuous reservoirs of *Plasmodium* sp., which are not treated with antimalarics due to the lack of an accurate diagnosis because of factors like low parasitaemia level and absence of malaria symptoms. The goal of this study was to evaluate the performance of a SemiNested-Multiplex PCR (SnM PCR), with expert laboratory microscopy used as the reference standard, during an active surveillance, in a malaria endemic Region in the northwest of Peru. The study was carried out during the period of August 2007 and January 2009 in 3 natives Peruvian communities: Guayabal, Shebonal, Kamientsa, located in the Rio Santiago District, Condorcanqui Province, at border with Ecuador. Fingerprick blood samples were taken from each of the 263 collateral individuals and used to prepare thick and thin blood films and filter papers samples for Microscopy and PCR (18ssrRNA) analysis, respectively. The PCR had a sensitivity of 83.30% and specificity of 94.80%. VPP and VPN values were 43.50% and 99.20%, respectively. For species detection, sensibility and specificity were 85.7%, 98.4% and 80.0%, 96.5% for *P. falciparum* and *P. vivax* repectively. The media level of parasitaemia for *P. vivax* was 2455.6 parasites/ul and 16.14 parasites/ul for *P. falciparum*. Additionally, microscopy detected a significant percentage (85.72%) of *P. falciparum* infections at gametocyte stage. In conclusion, the low values of sensitivity and VPP for PCR are due to the performance of Microscopy in this area. A high percent of malaria infections detected by PCR were considered false positive because of the low parasitaemia level missed by the local microscopist. Larger active surveillance studies, using sensitive tools like PCR, need to be carried out in order to determinate the real prevalence of asymptomatic malaria carriers in this Province, were high transit of travelers and natural persons between Peru and Ecuador is common.

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TARGET PROTEINS OF THE CYTOSOLIC THIOREDOXIN IN *PLASMODIUM FALCIPARUM*

Shin-ichiro Kawazu[1], Hitoshi Takemae[2], Kanako Komaki-Yasuda[2], Shigeyuki Kano[2]

[1]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan, [2]Research Institute, International Medical Center of Japan, Tokyo, Japan

In *Plasmodium falciparum*, which actively develops and proliferates within human and insect hosts, thioredoxin (Trx) and glutathione system are considered to play essential roles to keep the cellular redox homeostasis. Trx is known to regulate a number of phenomena in the cell by controlling the activity of various enzymes through reduction of their disulfide bridge. In this study, we examined the target proteins of the cytosolic Trx (PfTrx1), in the parasite cell with the Trx-affinity chromatography. Based on the reduction pathway of the proteins by Trx, we generated the cysteine mutant PfTrx1, which capture the target protein as a mixed disulfide intermediate. A number of proteins were captured with the PfTrx1(C33S) immobilized resin, and they were eluted by DTT treatment. To confirm the mixed disulfide-based acquisition of the target proteins in our system, the insulin reduction activity of the recombinant PfTrx1 protein was examined. The PfTrx1(C33S) immobilized resin-captured proteins were trypsin-digested and analyzed on a liquid chromatography-mass spectrometry system. The analysis of sequence data against database assigned 24 proteins. The similar analysis with the PfTrx1(C30S/C33S)-captured proteins assigned 5 proteins; four of which were captured with the PfTrx1(C33S) immobilized resin. The potential Trx-target proteins involved those in various pathways, including redox cycle, protein biosynthesis, energy metabolism and signal transduction. We captured 4 enzymes in glycolysis pathway (hexokinase, glyceraldehyde-3-phoshate dehydrogenase (GAPDH), phosphoglycerate mutase and L-lactate dehydrogenese) as the Trx-targets, and we found that PfTrx1 enhanced the activity of PfGAPDH. The results in this study indicate diversity and physiological significance of the Trx-mediated redox-regulation in the malaria parasite cell.

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USE OF MICROSATELLITE MARKERS TO DISTINGUISH RECRUDESCENCE FROM REINFECTION IN *PLASMODIUM VIVAX* INFECTIONS FROM THE PERUVIAN AMAZON BASIN

Valeria R. Soberon[1], **Carola J. Salas**[1], Andrea M. McCollum[2], Meddly Santolalla[1], Salomon Durand[1], Venkatachalam Udhayakumar[2], Carmen M. Lucas[1], David J. Bacon[1]

[1]United States Naval Medical Research Center Detachment, Lima, Peru, [2]Centers for Disease Control and Prevention, Malaria Branch, Atlanta, GA, United States

Malaria is endemic in the Peruvian Amazon Region and about 80% of the cases reported are caused by *Plasmodium vivax*. The main drugs used to treat *vivax* malaria are chloroquine (CQ), which acts against

the blood-stage parasites, and primaquine (PQ), which acts on the hypnozoite stage to prevent relapse. In some instances, symptoms re-appear after treatment. In clinical trials it is important to identify whether the re-appearance is due to: 1) blood-stage parasite recrudescence, 2) hypnozoite relapse or 3) a new infection. In low transmission and hypoendemic regions, such as the Peruvian Amazon basin, the probability of a patient being re-infected by a mosquito carrying the same parasite strain at a different time is highly unlikely. Thus, new infections are expected to be caused by genetically different *P. vivax* strains, whereas recrudescence and relapse should be caused by the same initial strain. We analyzed 6 neutral microsatellite loci in *P. vivax* isolates from 3 different localities (n=277) in the Peruvian Amazon basin to assess the genetic diversity of the populations. Loci were found to be highly polymorphic (Hz=0.72272±0.07054) in all 3 localities. We further analyzed 30 samples from a clinical study evaluating the efficacy of CQ plus different regimens of PQ at the beginning of the treatment (D0) and on the day of treatment failure (DF). All 6 neutral microsatellite loci were analyzed, as well as 5 polymorphic antigenic genes: *Pvama*, *Pvdbp*, *Pvcsp*, *Pvmsp1* and *Pvmsp3* for D0 and DF samples. Out of the 30 samples, 8 samples were identical in all 6 microsatellite loci from the D0 and DF pairs. The remaining 22 samples were different in at least 1 microsatellite locus. On the other hand, 12 samples out of 30 were genetically identical for D0 and DF for the polymorphic antigenic genes. Of those 12 samples, 6 had different D0 and DF haplotypes at one or more microsatellite loci. Thus, the neutral microsatellite markers are more sensitive for detecting differences between D0 and DF samples.

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GENETIC DIVERSITY ON RE-EMERGED *PLASMODIUM VIVAX* IN SOUTH AND NORTH KOREA

Yien Kyoung Choi[1], Kyung Mi Choi[1], Inho Park[2], Yeon Joo Kim[1], Shin Hyung Cho Cho[1], Jae Ran Yu[1], Jung Yeon Kim[1]

[1]National Institute of Health, Korea Centers for Disease Control and Prevention, Seoul, Republic of Korea, [2]Armed Forces Seoul Hospital, Seoul, Republic of Korea

Since 1993, malaria was re-emerged in DMZ region in Korea, the distribution of risk areas have varied and the portion of patients was changed. Current malaria in Korea seems to be endemic. In that regards, we have investigated the polymorphism of overall *Plasmodium vivax* strains based on MSP-1 and CSP genes in the DMZ, the endemic areas of South Korea and North Korea. At first, we analyzed PvMSP-1 gene from *P.vivax* isolates (n=632) of South Korean patient during 1996-2007, an extended period of time after its reemergence to its current status, indicating that the majority of the isolates recently collected were completely different in comparison with the samples in the initial reemergence period. There was initially only one subtype (recombinant) present but its subtypes have been varied since 2000. We investigated the genetic diversity based on MSP-1 and CSP genes in recent sample from the South, North Korea and DMZ patients. In results, we identified six of allelic sub-types: S-a, S-b and S-c (Sal-1), B-1, B-2 (Belem), and one recombinant sub-type. The results from South Korean patients, more than half of parasites were belong to Sal I types (Sal-1: 52%, Belem: 26%, Recombinant: 22%). However, MSP-1 diversity in malaria samples originated from North Korea, showed the majority of these samples also comprised the recombinant strain and the sequence including Q repeat region is same (Recombinant: 61%, Sal-1: 26%, Belem: 13%). A similar variation characteristic was observed via CSP genes analysis. 5 subtypes were identified and 2 CSP subtypes were newly found in this study. All the sequences of MSP-1 and CSP genes obtained in this study were phylogenetically analyzed. In addition, we analyzed the mixed infections with several subtypes of malaria parasite in the *P. vivax* population of three different loci (North Korea, DMZ and low endemic area of South Korea). Higher percentage (26%) of mixed infection rates were observed in North Korean patients. Genetic variation with multiple concurrent infections and the high transmission rates may help perpetuate malaria in the South and North Korea population. The present study indicated that the diversity might be more complicated every year and implied that vivax malaria in south Korea is getting indigenous although it has been largely influenced by North Korea in the past.

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LISP1 IS IMPORTANT FOR THE EGRESS OF *PLASMODIUM* PARASITES FROM LIVER CELLS

Tomoko Ishino[1], Bertrand Boisson[2], Yuki Orito[3], Céline Lacroix[2], Emmanuel Bischoff[2], Céline Loussert[2], Chris Janse[4], Robert Ménard[2], Masao Yuda[3], Patricia Baldacci[2]

[1]Ehime University, Toon, Japan, [2]Institut Pasteur, Paris, France, [3]Mie University, Tsu, Japan, [4]Leiden University Medical Centre, Leiden, The Netherlands

Most Apicomplexa are obligatory intracellular parasites that multiply inside a so-called parasitophorous vacuole (PV) formed upon parasite entry into the host cell. *Plasmodium*, the agent of malaria and the Apicomplexa most deadly to humans, multiplies in both hepatocytes and erythrocytes in the mammalian host. Although much has been learned on how Apicomplexa parasites invade host cells inside a PV, little is known of how they rupture the PV membrane and egress host cells. Here, we characterize a *Plasmodium* protein, called LISP1 (liver specific protein 1), which is specifically involved in parasite egress from hepatocytes. LISP1 is expressed late during parasite development inside hepatocytes and locates at the PV membrane. Intracellular parasites deficient in LISP1 develop into hepatic merozoites, which display normal infectivity to erythrocytes. However, LISP1-deficient liver stage parasites do not rupture the membrane of the PV and remain trapped inside hepatocytes. LISP1 is the first *Plasmodium* protein shown by gene targeting to be involved in the lysis of the PV membrane.

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A HIGHLY SENSITIVE REAL-TIME REVERSE TRANSCRIPTION-PCR ASSAY FOR DETECTION OF *PLASMODIUM FALCIPARUM* GAMETOCYTES USING A SINGLE AMPLIFICATION STEP

Laura Dickson[1], Ann Stewart[2], Shirley Luckhart[1]

[1]University of California, Davis, Davis, CA, United States, [2]United States Military Malaria Vaccine Program, United States Army Medical Research Unit-Kenya, Kisumu, Kenya

A real-time RT-PCR assay without nested primers has been developed to use as an efficient and highly sensitive assay for detection of gametocytes in patient blood. The *Plasmodium falciparum* Pfs25 protein is produced only in stage V gametocytes and, therefore, has potential to be a reliable target for gametocyte detection in clinical samples. The assay was designed to amplify Pfs25 cDNA prepared from RNA isolated from dried human blood on filter paper and was validated in the presence of potentially confounding human DNA from WBCs. The assay can reliably detect down to 0.25 gametocytes/microliter human blood with an amplification efficiency of 98%. Redesigned primer and probe sequences, the use of filter paper with proper storage, and a single amplification step enhanced detection sensitivity. We propose that this assay can be used to identify infectious reservoirs in populations and for transmission studies in the field.

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SEASONAL VARIATION OF *PLASMODIUM FALCIPARUM* APICAL MEMBRANE ANTIGEN 1 (*AMA-1*) HAPLOTYPES IN CHILDREN LIVING IN MALARIA ENDEMIC AREA OF BURKINA FASO

Soulama Issiaka[1], Nebie Issa[1], Bougouma C. Edith[1], Diarra Amidou[1], Sanon Souleymane[1], Tiono B. Alfred[1], Ouedraogo Alphonse[1], Yaro B. Jean Baptiste[1], Ouedraogo Espérance[1], Gansane Adama[1], Lankoande Malik[1], Konate T. Amadou[1], Sirima B. Sodiomon[2]

[1]Centre National de Recherche et de Formation sur le Paludisme, Ouagadougou, Burkina Faso, [2]Centre National de Recherche et de Formation sur le Paludisme/Groupe de Recherche et d'Action en Santé, Ouagadougou, Burkina Faso

The antigenic variation in *Plasmodium falciparum* is known to hamper malaria vaccine development because the antibodies elicited against one allele of an antigen may not necessarily inhibit the invasion of the parasite that can express different alleles of the antigen. AMA1, one of the potential components of asexual stage malaria vaccine, presents some extent of variation across the entire AMA1 sequence. Therefore, the assessment of *ama*-1 gene polymorphism from natural *P. falciparum* populations could be useful before initiating any vaccine trial using this antigen. The present study describes the seasonal extent of *ama*-1 domain I haplotypes diversity in *Plasmodium falciparum* parasite isolates in children under five years in Burkina Faso. Blood filter papers were collected from 133 and 144 children under five years of age during the low and high malaria transmission seasons, respectively. Parasite DNA was extracted by QIAGEN Kits and the haplotypes diversity assessed by a nested PCR following by digestion (enzyme restriction). Prevalences of AMA-1 haplotypes K1, 3D7 and HB3 during the low season were 31.8%, 25.2% and 43.0% respectively, while in high transmission season the prevalences were 17.2%, 33.8% and 49.0% respectively. A statistically significant difference (p=0.003) was observed between low and high transmission in K1 haplotypes distribution. However no seasonal difference was observed in 3D7 (p=0.1) and HB3 (p=0.3) haplotypes prevalences. These results suggest that AMA-1 K1 haplotypes prevalence may be affected by the level of malaria transmission, while 3D7 and HB3 haplotypes remained unchanged. This finding must be taken into account when designing malaria vaccine trial with this blood stage antigen.

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PROTOXIN CRY1AC INDUCES PROTECTION AGAINST *PLASMODIUM BERGHEI* ANKA AND *P. CHABAUDI* AS IN CBA/CA MICE

Martha Legorreta-Herrera, Rodrigo Oviedo-Meza, Leticia Moreno- Fierros

Universidad Nacional Autonoma de Mexico, Distrito Federal, Mexico

No malaria vaccine has provided a strong and lasting immune response due to different reasons for example: a short immunological memory, high antigenic variation, lack of knowledge about the protective immune mechanisms against *Plasmodium*, etc. The tendency in the development of new vaccines against malaria is to use the complete parasite. On the other hand, *Bacillus thuringensis* produces the potent toxin Cry1Ac which is a strong immunogen. This molecule induces an immune activation and protection against protozoa as *Naegleria flowlery* in a murine experimental model. In this work we analyzed the effect of Cry1Ac on the infection with *P. chabaudi* AS and *P.berghei* ANKA in CBA/Ca mice. Groups of CBA/Ca mice were weekly ip injected with 5 µg of Cry1Ac protein for 4 weeks, 24 h after last injection mice were iv infected with *P. chabaudi* AS or *P. berghei* ANKA. Two additional groups of mice not infected were administered with PBS or with the same amount of Cry1Ac protein as controls. Parasitaemia was evaluated daily in Giemsa stained thin slides, survival, levels of cytokines and levels of antibodies were also measured in serum. Mice treated with Cry1Ac developed lower parasitaemias than mice administered with PBS, in particular on day 8 after infection. *P. berghei* infected mice treated with Cry1Ac showed increased levels of IgM, IFN-γ and TNF-α compared to PBS treated mice. *P. chabaudi* AS infected mice treated with Cry1Ac increased the levels of total IgG. Our results show that Cry1Ac induces protection against *Plasmodium* infection detected as a higher survival, lower levels of parasitaemia and by increasing the levels of antibodies. The mechanism of action of Cry1Ac is not well known but it is possible that activates the innate immune response that allows decreasing the level of parasitaemia, and then the immune system could better deal with the remaining parasites. The knowledge of how this kind of immunogens works could help to develop efficient vaccines against malaria.

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THE IDENTIFICATION OF MOLECULAR MARKERS FOR DORMANCY IN *PLASMODIUM FALCIPARUM*

Alexis N. LaCrue, Dennis E. Kyle

University of South Florida, Tampa, FL, United States

Artemisinin compounds are fast-acting anti-malarial drugs that are effective against multi-drug-resistant *P. falciparum*; however, these compounds result in frequent recrudescence when administered alone. Our studies suggest that artemisinin drugs arrest ring-stage parasite development and that these dormant ring-stage parasites are responsible for this recrudescence. Distinguishing dormant rings from newly invaded merozoites, tiny rings, and Howell-Jolly bodies in giemsa stained blood smears can be difficult, therefore, in these studies we used real-time QPCR and fluorescent *in situ* hybridization (FISH) to find potential markers for dormancy in an effort to simplify the identification of dormant parasites. Using the *P. falciparum* W2 strain, preliminary real-time QPCR data suggests that genes involved in important parasite metabolic pathways are up-regulated in dormant parasites and FISH analysis has revealed three possible genes that are highly expressed by dormant parasites as compared with ring-stage parasites and free merozoites. Future studies include evaluating the dormant parasite markers on slides prepared from *P. falciparum*-infected patients. Ultimately, the findings from this research may help with determining if there is a correlation between the prevalence of dormant rings in peripheral blood and recrudescence.

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CLINICAL LABORATORY REFERENCE RANGES DERIVED FROM RURAL POPULATION OF HEALTH DISTRICT OF SAPONÉ IN BURKINA FASO DURING MALARIA HIGH TRANSMISSION SEASON

Adama Gansane[1], Amidou Diarra[1], Souleymane Sanon[1], Issiaka Soulama[1], Noëlie B. Henry[1], Amathe Ouedraogo[1], Alphonse Ouedraogo[1], Jean B. Yaro[1], Espérance Ouedraogo[1], Andre L. Ouedraogo[1], Edith C. Bougouma[1], Amadou T. Konate[1], Alfred B. Tiono[1], Sodiomon B. Sirima[2], Issa Nebie[1]

[1]Centre National de Recherche et de Formation sur le Paludisme (CNRFP), Ouagadougou, Burkina Faso, [2]Centre National de Recherche et de Formation sur le Paludisme (CNRFP), Groupe de Recherche et d'Action en Santé (GRAS), Ouagadougou, Burkina Faso

The reference intervals of biological parameters currently used in many countries of Africa are derived from populations living in Europe or America and it is likely that the distribution of these values in malaria endemic countries may not be identical to those from developed countries. In order to plan the conduct of malaria vaccine trials in Sapone Health District, a malaria endemic area in Burkina faso, we conducted a survey to establish the biological reference range for local population. In 2008, a community cross-sectional survey was conducted at the peak of malaria transmission season in 14 villages randomly selected in the Health District of Sapone. After written informed consent and clinical examination, blood samples were obtained from participants aged from 6 months to 45 years for full blood count and biochemistry analysis. The

full blood count and biochemistry analysis were performed respectively with an haematology and chemistry analyzers in the clinical lab of CNRFP involved in CAP proficiency testing. Data were analysed with Stata 9.0 and biological reference intervals were estimated using the 2.5 and 97.5 centiles by different age group (0.5-1, 1-3, 3-6, 6-10, 10-15 years) and gender for adults (>15 years). Extremes values of biological parameters were excluded in analysis. A total of 1304 participants fulfilled inclusion criteria were screened. Among them, 364 participants were excluded after clinical examination. During analysis 47 children were excluded for biochemistry blood collection failure and 45 for haematology blood collection failure. The 95% reference intervals for RBC, hemoglobin, WBC, hematocrit, platelets, lymphocyts, neutrophils, basophils, eosinophils, glucose, albumine, lactate, urea, alkaline phoshatase, AST, ALT, creatinine and electrolytes were estimated for each age group and gender and compared to those from Europe and America. In conclusion, reference intervals of haematological and biochemical indices based on results from population of developed countries of the same age are not in agreement with the estimated values for populations of the Health District of Saponé in Burkina Faso during the malaria high transmission season. These findings will support implementation of malaria vaccines trials in this area using site-specific biological references intervals for enrolment and monitoring of patients during malaria high transmission season.

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A POTENT MALARIA TRANSMISSION BLOCKING VACCINE BASED ON CODON-HARMONIZED PFS48/45

Debabani Roy Chowdhury[1], Evelina Angov[2], Thomas Kariuki[3], Nirbhay Kumar[1]

[1]Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, United States, [2]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [3]Institute of Primate Research, National Museums of Kenya, Nairobi, Kenya

Malaria caused by *Plasmodium falciparum* is responsible for nearly 1 million deaths annually. Although much progress has been made in the recent past, the development of a safe, effective and affordable malaria vaccine has remained a challenge. A vaccine targeting sexual stages will not only reduce malaria transmission by female anopeline mosquitoes, but also reduce spread of parasites becoming non-responsive to vaccines targeting pre-erythrocytic and erythrocytic asexual stages. Towards this end, we focused our studies on Pfs48/45, a protein expressed in the sexual stages developing within an infected person and one of the most promising transmission-blocking vaccine targets. Functional immunogenicity of Pfs48/45 protein requires proper disulfide bond formation, consequently evaluation of immunogenicity of recombinant form of full length Pfs48/45 has been hampered by difficulties in expressing properly folded protein to date. Here we present a strategy involving harmonization of the codons for successful recombinant expression of Pfs48/45 in *Escherichia coli*. The purified protein, designated CH-rPfs48/45, was recognized by monoclonal antibodies directed against reduction-sensitive conformational epitopes in the native protein. Immunogenicity evaluation in mice revealed potent transmission blocking activity in membrane feeding assays of antisera elicited by CH-rPfs48/45 formulated in three different adjuvants, i.e. Alum, Montanide ISA-51 and complete Freund's adjuvant. More importantly, CH-rPfs48/45 formulated with Montanide ISA-51 when administered to nonhuman primates (Olive baboons, *Papio anubis*) resulted in uniformly high antibody responses (ELISA titers > 2 million) in all five animals, which in membrane feeding assays displayed greater than 93% blocking activity after a single immunization reaching nearly complete blocking after a booster dose of the vaccine. High antibody titer and transmission blocking activity were retained in these animals for more than 6 months post last immunization. The relative ease of expression and induction of potent transmission blocking antibodies in mice and nonhuman primates provide the compelling rationale and basis for development of a CH-rPfs48/45 based malaria transmission blocking vaccine.

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ABNORMALITIES OF HAEMOGLOBIN AND *PLASMODIUM FALCIPARUM* MALARIA IN UNDER FIVE CHILDREN LIVING IN A HIGH AND SEASONAL MALARIA TRANSMISSION AREA OF BURKINA FASO

Edith C. Bougouma[1], Alfred Tiono[1], Amidou Diarra[1], Alphonse Ouedraogo[1], Issiaka Soulama[1], Adama Gansane[1], Jean Baptiste Yaro[1], Espérance Ouédraogo[1], Souleymane Sanon[1], Malick Lankouande[1], Amadou T. Konaté[1], Issa Nébié[1], Sodiomon B. Sirima[2]

[1]Centre National de Recherche et de Formation sur le Paludisme (CNRFP), Ouagadougou, Burkina Faso, [2]Centre National de Recherche et de Formation sur le Paludisme (CNRFP)/Groupe de Recherche et d'Action en Santé, Ouagadougou, Burkina Faso

Susceptibility of the human host to malaria infection has been reported to be influenced by some genetic factors which could be confounders if not taken into account in the assessment of the efficacy of future malaria vaccine candidates. This study aimed at assessing the relationship between abnormal haemoglobin genotypes and malaria infection in children in a site being characterised for future malaria vaccine trials. The study was carried out in four rural villages. It consisted of a combination of 2 cross-sectional and one longitudinal survey. During each cross-sectional survey, each child was clinically examined, thick and thin blood films were prepared for malaria parasites diagnosis and an additional blood was taken to determine the haemoglobin genotypes by PCR. A longitudinal survey was performed by active case detection to record malaria clinical episodes. The prevalence of Haemoglobin genotype was: AA (73.2%); AC (15.0%), AS (8.2%) and CS (1.1%) and SS (0.2%). At enrolment, during the first cross-sectional survey, the *P. falciparum* index was 64.7 in the normal haemoglobin group and 58.7% in the abnormal group. The geometric mean of parasite density was significantly higher in abnormal than in normal group (1556 vs 1788 Parasites/µl ($p<0.0001$). The same trend was observed during the second cross-sectional survey at the peak of malaria high transmission season. The overall malaria incidence was 0.7 child days at risk during the low transmission season and 1.8 child days at risk during the high transmission season. The incidence rate between the haemoglobin genotypes was observed to be 1.6 and 0.7 during the high and low transmission seasons respectively for the abnormal group. This was observed to be 1.4 and 0.6 for the normal haemoglobin during the high and low transmission seasons respectively. The difference in malaria incidence between the normal and the abnormal groups was significant ($P = 0.046$) during the high transmission season. However, no significant difference was observed during the low transmission season ($P = 0.05$). In conclusion, these findings suggest that whatever the season the presence of abnormal haemoglobin positively influence malaria infection in children living in malaria endemic areas and this should be taken into account when interpreting results of clinical trial studies

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CELL TRAVERSAL PROTEIN FOR OOKINETES AND SPOROZOITES (CELTOS) FROM *PLASMODIUM FALCIPARUM* ELICITS PROTECTIVE IMMUNITY IN MICE AGAINST HETEROLOGOUS CHALLENGE WITH *P. BERGHEI*

Elke S. Bergmann-Leitner, Kari M. Laquer, Elizabeth H. Duncan, Tatyana Savranskaya, Ryan M. Mease, Mark E. Polhemus, Christian F. Ockenhouse, Evelina Angov

Walter Reed Army Institute of Research, Silver Spring, MD, United States

The *Plasmodium* protein Cell-traversal protein for ookinetes and sporozoites (CelTOS) has been documented to play an essential role in cell traversal of host cells in both, mosquito and vertebrates and is required for successful infections. CelTOS is highly conserved among the *Plasmodium* species indicating that polymorphisms are incompatible with the functionality of the protein. We previously reported on the efficacy of *E. coli* expressed recombinant *P. berghei* CelTOS/Montanide

ISA 720 against a subcutaneous challenge with homologous *P. berghei* sporozoites. Based on these results we developed a codon-harmonized recombinant *P. falciparum* CelTOS that can be produced at high levels in an *E. coli* expression system. We immunized Balb/c and ICR mice with the recombinant protein adjuvanted with Montanide ISA-720 or with a pCI-TPA plasmid encoding the *P. falciparum* CelTOS (epidermal delivery by gene-gun) to characterize their abilities to induce protective responses against a heterologous *P. berghei* challenge. In each of three challenge experiments, fewer *P. falciparum* CelTOS/ISA 720 immunized mice developed malaria after heterologous *P. berghei* challenge than did control mice. Humoral and cellular immune responses induced by the protein or plasmid immunizations will be assessed in an effort to establish immune correlates. Moreover, we will report on the functional impact of CelTOS-specific antibodies on the motility and infectivity of sporozoites. These results emphasize the potential for use of this novel antigen as a candidate for pre-erythrocytic vaccine development.

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INACTIVATED *ESCHERICHIA COLI* EXPRESSING *PLASMODIUM BERGHEI* CSP EXPRESSED FROM DIFFERENT CELLULAR LOCALIZATIONS INDUCE DIFFERENTIAL IMMUNITY

Elizabeth Deriso[1], Elke S. Bergmann-Leitner[1], Jessica Whittington[2], Elizabeth H. Duncan[1], Katharine Boyle[1], Tatyana Savranskaya[1], Timothy Alefantis[2], Paul Grewal[2], Vito DelVecchio[2], Evelina Angov[1]

[1]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]Vital Probes, Inc., Mayfield, PA, United States

Inactivated bacteria (GeMI-Vax) are biological particles endowed with intrinsic adjuvant properties used to express and deliver a target antigen to different cellular compartments. We used the rodent *P. berghei* malaria as a model system to study pre-erythrocytic-stage vaccine efficacy in mice using the circumsporozoite protein (PbCSP) as the target delivered by GeMI-Vax from various localizations in *E. coli: (1)* lumen of the cytosol, (2) periplasmic space, and (3) outer membrane. We sought to investigate the effect of the different target antigen localizations on the quality and quantity of the resulting immune responses. We determined vaccine efficacy based on sterile protection against a subcutaneous challenge with *P. berghei* sporozoites. Immunization with the outer membrane construct resulted in 57% sterile protection, while the periplasmic and cytosolic constructs had only 42% and 14% efficacy, respectively, compared to empty GeMI-Vax. When analyzing the humoral immune responses by ELISA, only the outer membrane construct produced a statistically significant concentration of antibodies reacting with full length CSP protein. The antibody response was mainly directed to the central repeat region and the C-terminus. Cellular responses induced by the various constructs were evaluated by IFNγ and IL-4 specific ELISpot assays. Dose and localization of the antigen was able to modulate the type of immune response induced by the different GeMI-Vax constructs. We conclude that the GeMI-Vax system presents a valuable model system for studying the effects of antigen localization on the type and specificity of immune responses and for exploring the potential of target antigens as vaccine candidates in an adjuvant free system.

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HETEROLOGOUS PRIME-BOOST STRATEGIES USING DIFFERENT ALLELES OF $MSP1_{42}$ TO OVERCOME ALLELE-SPECIFIC IMMUNITY

Evelina Angov, Elizabeth H. Duncan, Kari M. Laquer, Ryan M. Mease, Elke S. Bergmann-Leitner

Walter Reed Army Institute of Research, Silver Spring, MD, United States

Plasmodium falciparum MSP1 is one of the leading erythrocytic stage malaria antigens under consideration as a vaccine constituent. Although it has been implicated in the primary association of merozoites with erythrocytes and results from several preclinical and seroepidemiological studies have shown that antibodies to $MSP1_{42}$, particularly to the $MSP1_{19}$, may be associated with protection and/or reduced parasite densities, confounding factors that exist such as pre-existing immunity, concurrent exposure and allelic heterogeneity have not been completely addressed. In malaria endemic areas, multiple strains of *P. falciparum* circulate at any given time, giving rise to complex immune responses. Better understanding of this interplay among antigenic specificities may enable improved vaccine design and suggest more effective vaccination regimens. Here we report results relevant to the potential for recombinant $MSP1_{42}$ subunit vaccines to prime and boost allele-specific and cross-reactive recall responses. Balb/C mice were immunized with various recombinant *P. falciparum* $MSP1_{42}$ proteins that represent the three major alleles of $MSP1_{42}$, 3D7, FVO, Camp/FUP, as well as a novel chimeric molecule formed between $MSP1_{33}$ 3D7and $MSP1_{19}$ FVO, here designated $MSP1_{42}$ 3DV. These immunization strategies were designed to evaluate T cell and B cell recall responses induced by homologous or heterologous boosting (for example, prime 3D7:boost 3D7 or prime 3D7:boost FVO). We will report (1) allele-specific antibody responses to each of the subunits of $MSP1_{42}$; p33, p19, EGF-like domain 1 and 2, induced by both homologous and heterologous immunization strategies measured by ELISA and by bead-based luminex, (2) MSP1-specific functional antibody activities against the various alleles measured by quantitative pLDH GIA, and (3) Th1 and Th2 cellular responses by IFNγ and IL-4 ELISpot assays.

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EFFECT OF HISTIDINE AFFINITY TAGS ON *PLASMODIUM FALCIPARUM* MSP1-42 PROTEIN STRUCTURE AND INDUCTION OF IMMUNITY

Farhat Khan[1], **Ryan M. Mease**[1], Patricia Legler[2], Elke S. Bergmann-Leitner[1], Evelina Angov[1]

[1]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]Walter Reed Army Institute of Research, Biochemistry, Silver Spring, MD, United States

Purification process development for malarial antigens has been greatly facilitated by incorporation of affinity tags into cloned sequences. These affinity tags have been introduced to primarily facilitate the solubilization and purification of these target molecules. However, these metal binding tags may influence the protein's solubility and susceptibility to aggregation, and alter immunogenicity by binding metals ions *in vivo* or during purification. Merozoite Surface Protein 1 (MSP1), an erythrocytic stage protein of *Plasmodium falciparum* and a potential candidate for malaria vaccine development, was used to evaluate the impact of an affinity-tag on protein structure and ultimately, induction of immunity. We cloned ($MSP1_{42}$) of *Plasmodium falciparum* FVO and 3D7 strains with and without the presence of a histidine tag. We examined the effects of such an affinity tag on bacterial protein expression levels, protein solubility and helical structure. We then immunized Balb/c mice with His-tagged or His-tag free recombinant protein of each strain adjuvanted with Montanide ISA-720. Recombinant proteins produced will be characterized by SDS-PAGE, circular dichroism, immunoblotting using disulfide-dependent conformational mAbs; and ELISA and pLDH growth inhibition assay for induction of antigen specific and functional antibodies against the parasite. We will analyze the effect of removing the His-tag from MSP1-42 recombinant proteins on their structure, total expression levels, and induced immunity in mice.

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IMMUNOGENECITY OF APICAL MEROZOITE ANTIGEN-1 CONJUGATES IN MICE

Christopher G. Rowe, David S. Jones, David Narum, Rich Shimp, Karine Reiter, Kelly Rausch, Emma Barnafo, Lynn Lambert, Kazutoyo Miura, Joan Aebig, Feng Qian, Louis Miller

Malaria Vaccine Development Branch, Rockville, MD, United States

The apical merozoite antigen-1 (AMA1) is important in the invasion of merozoites into red blood cells, and it is a leading blood-stage vaccine candidate for malaria. Recombinant AMA1 was chemically cross-linked with itself to make self-conjugates (AMA1-AMA1). It was also cross-linked with other proteins to make mixed conjugates. Mixed conjugates AMA1-EPA and AMA1-Pfs25 were prepared using recombinant *Pseudomonas aeruginosa* exoprotein A (EPA) and recombinant Pfs25, an antigen of interest for developing a transmission blocking vaccine. The proteins were cross-linked using thioether chemistry or adipic acid dihyrazide (ADH) cross-linking chemistries. Thioether conjugation involves the reaction of a thiolated protein with a maleimidylated protein. The ADH chemistry involves cross-linking by amide bond formation between protein carboxylic acids and ADH. The average molecular weights of the conjugates, ranging from 430 kDa to 20 MDa, depended both on the protein concentrations and the concentrations of linking groups used in the cross-linking reactions. Immunogenicity was tested in out-bred mice, Swiss-Webster or CD-1, by dosing subcutaneously on days 0, 14, and 28 with conjugates adsorbed on alum or in saline solution. In general, all conjugates retained immunogenicity, as determined by ELISA titers compared with titers obtained using unmodified AMA1 control formulations. Significant increases in titer were observed in some groups that received low doses (0.02 µg of AMA1-EPA/alhydrogel and 1.0 µg of the AMA1-ADH-AMA1/saline). The CD-1 mice exhibited significantly higher antibody titers than did the Swiss-Webster mice. We have developed procedures to prepare cross-linked AMA1 conjugates of various size using two different conjugation chemistries without loss of immunogenicity. Future studies will be designed to determine whether conjugation will improve the poor immunogenicity of AMA1, or other poorly immunogenic antigens, in humans.

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DEVELOPMENT OF A RECOMBINANT VACCINE BASED ON THE CIRCUMSPOROZOITE PROTEIN (CSP) OF *PLASMODIUM FALCIPARUM*

Jennifer Carter, Joshua Clayton, Aline Alexanian, Evelina Angov, Sheetij Dutta

Walter Reed Army Institute of Research, Silver Spring, MD, United States

Circumsporozoite protein encapsulates the surface of the invading sporozoite stage of the malaria parasite *Plasmodium falciparum*. A CSP based vaccine has been shown to protect adults and young children against malaria infection in endemic areas. Based on this protection data we wanted to develop a near-full length CSP that can be formulated and tested in combination with other malaria proteins and novel adjuvant systems. The CSP of *P. falciparum* contains a signal sequence, N-terminal region, followed by multiple repeats, a cysteine-rich C-terminal region and a GPI anchor sequence. Since, the N-terminal processing site of mature CSP on the parasite is not known, expression in *E. coli* was initiated with multiple constructs that either contain or lack the N-terminal cysteine residue. We also tested two codon optimization strategies and varying the number of repeats. Based on product stability one of the constructs was selected for further process development and yield optimization. A downstream process that includes two rounds of chromatography was developed to purify the protein. The final product was soluble and highly purified. The recombinant protein was also subjected to a series of biophysical characterizations including analytical size-exclusion chromatography, dynamic light scattering, native polyacrylamide gel electrophoresis, mass spectroscopy and electron microscopy. The biochemical and biophysical data showed an interesting pattern that may reflect upon the structure of the CSP. We are now conducting assays to determine if the properties of the recombinant protein also reflect those of the native CSP present on the sporozoite surface. Ongoing studies will shed light on the structure of the CS protein and how it might affect its immunogenicity.

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ROUTES OF MALARIA VACCINE AND CHALLENGE ADMINISTRATION: TRANSITION FROM MOSQUITO TO NEEDLE AND SYRINGE

Nicole M. Thomas[1], Judith Epstein[1], Sumana Chakravarty[2], Stephen L. Hoffman[2]

[1]Naval Medical Research Center, United States Military Malaria Vaccine Program, Silver Spring, MD, United States, [2]Sanaria Incorporated, Rockville, MD, United States

Humans can be successfully immunized against malaria by exposure to the bite of irradiated mosquitoes carrying *Plasmodium falciparum* sporozoites. Although highly protective, administration of a vaccine by mosquito bite is not feasible for widespread use. A radiation-attenuated, vialed and cryopreserved *P. falciparum* sporozoite vaccine (the PfSPZ Vaccine) has been developed, and is currently being evaluated in a Phase 1/2a clinical trial. The vaccine is being administered with a needle and syringe by intradermal (ID) or subcutaneous (SC) routes. To test for vaccine efficacy, the volunteers will be challenged by the bite of mosquitoes carrying infectious sporozoites. This efficacy can only be assessed in clinical centers with access to sporozoite-infected mosquitoes. If sporozoites could be vialed and inoculated by needle and syringe malaria challenge studies could be done at any clinical testing site. Developing the technology to manufacture the PfSPZ Vaccine has enabled the primary developer, Sanaria, Inc, to manufacture and vial fully infectious *P. falciparum* sporozoites (PfSPZ Challenge) in compliance with current Good Manufacturing Practices. It is anticipated that the first clinical trial of challenge of volunteers with PfSPZ Challenge will occur in 2010. In making this transition from mosquito to needle and syringe, it is important to be aware of experience with inoculation of sporozoites by needle and syringe. There is literature from 1926 to the 1960s in which intravenous (IV), ID, and SC routes were successfully used to infect patients with malaria parasites for the treatment of tertiary syphilis and testing of anti-malarials. In one report 100% of patients were infected by the ID inoculation of 10 *P. vivax* sporozoites. Plans for establishing a new challenge model and the data upon which these plans are based will be discussed.

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ENTOMOLOGICAL SUPPORT FROM PERU TOWARD DEVELOPMENT OF A SAFE AND REPRODUCIBLE *PLASMODIUM VIVAX* CHALLENGE SYSTEM IN THE U.S. MILITARY MALARIA VACCINE PROGRAM

Paul C. Graf[1], Kirk D. Mundal[1], **Carmen M. Lucas**[1], David J. Bacon[1], Benjamin J. Espinosa[1], Arturo Alvarado[2], Rosa Santillan Vadivia[2], Lourdes Jimenez Campos[3], Rommel Garrido Gonzales[3], Walter Vegas Olaya[2], Edwar Pozo Suclupe[2], Chris F. Ockenhouse[4], Thomas L. Richie[5], David J. Fryauff[5]

[1]Naval Medical Research Center Detachment, Lima, Peru, [2]Sub Region of Health Luciano Castillo Colonna, Sullana, Peru, [3]Bellavista Health Center, Sullana, Peru, [4]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [5]Naval Medical Research Center, Silver Spring, MD, United States

Plasmodium vivax causes the majority of malaria in deployed U.S. forces and is the most widely distributed of the four human malarias. It is generally less severe than *P. falciparum* but its relapsing character makes it a difficult and tenacious parasite to treat and control. While the U.S. military has had a safe human challenge system for *P. falciparum* for 20 years, owing to our ability to culture that parasite, *P. vivax* cannot be

cultured and no comparable challenge model exists for testing drugs and vaccines. We are attempting to overcome this barrier by large-scale production of a clean, laboratory colony of the local vector mosquito, *Anopheles albimanus*, in a region of northwestern Peru with nearly pure *P. vivax* endemicity. Colony mosquitoes artificially blood-fed and infected with local strains of *P. vivax* could support challenge studies. After 15 generations, our colony produces ~2500 female mosquitoes/week, with the important character of avid (~30%) feeding through an artificial membrane device. Informed, consenting patients with vivax malaria at the local clinic provide a sample of venous blood before receiving standard chloroquine + primaquine treatment. Part of the blood is immediately fed via the membrane system to a batch of colony mosquitoes; the remaining aliquot is tested to confirm *P. vivax* and to rule out other malaria species, chagas, syphilis, HIV, HTLV-1 and -2, HBV, and HCV. Mosquitoes are destroyed if any other pathogen besides *P. vivax* is detected. Serving a population of 27,350, the local Sullana health clinic has not recorded *P. falciparum* since 2004, but *P. vivax* has steadily risen with ~151 cases/mo during 2008. Thus far, blood testing has cleared 22/25 batches fed on donor blood; exclusions were due to syphilis (2) and HIV (1). Dissections of mosquitoes on day 7 found 57.6% to be oocyst-positive and on day 14 found 11.3% to be sporozoite-positive. Measures are being taken to increase the blood feeding rate, survivorship, and the infection rate of our colony-reared mosquitoes so that this resource can support challenge trials.

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PFMSP3 N-TERMINUS AS A VACCINE TARGET: CROSS-REACTIVE ANTIBODIES IN A HYPOENDEMIC TRANSMISSION ENVIRONMENT

Stephen J. Jordan[1], Ana L. Oliveira[1], Robert A. Oster[1], Oralee H. Branch[2], Julian C. Rayner[3]

[1]University of Alabama at Birmingham, Birmingham, AL, United States, [2]New York University, New York, NY, United States, [3]Wellcome Trust Sanger Institute, Cambridge, United Kingdom

Plasmodium falciparum Merozoite Surface Protein 3 (PfMSP3) is a strong candidate for inclusion in a blood stage vaccine cocktail. Like many merozoite surface proteins, PfMSP3 is polymorphic and vaccine development to date has focused largely on the C-terminal domain, which is more conserved. However field data suggests that the PfMSP3 N-terminus is much more immunogenic than the C-terminus, and antibodies against the N-terminal domain can correlate with protection from severe malaria. Given that the PfMSP3 N-terminus is polymorphic, it will only be useful as a vaccine target if cross-reactive antibodies can be generated to provide protection against different antigenic variants. To establish whether such cross-reactive antibodies develop *in vivo*, we have conducted a systematic study of antibody dynamics generated against each PfMSP3 domain in individuals living in a malaria-hypoendemic environment in the Peruvian Amazon. ELISA assays were carried out using two different PfMSP3 N-terminal antigens, based on the currently circulating genotypes present at the study site, as well as a PfMSP3 C-terminal antigen conserved in both alleles. Given the low transmission dynamics (less than one infection per person per year), individuals are usually infected with clonal *P. falciparum* infections spaced many months apart. All the infection samples used in the study have been previously genotyped for PfMSP3 allele and sequence diversity, allowing us to compare the immune response against both the currently infecting PfMSP3 antigen sequence and a PfMSP3 antigen that the individual has not been exposed to for at least one year. By measuring the strength, isotype and longevity of antibody responses against each antigen, we have found that there is a level of cross-reactivity between PfMSP3 N-terminal alleles that is equivalent to the reactivity against the PfMSP3 C-terminal antigen. These sera samples are also being used in *in vitro* assays to measure the effectiveness of the cross-reactive antibodies to induce cross-protection between different strains of *P. falciparum* parasites. This systematic study of anti-PfMSP3 domain-specific antibody responses is an important step in the go/no-go decisions that will inform future development of PfMSP3 as a vaccine target.

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IDENTIFICATION OF NOVEL BLOOD-STAGE VACCINE CANDIDATES AGAINST *PLASMODIUM FALCIPARUM* BY HIGH-THROUGHPUT IMMUNOSCREENING

Satoru Takeo[1], Hirokazu Sakamoto[1], Takamasa Kaneko[1], Mayumi Tachibana[1], Kazutoyo Miura[2], Sudhir Varma[3], Jetsumon Sattabongkot[4], Motomi Torii[1], Takafumi Tsuboi[1]

[1]Ehime University, Ehime, Japan, [2]MVDB/NIAID/National Institutes of Health, Rockville, MD, United States, [3]BCBB/NIAID/National Institutes of Health, Bethesda, MD, United States, [4]Armed Forces Research Institute of the Medical Sciences, Bangkok, Thailand

The reference genome sequence of *Plasmodium falciparum* (Pf) has opened the way to discovering novel malaria vaccine candidates amongst ~3,000 genes coding for unknown proteins. High-throughput system is crucial to identify candidate molecules, from recombinant protein synthesis to immunoscreening with a number of malaria immune serum samples. Here we exploited a high-throughput protein interaction assay system in combination with upstream wheat germ cell-free protein synthesis. We launched a proof of principle study with 170 biotinylated recombinant Pf putative merozoite stage proteins and applied them to the assay system on a tiny scale of 6 micro L human serum sample / 1x 384 well reaction plate. We focused on the difference in reactivity of serum samples against individual proteins between Pf asymptomatic and symptomatic groups (~15 separate serum samples each from Thai residents). This approach singled out <10 proteins with higher reactivity to asymptomatic sera than symptomatic group including an unknown protein as well as widely-known vaccine candidate antigens, making further candidate discovery study with more number of recombinant proteins feasible.

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ANOPHELES STEPHENSI D7 L: A SALIVARY GLAND PROTEIN WITH BOTH ANTI-INFLAMMATORY AND ANTIHEMOSTATIC EFFECT

Patricia H. Alvarenga, Eric Calvo, Ivo M. Francischetti, Anderson Sa-Nunes, Jose Marcos Ribeiro, John F. Andersen

National Institute of Allergy and Infectious Diseases/National Institutes of Health, Rockville, MD, United States

In order to feed on blood, disease-transmitting mosquitoes need to inhibit host hemostatic and inflammatory responses. One way to do this is to bind small-molecule effectors of host responses using specific salivary proteins. A highly expressed group of proteins, the "D7" family, carry out this function in the salivas of Aedes and Anopheles. We are currently elucidating the functions of these proteins in feeding and disease transmission using physical measurements of ligand binding, cellular bioassays, and X-ray crystallography. This study was undertaken to characterize *An. stephensi* D7 L, a member of the D7 family whose ligand-binding specificity is unknown. Screening of potential ligands using isothermal titration calorimetry (ITC) showed that *An. stephensi* D7L bound the cysteinyl leukotrienes (cys LTs) LTC4, LTD4 and LTE4 with very high affinities. Unlike other members of the family, it also bound thromboxane A2 (TXA2) analogs (U46619 and carbocyclic TXA2) with high affinity and was unable to bind biogenic amines. *An. stephensi* D7 L abrogated LTC4-induced contraction of guinea pig ileum ex-vivo. *In vivo* cell migration assays revealed no effect on leukocyte migration to the peritoneum in the presence of the protein. This is consistent with an observed lack of LTB4 binding in ITC experiments. Biological relevance of *An. stephensi* D7 L binding to TXA2 was demonstrated by its ability to inhibit platelet aggregation induced by both collagen and U46619 and to inhibit U46619-induced contraction of rat aorta preparations. Large crystals of both the wild-type protein and its selenomethionine derivative have been obtained, which we hope will allow solution of its structure in

the presence and absence of ligands. In conclusion, the results suggest that An. *stephensi* D7L is important in preventing itching, pain and erythema formation induced by cys LTs, as well as platelet aggregation induced by TXA2, and vasoconstriction induced by both. This is the first description of binding thromboxane A2 analogs by a D7 protein, demonstrating the evolution of new functions within the protein family.

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ROLE OF GLUTAMINE SYNTHETASE AND GLUTAMATE SYNTHASE (GS/GLTS) IN *AEDES AEGYPTI* FAT BODY METABOLISM

Patricia Scaraffia, Vicki Wysocki, Roger Miesfeld

University of Arizona, Tucson, AZ, United States

We have previously investigated the ammonia metabolism in mosquito whole body by using an *in vivo* approach. In this study we show how *Aedes aegypti* fat body responds to an ammonia challenge by using an *in vitro* approach. The studies have been performed using stable isotope labeling and mass spectrometry techniques. The kinetic of incorporation of $^{15}NH_4Cl$ into specific amino acids has been investigated during a time course by incubating fat body in a *Aedes* saline solution enriched with glucose, $^{15}NH_4Cl$ or ^{15}N-glutamine with ^{15}N in either the amide, amine nitrogen or in both amide and amine nitrogen atoms. The results indicate that fat body detoxifies ammonia by fixing ^{15}N into glutamate by glutamine synthetase (GS), which leads to a fast increase of [5-^{15}N]-Gln concentration during the time course studied. Part of [5-^{15}N]-Gln is then metabolized by glutamate synthase (GltS) yielding to [^{15}N]-Glu and [^{14}N]-Glu. The [^{15}N]-Glu produced by GltS can also be used by GS to fix other labeled ammonia and produce [2, 5-^{15}N]-Gln, which can be converted in two [^{15}N]-Glu molecules through GltS. Although alanine aminotransferase can catalyzes the transfer of the amino group of [^{15}N]-Glu to pyruvate for producing [^{15}N]-Ala, the data indicate that most of the [^{15}N]-Glu is used to synthesize [^{15}N-Pro] by pyrrolidine carboxylase synthase and pyrrolidine carboxylase reductase. Experiments performed using [2, 5-$^{15}N_2$]-Gln or [5-^{15}N]-Gln or [2-^{15}N]-Gln demonstrate that a significant amount of the glutamine is indeed utilized for proline synthesis through GltS. In the presence of [2, 5-$^{15}N_2$]-Gln, the concentration of [^{15}N]-Pro increase almost twice as much as in the presence of [5-^{15}N]-Gln or [2-^{15}N]-Gln. Furthermore, the participation of GltS in the metabolism of glutamine and its role in proline synthesis is also demonstrated by the fact that in the presence of azaserine, an inhibitor of GltS, the [^{15}N]-Pro concentration is significantly reduced. The results show that GS/GltS pathway plays an essential role for ammonia detoxification in *Ae. aegypti* fat body.

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MOLECULAR ANALYSIS OF PROTEASE FUNCTION IN THE MIDGUT OF BLOOD FED *AEDES AEGYPTI* MOSQUITOES

Jun Isoe, Alberto Rascon, Susan Kunz, Roger Miesfeld

University of Arizona, Tucson, AZ, United States

Inhibiting blood meal metabolism in vector mosquitoes could provide a novel strategy to control mosquito populations in areas of high pathogen transmission. Since 98% of the dry weight of the blood meal consists of protein, we reasoned that selective inhibition of major midgut proteases could result in a metabolic crisis for anautogenous mosquitoes that must obtain blood meal nutrients for reproduction. To identify which *Ae. aegypti* midgut proteases have the greatest potential as inhibitor targets, we used bioinformatic approaches to select the most highly expressed protease genes, and then systematically knocked-down their expression using RNAi in blood fed mosquitoes. Quantitative real-time PCR (QRTPCR) identified 5G1, CxLT, LPSP-1 (late trypsin), and SC-1, as the most highly induced protease genes in the mosquito midgut at 6-36 hours post bloodmeal (PBM). Three day old mosquitoes were injected with double stranded RNA (dsRNA) specific for each of the four proteases, or a control dsRNA (LUC), and midgut tissue of individual mosquitoes was analyzed at 24 hr PBM for knock-down efficiency at the RNA and protein (except SC-1) levels. We also used these same midgut samples to measure serine protease activity with the BApNA assay, and the extent of endogenous albumin protein degradation using an anti-BSA antibody. Our optimized single mosquito RNAi methods revealed that 90% of injected mosquitoes had a >90% knock-down of midgut protease expression based on QRTPCR and Western blotting. Moreover, knock-down of 5G1 and CxLT expression resulted in significant decreases in BApNA activity and albumin protein digestion compared to LUC controls, whereas, blocking LPSP-1 and SC-1 expression had no effect on these same parameters. The central role of 5G1 and CxLT in digestion was confirmed when we examined oocyte maturation and fecundity in dsRNA-injected mosquitoes and found that ovaries were under developed at 48 hrs PBM, and the number of oviposited eggs at 96-120 hrs PBM was reduced by up to 50% in mosquitoes injected with 5G1 and CxLT dsRNA, but not LUC dsRNA.

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BIOCHEMICAL ANALYSIS OF BLOOD MEAL-INDUCED MIDGUT PROTEASES IN *AEDES AEGYPTI* MOSQUITOES

Alberto Rascon, Jun Isoe, Roger Miesfeld

University of Arizona, Tucson, AZ, United States

Mosquito midgut proteases degrade blood meal proteins into amino acids that are required for protein synthesis and metabolic energy. Although the expression pattern of midgut proteases in blood fed *Ae. aegypti* mosquitoes is well understood, little is known about the biochemical properties of these essential enzymes. Digestion during the first few hours post-bloodmeal (PBM) is mediated by the early phase trypsin, whereas, late phase proteases are required to complete blood meal digestion from 12-36 hours PBM. Our biochemical studies are focused on determining the kinetic activity and substrate specificity of the 5G1, CxLT, and LT late phase proteases using recombinant protein expressed in *E. coli*. Initial studies using midgut protease cDNA sequences cloned into the pET28a expression vector produced high yields of protein, however, it was mostly insoluble. To solve this problem, we cloned cDNA sequences encoding both the zymogen and mature forms of the proteases into the pMALc4E vector, which increases protein solubility by fusing the maltose binding protein (MBP) to the N-terminus of the protein of interest. We have so far produced large amounts of soluble pMAL fusion proteins (>2 g/L), and have used amylose resin to purify several of the proteases to homogeneity. The recombinant zymogen and mature forms of the 5G1 protease are being used initially to quantitate *in vitro* trypsin activity by BApNA assays as 5G1 has been shown to have the highest *in vivo* level of trypsin activity based on RNAi experiments. We have also begun to explore methods to activate the full-length zymogen forms of all three purified enzymes using *in vitro* conditions for autocatalysis, or by incubating recombinant zymogen protein with mosquito midgut protein extracts, which could lead to the identification of a blood meal-induced proteolytic cascade. The objective of these studies is to determine what role 5G1, CxLT, and LT have in the digestive process, and if one or more of these proteases may be suitable as mosquito-selective targets for vector control.

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FATTY ACID SYNTHASE (FAS1) AND THE CD36 FATTY ACID TRANSPORT PROTEIN ARE REQUIRED FOR THE CONVERSION OF BLOOD MEAL PROTEINS INTO STORED LIPIDS IN *AEDES AEGYPTI* MOSQUITOES

Amy Alabaster, James Morton, **Roger Miesfeld**

University of Arizona, Tucson, AZ, United States

Aedes aegypti mosquitoes are responsible for the transmission of Yellow Fever virus and Dengue virus, both of which are human pathogenic viruses that are increasing in prevalence worldwide and can cause fatality. While broad-spectrum insecticides have provided a means to control mosquito populations in areas where pathogens are readily transmitted, collateral damage to other organisms in the environment can occur. To develop a more selective approach to mosquito control, we are using RNAi

methods to first determine which enzymes are required for blood meal metabolism, and then utilizing bioinformatic and biochemical analyses to select candidate proteins for future high throughput drug screens. The long term goal is to identify small molecule inhibitors that block blood meal metabolism in vector mosquitoes, and either cause feeding-induced death to prevent disease transmission, or significantly reduce fecundity as a form of population control. In addition to an acute metabolic need to rapidly digest blood meal proteins, and to detoxify ammonia resulting from transamination reactions, *Ae. aegypti* mosquitoes convert ~15% of the scavenged carbon into newly synthesized lipids for egg development and energy storage. We have now used our approach to screen lipid metabolizing enzymes in blood fed *Ae. aegypti* by knocking-down the expression of fatty acid synthase (FAS1) and the CD36 fatty acid transporter genes. In the case of FAS1, we were able to achieve 87% knock-down of FAS1 transcripts in the fat body at 48 hr post-feeding, as compared to mosquitoes injected with a dsRNA control (LUC). This level of FAS1 knock-down was associated with a 45% decrease in follicle length and a 35% decrease in oviposited eggs. Similarly, a 93% decrease in CD36 transcript levels led to a 33% decrease in follicle length and a 24-48 hr delay in oviposition. Quantitative metabolic labeling studies are currently underway to determine what effect FAS1 and CD36 knock-downs have on the conversion of ^{14}C-protein into ^{14}C-lipid in the fat body and ovaries of blood fed mosquitoes.

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AN RNAI-BASED FORWARD GENETIC TOOL FOR ANALYSIS OF MOSQUITO CELLULAR RESPONSES TO DENGUE VIRUS INFECTION

Baojun Yang, Qi Li, C. Bruce Cropp, **Hongwei Li**

University of Hawaii, Honolulu, HI, United States

Dengue is the most prevalent mosquito-borne viral disease in humans, and epidemic in tropical and subtropical regions worldwide with about 50-100 million cases of infection annually. Transmission of dengue virus (DENV) is mediated by *Aedes* mosquitoes, including the primary vector *Ae. aegypti* and the secondary vector *Ae. albopictus*. Development of new strategies to interrupt DENV transmission will largely rely on further understanding of molecular DENV-mosquito interactions. However, there is still no genetic tool available for functional genomic studies of mosquito responses to DENV. In this study, we have developed an RNAi-based forward genetic approach for identification of mosquito genes that affect DENV infection. A plasmid-based dsRNA expression system has been established in *Drosophila* and mosquito cells, and dsRNAs synthesized by T7 RNA polymerase can efficiently knockdown expression of a transiently expressed reporter or an endogenous gene. Based on the dsRNA expression vector, subtractive cDNA libraries were constructed from DENV-infected *Ae. albopictus* C6/36 cells, the most common used mosquito cell line for DENV. In our initial assay, genes that are differentially expressed by DENV infection were identified and analyzed, including proteins involved in vesicle transportation, cellular metabolism, antioxidation, cellular trafficking, and also putative transmembrane proteins. Our results indicate that the dsRNA expression system can be used as a powerful tool for functional genomic studies of DENV-mosquito interactions, and would also be useful in genome-wide studies of specific cellular pathways in mosquitoes and other arthropod vectors.

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INSULIN SIGNALING IN THE MIDGUT OF *ANOPHELES STEPHENSI* MOSQUITOES IMPACTS LIFESPAN

Michael A. Riehle, Laurel Watkins de Jong, Vanessa Corby-Harris, Rolf Ziegler

University of Arizona, Tucson, AZ, United States

The insulin/insulin growth factor 1 signaling (IIS) cascade is a key regulator of lifespan and reproduction in a wide range of organisms, including mosquitoes. To assess what role the IIS cascade in the mosquito's midgut plays in lifespan, we genetically engineered *An. stephensi* mosquitoes to have either increased or decreased insulin signaling specifically in this tissue. This was accomplished by linking either an active form of the IIS molecule Akt or the IIS inhibitor PTEN to the midgut specific carboxypeptidase promoter. Female mosquitoes engineered with AsteAkt or AstePTEN6 expressed the transgene only in the midgut, both before and during a reproductive cycle. The AsteAkt transgene was capable of phosphorylating and activating FOXO, a downstream component of the IIS cascade, indicating that it was not simply overexpressed, but physiologically active. To assess what impact this increased insulin signaling had on the mosquito's lifespan, a series of mortality studies were performed. As expected, a significant reduction in lifespan was observed in transgenic mosquitoes overexpressing AsteAkt. Transgenic mosquitoes had higher mortality rates than wild-type mosquitoes when given either sugar alone or weekly bloodmeals supplemented with sugar. Transgenic mosquitoes given weekly bloodmeals had an average lifespan of only 15 days, compared with 20 days in wild-type mosquitoes, a reduction of 25%. Likewise, 90% mortality occurred at day 31 in transgenic mosquitoes compared to day 40 in wild-type mosquitoes, a 23% reduction. A similar increase in mortality rate was observed in transgenic mosquitoes fed sugar only. Thus, increased insulin signaling in the midguts of *An. stephensi* mosquitoes leads to a significant reduction in lifespan and may have potential as a novel control strategy in the future.

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BIOMARKERS OF PHYSIOLOGICAL AGE IN *ANOPHELES STEPHENSI*

Vanessa Corby-Harris, Michael A. Riehle

University of Arizona, Tucson, AZ, United States

Parasites with complex life stages, such as malaria, require relatively long extrinsic incubation periods (EIP; 10-12 days) relative to the lifespan of their hosts (14-20 days). Due to this EIP, the oldest mosquitoes are the primary transmitters of disease. Accurately assessing the age structure of a vector population is important, as it reflects that population's ability to transmit disease. Age-related changes such as the accumulation of age related pigments (e.g., lipofuscin), age-related oxidative damage to the mitochondria (e.g., aconitase activity), and differences in gene expression have been proposed as being biomarkers of physiological age in invertebrates. We tested whether these biomarkers correlated with physiological aging in *Anopheles stephensi*. Specifically, we tested if 1) lipofuscin accumulated, 2) aconitase activity increased, and 3) the expression of key aging genes changed with respect to mosquito age, blood feeding, and mutations in the insulin signaling pathway, an important regulator of aging in invertebrates. This data will allow us not only to understand the processes underlying aging in invertebrates, but also to develop strategies for assessing the transmission potential of mosquito populations.

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THE MOSQUITO HOMOLOGUE OF METHOPRENE-TOLERANT PROTEIN IS A TRANSCRIPTIONAL REGULATOR MODULATED BY INSECT JUVENILE HORMONE

Jinsong Zhu, Meng Li, Jeff Busche, Xing Zhang

Virginia Tech, Blacksburg, VA, United States

Insect juvenile hormone (JH) in mosquitoes controls many important developmental and physiological processes, such as metamorphosis, behavior development in adults and egg production. Synthetic analogues of this hormone are widely used as pesticides because they prevent immature mosquito larvae from becoming disease-spreading adults. While the increasing tolerance to these pesticides is observed in mosquito population in the field, the nature of receptor and molecular targets of this hormone remains largely unknown. The *Methoprene-tolerant* (*Met*) gene, encoding a bHLH-PAS protein, has been implicated in the juvenile hormone regulatory pathways in fruit flies and the red flour beetles. To

elucidate function of the mosquito Met homologue, we knocked down its expression in the adult female mosquitoes by utilizing double stranded RNA-mediated gene silencing. The results indicated that Met in the midgut of newly emerged female adults was required for JH-stimulated transcription of genes encoding early trypsin and Krüppel homolog 1, but not for several other JH target genes in the same tissue, suggesting existence of multiple regulatory pathways in response to JH. Yeast two hybrid screening using the bHLH-PAS domain of Met as bait yielded a cDNA clone encoding a transcriptional factor, tentatively named MIF. The bHLH-PAS domain of Met protein, when fused with the GAL4 DNA binding domain, activates expression of a UAS-luciferase reporter gene in *Drosophila* L57 cells only in the presence of JH or its analogues. The reporter activity is further boosted in a JH-dependent manner when the Met-GAL4 fusion is expressed in cells together with MIF. RNAi knockdown of MIF also leads to significant downregulation of the early trypsin gene in the midgut of newly emerged mosquitoes. Together these data suggest that Met is a transcriptional regulator whose activity is modulated by direct binding of JH.

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MOLECULAR BASIS OF THE ESSENTIAL AMINO ACID ABSORPTION IN VECTOR MOSQUITOES

Dmitri Y. Boudko[1], Melissa M. Miller[2], Dmitry A. Voronov[3], Ella A. Meleshkevitch[1]

[1]Rosalind Franklin University, North Chicago, IL, United States, [2]The Whitney Laboratory, University of Florida, St. Augustine, FL, United States, [3]Institute for Information Transmission Problems, Russian Academy of Sciences, Moscow, Russian Federation

The membrane transport of L-phenylalanine, L-tryptophan and L-methionine is essential in mosquito. We have discovered and partly characterized the unique population of essential amino acid transporters in two vector mosquitoes, *Aedes aegypti* and *Anopheles gambiae*. The identified transporters belong to the Nutrient Amino acid Transporters (NATs) subfamily of the Neurotransmitter Sodium Symporter or SoLute Carrier 6 family (NSS or SLC6). NAT-SLC6 possesses striking paralogous divergence, clustering together 7 Anopheles and 9 Aedes NATs, whereas 6 mammalian and 6 nematode NATs form upper and lower paralogous clusters, respectively. In addition to ubiquitous broad spectra neutral amino acid transporters (B0-type system) that exist in mammals and insects (AeAAT1, AeNAT1, AgNAT1), mosquito-specific NAT population includes several unique narrow substrate spectra transporters that so far unknown in other metazoan organisms. Specifically, we have cloned 3 mosquito NATs with narrow selectivity for either: Phe/phenol-branched substrates (AgNAT8), Trp/indole-branched substrates (AgNAT6), or Met + Cys/ sulfur-containing substrates (AeNAT5). The mosquito NATs were localized ubiquitously with elevated expression in the apical membrane of the posterior gut, where they absorb digested nutrient amino acids, and in specific central and sensory neurons of the insect nervous system, where they supply essential precursors of neurotransmitters. Evidently, narrow spectra NATs have evolved to enhance competitive absorptions of the most underrepresented essential substrates. Based on the functional and expression data mosquito NATs are critical substrate providers in larval development and blood meal processing. They also play important roles in cuticle and egg chorion tanning, peritrophyc membrane polymerization, salivary enzyme secretion, immune responses and free-radical defenses. The paralogous divergence and essential roles of NATs specify them as conceivable targets for the selective control of arthropod vector and pest organisms.

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PROTEOLYTIC PROCESSING OF *ANOPHELES* SGS: CANDIDATE RECEPTORS FOR SALIVARY GLAND INVASION BY *PLASMODIUM* SPOROZOITES

Jonas G. King, Julián F. Hillyer

Vanderbilt University, Nashville, TN, United States

Malaria kills millions of people each year and exacerbates economic troubles in endemic regions. Invasion of the female mosquito's salivary glands is an important *Plasmodium*-mosquito interaction that is required for transmission to vertebrates. A large membrane-bound protein named AaSGS1 was recently shown to be important during the invasion of *Aedes aegypti* salivary glands by *Plasmodium gallinaceum* sporozoites and was reported to localize only on the basal surface of the salivary glands. Four homologs were identified bioinformatically in the *Anopheles gambiae* genome and named AgSGS2, 3, 4 and 5. We previously showed that the expression of AgSGS4 and AgSGS5 is specific to salivary glands in adult females and that AgSGS4 and 5 proteins localize primarily in the distal-lateral lobes, the region sporozoites preferentially invade. Here we show that *Anopheles* SGS are not exclusively localized on the basal side of the salivary glands as previously believed, but instead are proteolytically processed and released into the hemocoel and saliva. Western analyses using antibodies recognizing different regions of the protein showed that a ~245 kDa N-terminal fragment of AgSGS4, and not AgSGS5, is released into the hemolymph within hours of thoracic injury. Similarly, a ~245 N-terminal fragment of AgSGS5, and not AgSGS4, is released into the hemolymph after blood feeding. Induced salivation followed by Western analyses showed that a 300 kDa fragment similar to that observed in salivary gland lysate is present in the saliva, suggesting a role in blood feeding. The masses of observed fragments correspond to bioinformatically predicted serine protease cleavage sites that suggest that SGSs may function in immunity and wound healing. Hypothesized SGS functions will be outlined, along with ongoing experiments exploring the role of AgSGS in *Plasmodium-Anopheles* interactions.

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EFFECTS OF HUMAN IGF1 ON INSULIN SIGNALING IN THE MALARIA VECTOR *ANOPHELES STEPHENSI*

Anna Drexler, Kong Cheung, Nazzy Pakpour, Shirley Luckhart

University of California, Davis, Davis, CA, United States

Malaria remains a great public health challenge and new targets to interrupt transmission are crucial to the future of malaria control. Severe malaria changes the composition of human blood, disrupting nutrient metabolism and altering levels of regulatory factors like insulin and insulin-like growth factor 1 (IGF1). The highly conserved insulin/IGF-like signaling (IIS) pathway regulates metabolism, development, lifespan, and immunity. Previous studies in our laboratory have shown that human insulin ingested in the blood meal stimulates endogenous mosquito IIS, attenuates lifespan and enhances a key immune response: the production of reactive oxygen species (ROS) for anti-parasite defense. Since human IGF1 (hIGF1) occurs at higher concentrations than human insulin and is closely tied to stress responses, we are interested in the effects of hIGF1 on mosquito physiology and the development of malaria parasites in the mosquito. Activation levels of IIS proteins were determined by Western blotting analyses of cultured mosquito cells and of mosquito midgut tissue. Preliminary results showed that hIGF1 activated mosquito Akt and FOXO, two key IIS molecules. Similarly, mosquitoes fed hIGF1 in the blood meal showed increased levels of activated Akt and FOXO in midgut tissue. Effects of hIGF1 treatment on parasite growth and infectivity were determined by flow cytometry and histological examination of infected mosquito midguts, respectively. Preliminary results indicated that hIGF1 does not induce growth of cultured *Plasmodium falciparum*, but that ingested hIGF1 may alter mosquito responses to malaria parasite infection. The IIS pathway is a promising target for transmission control and could be used to reduce mosquito lifespan or to enhance innate mucosal immune

processes to kill malaria parasites in the midgut. These studies enhance our understanding of the effects of human blood factors on malaria parasite transmission and will ultimately support the development of novel strategies for control.

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GENETIC STRUCTURE OF *AEDES AEGYPTI* (DIPTERA: CULICIDAE) USING MICROSATELLITE AND MITOCHONDRIAL MARKERS IN CAMEROON (CENTRAL AFRICA)

Basile Kamgang[1], Cécile Brengues[2], Frédéric Simard[3], Jean Pierre Herve[2], Christophe Paupy[1]

[1]Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale, Yaoundé, Cameroon, [2]Institut de Recherche pour le Développement, Montpellier, France, [3]Institut de Recherche pour le Développement, Bobo-Dioulasso, Burkina Faso

Aedes aegypti is considered as the worldwide vector of dengue, yellow fever and chikungunya. In West-Central Africa, the species is summarized as sylvan, breeding far from human dwellings and biting preferentially animals rather than humans. Therefore this mosquito is not considered as an efficient vector. However, its biology in this area is actually much more complex since this mosquito occurs in a wide spectrum of environments ranging from sylvan to urban. Also its diversity and its implication in transmission of arboviruses are probably largely underestimated. In order to better assess the diversity of *Ae. aegypti* in Central Africa, we undertook a study on the genetic diversity and structure of 19 populations originated from Cameroon. Samples were collected in 2007 according to North-South and West-East geographical transects representing all main bioclimatic regions. In each location, specimens were collected as larvae/pupae and reared to adult stage. In addition to morphological characters (McClelland's criteria), we used seven microsatellite and two mitochondrial markers (COI and ND4). All microsatellites were found polymorphic. Across all loci, allelic richness and heterozygozity ranged respectively from 2.6 to 4.0 (3.4 +/- 0.4) and from 0.33 to 0.52 (0.45 +/- 0.05), suggesting high level of genetic diversity across populations. Analysis from microsatellite data also revealed a strong genetic structure of populations (Fst =0.080, P<10-6); using the bayesian approach results suggested two genetic clusters. The analysis of COI and ND4 genes resulted in the detection of 30 (S= 50; ϖ = 0.090) and 10 (S= 18; ϖ = 0.002) haplotypes, respectively, confirming thus the high level of diversity in Cameroonian populations in comparison with data previously described outside Africa. The phylogeographical analysis of mtDNA polymorphism suggested the possible existence of two lineages across cameroonian populations of *Ae. aegypti* and recent demographic changes. The origin of these lineages and their implications on the epidemiology of arboviruses in Cameroon are discussed.

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MICROGEOGRAPHIC GENETIC DIVERSITY OF THE MALARIA VECTOR *ANOPHELES DARLINGI* FROM CORDOBA AND ANTIOQUIA, COLOMBIA

Lina A. Gutiérrez[1], Giovan F. Gómez[1], John J. González[2], Martha I. Castro[2], Shirley Luckhart[3], Jan E. Conn[4], Margarita M. Correa[1]

[1]Grupo de Microbiología Molecular, Escuela de Microbiología, Universidad de Antioquia, Medellín, Colombia, [2]Unidad de Entomología, Laboratorio de Salud Pública, Departamento de Córdoba, Montería, Colombia, [3]Department of Medical Microbiology and Immunology, University of California, Davis, CA, United States, [4]Griffin Laboratory, Wadsworth Center, New York State Department of Health, Albany, NY, United States

Anopheles darlingi competence for transmitting *Plasmodium falciparum*, *P. vivax* and *P. malariae* has been documented. It is an important malaria vector in various endemic regions such as Antioquia and Cordoba, two states in western Colombia. Because knowledge of vector population genetics is important for malaria control programs, this study was conducted to test genetic variation of *An. darlingi* at the microgeographic scale (~100 km), from five localities in Cordoba and Antioquia. Eight microsatellite loci and *COI* gene sequences were analyzed. Estimated F_{ST} indicated moderate to low genetic differentiation (MS F_{ST}= 0.01343; *COI* F_{ST} =0.02457) between states and estimates of *COI* nucleotide divergence were similar among populations from both states. mtDNA data agreed with microsatellite results showing the existence of demographic equilibrium with no signal of bottleneck and a common demographic history between *An. darlingi* populations from Cordoba and Antioquia. A neighbor-net network showed that *An. darlingi* populations from Colombia are genetically closer to Central American populations and more differentiated from populations of Brazil, Peru and French Guiana. This study suggests the existence of high gene flow between *An. darlingi* populations of Cordoba and Antioquia and therefore, integrated vector control strategies could be developed for this region.

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MOSQUITO VISION: MOLECULAR EVOLUTION AND FUNCTIONAL CHARACTERIZATION OF THE OPSINS IN *ANOPHELES GAMBIAE* AND *AEDES AEGYPTI*

Gloria I. Giraldo-Calderon, Michael J. Zanis, Catherine A. Hill

Purdue University, West Lafayette, IN, United States

A novel strategy to reduce incidence of vector-borne diseases involves manipulating mosquito vision to disrupt mating, host detection or oviposition such that their populations and vector capacity decline. Developing an understanding of mosquito vision at the molecular level is fundamental to explore the feasibility of this approach. Opsins are G-protein coupled receptors (GPCRs) that interact with photons to initiate a phototransduction signaling cascade, involved with a visual response. Typically, insects have three classes of opsins to visualize ultraviolet, short, or long light wavelengths. Gene expression of arthropod opsins is detected in the eye and that of the related gene pteropsin, which is believed to regulate circadian rhythm, is detected in the brain. Previously, we identified 11 and 10 opsin genes in *Anopheles gambiae* and *Aedes aegypti*, respectively, as part of the complete genome sequencing projects. Here, newly-available expressed sequence tag (EST) data and homology-based analyses were used to improve opsin annotations. An extensive reverse transcriptase PCR (RT-PCR) study revealed sex and stage specific expression of opsins in *A. aegypti*. Expression of all opsins except *op7* and *op11* was confirmed in *A. gambiae* adult using a real time quantitative RT-PCR approach. *In situ* hybridization of *op3* and *op8* in *A. aegypti* was consistent with that reported for orthologs in the *Drosophila melanogaster* eye. Phylogentic analyses were conducted using ~300 opsin gene sequences from invertebrates and vertebrates and used to predict the function of each mosquito opsin. Our phylogenetic analyses indicate that a complex pattern of duplication events underlie the radiation of the opsin gene family in insects, been this novel paralogous gene lineages with respect to vertebrates. The long wavelength opsin gene family has expanded in mosquitoes; perhaps this is tied to their visual needs during periods of low light intensity when they are most active. Future research is aimed at characterizing the spectral sensitivity of mosquitoes opsins to confirm their functions.

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POPULATION DEMOGRAPHY OF MALARIA VECTOR *ANOPHELES* (*ANOPHELES*) *PSEUDOPUNCTIPENNIS* FROM ARGENTINA

María J. Dantur Juri[1], Monica J. Prado Izaguirre[2], Juan C. Navarro[2], Guillermo L. Claps[1], Jan E. Conn[3]

[1]Instituto Superior de Entomologia, Facultad de Ciencias Naturales e I.M.L., Universidad Nacional de Tucumán, San Miguel de Tucumán- Tucumán, Argentina, [2]Laboratorio de Biología de Vectores, Instituto de Zoología Tropical, Universidad Central de Venezuela, Caracas, Venezuela, [3]Griffin Laboratory, Wadsworth Center, New York State Department of Health, Albany, NY, United States

Anopheles pseudopunctipennis is an important malaria vector in some areas of America, where this disease is still a problem in public health. Its wide geographical distribution includes several ecologically distinctive areas, only some of which are endemic for malaria transmission. Some markers provide evidence for *An. pseudopunctipennis* as a complex. In the present study, we analyzed the population structure and demography of *An. pseudopunctipennis* in two regions of NW Argentina, northern and southern, using cytochrome oxidase subunit I (COI). We identified 41 haplotypes, of which seven were shared among all samples localities, comprising 77% of all individuals examined. A statistical parsimony network showed that haplotype A was the most common and widespread and considered ancestral. The F_{ST} values were the highest between two southern localities. The Neighbor Joining tree based on the F_{ST} distances was poorly resolved, and did not detect any structure among the NW Argentinian populations. San Roquito (Tartagal) and Potrero Las Tablas were the populations with the highest haplotype diversities in both areas (0.805 and 1.000, respectively). Nucleotide diversity was similar in all populations. The highest average number of nucleotide differences (*K*) was observed for San Roquito Tartagal (0.00179) and Tucumán Sur (0.00262). G_{ST} and N_M values showed the pairwise genetic differentiation and the gene flow between different populations. Similar to the F_{ST} results, G_{ST} detected the highest values between two southern localities. The mismatch distribution in Aguas Blancas and Yuto (northern) and Rosario la Frontera and Tucumán Sur (southern) a smooth unimodal distribution characteristic of a population expansion. The time of the expansion of *An. pseudopunctipennis* varies among localities but all are during the Pleistocene. In the future we hope to carry on an extensive study including samples of *An. pseudopunctipennis* from different countries of America to evaluate the possible presence of lineages influenced geographic barriers.

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GENE EXPRESSION PROFILE ASSOCIATED WITH BLOOD FEEDING AND *PLASMODIUM FALCIPARUM* INFECTION IN THE MALARIA VECTOR *ANOPHELES FUNESTUS*

Mamadou B. Coulibaly[1], Brehima Diallo[1], Amadou Guindo[1], Mohamed M. Traore[1], Sekou A. Traore[1], Mamadou Konate[1], Jennifer M. Anderson[2], Neil F. Lobo[3], Nora J. Besansky[3], Jose M. Ribeiro[2], Sekou F. Traore[1]

[1]Malaria Research and Training Center, Bamako, Mali, [2]National Institutes of Health, Bethesda, MD, United States, [3]University of Notre Dame, Notre Dame, IN, United States

Anopheles funestus is the second major malaria vector in Mali and plays a primary role in certain other African countries, yet there are very few studies on it compared to *Anopheles gambiae*. Genetic control of malaria necessitates identifying genes important for vector reproduction and vector-parasite interaction. To identify those genes we proposed to establish a gene expression profile associated with blood feeding and *Plasmodium falciparum* infection in *An. funestus*. Mosquitoes were collected from Niono, Mali and identified to species. The first generation progeny were stored in RNAlater at different developmental stages (eggs, larvae, pupae, adults). Adults were collected at different abdomen stages (unfed, sugar fed, non-infected blood fed and infected blood fed). RNA was extracted from each category and used to construct a normalized cDNA library from which clones were sequenced. Bioinformatics tools were used to analyze the sequences. 3739 high quality ESTs were produced with an average length of 641 nucleotides. These were assembled into 2962 contigs of which 2149 were singletons. Comparison of the sequences with the only existing database (dbEST) for *An. funestus* revealed 1312 ESTs not described before. These results will contribute to the understanding of the *An. funestus* genomics. Additional sequencing will be conducted and selected annotated sequences will be used to generate a microarray chip for expression studies. Quantitative real-time PCR will then be used for confirmation.

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ROBUST SALIVARY GLAND-SPECIFIC GENE EXPRESSION IN TRANSGENIC *AEDES AEGYPTI*: DIVERGENT EXPRESSION OF TWO TRANSGENES DRIVEN BY A BI-DIRECTIONAL PROMOTER

Geetika Mathur, Osvaldo Marinotti, Danielle Alvarez, Anthony A. James

University of California Irvine, Irvine, CA, United States

The use of genetically-modified mosquitoes for population replacement is one of the proposed control strategies for the vector-borne diseases. It is hypothesized that replacing the existing vector population with one that is unable to transmit the pathogen will result in less disease. One of the important components of population replacement is the regulated expression of anti-pathogen effector molecules in a sex- and tissue-specific manner using *cis*-regulatory DNA sequences to control gene expression. The *Aedes aegypti 30K a* and *30K b* genes encode 30kDa proteins that were determined by transcriptome analyses to be expressed exclusively in the salivary glands of adult female *Ae. aegypti*. The two genes are located on the same chromosome, separated by only 263 base pairs, and their mRNAs are transcribed divergently. The putative control and promoter DNA positioned between the two genes was analyzed functionally in transgenic Ae. *aegypti* carrying constructs varying in the amount of *30K* gene DNA included. Analyses revealed that jointly, the 263bp intergenic region, 5'UTRs and 3' UTRs of both *30K* genes are sufficient to express simultaneously two different transgene products in the distal-lateral lobes of the female salivary glands. qRT-PCR on the *30K b* transgene transcript shows that the expression level is equivalent to that observed for the endogenous gene. Transgenic mosquitoes generated expressing an anti-dengue effector molecule (RNAi inducing effector gene *Mnp*- PNAS 103: 4198-4203, 2006) are being tested for their effect on DENV2 replication.

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PHYLOGENETIC RELATIONSHIP AND POPULATION STRUCTURE OF THE NEOTROPICAL MALARIA VECTOR *ANOPHELES MARAJOARA* (DIPTERA: CULICIDAE) USING MULTIPLE MOLECULAR MARKERS

S. N. McKeon[1], M. A. Lehr[2], C. W. Kilpatrick[2], R. C. Wilkerson[3], M. A. Sallum[4], J. B. Pereira[5], J. E. Conn[6]

[1]School of Public Health, State University of New York-Albany, Slingerlands, NY, United States, [2]College of Arts and Sciences, University of Vermont, Burlington, VT, United States, [3]Walter Reed Army Institute of Research, Smithsonian Institution, Washington, DC, United States, [4]Faculdade de Saúde Pública, Universidade de Sao Paulo, Sao Paulo, Brazil, [5]Laboratorio de Fisiologia e Controle de Artropodes Vetores, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, [6]The Wadsworth Center, Griffin Laboratory, New York State Department of Health, Albany, NY, United States

Previous phylogenetic analysis based on the complete (1537bp) mtDNA *COI* gene sequences, suggest that *A. marajoara* is paraphyletic. To test this hypothesis and to investigate Amazonian population structure, partial *COI* sequences from 14 Brazilian localities (7 Amazon River, the inland locality Itaituba and 6 northeastern localities) were used. Additionally, subsets

of the westernmost localities (7 riverine localities and Itaituba) were examined with nDNA *white* gene and ribosomal DNA internal transcribed spacer 2 (ITS2) sequences. Of 265 *COI* sequences, both Bayesian Inference and a median-joining network identified two *A. marajoara* lineages with significant differentiation, and time to coalescence estimates lineage divergence during the Pleistocene. Lineage 1 was present in all 14 localities, lineage 2 was restricted to the westernmost localities, and *A. albitarsis* B was found only in Itaituba, a new distribution record. However, three nDNA data sets (*white* gene sequences, ITS2 length, and the retention of the 4th *white* gene intron) detected a single lineage. Therefore, nDNA data support monophyly, whereas the *COI* data consistently depict two discrete lineages of *A. marajoara.* When the mtDNA data were examined for a molecular signature consistent with either the refugia or marine incursion hypotheses, we detected moderate nucleotide divergence and high haplotype diversity. Spatial analysis of molecular variance detected a geographical barrier between the westernmost and northeastern localities. The mismatch distributions for *A. marajoara* lineages were smooth and bimodal, but significant for sudden expansion. An estimation of the time since the expansion is during the Pleistocene (>100,000ya). The diversity within lineage 1 can be explained by isolation by distance (0.307, P<0.02). The estimated divergence of *A. marajoara* is recent, likely due to an interaction between the marine incursion and refugia, and possibly restricted to the mtDNA. There appears to be significant gene flow along the Amazon River, although a partial barrier has been detected near Rio Jari.

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MOLECULAR ANALYSIS OF THE *ANOPHELES GAMBIAE* S.S. CHROMOSOME 2RB INVERSION DISTAL BREAKPOINT AREA

Djibril M. Sangare[1], Neil F. Lobo[2], Maria Sharakhova[3], Maria Unger[2], Sekou F. Traore[1], Nora J. Besansky[2], Frank H. Collins[2]

[1]MRTC/FMPOS, Bamako, Mali, [2]Eck Institute for Global Health, University of Notre Dame, South Bend, IN, United States, [3]University of Virginia Tech, Blacksburg, VA, United States

The 2Rb chromosomal inversion is highly polymorphic, widespread and occurs in association with the 2Rj, 2Rc and 2Ru inversions in *Anopheles gambiae* s.s, a major malaria vector in Africa. Knowledge of the molecular structure of the 2Rb will lead to the development of PCR-based diagnostic assays that can be used to identify 2Rb chromosomal rearrangements in adult mosquitoes without the need for karyotyping. Also, Molecular analysis of 2Rb inversion breakpoints is essential for a better understanding of its chromosomal rearrangement mechanisms. We performed in situ hybridization and sequence analyses of cloned DNA to identify and characterize the 2Rb inversion breakpoints areas on chromosome 2Rbc/bc, (Mopti strain), on 2Rjcu/jcu (Bamako strain), and on 2Rb/b (Savanna strain). The 2Rb distal breakpoint contained an assembly of unique sequences and repetitive elements, including complete and degenerated transposable elements. FISH and PCR analyses of 2Rb breakpoints provided a promising molecular assay for diagnoses in individual mosquitoes. PCR amplification of the 2Rb distal breakpoint confirmed the molecular structure (4085 bp of unique and repetitive sequences) and led to the first step in molecular karyotyping of the 2Rb inversion. The study described here has provided groundwork for future molecular investigations (gene expression profile and sequence variation) of the 2Rb inversion breakpoints. An interesting outcome of this work is that 2Rb+ may not be orthologous on a molecular level in the different chromosomal arrangements in which it is observed within individuals of the Bamako, Mopti, Savanna, Forest and Bissau chromosomal forms of *An. gambiae* s.s.

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LINEAGE DIVERGENCE IN THE NEOTROPICAL MALARIA VECTOR *ANOPHELES (NYSSORHYNCHUS) NUNEZTOVARI* AND ITS SISTER TAXON *AN. GOELDII* (DIPTERA: CULICIDAE) BASED ON NUCLEAR WHITE AND MTDNA COI SEQUENCE ANALYSIS

Richard C. Wilkerson[1], Victoria Spindler[2], Maria Anice Sallum[3], Marinete M. Povoa[4], Margarita M Correa[5], Lina A. Gutierrez[5], Yadira Rangel[6], Jan E. Conn[7]

[1]Walter Reed Army Institute for Research, Smithsonian Institution, Washington, DC, United States, [2]School of Public Health, SUNY-Albany, Albany, NY, United States, [3]Faculdade de Saude Publica, Universidade de Sao Paulo, Sao Paulo, Brazil, [4]Programa de Pesquisas em Malaria, Instituto Evandro Chagas, Belem, Brazil, [5]Escuela de Microbiologia, Universidad de Antioquia, Medellin, Colombia, [6]Instituto de Zoologia Tropical, Universidad Central de Venezuela, Caracas, Venezuela, [7]The Wadsworth Center, New York State Department of Health, Albany, NY, United States

Recent published analysis using the single copy nuclear white gene detected five lineages in *Anopheles nuneztovari* from northern South America. We chose 77 samples from different localities in Bolivia, Brazil, Colombia, Ecuador and Venezuela to test the hypothesis of five lineages, using the white gene, and the complete mtDNA COI gene sequence, with *An. aquasalis* as the outgroup. Using sequence homology, our sequences were compared with those in GenBank, and all 77 samples were confirmed as *An. nuneztovari* or *An. goeldii*. The Bayesian Inference (BI) for the combined COI and white sequences detected nine lineages, all supported above 70%, eight of which are *An. nuneztovari*, and one of which is An. goeldii. An. goeldii appears to be restricted to Bolivia and Brazil whereas the *An. nuneztovari* lineages are much more broadly distributed. When all white gene sequences (n=148), including those previously published, were analyzed, BI detected six lineages, five *An. nuneztovari*, and one An. goeldii. In this analysis, samples of An. goeldii and *An. nuneztovari* were sympatric in Altamira, Para, Brazil. In addition, *An. nuneztovari* lineages were sympatric in Solano, Tachira, Venezuela and in Coca, Napo, Ecuador. A statistical parsimony analysis (SPA) of the white gene (95% level) detected three *An. nuneztovari* lineages, partially congruent with the BI, and 12 individuals (including *An. goeldii*), which could not be connected. There is sympatry of two of the *An. nuneztovari* lineages in Colombia and in Ecuador. Overall, our results find that 1) *An. goeldii* and *An. nuneztovari* had separate evolutionary histories; 2) An. nuneztovari is much more broadly distributed and consists of multiple lineages; 3) *An. goeldii* is geographically restricted to Bolivia and Brazil; 4) retention of ancestral polymorphisms or introgression may have occurred in the mitochondrial genome of *An. nuneztovari* and *An. goeldii*; and 5) the original five white gene lineages of *An. nuneztovari* are supported by BI but not by SPA.

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POPULATION CYTOGENETICS OF *ANOPHELES MOUCHETI* AND *ANOPHELES NILI*

Maria Sharakhova[1], Christophe Antonio-Nkondjio[2], Frederic Simard[3], **Igor Sharakhov**[1]

[1]Virginia Tech, Blacksburg, VA, United States, [2]Malaria Research Laboratory OCEAC, Yaounde, Cameroon, [3]Institut de Recherche en Sciences de la Santé (IRSS), Bobo-Dioulasso, Burkina Faso

Identification and characterization of polymorphic inversions in mosquitoes provide valuable tools for studying variations in natural populations. Anopheline mosquitoes are renowned for the presence of polytene chromosomes and chromosomal inversions. This provides the opportunity to develop cytogenetic research tools for taxonomic and population genetics studies of the neglected malaria vectors *Anopheles moucheti* and *An. nili.* The *An. moucheti* and *An. nili* females were collected at several locations in Cameroon. Chromosomal analysis of *An. moucheti* collected in Olama and Lepse revealed the presence of polymorphic inversions.

At least four different inversions were identified on two chromosomal arms. Two rearrangements found on 2R chromosome arm were the most frequent. Thus, *An. moucheti* is a chromosomally polymorphic species and, therefore, inversions can be used for studying population structure in this species. Chromosomal analysis of *An. nili* collected at three different locations identified only a single inversion 2Ra in a mosquito from Magba. Thus, *An. nili* populations from Cameroon have a low level of inversion polymorphism. The location of rearrangements on 2R in *An. moucheti* and *An. nili* is in agreement with the fact that 2R is enriched with polymorphic inversions in *An. gambiae* and *An. funestus*. We developed a cytogenetic map for *An. nili* polytene chromosomes. The chromosomes were divided by 46 regions in accordance with the *An. gambiae* and *An. funestus* maps. In order to determine the chromosome homologies among distant mosquito species, we mapped *An. gambiae* cDNA probes to the *An. moucheti* and *An. nili* chromosomes. The analysis demonstrated that the *An. nili* chromosomes have the *An. stephensi*-like arm association (2+5; 3+4). Availability of polytene chromosome maps and polymorphic inversions will further stimulate population genetics studies of these neglected malaria vectors.

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IRRITANT AND REPELLENT BEHAVIORAL RESPONSES OF *AEDES AEGYPTI* MALE POPULATIONS DEVELOPED FOR RIDL® DISEASE CONTROL STRATEGIES

Nicole Achee[1], Monthathip Kongmee[2], Cecilia Coscaron-Arias[1], John Grieco[1], Camilla Beech[3], Derric Nimmo[3], Luke Alphey[3], Ann Kramer[3]

[1]Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [2]Kasetsart University, Bangkok, Thailand, [3]Oxitec, Oxford, United Kingdom

A recent advance in Sterile Insect Technique (SIT) has been the development of RIDL® (**R**elease of **I**nsects carrying a **D**ominant **L**ethal) technology in *Aedes aegypti*. When male insects mate with a wild type female the offspring inherit a copy of the RIDL gene and die as pupae. One of the main benefits over traditional SIT is that RIDL insects are bred to be sterile, but can live and reproduce normally when fed a diet containing a supplement which represses the lethal phenotype. This control over the lethality allows the insects to be reared in large numbers and negates the need to sterilize the insects with radioactive isotope irradiators which are expensive, inconvenient and hazardous. As the success of this strategy will include, among other factors, the ability of RIDL males to interact with natural female populations during mating processes, it is important to quantify potential behavioral changes that may occur upon exposure to chemical irritants (i.e. bed net treatments) and repellents (i.e. indoor residual sprays), that may be simultaneously in use. If a RIDL male behaves in a different way to these chemicals compared to a male from the natural population this may affect its ability to find and mate with females. This is especially true if mating occurs primarily indoors as repellents prevent mosquito house-entry and irritants decrease the length of time that a mosquito spends inside homes. Both actions may reduce the probability of mating success and may indicate adaptations or incompatibilities of the technique with other methods of control. We report a comparison of the behavioral responses of males from a RIDL strain of *Ae. aegypti* and a wild-type strain to varying doses of standard chemical irritants and repellents using the previously described HITSS laboratory assay system.

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MOSQUITOMAP AND THE MAL-AREA CALCULATOR: NEW WEB RESOURCES FOR GLOBAL MOSQUITO SPECIES DISTRIBUTION AND VECTOR-BORNE DISEASE RISK ASSESSMENT

Desmond H. Foley, Richard C. Wilkerson

Walter Reed Army Institute of Research, Suitland, MD, United States

We introduce MosquitoMap, an online geospatially referenced clearinghouse for mosquito species collection records and species distribution models. Users can pan and zoom to anywhere in the world to view the locations of past mosquito collections and the results of models that predict the geographic extent of individual species. Collection records are searchable and downloadable, users can map and upload their own georeferenced collection data or distribution models, and all contributions are fully acknowledged. MosquitoMap includes a vector-borne disease risk analysis tool called the Mal-area calculator. This tool combines models of mosquito vector distribution and pathogen suitability to determine the extent of the area where these organisms co-occur. The vectorial importance of each species is adjustable, the area of interest is resizeable and different locations can be compared to determine relative risk. MosquitoMap is designed to preserve and make available the results of past collecting and distribution modeling activity. MosquitoMap currently has over 100,000 collection records for over 1100 species level taxa, and a selection of mosquito species distribution models. However, the utility of MosquitoMap will increase as more records and distribution models are added. Contributions are encouraged, especially from individuals and organizations with digitized, georeferenced records and those involved in ongoing mosquito surveillance.

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TARGETED METHOD FOR THE DETECTION OF EASTERN EQUINE ENCEPHALITIS VIRUS FROM MOSQUITOES IN FLORIDA

Christy L. Ottendorfer[1], Gregory S. White[2], Hassan K. Hassan[1], Kevin J. Hill[1], Lillian M. Stark[3], Thomas R. Unnasch[1]

[1]GHIDR Program, University of South Florida, Tampa, FL, United States, [2]University of Alabama at Birmingham, Birmingham, AL, United States, [3]Florida Department of Health, Bureau of Laboratories-Tampa, Tampa, FL, United States

Eastern equine encephalitis virus (EEEV) is a serious arboviral infection of humans and horses, with case mortality rates approaching 50%. In Florida, EEEV maintains year-round enzootic transmission foci monitored by a statewide sentinel chicken surveillance program. During 2008 - 2009, historical sentinel seroconversion rates and mosquito collection data were used to place mosquito traps for year round surveillance. Furthermore, EEEV seroconversions were used to target mosquito collection and cloacal sampling of chickens for up to 2 weeks at sites with recent transmission activity for virus isolation. County agencies trapped mosquitoes using either CDC light traps or pickle jar traps, baited with CO_2, 1 - 2 nights per week. *Culex nigripalpus* was the most abundant species trapped. Mosquitoes belonging to several genera (*Aedes, Anopheles, Coquillettidia, Culiseta, Culex, Ochlerotatus, Uranotaenia*) were speciated, pooled in groups of 50, and processed for molecular RT-PCR and Vero cell culture assays. EEE viral RNA was detected in pools of *Cx. erraticus* and Cx. *quinquefasciatus*. Other virus(es) were cultured from both mosquito pools and cloacal swabs of sentinel chickens at targeted EEEV sites. Further characterization of these viral isolates is needed as they were negative by a standard RT-PCR panel used for identification of suspected arboviral isolates in Florida. The targeted method successfully detected EEEV and resulted in the isolation of additional virus(es) from mosquitoes collected at sites with recent sentinel seroconversions. This technique enhanced surveillance and characterization of arboviral pathogens in Florida.

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A SURVEY OF TWO SPECIES IN THE MINIMUS GROUP FROM MALARIA ENDEMIC AREAS IN WESTERN THAILAND

Monthathip Kongmee[1], Nicole Achee[2], Penny Masuoka[2], Theeraphap Chareonviriyaphap[1]

[1]Kasetsart University, Bangkok, Thailand, [2]Uniformed Services University of the Health Sciences, Bethesda, MD, United States

Members of the *Anopheles minimus* complex play a significant role in malaria transmission in Thailand. At least three species within this complex are found in Thailand, especially along the Thai-Mynamar border. The objective of this study was to survey the two species within the Minimus group (*An. minimus* and *An. harrisoni*) and genetically related species, *An. aconitus*, *An. pampani*, and *An. varuna* from two villages of Kanchanaburi Province, western Thailand. Bi-monthly larval surveys were carried out in these locations during a two year period (2006-2008). Habitat characteristics and surrounding environmental factors that may affect target mosquito populations were also collected. Survey results revealed that all five species were present at both locations but with site-specific density variations: the majority of *An. harrisoni* in one village and *An. minimus* in the other. Remote sensing and GIS technology was used to identify predictors of species-specific habitat distribution. Details on this finding will be discussed.

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ANOPHELES ARABIENSIS FEEDING BEHAVIOR AND ITS RELATIONSHIP TO THE DEMOGRAPHICS OF INSECTICIDE-TREATED BED NET USE IN MACHA, ZAMBIA

Douglas E. Norris, Laura C. Norris, Christen M. Fornadel

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, United States

The Macha region, in rural Southern Zambia, was formerly hyperendemic for *Plasmodium falciparum* malaria. In 2007, distribution campaigns provided a large number of insecticide-treated bed nets (ITNs) to the resident population. This study examined bed net usage in Macha by population demographics and the effect of bed net use on mosquito foraging. *Anopheles arabiensis* mosquitoes were collected by indoor CDC traps in two villages in the Macha area. Blood meals from humans were identified by X and Y chromosome markers as coming from male or female hosts. Census data on bed net usage was taken once a month at each house in the villages where CDC collections occurred. Nearly one third of *An. arabiensis* caught in CDC traps were blood-fed, mostly from human hosts, despite the reported use of bed nets by all those sleeping in houses where trapping occurred. This finding suggests that either ITNs are being used improperly, some level of insecticide resistance may occur in the vector population, and/or these mosquitoes are more endophilic than previously considered. Overall, 75% of people in the villages reported sleeping under a bed net the previous night. Males were less likely to sleep under a bed net, and were also more likely to be the source of *An. arabiensis* blood meals. Surveys revealed that children under 5 years of age were most likely to sleep under a bed net, while older children were less likely to do so. Although bed net coverage of young children is a priority, it is important to expand bed net coverage to the entire population in order to decrease transmission of *Plasmodium* from gametocyte carriers to mosquitoes.

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EVIDENCE OF PASSIVE DISPERSAL OF *ANOPHELES GAMBIAE* IN THE EARLY ADULT PHASE

Nicholas C. Manoukis[1], Nafomon Sogoba[2], Moussa Diallo[2], José M. Ribeiro[1]

[1]Laboratory of Malaria and Vector Research, NIAID/National Institutes of Health, Bethesda, MD, United States, [2]Malaria Research and Training Center, Faculty of Medicine, Bamako, Mali

Malaria vector control measures would benefit from an improved understanding of the mode and extent of dispersal by anophelines at various stages of the life cycle. For adults emerging from the larval phase, it would be helpful to understand if there are medium- or long-range attractants that draw the mosquitoes to human habitations for eventual feeding. To test if such factors might exist in the field, we constructed a novel apparatus for examining directional dispersal in emergent insects, in the form of a large enclosure with exit traps in four or eight directions. To validate the ability of this apparatus, which we call a directometer, to detect biases in dispersal direction, we conducted laboratory tests with carbon dioxide as an attractant. These tests indicate that the directometer does detect differences in dispersal direction when attractants are present. We then conducted a field study testing directional dispersal of *An. gambiae* following emergence by placing multiple directometers around the village of Donéguébougou, Mali. The results of those experiments indicate passive dispersal of *An. gambiae* immediately following emergence of adults from the pupal stage, with an unexpectedly clear positive relationship between wind direction and dispersal, regardless of the position of the village. We discuss these results in the context of the life history of the mosquito as well as implications for malaria vector control. Finally, we present some of the limitations of the current directometer design and plans for its improvement.

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OPTIMIZATION OF AN AUTOMATED COUNTING DEVICE FOR USE IN VECTOR BEHAVIOR STUDIES

Monthathip Kongmee[1], Philipp Kirsch[2], Hortance Manda[3], John Grieco[3], Theeraphap Chareonviriyaphap[1], Nicole Achee[3]

[1]Katsetsart University, Bangkok, Thailand, [2]Aptiv Inc., Portland, OR, United States, [3]Uniformed Services University of the Health Sciences, Bethesda, MD, United States

The use of an automated counting system for mosquito behavioral studies could provide advantages over manual observation by increasing data accuracy and eliminating confounding factors such as movement, carbon dioxide and heat. However, the accuracy of such a system is dependent upon optimization of the device under laboratory conditions.We report on a laser sensor system that was designed to be integrated with a laboratory assay used to quantify responses of *Aedes aegypti* upon exposure to irritant chemicals. The counting system consists of 2 components: a laser source and detector (model LV-H300) and amplifier (LV-51M). The system produces a light curtain that mosquitoes must pass through when exiting the assay chamber. Interruptions in the 30 mm high light curtain, due to the passage of mosquitoes, are recorded as exiting events. The system implements a 10-minute assay sampling period by automatically stepping through 30 sec timed intervals (i.e., a total of 20 intervals). Optimization of the system included identifying light curtain detection thresholds set midway between the calibration (no object) and sample object (mosquito) light levels. This was performed using female *Ae. aegypti* of varying ages and physiological states under different ambient light conditions. Pros and cons of the system will be presented.

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STRUCTURE AND PUTATIVE FUNCTIONS OF THE ANTERIOR AND POSTERIOR LARVAL INTIMAL REMNANTS IN THE MOSQUITO ALIMENTARY CANAL

Christine E. Leistner[1], Kriangkrai Lerdthusnee[2], William S. Romoser[3]

[1]International Development Studies, Ohio University, Athens, OH, United States, [2]Department of Entomology, Armed Forces Institute of Medical Research, Bangkok, Thailand, [3]Tropical Disease Institute, OUCOM, Ohio University, Athens, OH, United States

Non-cellular linings are present in the insect alimentary canal in the form of the foregut and hindgut intimas and the midgut peritrophic matrix surrounding the food bolus. In mosquitoes during the pharate pupal stage and larval-pupal ecdysis, the fore- and hindgut intimas are shed, but remnants remain attached to cells at the foregut-midgut and midgut-hindgut junctions. The term larval intimal remnant (LIR) describes these structures, the anterior LIR (ALIR) and the posterior LIR (PLIR). In newly emerged pupae, the ALIR is directed anteriorly, like a windsock, into the developing adult foregut lumen and the PLIR is directed posteriorly into the developing adult hindgut lumen. Using stained serial paraffin sections, we have identified these structures in representative species of *Aedes, Culex, Anopheles* and *Psorophora*. Putative barrier functions of the ALIR and PLIR are suggested by their structure and locations in the mosquito gut. For example, during the foregut development, apparent adult esophageal and diverticular intimal precursor material accumulates extra-cellularly in the developing foregut lumen. This material is later absorbed by the developing esophageal and diverticular epithelial cells and deposited as the adult intima. The ALIR would isolate this material in the foregut lumen and prevent leakage into the developing midgut lumen. The PLIR would likewise be a barrier between the developing adult midgut and hindgut lumens. During pupal-adult ecdysis (adult emergence) the ALIR reverses direction due to the swallowing of molting fluid, and then gas, from the exuvial space prior to exit from the exuvia. Subsequently, due to structure and location, the ALIR would function as a one-way valve, working in conjunction with the sphincter muscles located between the walls of the intussuscepted esophageal epithelium. Likewise, the PLIR would have an analogous function at the midgut-hindgut junction. Preliminary evidence suggests that the LIRs may persist in the adult stage where they would continue the roles of one-way valves.

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EVALUATION OF *AEDES AEGYPTI* RESTING PREFERENCES IN EXPERIMENTAL HUTS IN IQUITOS, PERU

Fanny Castro-Llanos[1], Hortance Manda[2], Amy Morrison[1], Victor Lopez[1], Nicole L. Achee[2], John Grieco[2], Kirk Mundal[3]

[1]United States Naval Medical Research Center Detachment, Iquitos, Peru, [2]Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [3]United States Naval Medical Research Center Detachment, Lima, Peru

Dengue is currently a major public health problem in the tropics with *Aedes aegypti* being the primary vector. Preventive measures targeting the adult stage have proven to be the most effective method for reducing disease transmission. However, adult control strategies have historically focused on the toxic action of chemicals and are becoming increasingly ineffective due to insecticide resistance. For this reason, development of a "push-pull" strategy to reduce *Ae. aegypti* inside homes is underway to identify novel approaches to adult vector control with the aim of minimizing treatment coverage and chemical dose. This approach will include targeting preferred indoor resting sites with irritant chemicals. An understanding of changes in resting patterns of *Ae. aegypti* to various surfaces and colors is of paramount importance to the success of the strategy. This study, conducted in Iquitos Peru, is part of the field validation component of the development of the "push-pull" strategy. We report on changes in *Ae. aegypti* resting preferences in response to material texture, color, coverage ratios and configurations (vertical versus horizontal) using a mark-release-recapture technique in experimental huts. The results from this study will be used to validate laboratory findings and generate baseline data to guide the optimum placement of insecticide treated materials inside the huts to promote premature house exiting by *Ae. aegypti*.

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SUSCEPTIBILITY OF *AEDES AEGYPTI* TO ORAL INFECTION WITH MAYARO VIRUS

Kanya C. Long, Charles E. McGee, Konstantin A. Tsetsarkin, Stephen Higgs, Robert B. Tesh

University of Texas Medical Branch, Galveston, TX, United States

Mayaro virus (MAYV) is a forest-associated, mosquito-borne αvirus currently circulating in South America that causes fever, arthralgia and rash in infected people. In Iquitos, an urban area in the Amazon region of Peru (population ~350,000), cases of Mayaro fever are identified every year through a clinic-based surveillance network. Since transmission of the virus to humans is occurring regularly, and because of the ubiquity of *Aedes aegypti* in Iquitos, we assessed the potential for *Ae. aegypti* to serve as an urban vector for MAYV, an alternate to its suspected sylvan vector *Haemagogus janthinomys*. Studies demonstrate 100% infection (24/24, each dilution) in a colonized strain of *Ae. aegypti* from Iquitos at blood meal titers of 7.95, 6.52 and 5.95 $\log_{10}TCID_{50}$/ml. Infection rates were dose-dependent, with 71% infection (17/24) at 4.95 and 38% (9/24) at 3.52 $\log_{10}TCID_{50}$/ml. This contrasted with a 0% infection rate (0/24) in colonized *Cx. p. quinquefasciatus* from Houston at the highest titer blood meal. The 50% oral infectious dose (OID_{50}) of MAYV in *Ae. aegypti*$_{Iquitos, colonized}$ was determined to be 3.87 $\log_{10}TCID_{50}$/ml (95% confidence interval of 3.45-4.17 $\log_{10}TCID_{50}$/ml). Future studies will be performed with F_2 generation *Ae. aegypti* from eggs collected in Iquitos to establish their ability to be infected by and to transmit MAYV. These data on the infectivity and transmissibility of MAYV in *Ae. aegypti* will increase our understanding of the potential for MAYV to develop an urban cycle of transmission.

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THE RELATIONSHIP BETWEEN HOST ABUNDANCE AND PER-HOST FEEDING DENSITY OF *CULEX QUINQUEFASCIATUS* SAY (DIPTERA: CULICIDAE)

Ivo M. Foppa[1], Jerrilynn L. Moore[1], Ricardo Cortez[1], Angela C. Gallegos[2], Dawn M. Wesson[1]

[1]Tulane University, New Orleans, LA, United States, [2]Occidental College, Los Angeles, CA, United States

The rate at which mosquito vectors feed on individual virus amplification hosts (per-host feeding rate=PHFR) is an important driver of mosquito-borne transmission. Given the biological parameters of transmission, such as vector competence, duration and level of host viremia, mosquito survival and transmission probabilities, the basic reproductive number is a linear function of the PHFR. Despite its epidemiological importance, the relationship between the spatial distribution of vertebrate hosts and PHFR is poorly understood. Here, we present results from an ongoing experimental study of PHFRs of Cx. quinquefasciatus mosquitoes when simultaneously presented with two levels of host abundance. We compare these findings with insights from simulation studies that explicitly model odor plume dispersal and odor-dependent host seeking of mosquitoes. This comparison of experimental data with simulation results will help us determine whether PHFRs are largely a function of the "odorscape" host-seeking Cx. quinquefasciatus mosquitoes experience. We expect from these studies important insights into the texture of the epizootiological fabric that underlies the transmission dynamics of West Nile virus and similar mosquito-borne ornithoses.

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EFFECT OF DENGUE II VIRUS INFECTION ON PROTEIN EXPRESSION IN THE HEAD AND THORAX OF *AEDES AEGYPTI* MOSQUITOES

Christopher N. Mores, Daniel M. Chisenhall

Louisiana State University, Baton Rouge, LA, United States

Dengue virus has been shown to alter transcript levels in *Aedes aegypti* mosquitoes; however the impact of infection on protein expression is less well characterized. In order to determine the effects of viral infection on protein expression in mosquitoes, colony *Ae. aegypti* (Rockefeller) were fed either a plain blood meal of bovine blood in citrate warmed to 37C or a blood meal of bovine blood in citrate mixed with dengue II stock virus (strain 16803) also warmed to 37C. The mosquitoes were sorted by blood-fed status and the individuals who had fed were dissected 10 days later. For each individual mosquito, the head and thorax were combined, and abdomen placed separately in 200 µL 2-D rehydration buffer. In order to determine dissemination the legs for each were placed in 900 µL of BA-1 for viral RNA extraction and detection via qRT-PCR. The samples were then loaded at a standardized load of 30 µg of protein per gel on a 3-10 nonlinear 11 cm IPG strip and then loaded onto a pre-cast 12.5% Tris-HCl second dimension gel. The resulting gels were stained with Sypro Ruby, imaged on a Gel Doc system, and analyzed using PDQuest image analysis software. Based on comparisons between blood-fed mosquitoes and dengue II infected mosquitoes (as verified by a positive qRT-PCR result from legs, indicating a disseminated infection), a total of 82 proteins were differentially expressed by at least 2-fold between the groups. 3 proteins were more highly expressed in virus-infected mosquitoes than in the blood-fed controls, while 37 proteins were under expressed in the virus-infected mosquitoes compared to the blood-fed controls. Of these 82 proteins, 34 were statistically significant with a 99% confidence interval using the partial least squares method. Statistically significant proteins were excised from the gels and analyzed via LC-MS/MS to determine their identity, in order to help elucidate possible mechanisms associated with the changes to the proteome observed due to viral infection.

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ENVIRONMENTAL FACTORS ASSOCIATED WITH THE MALARIA VECTORS *ANOPHELES GAMBIAE* S.L AND *ANOPHELES FUNESTUS* IN KENYA

Louise A. Kelly-Hope[1], F. Ellis McKenzie[2]

[1]Liverpool School of Tropical Medicine, Liverpool, United Kingdom, [2]Fogarty International Center, National Institutes of Health, Bethesda, MD, United States

In sub-Saharan Africa *Plasmodium falciparum* malaria is primarily transmitted by the mosquito species complexes *Anopheles gambiae* and An. funestus. To better understand the environmental factors influencing these species, we retrospectively analysed abundance, distribution and transmission data from a south eastern Kenyan study, and compared climate, vegetation and elevation data derived from remote-sensed satellite sources in key locations. *An. gambiae* s.s and An. funestus were the main vectors found in this region and their geographical distributions differed over the 200km study area. Transmission by *An. gambiae* s.s was found to be widespread whereas An. funestus was mostly confined to the southern lowlands of the study region. Our descriptive and statistical analyses of environmental factors indicated that locations with high *An. gambiae* s.s abundance and transmission rates had higher rainfall and elevations, but significantly lower temperatures, humidity and vegetation/greenness than those locations where An. funestus predominated. Bivariate correlation analyses found that An. funestus abundance and transmission rates were significantly positively associated with temperature and humidity, but significantly negatively associated with rainfall. These finding suggest that different malaria vectors are driven by different environmental factors. A better understanding of the specific ecological parameters of each malaria mosquito species will help define their current distributions and how they may affected in the future with the impact of climate change.

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TEMPORAL ASSOCIATIONS BETWEEN *CULEX TARSALIS* ABUNDANCE AND WESTERN EQUINE ENCEPHALOMYELITIS VIRUS TRANSMISSION

Christopher M. Barker[1], Bruce F. Eldridge[1], Wesley O. Johnson[2], Bborie K. Park[1], William K. Reisen[1]

[1]University of California, Davis, CA, United States, [2]University of California, Irvine, CA, United States

Definition of thresholds for arbovirus transmission risk or targets for vector control requires an understanding of the relationship between vector abundance and the intensity of arbovirus transmission. Using an extensive surveillance dataset with observations from sentinel chicken flocks and mosquito traps paired in time and space, models were developed to predict the probability of seroconversion for western equine encephalomyelitis virus (WEEV) based on the relative abundance of the principal vector, *Culex tarsalis*. Higher temperatures within two time windows (49-36 and 21-8 d) prior to chicken sampling dates were associated with increased WEEV transmission risk, except in the hot Coachella Valley, where mean maximum temperatures 49-36 d prior to the sampling date were associated with reduced risk. After adjustments for confounders, the abundance of *Cx. tarsalis* 42-29 d prior to the date of chicken sampling was credibly associated with WEEV transmission risk in both the Central and Coachella Valleys, and a doubling of relative *Cx. tarsalis* abundance was associated with a 58% increase in the odds of seroconversion. The critical time windows identified in our study highlight the need for surveillance of vector populations and forecasting models to guide proactive vector control measures prior to the detection of virus transmission.

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UNDERSTANDING THE EFFECTS OF CLIMATE ON MALARIA TRANSMISSION

Krijn Paaijmans, Simon Blanford, Andrew Bell, Andrew Read, Matthew Thomas

Pennsylvania State University, University Park, PA, United States

Climate change has the potential to affect the dynamics and distribution of malaria. However, our ability to quantify risk is limited due to the poorly-specified relationship between transmission and environmental parameters. Here we show how the influence of temperature fluctuations and extreme events can be as, or more important than changes in mean conditions for malaria transmission. We investigated the effects of mean temperature and temperature fluctuation on key aspects of mosquito and parasite life history using a combination of empirical and theoretical approaches. We find that, in general, temperature fluctuation reduces the impact of increasing mean temperatures. Specifically, we show that diurnal temperature fluctuation around warmer mean temperatures slows processes such as larval development and parasite incubation, whereas fluctuation around cooler mean temperatures speeds up these processes, compared with constant temperatures. These effects suggest that by ignoring fluctuation, we may currently be overestimating malaria risk in warmer environments, and underestimating risk in cooler environments. We further show that the effects of temperature fluctuation are important for understanding the dynamics of seasonal malaria in areas such as the Kenyan Highlands. To better understand the ecology of mosquitoes and malaria we need to consider not just basic measures of ambient temperature, but the fine-scale thermal environment in which the vector-parasite interaction is actually played out. Effects of short-term temperature fluctuations are not widely considered but appear central to understanding current malaria transmission and for evaluating consequences of future climate change.

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BITING BEHAVIOR OF *ANOPHELES MINIMUS* THEOBALD (DIPTERA:CULICIDAE) IN NORTHERN PROVINCE, A SITE OF HIGH MALARIA INCIDENCE IN THAILAND

Wannapa Suwonkerd[1], Nanatawan Suwannachote[1], Theeraphap Chareonviriyaphap[2]

[1]Office of Disease Prevention and Control, Ministry of Public Health, Chiang Mai, Thailand, [2]Faculty of Entomology, Department of Agriculture, Kasetsart University, Bangkok, Thailand

Anopheles minimus Theobald (Diptera:Culicidae) is the major vector of malaria in Thailand. Mae Hong Son province located along Thai-Myanmar border has reported high malaria incidence in Thailand over two decades. This study aims to understand blood feeding behavior of local malaria vector. Mosquito collections were conduct successively 9 months in hot (Feb-April 2006), rain (May-July 2006) and cool season (November 2006 -January 2007). A total 2,622 of 14 anopheline species were encountered. *Anopheles minimus* sensu lato was the most predominant collected species (60.45%) followed by Anopheles willmori. Attempted blood feeding by *An. minimus* s.l. occurred throughout the night, with consistently high during 2400-0300 hrs. Results indicated that *An. minimus* show site specific different host-seeking behavior, data is essential for improvement of malaria control strategies, and its proof useful for health education to the malaria risk group.

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COMPARATIVE KINETICS OF SOUTHEAST ASIAN DENGUE VIRUSES WITHIN AND BETWEEN SEROTYPES

Rebecca C. Christofferson, Christopher N. Mores

Louisiana State University, Baton Rouge, LA, United States

Dengue viruses (DENV) have been circulating in Southeast Asia, and significant outbreaks have been recurring, since at least the 1950s; with severe cases often manifesting as the hemorrhagic illness. The observed increase in dengue transmission is associated with the sequential or co-circulation of multiple serotypes. The mechanisms by which co-circulation of dengue strains impacts transmission dynamics is obscure. It may be that enhanced vector competence for individual viral strains is associated with a reduction in the extrinsic incubation period (the interval between a vector acquiring a pathogen and its being able to transmit the pathogen) of other virus strains. Accordingly, we sought to detect temporal differences in the infection and dissemination rates (defined as our ability to detect virus from the legs of mosquitoes whose bodies were positive for dengue virus following oral exposure to the virus) for dengue virus serotypes and genotypes in the mosquito vector. *Aedes aegypti* (Rockefeller) mosquitoes were artificially fed one of four serotypes (eight strains) of Southeast Asian-origin dengue virus strains in rabbit blood containing Alsever's anticoagulant (DENV-1: WestPac74, JKT 85-1416; DENV-2: 16803, 1232; DENV-3: JKT-86-5283, CH5548904500; DENV-4: 1228, LN 634441). Engorged females were held at 28C and sampled at five, seven, and nine days post infection. Infection and dissemination rates, and body titers were calculated. Preliminary data analysis suggests that differences exist in dissemination rates within and between at least some of the virus strains. Such variation could significantly impact estimates of vector competence. Here we describe the kinetics of eight dengue viruses, both within and between the four serotypes.

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EVALUATION OF A NEW LONG LASTING INSECTICIDAL NET (NETPROTECT®) ON INDOOR RESTING ACTIVITY AND ON HUMAN MALARIA INCIDENCE IN VILLAGES IN WESTERN KENYA

Ojera Odhiambo[1], John Vulule[2], L. Odeny[2], N. Mulaya[2], E. D. Kokwaro[2], **Ole Skovmand**[3]

[1]Kenyatta University, Nairobi, Kenya, [2]KEMRI, Kisumu, Kenya, [3]Intelligent Insect Control, Castelnau le Lez, France

Long Lasting Insecticidal bednets have been shown to be effective tools for personal protection against malaria when properly used. Netprotect® was developed on the known advantages of the two first long lasting bednets using the fine mesh as in a polyester net, but the strength and incorporation technology of a polyethylene net. This net was tested in a dispersed village (4000 people in 18,5 km²) at the border of the Yala Swamp, 70 km west of Kisumu. The study was quasi-experimental design carried out in a dispersed village at Yala Swamp near Lake Victoria. Intervention and control areas were 1,5 km apart. 2 x 150 households were randomly selected within the intervention and control areas. At least 3 LLIN were hung in selected households by trained assistants, total 450 nets. Indoor and outdoor resting *Anopheles* were counted monthly for 6 month by pyrethrum spray. Mosquitoes were morphologically determined as *An. gambia s.l.* or *funestus*. Malaria incidences at the local clinic were followed over 7 months for people identified from the intervention or control households. The physical state of the net was examined on randomly selected samples after 1 and 2 years. Results: Before the intervention, just 5% of all households in the village possessed any bed net, and only nets given to pregnant women were insecticidal. 352 malaria cases were confirmed as belonging to the two areas, 75 from the LLIN area and 277 from the control area. Malaria cases per week increased in the rainy season in the control area but remained stable and lower in the LLIN area. For both species, the ratio of indoor to out door resting was higher in the control area than in the intervention area. The acceptance of net use was high, but 10% of the nets disappeared from the intervention area during the study, mostly sold to generate income. After the 6 months, nets were also distributed in the control area. Among the nets distributed in the intervention area, many nets had holes, especially burn holes where food was prepared very close to the net.

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CD36 DEFICIENCY AND RESISTANCE TO MYCOBACTERIAL INFECTION

Michael Hawkes, Xiaoming Li, Maryanne Crockett, Angelina Diassiti, Constance Finney, Gundula Min-Oo, W Conrad Liles, Jun Liu, Kevin Kain

University of Toronto, Toronto, ON, Canada

Members of the CD36 scavenger receptor family have been implicated as sensors of microbial products, mediating phagocytosis and inflammation in response to a broad range of pathogens. Here, we investigated the role of CD36 in host response to mycobacterial infection. Using an *in vivo* model of *M. bovis* Bacillus Calmette-Guérin (BCG) infection in $Cd36^{+/+}$ and $Cd36^{-/-}$ mice, we show that mycobacterial burden in liver and spleen is reduced (83% lower peak splenic colony forming units, $p<0.001$), as well as the density of granulomas, and circulating tumor necrosis factor (TNF) levels in $Cd36^{-/-}$ animals. The mechanism underlying this observation was investigated *in vitro* using peritoneal macrophages of $Cd36^{-/-}$ and $Cd36^{+/+}$ mice infected with *M.tb*, BCG and *M. marinum*. Consistently, intracellular growth of all three mycobacterial species was reduced in $Cd36^{-/-}$ relative to wild type $Cd36^{+/+}$ macrophages. This difference was not attibutable to alterations in mycobacterial uptake, macrophage viability, rate of macrophage apoptosis, production of reactive oxygen and nitrogen species, TNF or interleukin-10. Together, these data indicate that CD36 deficiency confers relative resistance to mycobacterial infection, and that

this phenomenon is best explained by reduced intracellular survival of mycobacteria in the *Cd36*$^{-/-}$ macrophage.

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PITFALLS IN DIAGNOSING CNS TUBERCULOSIS

Deborah Asnis, Tamar Toronjadze, Robert Rilpuou, Anil Kapoor, Jordan A. Kazakov, Chiminyan Sathyakumar

Flushing Hospital Medical Center, Flushing, NY, United States

In 2006, the CDC reported 13,779 cases of Tuberculosis (TB) in the USA with < 1% affecting the central nervous system (CNS). During 2008, there were 2,889 (21%) extrapulmonary TB cases; of these 170 (5.9%) were meningeal TB. CNS TB presents as: 1) meningitis, 2) intracerebral tuberculoma, and 3) spinal arachnoiditis. TB meningitis results from the rupture of a subependymal tuberculous focus into the CSF. The tubercular protein enters the subarachnoid space and causes intense inflammatory response with arachnoiditis, vasculitis with thrombosis/infarction and hydrocephalus. TB meningitis presents as a subacute illness which progresses through 3 stages: 1) prodrome - malaise, headache and low grade fever, 2) meningitis- confusion, lethargy, cranial nerve palsies and 3) paralytic- hemiparesis, coma. Death usually occurs within 2 months without treatment. A 64 y/o Korean man who just returned from Texas presented in the summer with worsening left occipital headache for 3 days, chills and no fever, cough or weight loss. Physical exam was normal and he was afebrile. CT/MRI brain were unremarkable. Lumbar puncture (LP) was done - see table. A PPD/HIV/encephalitis panel (NY State DOH)/ Lyme antibody and CSF bacterial/fungal/mycobacterial cultures were negative. Aseptic meningitis was diagnosed. On day 6, he became febrile and delirious. Repeat MRI showed linear enhancement in the internal auditory canals. Repeat LP was done- see table. Empiric TB treatment with rifampin/INH/pyrazinamide/ethambutol (RIPE) and dexamethasone were started. He improved within 24 hours and was discharged home on treatment. Diagnosing CNS TB can be elusive because most tests have low yield: positive PPD in 31-61%, CXR findings of old TB in 44-88%, CSF AFB smear positive in 37% (87% if 4 taps), positive mycobacterial culture in 40-83%. Rapid nucleic acid amplification testing, a PCR technique, has 56% sensitivity and 98% specificity. CT/MRI may show basilar arachnoiditis, cerebral edema/infarction or hydrocephalus. Early diagnosis is essential because mortality ranges from 15 to 40% in untreated disease. NYC DOH initially screened the second LP with the fluorochrome stain (FS) and found it negative. Because of our high suspicion of TB, direct mycobacterial antigen test was done and was found positive. A Ziehl Nielsen stain was also positive; repeated FS was negative. A false negative FS can be seen if tap water, which contains chloride, is used on the slide. Possibly an extraneous protein in the spinal fluid acted as inhibitor. Hence,if there is a high clinical suspicion of TB, FS should not be the only screening test done on a sample.

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MOLECULAR CHARACTERIZATION OF HUMAN METAPNEUMOVIRUS ISOLATES FROM PERU, HONDURAS AND COLOMBIA: 2008-2009

Maria E. Gamero[1], Merly Sovero[1], Josefina Garcia[1], Jane Rios[1], Ivette Lorenzana[2], Ana E. Arango[3], Victor Ocaña[4], Gloria Chauca[1], Jose L. Huaman[1], V. Alberto Laguna-Torres[1], Patricia V. Aguilar[1], Tadeusz Kochel[1]

[1]Naval Medical Research Center Detachment (NMRCD), Lima, Peru, [2]Universidad Nacional Autonoma de Honduras, Tegucigalpa, Honduras, [3]Grupo de Inmunologia, Universidad de Antioquia, Medellin, Colombia, [4]Centro de Salud Pachitea/Ministerio de Salud, Piura, Peru

Human metapneumovirus (hMPV) is a newly discovered member of the family *Paramyxoviridae* responsible for acute respiratory tract infections in young children, elderly patients, and immunocompromised hosts. Based on phylogenetic analysis, two major genotypes of hMPV have been recognized (A and B) each with two subtypes (A1, A2; B1, B2). To date, studies have demonstrated the presence of hMPV in Argentina, Brazil, Chile and Peru; however, circulation of the virus in Central America and other South American countries remains unknown. As part of a passive surveillance for influenza-like illness (ILI) in sites of central (Honduras, Nicaragua, El Salvador) and South America (Argentina, Bolivia, Colombia, Ecuador, Peru, Paraguay and Venezuela) we have investigated what hMPV viruses were in circulation, during March 2008 to February 2009. Samples were cultured and analyzed for different respiratory viruses using MDCK, Vero E6, Vero 76 and LLCMK2 cell lines, each sample were inoculated in the four cell lines. Samples positive to hMPV previously identified by direct inmunofluorescence assay were further confirmed by RT-PCR and sequencing of the nucleoprotein (N). Phylogenetic analyses were carried out to molecularly characterize the virus isolates. A total of 4,183 samples from ILI patients were analyzed by immunofluorescence and 13 hMPV were isolated. From those, in six we performed phylogenetic analyses. Four isolates were obtained from Peru, one from Colombia, and one from Honduras. Phylogenetic analyses based on the N gene revealed that the isolates from Peru belongs to subtypes A (n=3) and B (n=1). Sample from Honduras was subtype B and from Colombia was subtype A. Our study revealed for the first time the circulation of HMPV in Colombia, Honduras and the continuous circulation of HMPV in Peru. Thus, to our knowledge, this is the first report of HMPV subtype A in Peru. Future studies will be conducted to define the role of Human Metapneumovirus in the epidemiology of respiratory infections.

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DF152 AS A NEW MOLECULE TO TREAT TUBERCULOSIS AND OTHER NEGLECTED MYCOBACTERIAL INFECTIONS

Anny Fortin[1], Shahid Soomro[1], Paul Stoppie[1], Sarah Cauwenbergh[1], Sabine Ruesch-Gerdes[2], Frans Herwig Jansen[1]

[1]Dafra Pharma R&D bvba, Turnhout, Belgium, [2]National Reference Center for Mycobacteria, Research Center Borstel, Borstel, Germany

Tuberculosis (TB) is a leading cause of death in the developing world, but is now considered a worldwide problem as multidrug-resistant TB (MDR-TB) is spreading at an alarming rate. "Super strains" of MDR-TB and extensively drug-resistant TB have been reported in 50 countries including the U.S., England, Japan, Italy and Norway. Novel drugs are continuously needed to fight these new strains, and it is important to maintain new discovery programs to fill the early-stages pipeline. We have recently performed the synthesis of a series of new anti-mycobacterial compounds derived from a previously described molecule (VUF 8514; internally renamed DF152). The goal of this work was to identify novel, rapid-acting and highly potent anti-mycobacterial drugs that would be safer than existing treatments and effective against MDR-TB strains. DF152 is made of a 2-pyridyl isoquinoline moiety attached to an amidine group, making it a novel class of anti-mycobacterial compound. This lead structure is found to be very active against *M.tuberculosis* strains, but also highly cytotoxic for mammalian cells. Chemical optimization of this parent molecule was performed with the aim of improving its physical and anti-microbial properties, and improving its safety profile. This operation led to the production of a series of five derivatives, including halogen substitutions on the isoquinoline moiety of the parent compound and the amide derivatives. All six compounds were designed to be easily synthesized. The five derivatives of DF152 were tested for *in vitro* efficacy and *in vitro* cytotoxicity together with the parental molecule. Overall, our results provide evidence that modifications of DF152 can indeed considerably improve the safety profile of the DF152 while retaining overall excellent anti-mycobacterial efficacy. Two of the derivatives are currently further characterized *in vivo*, while additional modifications of DF152 are underway to identify new lead compounds.

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ASSOCIATION BETWEEN HTLV-1 INFECTION AND TUBERCULOSIS: EPIDEMIOLOGICAL AND CLINICAL ASPECTS

Maria de Loudes S. Bastos[1], Beth Osterbauer[2], Daniel L. Mesquita[3], Carlos A. Carreira[3], Maria Juliana Albuquerque[3], Leandro Silva[3], Daniele N. Pereira[3], Lee Riley[2], Edgar M. Carvalho[4]

[1]Hospital Especializado Octavio Mangabeira, Salvador - BA, Brazil, [2]University of California at Berkley, Berkley, CA, United States, [3]Escola Bahiana de Medicina e Saúde Pública, Salvador - BA, Brazil, [4]Federal University of Bahia, Salvador - BA, Brazil

A few reports have suggested that HTLV-1 may influence immunological response and therefore, clinical course of tuberculosis in co-infected individuals. The aims of this study are: 1) to determine the prevalence of HTLV-1 infection among hospitalized patients in Salvador, Brazil, a region endemic for both HTLV-1 infection and latent tuberculosis infection; 2) to evaluate if HTLV-1 may influence clinical and bacteriological aspects of tuberculosis. A cross-sectional study was conducted at a pulmonary disease hospital between September 1st, 2006 to August 31st, 2007. Study participants were interviewed and tested for HTLV-1 infection and current or past episode of tuberculosis. More recently, a cohort study was started aimed to involve 30 patients with tuberculosis and HTLV-1 and 30 patients with tuberculosis without HTLV-1. Of 607 participants recruited for the cross-sectional study 360 (59.3%) had current or past history of tuberculosis and 50 (8.2%) had HTLV-1 infection; 39 (6.4%) had both. After controlling for confounding variables, we found that the odds of patients with a positive HTLV-1 test having tuberculosis were 2.57 times the odds (95%, CI : 1,23, 5:35) in those who tested negative for HTLV-1 infection. Moreover, patients with HTLV-1 and tuberculosis had more admissions due to tuberculosis. Regarding the ongoing cohort study, we have already enrolled 10 patients in each group and are comparing the size of the skin test with PPD, microbiological and serological data in the two groups. In a region endemic for both tuberculosis and HTLV-1 infection, HTLV-1 infection increases the risk of *Mycobacterium tuberculosis* infection. Moreover, tuberculosis in HTLV-1 infected subjects tends to be more severe than in HTLV-1 seronegative population. The presence of HTLV-1 infection may influence tuberculosis transmission and therefore epidemiology and clinical manifestations of the disease in this community.

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IDENTIFICATION OF ENTEROVIRUS ISOLATIONS IN CHILDREN WITH INFLUENZA-LIKE ILLNESS IN SOUTH AMERICA

Jose L. Huaman[1], Maria E. Gamero[1], Ana E. Arango[2], Washington Aleman[3], Wilson Chicaiza[4], Gloria Rey[5], Nicolas Aguayo[6], Guillermo Comach[7], Cecilia Rivera[1], Gloria Chauca[1], V. Alberto Laguna-Torres[1], Melvin Barrantes[8], Tadeusz Kochel[1]

[1]Naval Medical Research Center Detachment, Lima, Peru, [2]Grupo de Inmunovirologia, Universidad de Antioquia, Medellin, Colombia, [3]Hospital Vernaza, Guayaquil, Ecuador, [4]Hospital Vozandes, Quito, Ecuador, [5]Instituto Nacional de Salud, Bogota, Colombia, [6]Asociacion Rayos de Sol, Asuncion, Paraguay, [7]Laboratorio Regional de Diagnostico e Investigación del Dengue y otras Enfermedades Virales, Maracay, Venezuela, [8]Hospital Materno Infantil San Francisco Solano, Buenos Aires, Argentina

Children are extremely susceptible to respiratory infections; they have an average of two to seven acute respiratory infections each year. Adenoviruses, Respiratory Syncytial Virus, Influenza Viruses, and Enteroviruses are among the viruses infecting children worldwide. The genus Enterovirus is one of the nine genera of the Family *Picornaviridae* and comprises five groups: Poliovirus, Coxsackie A, Coxsackie B, Echoviruses and Enteroviruses (68-71). During March 2008 to February 2009, we enrolled 2724 children (age ≤ 15 years) with influenza-like illness as part of a passive influenza surveillance at sites in Argentina (n = 326) Colombia (n = 257), Ecuador (n = 216), Peru (n = 1769), Paraguay (n = 89) and Venezuela (n = 67). Virus isolation was carried out by inoculation in LLCMK2 and VERO E6 cell lines. After 10 days, viral identification was performed by indirect immunofluorescence using polyclonal anti-enterovirus antibody. Monoclonal antibodies were used to further classify positive cultures by group and serotype. A total of 62 (2.3%) samples were positive for enterovirus by isolation; 79% (49/62) of them were identified as Coxsackie B, 14.5% (9/62) were identified as Enterovirus 71 and 6.5% (4/62) were identified as Echovirus. Our results indicate that Coxsackie B viruses are the most common Enteroviruses involved in respiratory infections of children in these study sites in South America. Coxsackie B viruses were circulating at all study sites, with the greatest frequency observed at the study site in Argentina.

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THE PITFALLS IN DIAGNOSING CENTRAL NERVOUS SYSTEM MENINGITIS

Robert Rilpuou, Deborah Asnis, Tamar Toronjadze, Jordan Kazakov

Flushing Hospital Medical Center, Flushing, NY, United States

In 2006, the CDC reported 13,779 cases of Tuberculosis (TB) in the USA with < 1% affecting the Central Nervous System (CNS). During 2008, there were 2,889 (21%) extrapulmonary TB cases; of these 170 (5.9%) were Meningeal TB. CNS TB presents as: 1) Meningitis, 2) Intracerebral Tuberculoma, and 3) Spinal Arachnoiditis. TB meningitis presents as a subacute illness which progresses through 3 stages: 1) prodrome - malaise, headache and low grade fever, 2) meningitis- confusion, lethargy, cranial nerve palsies and 3) paralytic- hemiparesis, coma. Death usually occurs within 2 months without treatment. A 64 y/o man presented with worsening left occipital headache for 3 days, no fever/chills, cough or weight loss. Physical exam was normal and he was afebrile. CT/MRI brain were unremarkable. Lumbar puncture (LP) was done - see table. A PPD/HIV/encephalitis panel (NY State DOH)/Lyme antibody and CSF bacterial/fungal/mycobacterial cultures were negative. Aseptic meningitis was diagnosed. On day 6, he became febrile and delirious. Repeat MRI showed linear enhancement in the internal auditory canals. LP was repeated. Empiric TB treatment with rifampin/INH/pyrazinamide/ethambutol and dexamethasone were started. He improved within 24 hours and was discharged home. Diagnosing CNS TB can be elusive because most tests have low yield: positive PPD in 31-61%, CXR findings of old TB in 44-88%, CSF AFB smear positive in 37% (87% if 4 taps), positive mycobacterial culture in 40-83%. Rapid nucleic acid amplification testing, a PCR technique, has 56% sensitivity and 98% specificity. CT/MRI may show basilar arachnoiditis, cerebral edema/infarction or hydrocephalus. Early diagnosis is essential because mortality ranges from 15 to 40% in untreated disease. NYC DOH initially screened the second LP with the Fluorochrome stain (FS) and found it negative. Because of our high suspicion of TB, direct mycobacterial antigen test was done and was positive. A Ziehl Nielsen stain was also positive; repeated FS was negative. A false negative FS can be seen if tap water, which contains chloride, is used on the slide. Possibly an extraneous protein in the spinal fluid acted as inhibitor. Hence, if there is a high clinical suspicion of TB, FS should not be the only screening test done on a sample.

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DEVELOPMENT OF A SINGLE DOSE, POX-VIRUS BASED VACCINE FOR HIGHLY VIRULENT AVIAN INFLUENZA VIRUSES (H5N1)

Jeremy C. Jones[1], Tim D. Powell[2], Brock Bakke[3], Joseph N. Brewoo[1], Charalambos D. Partidos[1], Dan T. Stinchcomb[2], Jorge E. Osorio[3]

[1]Inviragen, Inc., Madison, WI, United States, [2]Inviragen, Inc., Fort Collins, CO, United States, [3]University of Wisconsin, Madison, WI, United States

The emergence of avian influenza viruses of the subtype H5N1 in human populations poses a significant public health threat. Current H5N1 vaccines are poorly immunogenic and difficult to produce on a

large scale by traditional methods. We have optimized the expression of H5N1 influenza hemagglutinin (HA) in the poxvirus vector, modified vaccinia virus Ankara (MVA). Various molecular signals were used to effect expression levels, post-translational cell trafficking and immune responses. Our first vaccine construct (MVA/HA1) was administered to mice with one intradermal (ID), one intramuscular (IM), or two intramuscular doses. Challenge by a lethal dose of VN/1203 was conducted 9 weeks post-vaccination. All vaccinated mice survived. However, mice vaccinated with a single ID dose recovered nearly all weight lost 6 days earlier than mice vaccinated with a single IM dose. Intradermal vaccinated mice showed less severe clinical scores than IM mice and lower lung virus titers on day 4 post-challenge. Thus, our MVA influenza vaccines protect against lethal challenge, and ID immunization is the ideal route of administration. Dose titration experiments were preformed utilizing a number of optimized constructs expressing the avian H5 HA protein. Mice were ID vaccinated with a single dose of each construct at three concentrations (5x107, 5x106, or 5x105 pfu/mouse) and challenged 11 weeks post-vaccination. Two candidate vaccines emerged from these studies, each demonstrating 100% survival at even the lowest tested concentration. Safety of the MVA/HA vaccines was assessed by intraperitoneal administration of 1x108 pfu of each construct to SCID mice. Mice were monitoring for weight loss and pox virus lesions over 10 weeks. No mice receiving the MVA vaccine constructs displayed clinical signs of disease as compared to a group administered the Wyeth vaccinia strain. Thus, we have developed MVA-based pandemic influenza vaccines that provide efficacy after a single dose, are inexpensive to manufacture and are safe in immune-compromised animals.

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ANTIBIOTIC SUSCEPTIBILITY AND SEROTYPES OF *STREPTOCOCCUS PNEUMONIAE* FROM INVASIVE DISEASE IN RURAL THAILAND, 2005-2009

Prasert Salika[1], Leelaowadee Sangsuk[2], Possawat Jorakate[1], Anek Kaewpan[1], Duangkamon Siludjai[1], Sopida Pookit[2], Surang Dejsirilert[2], Sumalee Boonmar[1], Leonard Peruski[1]

[1]International Emerging Infections Program, Nonthaburi (Bangkok), Thailand, [2]National Institute of Health, Ministry of Public Health, Nonthaburi (Bangkok), Thailand

Invasive Pneumococcal Disease (IPD) is a major cause of morbidity and mortality in young children and older adults worldwide but little is known about IPD in the tropical climate of Southeast Asia. Using well-characterized, unique isolates of *Streptococcus pneumoniae* obtained from blood cultures of patients with invasive disease in rural Thailand from 2005-2099, we determined the serotype distribution and antimicrobial resistance patterns common to IPD in this setting. From 2005-2009 invasive pneumococci were isolated from blood cultures collected from patients of all ages at 20 hospitals in the provinces of Sa Kaeo and Nakhon Phanom in Thailand. Each isolate was linked to a single episode of invasive disease. Isolates were identified as *S. pneumoniae* by biochemical tests, a commercial antigen assay, and a diagnostic real-time PCR assay. Antibiotic susceptibility was determined for chloramphenicol, clindamycin, co-trimoxazole, erythromycin, and oxacillin by disk-diffusion; susceptibility to penicillin and cefotaxime was determined by Etest. A multiplex PCR cascade was used to ascertain serotypes. Ninety-six unique isolates from patients with IPD have been characterized thus far for antibiotic susceptibility and serotype. Genotype analysis is currently underway. Of these isolates, 23 (24%) were from children <5 years, 51 (53%) were ≥5-59 years, and 22 (23%) from patients ≥60 years. Twenty-one (22%) were resistant to chloramphenicol, 20 (21%) to clindamycin, 20 (21%) to erythromycin, 52 (54%) to co-trimoxazole. MIC analysis revealed that 2 (2%) isolates displayed intermediate resistance to cefotaxime while 1 (1%) isolate displayed intermediate resistance to penicillin. The serotype coverage by the available 7-valent pediatric vaccine was only 55%, suggesting a limited utility. Resistance to commonly used drugs was extensive, limiting effective treatment to more costly ones of poor availability. Our collection of unique clinical isolates *S. pneumoniae* will be a useful tool in probing the pathogen ecology and virulence of invasive pneumococcal disease.

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SENTINEL HUMAN SURVEILLANCE FOR INFLUENZA AT HEALTH CARE FACILITIES IN KATHMANDU

Sanjaya K. Shrestha[1], Julie A. Pavlin[2], Khin S. Myint[2], **Bishnu K. Shrestha**[1], Robert V. Gibbons[2], Binob Shrestha[1], Kittinun Hussem[2], Bishnu B. Rayamajhi[1], Richard G. Jarman[2]

[1]Walter Reed/AFRIMS Research Unit Nepal, Kathmandu, Nepal, [2]Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

Influenza is a viral respiratory infection causing many epidemics worldwide. A swine-origin influenza (H1N1) virus outbreak in humans occurred in April 2009 causing the World Health Organization to raise the pandemic alert to level five. This strain poses a considerable human public health risk with great economic impact. We examined influenza samples collected from 630 Influenza-like Illness (ILI) cases enrolled through multiple health facilities at Kathmandu from January 2007 to December 2008. There were 362 (57%) male with majority (82%) between 15 and 59 years of age. Rapid diagnostic tests performed at field sites detected 75 (12%) FLU A and 48 (8%) FLU B. The specimens collected in viral transport media were forwarded to AFRIMS for RT-PCR which showed 158 (25%) positive for FLU A (93 FLU A/H1, 62 FLU A/H3 and 3 unsubtyped) and 83 (13%) FLU B. The US Air Force School of Aerospace Medicine laboratory in San Antonio, TX cultured 80 (16%) FLU A, 53 (10%) FLU B of 513 samples. We initiated real time PCR testing for influenza at WARUN in December 2007, the first laboratory in Nepal to have this capability. We tested 259 specimens showing 57 (22%) FLU A (34 A/H3 and 23 not subtyped) and 30 (12%) FLU B. The tests performed at WARUN were in very good agreement with the result of the same specimens performed at AFRIMS (Kappa=0.907), demonstrating excellent quality control. There were more cases during July-August. FLU B was prevalent throughout the year except in May. FLU A prevailed during July-September for both surveillance years. It is important to have a regular influenza surveillance program in Nepal to report the recent circulating strain in the region, and the ability to type and subtype influenza viruses greatly increases the capacity for rapid response to an influenza outbreak. Moreover, rapid diagnosis of viral influenza will decrease unnecessary use of antibiotics to treat respiratory tract infections caused by influenza viruses and advice on preventive measures to limit further spread.

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EMERGENCE OF DENGUE VIRUS SEROTYPE 4 IN NORTHERN PERU

Brett M. Forshey[1], Amy C. Morrison[2], Cristhopher Cruz[3], Claudio Rocha[2], Stalin Vilcarromero[2], Carolina Guevara[3], Daria E. Camacho[4], Araceli Alava[5], Cesar Madrid[6], Luis Beingolea[7], Victor Suarez[8], Guillermo Comach[4], Tadeusz Kochel[3]

[1]Naval Medical Research Center Detachment, Lima and Iquitos, Peru, [2]Naval Medical Research Center Detachment, Iquitos, Peru, [3]Naval Medical Research Center Detachment, Lima, Peru, [4]Laboratorio Regional de Diagnóstico e Investigación del Dengue y otras Enfermedades Virales, Maracay, Venezuela, [5]Instituto Nacional de Higiene y Medicina Tropical "Leopoldo Izquieta Perez", Guayaquil, Ecuador, [6]Hospital Naval de Guayaquil, Guayaquil, Ecuador, [7]Direccion General de Epidemiologia/ Ministerio de Salud, Lima, Peru, [8]Instituto Nacional de Salud, Lima, Peru

Dengue viruses have been an important source of morbidity in the Loreto Department of northeastern Peru since at least 1990, when dengue virus serotype 1 (DENV-1) was first identified in Iquitos. Since then the region has seen subsequent introductions of dengue virus serotype 2 (DENV-2) in 1995 and dengue virus serotype 3 (DENV-3) in 2001. Based on data from a clinic-based febrile surveillance system, between 2002 and early 2008 DENV-3 was shown to be the predominant serotype in circulation in both Iquitos and Yurimaguas in Loreto, with little evidence of DENV-

1 and DENV-2 circulation, and no evidence of dengue virus serotype 4 (DENV-4) transmission in either city. DENV-4 was first detected in febrile patients in the region in February 2008; by October 2008, DENV-4 had become the dominant serotype in the region, representing greater than 95% (170/176) of DENV isolates from febrile patients. In total, between October 2008 and April 2009, DENV-4 was isolated from over 400 febrile patients. Concomitant with the increase in DENV-4 was a decrease in DENV-3 transmission in the region: DENV-3 decreased from 176 isolates (100% of DENV isolates) and 420 isolates (100%) during peak months in 2006-2007 and 2007-2008, respectively, to fewer than 10 isolates (<5%) during the same period in 2008-2009. Molecular analysis of the envelope gene demonstrated that the DENV-4 strains collected in 2008 and 2009 were genetically similar to strains recently circulating in coastal Peru, Ecuador, and Venezuela, grouping together into a lineage within genotype II of DENV-4 that was distinct from DENV-4 strains previously described in Latin America.

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LAGUNA NEGRA VIRUS ASSOCIATED WITH HUMAN ILLNESS IN PARAGUAY

Vidal Felices[1], Nicolas Aguayo[2], V. Alberto Laguna-Torres[1], Patricia V. Aguilar[1], Cristhopher Cruz[1], Ivan Allende[3], Alma Barboza[2], Tadeusz Kochel[1]

[1]Naval Medical Research Center Detachment, Lima, Peru, [2]Asociacion Rayos de Sol, Asuncion, Paraguay, [3]Ministry of Health, Asuncion, Paraguay

Laguna Negra virus (LNV) was first reported as the cause of hantavirus pulmonary syndrome (HPS) in Paraguay in 1997. The virus was later shown to circulate in other South American countries including Argentina, Bolivia and Chile. Since the initial report of LNV, sporadic HPS outbreaks associated with LNV have been detected in Paraguay. Here, we described the observations of a recent LNV outbreak detected in Paraguay during July-September 2008. The patients were farmers who worked in the Chaco region, a known endemic area of LNV transmission. More than 25 cases were initially reported, with at least five fatalities. Six patients were confirmed as cases of LNV based on a polymerase chain reaction (PCR) that targets the S segment or serological analyses. Sequence analyses of the 331 base pair PCR products revealed a 96-98% nucleotide sequence homology to the LNV prototype strain 510B from Paraguay. Our investigations highlight the necessity for continuous monitoring of hantavirus pulmonary syndrome, to enhance rapid identification and treatment of cases and possibly nosocomial infections and more importantly to identify the potential emergence of novel reassortant hantaviruses.

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CHIKUNGUNYA VIRUS ANTIBODY IN TRAVELERS

Natasha K. Soodoo[1], Elizabeth D. Barnett[1], Abbie Stevenson[1], Stephen I. Pelton[1], Emad Yanni[2], Nina Marano[2], Lin H. Chen[3], Mary E. Wilson[4], Winnie W. Ooi[5], Laura Kogelman[6], Adolf W. Karchmer[7], Davidson H. Hamer[8]

[1]Boston Medical Center, Boston, MA, United States, [2]Centers for Disease Control and Prevention, Atlanta, GA, United States, [3]Mount Auburn Hospital, Cambridge, MA, United States, [4]Harvard School of Public Health, Boston, MA, United States, [5]Lahey Clinic, Burlington, MA, United States, [6]Tufts Medical Center, Boston, MA, United States, [7]Beth Israel Deaconess, Boston, MA, United States, [8]Boston University School of Public Health, Boston, MA, United States

Outbreaks of chikungunya virus infection have occurred in the Indian Subcontinent, Southeast Asia, East Africa, Indian Ocean, and Italy. Cases have been identified in travelers, and vector mosquitoes exist in many counties where the chikungunya virus has not yet been described. We standardized and used an ELISA technique to be used for research purposes since no assay was available for large scale screening of travelers. We then performed a serosurvey of chikungunya virus antibody in travelers presenting to Boston Area Travel Medicine Network (BATMN) clinics. BATMN is a research collaboration of 5 travel clinics in the greater Boston area seeing about 7,500 travelers per year in urban and suburban, academic, university-affiliated and independent facilities. Serum was collected from travelers who lived or traveled for ≥2 weeks to areas outside the US where the vector, *Aedes* spp., exist. The chikungunya antibody assay is a monoclonal antibody (MAb)-based capture ELISA following a protocol from A. Johnson et al. Chikungunya IgG antibodies were identified using a chikungunya S27 viral antigen strain prepared as β-propiolactone-inactivated sucrose-acetone extracts obtained from the CDC. A MAb capture alphavirus 1A4B-6 was used as a viral antigen capture vehicle. The cutoff was calculated as mean optical density (OD) with a positive/negative ratio of positive control serum to be ≥2.0. A reactive specimen was defined as having mean OD that was two-fold greater than the background. To date 46 specimens were tested for chikungunya IgG antibody, of which 2 were positive (4%) and 4 indeterminate (8%). The two positives were from individuals who lived in Nigeria and Thailand when outbreaks were known to have occurred. Two of the four indeterminates were from individuals born in Nigeria and India. The others were from US-born individuals who traveled to Nigeria and India during known outbreaks. This ELISA appears promising for use in identifying antibodies to chikungunya in travelers. Further confirmation of assay specificity is required. Additional specimens are being tested and a study of seroconversion to chikungunya virus following travel is underway to help elucidate the role of travel in chikungunya infection.

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ARBOVIRUSES CIRCULATING IN BOLIVIA 2008 - 2009

E. Roxana Caceda[1], Juan Sulca[1], Patricia V. Aguilar[1], Jorge Vargas[2], Yelin Roca[2], Carolina Guevara[1], Tadeusz Kochel[1]

[1]Naval Medical Research Center Detachment, Lima, Peru, [2]Centro Nacional de Enfermedades Tropicales, Santa Cruz, Bolivia

Arboviruses, arthropod-borne viruses, are the cause of hundreds of thousands of human cases throughout the world. There are more than 520 known arboviruses of which about 100 cause disease in humans. To better characterize the etiologic agents responsible for human febrile illnesses in Bolivia, a surveillance system was implemented at 3 sites (Concepcion, Santa Cruz and Eterazama). Serum samples were collected from febrile patients who presented at health clinics or hospitals with an undifferentiated febrile illness. Since January 2008 through January 2009, 817 samples were received from the surveillance sites in Bolivia and were tested for viral infection by virus isolation/immunofluorescence assay. Seventy-six virus isolates were recovered from these samples (9.3%) and were later identified as Dengue 3 (n=53; 6.5%), Dengue-1 (n=21; 2.6%) and Group C viruses (n=2; 0.25%). The Group C viruses were recovered from patients resident of Eterazama in Cochabamba whose main occupations were housewife (24 years old) and agricultural worker (47 years old). To date, Group C viruses have been detected in samples collected in Brazil, Trinidad, Panama, French Guiana, Suriname, Ecuador, Peru, Honduras, Mexico, Guatemala, United States and Venezuela. Thus, this is the first report of Group C viruses circulating in Bolivia. Due to the concurrent circulation of Dengue viruses and Group C within the same geographic area (Eterazama, Cochabamba) and the similarity in clinical symptoms among the patients, it is likely that Group C virus infections are mis-diagnosed or under diagnosed.

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CELLULAR RESPONSE TO RIFT VALLEY FEVER VIRUS INFECTION

Kimberly K. Gray, Michael R. Holbrook

University of Texas Medical Branch, Galveston, TX, United States

Rift Valley fever virus (RVFV) is an important pathogen in Africa and on the Arabian Peninsula. It belongs to the phlebovirus genus of the *Bunyaviridae* family. 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 very little data pertaining to the effect of infection on the host cellular response and how this correlates with pathogenesis. In an effort to characterize the cellular response to the vaccine strain of RVFV (MP-12), the phosphorylation of signaling proteins that are important in the cellular response and the secretion of antiviral and inflammatory cytokines in mouse derived immunomodulatory cells was examined. These data will be used to identify the host response to infection with MP-12 and identify possible mechanisms associated with disease pathogenesis. My initial efforts to characterize the effect of MP-12 infection on cellular response in mouse immunomodulatory cells have found that MP-12 can significantly inhibit inflammatory and Th1 associated cytokines. The concentrations of MCP, MIP-1β, and RANTES; along with that of IL-12, secreted in infected cells was similar to the levels that were secreted in mock infected controls. The suppression of these chemokines provides evidence that RVFV inhibits the immune response in infected cells. These data point to distinct mechanisms the virus may possesses for inhibiting the host response to infection in mice. Also, I have found that the phosphorylation of the pro-apoptotic proteins examined shows an increase over controls during the course of infection. I have found that JNK, p38, and p53 are phosphorylated after infection when compared to controls. Phosphorylation of STAT proteins is also significantly increased over mock infected controls at least 6 hours after infection.

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MATERNAL AND NEONATAL DEATHS ASSOCIATED WITH ACUTE JAUNDICE DURING PREGNANCY IN BANGLADESH: USING VERBAL AUTOPSY DATA TO ESTIMATE OF THE BURDEN OF HEPATITIS E INFECTION

Emily Gurley, Amal K. Halder, Peter K. Streatfield, H. M. Shahed Sazzad, M. Tarique Huda, M. Jahangir Hossain, Stephen P. Luby

International Center for Diarrhoeal Disease Research, B, Dhaka, Bangladesh

Hepatitis E virus (HEV) is primarily transmitted through the fecal-oral route in HEV endemic countries and provision of clean drinking water can prevent infections. HEV disease is endemic in South Asia where approximately 39% of all maternal and 38% of all neonatal deaths occur annually. Considering the poor health outcomes associated with HEV disease in pregnancy for women and newborns, estimating the population-based burden of death from HEV disease can help inform public health interventions for maternal and child survival. We analyzed verbal autopsy data from 4 population-based studies conducted at the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B); including 2 data sets on maternal mortality and 3 on neonatal mortality. Each study covered a 2 to 5 year time period. We calculated the maternal mortality ratio per 100,000 live births and neonatal mortality ratio per 1,000 live births for maternal and neonatal deaths associated with acute onset jaundice during pregnancy. We investigated the seasonality of these deaths accounting for seasonality of births in Bangladesh. We then summarized the published literature on outcomes of pregnant women with hepatitis and other liver disease to estimate the proportion of maternal deaths associated with jaundice which might be due to HEV infection. We estimate that 19-25% of all maternal deaths and 7-13% of all neonatal deaths in Bangladesh are associated with jaundice in pregnant women representing a maternal mortality ratio of 61-109 per 100,000 live births and a neonatal mortality ratio of 2.2-8.4 per 1000 live births, resulting in approximately 2,300 to 4,100 maternal deaths and 8,100 to 31,000 neonatal deaths per year. Deaths in our analysis peaked in the post-monsoon period. In the published literature, 74% of deaths in pregnant women with liver disease in HEV disease endemic countries occurred in women with HEV. In conclusion, this analysis suggests that HEV could be a major cause of maternal and neonatal mortality in Bangladesh and other HEV disease endemic countries. If true, the provision of clean drinking water could prevent thousands of maternal and neonatal deaths each year in Bangladesh. Additional studies to better define the population-based incidence of death from HEV could assist in appropriately prioritizing improved water quality and vaccine development.

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EMERGING VIRAL ZOONOSES IN AZERBAIJAN: A CROSS-SECTIONAL STUDY

Danielle V. Clark[1], Afrail Ismayilov[2], Esmiralda Seyidova[2], Ayten Hajiyeva[3], Sevinj Bakhishova[2], Huseyn Hajiyev[2], Tahir Nuriyev[4], Saleh Piraliyev[4], Sadigulla Bagirov[4], Afag Aslanova[5], Amanda Lane[6], Maqsud Qasimov[2], Matthew J. Hepburn[6]

[1]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]Anti-Plague Station, Baku, Azerbaijan, [3]Republican Veterinary Laboratory, Baku, Azerbaijan, [4]Center for Hygiene and Epidemiology, Quba, Qusar, Xachmaz, Azerbaijan, [5]Raytheon Technical Services Company, Baku, Azerbaijan, [6]United States Army Research Institute for Infectious Diseases, Fort Detrick, MD, United States

Viral zoonoses such as West Nile virus (WNV), Tick-borne Encephalitis (TBE), and hantavirus are often undetected due to mild and non-specific clinical presentations and unavailability of diagnostic tests. Large disease outbreaks and severe clinical manifestations of these infections are fairly rare, but can be associated with severe morbidity. Historical data suggest these infections occur in the region, but there is a paucity of current information on zoonotic viruses in Azerbaijan. A two-stage, probability-proportional to size sampling design was used to select 40 villages in northern Azerbaijan with populations <500 people. Informed consent was provided, and samples were obtained from 793 volunteers and tested by ELISA for IgG antibodies to WNV, TBE and hantaviruses. Information was collected on demographics, history of clinical symptoms, and potential exposures. WNV was the most prevalent viral zoonosis (12%), followed by hantavirus (4.2%) and TBE (0.4%). Gender and regional differences were significant for WNV. Risk factors associated with WNV seropositivity included contact with cattle (POR=2.0, p=0.02), barns/animal pens (POR=2.1, p=0.03), and assisting in animal birth (POR=1.7, p=0.02). Controlling for age and gender, volunteers seropositive for WNV had significantly higher odds of ever having a seizure (POR=3.1, p=0.006) and depression (POR=2.4, p=0.02). Hantavirus seropositivity was associated with sweeping around the home (POR=2.4, p=0.04). In concluaion, this study provides the first report of viral zoonoses in Azerbaijan in the last 20 years. This information will be utilized to guide prevention and disease surveillance efforts, as well as inform physicians on the clinical presentations of these infections. In particular, clinicians should be aware of the association between neurologic and neuropsychiatric symptoms and WNV.

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PHYLOGENY OF EASTERN EQUINE ENCEPHALITIS VIRUS IN FLORIDA

Gregory S. White[1], Lillian M. Stark[2], Christy L. Ottendorfer[3], Thomas R. Unnasch[3]

[1]University of Alabama at Birmingham, Birmingham, AL, United States, [2]Florida Department of Health, Bureau of Laboratories-Tampa, Tampa, FL, United States, [3]GHIDR Program, University of South Florida, Tampa, FL, United States

The state of Florida has more documented Eastern Equine Encephalitis virus (EEEV) activity than any other state. Furthermore, it has been recently suggested that Florida may serve as a reservoir from which EEEV might be periodically introduced into the Northeastern USA. To study the evolutionary patterns of the EEEV in Florida, the majority of the genome (~11.6 kb) was sequenced from seven isolates from north Florida (Volusia County). These data were used to identify 4 regions in the genome exhibiting high divergence among the Florida isolates. Over 20 additional Florida EEEV isolates, chosen from distinct years and geographical areas, were then sequenced in these 4 regions, together encompassing roughly 3kb. A phylogenetic analysis of these data revealed the existence of

three distinct clades in Florida. Two clades showed temporal and spatial clustering, while the third contained strains from different locations and years. When published partial genomic sequences (roughly 1kb) derived from isolates Northeastern USA were analyzed with the corresponding regions from the Florida isolates, some, but not all of the NE isolates grouped with particular isolates from Florida. In these cases, the isolation of the Florida isolate predated the NE isolate, supporting the hypothesis that these particular NE isolates may have been derived from Florida, implicating Florida as a potential source for the introduction of EEEV into the Northeastern USA.

1003

BASELINE KAP SURVEY: AVIAN INFLUENZA IN THE INCIDENCE AREAS OF TANGERANG, BANTEN PROVINCE, INDONESIA

Lisa Safira, Alyya S. Siregar

Faculty of Medicine, Pembangunan Nasional Veteran Jakarta University, Jakarta Selatan, Indonesia

Avian influenza is an infection caused by A type avian influenza viruses. These influenza viruses occur naturally among birds. However, avian influenza is very contagious among birds and can cause some domesticated birds, including chickens, ducks, and turkeys, very sick and kill them. Influenza virus type A has two types of glycoprotein on its surface. These glycoproteins are haemagglutinine H (H1 to H16) and Neuraminidase N (N1 to N9). All of these sub types may be transmitted among animals, but some sub types can be transmitted from animal to human, especially H5N1 which can be transmitted to human by infected poultry. Total reported human AI cases in Indonesia since June 2005 until October 2008 were 137 with 112 deaths distributed in 12 provinces (Case Fatality Rate or CFR= 81,8%.). There were 25 confirmed cases of Avian Influenza in Tangerang areas (Municipality and District) and all cases end with deaths. To determine the state of the awareness in the community where the H5N1 incidences occurred, a KAP study was initiated. This study was conducted in two villages in Tangerang area where AI cases were found, i.e. Panunggangan village, sub district of Pinang and Gondrong village, sub district of Cipondoh. A total of 764 people were interviewed with standardized questionnaires. The results showed that the knowledge of the respondents towards Avian Influenza was not too good. The respondents know how the birds get infected (40.58%), the sudden death symptom of the infected birds (46.86%), how humans get infected by contact of the sick birds (66.7%) and the fever symptom of the infected human (82.64%). The respondent practices also not really support the prevention towards AI infection among themselves and their family. They know how to cook the birds properly (90.97%), but only 43.32% would not eat the sick birds, and only 38.48% would wash hands after handling the birds though almost all respondents admitted that washing hands before eating has become their daily habit. For the 140 poultry farmers, they will keep the bird cage clean (93. 57%), but only 49.29% who will kill and burry the suspected sick birds (49,29%), and only 95 farmers (67.857%) would report whether their birds might get infected by AI.

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A COMPARISON OF THE INFECTION DYNAMICS OF HOUSE SPARROW AND COTTON RATS WITH NORTH AND SOUTH AMERICAN EASTERN EQUINE ENCEPHALITIS VIRUS

Nicole Arrigo[1], Douglas M. Watts[2], Patrick C. Newman[1], Scott C. Weaver[1]

[1]University of Texas Medical Branch, Galveston, TX, United States, [2]University of Texas El Paso, El Paso, TX, United States

Eastern equine encephalitis Virus (EEEV) is an arbovirus in the genus *Alphavirus*, family *Togaviridae*. The EEEV antigenic complex includes 4 major subtypes/lineages; three circulate in Central/ South America (SA EEEV) and are associated with equine disease, and one occurs in North America, the Caribbean, and Mexico (NA EEEV) and is associated with severe human and equine neurological disease. Despite the epidemiologic dichotomy between NA and SA EEEV, little is known about their ecological differences. It remains unclear whether SA EEEV is ecologically more similar to its genetic sister, NA EEEV, which utilizes avian amplifying hosts, or to its genetic and sympatric cousin, VEEV, which generally utilizes mammalian amplifying hosts. In order to clarify the role of mammalian and avian species in the transmission of SA EEEV, the infection dynamics of cotton rats (*Sigmodon hispidus*) and house sparrow (*Passer domesticus*) with NA and SA EEEV were compared. Cotton rats collected in Galveston, TX, and house sparrow collected in Houston, TX, were divided into experimental cohorts and subcutaneously inoculated with one of three EEEV viruses: FL93-939 (NA EEEV), C-49 (SA EEEV), or PE70 77U1104 (SA EEEV). Animals were bled daily for 7 days or until moribund and the blood tested by plaque assay to determine intensity and duration of viremia and PRNT to determine antibody response profiles. Viremia levels of SA EEEV PE70 were consistently higher in cotton rats, while those of NAEEEV were consistently higher in house sparrow. NA EEEV generally resulted in high mortality rates in all adult animals. Alternatively, SA EEEV resulted in no death or observable illness in cotton rats and intermediate levels in house sparrow. In conclusion, these results suggest that, although both cotton rats and house sparrow are capable of serving as competent hosts for both NA and SA EEEV, *Sigmodon hispidus*, and potentially other ground-dwelling mammals, may be better adapted to serve as reservoirs hosts in the enzootic transmission of SA EEEV. Alternatively, NA EEEV appears better adapted to avian reservoir species, particularly passerine species such as *Passer domesticus*.

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DENGUE SMS SURVEILLANCE PROJECT IN THE PHILIPPINES

Jacqueline Coberly[1], Richard Wojcik[1], Agnes D. Tomayao[2], Ilya A. Tac-an[3], John Mark S. Velasco[4], Sheri Lewis[1]

[1]JHU Applied Physics Laboratory, Laurel, MD, United States, [2]USAMC-AFRIMS/Philippines-AFRIMS Virology Research Unit, Cebu City, Philippines, [3]Cebu City Health Department/City Epidemiology Surveillance Statistics Unit, Cebu City, Philippines, [4]PAVRU, Manila, Philippines

Dengue fever poses a significant health threat in the Republic of the Philippines (RP). Current hospital-based surveillance is highly valid, but poorly suited for rapid identification of dengue 'hot spots' because of the delay associated with laboratory confirmation. The objective of this pilot study is to implement and evaluate a simple dengue surveillance protocol using Short Message Service (SMS) text messages to send daily, person-based dengue surveillance data from local health clinics (BHCs) to the city health office (CHO) in Cebu City, RP. Beginning 1 June 2009, BHCs in Cebu City, RP will identify all patients who meet the RP clinical case definition for dengue fever (age >6 mos, fever in the last 7 days, and any two of the following symptoms: headache, pain behind the eyes, rash, muscle or joint pain, anorexia, nausea, vomiting, abdominal pain, nose bleed or dark stool color.) Family and address codes, age, sex, date of onset and presenting signs/symptoms will be recorded for each patient meeting the case definition. Each day, the BHC doctor will send this information to the CHO in a single SMS text message. The first line of the SMS message will contain the date and clinic name. Each subsequent line will contain the required data for a single case. The SMS message will be transferred into an EpiInfo database, and the Electronic Surveillance System for the Early Notification of Community-based Epidemics (ESSENCE) Desktop Edition application will be used to identify statistically significant increases in reported clinical dengue cases. The time series for the SMS rapid surveillance system data will be compared with those produced by the RP hospital-based, sentinel dengue surveillance system. Success of the SMS system will be measured by the agreement between these two time series. This study will implement a more rapid, but less specific, surveillance method for dengue fever and compare this method to the standard sentinel surveillance system.

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PRESENTATION OF CD8+ EPITOPES IN THE CIRCUMSPOROZOITE PROTEIN OF *P. BERGHEI* IS STRICTLY TAP DEPENDENT

Ian A. Cockburn[1], Sze-Wah Tse[1], Andrea J. Radtke[1], Yun-Chi Chen[1], Photini Sinnis[2], Fidel Zavala[1]

[1]Johns Hopkins Malaria Research Institute, Bloomberg School of Public Health, Baltimore, MD, United States, [2]Department of Medical Parasitology, New York University, New York, NY, United States

Immunization with irradiated malaria sporozoites induces CD8+ T cell responses that contribute to protection against challenge with live parasites. Antigen-specific CD8+ T cell responses are primed in the lymph node draining the skin site of immunization and protect by killing infected hepatocytes in the liver. Thus the development and execution of protective immune responses by CD8+ T cells requires antigen presentation by at least two distinct cells types - dendritic cells in the draining lymph node and hepatocytes in the liver. We wanted to determine the antigen processing pathways used by these two cells in order to better understand which antigens might prime the immune response and which might be the target of protective responses. To address these questions we developed genetically modified *P. berghei* model in which sporozoites express a CS protein bearing five point mutations (*P. berghei* CS^{5M}) to create the K^b restricted epitope SIINFEKL. *P. berghei* CS^{5M} parasites induced robust SIINFEKL specific responses in wild type C57/B6 mice but not in TAP-/- animals showing that presentation of the CS protein by DCs is TAP dependent. Moreover SIINFEKL specific effector cells were able to kill parasites in the liver in wild type but not TAP-/- animals showing that presentation of CS by hepatocytes is also TAP dependent. The potential routes by which parasite antigens enter the TAP-dependent class I antigen-processing pathways in DCs and hepatocytes will also be discussed.

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PROFILING ANTIBODY RESPONSES TO *P. FALCIPARUM* INFECTION BY PROTEIN MICROARRAY: A STRATEGY FOR IDENTIFYING NOVEL MALARIA VACCINE TARGETS

Peter D. Crompton[1], Matthew A. Kayala[2], Boubacar Traore[3], Kassoum Kayentao[3], Aissata Ongoiba[3], Greta E. Weiss[1], Douglas M. Molina[4], Chad R. Burk[5], Michael Waisberg[1], Algis Jasinskas[5], Safiatou Doumbo[3], Didier Doumtabe[3], Younoussou Kone[3], David L. Narum[6], Xiaowu Liang[4], Ogobara K. Doumbo[3], Louis H. Miller[6], Denise L. Doolan[7], Pierre Baldi[2], Philip L. Felgner[5], Susan K. Pierce[1]

[1]Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, United States, [2]School of Information and Computer Sciences, Institute for Genomics and Bioinformatics, University of California, Irvine, CA, United States, [3]Malaria Research and Training Center, Faculty of Medicine, Pharmacy and Dentistry, University of Bamako, Bamako, Mali, [4]Antigen Discovery, Inc., Irvine, CA, United States, [5]Department of Medicine, Division of Infectious Diseases, University of California, Irvine, CA, United States, [6]Malaria Vaccine Development Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, United States, [7]Molecular Vaccinology Laboratory, Queensland Institute of Medical Research, Brisbane, Australia

Passive transfer studies indicate that antibodies against *P. falciparum (Pf)* play a critical role in controlling the blood stage of malaria. However, which of the over 5000 *Pf* proteins elicit the production of protective antibodies is unknown. Identifying this set of proteins could overcome a major hurdle in the effort to develop a malaria vaccine and provide insights into the mechanisms by which *Pf* antibodies protect against malaria. Using *Pf* genome sequence data and high-throughput cloning and *in vitro* protein expression methods we developed a protein microarray representing ~23% of the *Pf* proteome (1204 known and hypothetical proteins). In a longitudinal study in Mali of 225 individuals aged 2-25 years, plasma samples collected before and after the 6 month malaria season were probed against this microarray to identify an antibody signature associated with malaria immunity. Antibody reactivity against 49 proteins was higher in malaria-immune (no malaria episodes) vs. susceptible (≥1 malaria episodes) children. Combined antibody reactivity against the top 5 signature proteins had a 90.3% sensitivity and 86.3% specificity in discriminating malaria immune and susceptible children. This 5 protein panel performed better than the combined antibody reactivity to 5 leading malaria vaccine candidates (CSP, LSA-3, MSP-1, MSP-2, and AMA-1) in discriminating malaria-immune and susceptible children (AUC for top 5 signature proteins, 0.92; AUC for the 5 malaria vaccine candidates, 0.53). We are pursuing several of these proteins as potential malaria vaccine candidates by expressing the *P. yoelii* orthologues in *E. coli*. and assessing their immunogenicity and protective efficacy in a murine model of malaria. This approach, if validated in other epidemiological settings and in animal models, could prove to be a useful strategy for identifying novel malaria vaccine targets and for better understanding fundamental properties of the human immune response to *Pf*.

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VIVAX MALARIA VACCINE DEVELOPMENT: TAKING CUES FROM NATURE

Sope Olugbile[1], Akihide Takagi Takagi[1], Pu Liu[1], Malcolm Gardner[1], Ivan Dario Velez[2], Ruobing Wang[1]

[1]Seattle Biomedical Research Institute, Seattle, WA, United States, [2]Programa de Estudio y Control de Enfermedades Tropicales PECET, Universidad de Antioquia, Medellín, Colombia

Vivax malaria is one of the most neglected infectious diseases despite the estimated 100 million clinical cases annually and recent association with severe complications. A vaccine will form the bedrock for its control and no effective candidate has been found among the few being developed. The published genomes of common malaria parasites provide a potent tool for the discovery of novel candidates using innovative approaches. The erythrocytic vivax malaria parasite requires interaction with Duffy antigen expressed on the surfaces of red blood cells of Duffy positive (Fy+) individuals for its invasion. Individuals that do not express Duffy antigen on their red cells (Duffy negative, Fy-), are therefore 'resistant' to the erythrocytic stage but are still susceptible to the pre-erythrocytic (PE) stage against which they mount immunity. We exploited this as a concept to identify new vivax antigens. We selected 100 potential vivax PE proteins by comparing the vivax genome with the genomes, sporozoite / liver stage transcriptomes and proteomes of other Plasmodium species. The genes were cloned and transfected into donor antigen presenting cells (B cells) and cocultured with autologous PBMCs. Stimulation of memory T cells that had been primed by natural infections was then assessed by cytokine analyses. We identified new vivax PE antigens by comparing and contrasting responses in Fy+ and Fy- individuals. Some of these antigens were recognized at higher frequencies by Fy- individuals while some stimulate predominantly Th1, Th2, and/or Treg responses. These findings support the concept of antigen discovery through natural differential infection in human.

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DISCOVERING NOVEL PRE-ERYTHROCYTIC ANTIGENS FOR MALARIA VACCINES

Joao Carlos Aguiar[1], Jessica Bolton[1], Joyce Wanga[1], AnneMarie Urquhart[2], John B. Sacci[3], Keith Limbach[1], Takafumi Tsuboi[4], Chris Ockenhouse[2], Thomas L. Richie[1]

[1]Naval Medical Research Center, Silver Spring, MD, United States, [2]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [3]The University of Maryland School of Medicine, Baltimore, MD, United States, [4]Cell-Free Science and Technology Research Center, Ehime University, Matsuyama, Japan

The malaria vaccine antigen pipeline is insufficient, with only a few antigens representing less than 0.2% of the *P. falciparum* proteome

currently undergoing clinical testing. With only one or two exceptions, candidate vaccines based on these traditional antigens have induced little or no protection in humans. Nevertheless, there is evidence that an effective malaria vaccine is feasible, based on the high grade (>90%) sterile immunity induced in humans by immunization with the radiation-attenuated sporozoite vaccine (ISV). Because radiation-attenuated sporozoites do not develop into blood stage parasites, the primary immune mechanisms underlying ISV-induced protection are thought to be cell-mediated cytotoxic T cell responses induced solely by multiple antigens expressed in pre-erythrocytic stages. It should therefore be possible to identify these protective antigens, and to formulate a protective pre-erythrocytic stage sub-unit vaccine based on these antigens. To meet this objective, we have selected and cloned a panel of hypothetical *P. falciparum* pre-erythrocytic genes. Using a modified wheat germ cell-free expression system, we have achieved expression of 90% of the selected clones. To date the screening of these expressed recombinant proteins has identified 20 novel antigens reacting to the sera from ISV-immunized volunteers. Further characterization of several antigens has revealed their stage-specific expression and cellular localization within sporozoite and hepatic parasites. Ongoing screening for these antigens' ability to induce recall responses in PBMC's from ISV-immunized volunteers has thus far identified 2 novel antigens implicated in ISV-induced T cell-mediated immunity. Several *P. yoelii* orthologs have been tested and shown promising efficacy in the *P. yoelii* mouse challenge model. These protection data validate our overall approach as a feasible strategy to discover novel antigens for malaria vaccines.

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IL-2-PRODUCING EFFECTOR MEMORY AND CENTRAL MEMORY CD4⁺ T CELL SUBSETS ARE ASSOCIATED WITH PROTECTIVE IMMUNITY IN RTS,S-IMMUNIZED SUBJECTS

Joanne Lumsden[1], Robert J. Schwenk[1], Lisa Egner[1], Joe Cohen[2], Ripley Ballou[2], Opokua Ofori-Anyinam[2], Philippe Moris[2], Kent E. Kester[1], D. Gray Heppner[1], Urszula Krzych[1]

[1]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]GSK Biologicals, Rixensart, Belgium

RTS,S, the only successful sub-unit malaria vaccine, confers protection in approximately 50% of subjects experimentally challenged by bites from *Plasmodium falciparum* (Pf) infected mosquitoes. Previously, we showed that PBMC from subjects protected by RTS,S immunization had higher *ex-vivo* CS protein-specific IFN-γ ELISPOT responses than PBMC from non-protected subjects. In addition, using intracellular staining we demonstrated that the frequency of CS protein-specific CD4⁺ T cells expressing two or more immune markers among CD40L, IL-2, TNF-α and IFN-γ, were significantly higher in protected vs. non-protected persons. Recent studies of other vaccines or disease models indicate that short-term protection is mediated by effector/effector memory T ($T_{E/EM}$) cells while protracted protection may require long-lived central memory T (T_{CM}) cells. Moreover, polyfunctional T cells are more effective in conferring protection than T cells producing only one cytokine. In the present study we asked whether RTS,S-induces CD4⁺ $T_{E/EM}$ and/or CD4⁺ T_{CM} cells and if these could be more informative indicators of RTS,S-induced protective immunity. We analyzed PBMC from RTS,S-immunized, protected and non-protected subjects, for the presence of CS protein-specific CCR7⁻CD45RO⁺ CD4⁺ $T_{E/EM}$ and CCR7⁺CD45RO⁺ CD4⁺ T_{CM} cells and for their production of multiple cytokines. RTS,S induced both CD4⁺ $T_{E/EM}$ and CD4⁺ T_{CM} cells that could be recalled *in vitro* by Pf CS protein peptides to produce strong IL-2 responses. The frequency of CD4⁺ $T_{E/EM}$ as well as CD4⁺ T_{CM} cells producing IL-2 was associated with protection. In addition, the frequency of CD4⁺ $T_{E/EM}$ cells producing TNF-α was higher in protected than non-protected subjects. Finally, we identified a population of CD4⁺ $T_{E/EM}$ cells that produced both IL-2 and TNF-α and the frequency of these cells was also associated with protection.

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GENETICALLY ATTENUATED VACCINES INDUCE CONTACT-DEPENDENT CD8⁺ T CELL KILLING OF *PLASMODIUM YOELII* LIVER STAGE-INFECTED HEPATOCYTES

Megha Gupta[1], Adama Trimnell[1], Akihide Takagi[1], Thomas L. Richie[2], Stefan H. Kappe[1], Ruobing Wang[1]

[1]Seattle Biomedical Research Institute, Seattle, WA, United States, [2]Naval Medical Research Center, Silver Spring, MD, United States

Protracted sterile protection conferred by a *P. yoelii* genetically attenuated parasite (PyGAP) vaccine was found to be completely dependent on CD8⁺ T lymphocytes in the effector phase. Immunization studies with perforin and IFN-γ knock-out mice indicated that the protection was largely dependent on perforin as compared to IFN-γ. For the first time, we demonstrate contact-dependent killing of *Plasmodium* infected hepatocytes based on live visualization of interactions between CD8+ T cell and infected hepatocytes. Both liver and spleen CD8⁺ T cells from PyGAP immunized mice induced massive apoptosis of liver stage (LS)-infected hepatocytes *in vitro* without the release of detectable IFN-γ and TNF-α, which directly correlated with GAP vaccine efficacy *in vivo*. Most importantly, we demonstrated that CD8⁺ T cells isolated from naïve mice that had survived wild-type *P. yoelii* sporozoite infection targeted mainly sporozoite-traversed and uninfected hepatocytes, revealing an immune evasion strategy that might have been used by wild-type parasites to subvert host immune responses *in vivo*. In contrast, CD8⁺ T cells derived from PyGAP-protected mice are cytotoxic against both sporozoite-traversed and LS-infected hepatocytes. A controversial role of circumsporozoite protein (CSP) in host immune protection versus immune evasion against malaria is also highlighted by our findings.

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INTERACTIONS BETWEEN MUTUALIST *WIGGLESWORTHIA* AND TSETSE PEPTIDOGLYCAN RECOGNITION PROTEIN (PGRP-LB) INFLUENCE TRYPANOSOME TRANSMISSION

Jingwen Wang, Yineng Wu, Guangxiao Yang, Serap Aksoy

Yale University, New Haven, CT, United States

Tsetse flies, the sole vectors of African trypanosomes, have coevolved with mutualistic endosymbiont *Wigglesworthia glossinidiae.* Elimination of *Wigglesworthia* renders tsetse sterile, and also increases their trypanosome infection susceptibility. We show that a tsetse peptidoglycan recognition protein (PGRP-LB) is crucial for symbiotic tolerance and trypanosome infection processes. Tsetse *pgrp-lb* is expressed in the *Wigglesworthia*-harboring organ (bacteriome) in the midgut, and its level of expression correlates with symbiont numbers. Adult tsetse cured of *Wigglesworthia* infections have significantly lower *pgrp-lb* levels than corresponding normal adults. RNA interference (RNAi) mediated depletion of *pgrp-lb* results in the activation of the immune deficiency (IMD) signaling pathway and leads to the synthesis of antimicrobial peptides (AMPs), which decrease *Wigglesworthia* density. Depletion of *pgrp-lb* also increases host's susceptibility to trypanosome infections. Finally, parasitized adults have significantly lower *pgrp-lb* levels than flies, which have successfully eliminated trypanosome infections. When both PGRP-LB and IMD immunity pathway functions are blocked, flies become unusually susceptible to parasitism. Based on the presence of conserved amidase domains, tsetse PGRP-LB may scavenge the peptidoglycan (PGN) released by *Wigglesworthia* and prevent the activation of symbiont-damaging host immune responses. In addition tsetse PGRP-LB apparently has an anti-protozoal activity that confers parasite resistance. The symbiotic adaptations and the limited exposure of tsetse to foreign microbes may have led to the considerable differences in *pgrp-lb* expression and regulation noted in tsetse from that of closely related *Drosophila*. A dynamic interplay between *Wigglesworthia* and host immunity apparently is influential in tsetse's ability to transmit trypanosomes.

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THE MOSQUITO HEART: FUNCTIONAL MECHANICS AND ROLE IN MALARIA SPOROZOITE MIGRATION

Julian F. Hillyer, Jonas G. King, Justin D. Glenn

Vanderbilt University, Nashville, TN, United States

The migration of sporozoites from the mosquito midgut to the salivary glands is a step required for the completion of the *Plasmodium* life cycle. The mechanism for this migration is not understood, with possibilities including parasite-driven motility, hemolymph flow-based transport, or a combination of the two. Here, we will present qualitative and quantitative data analyzing the structure and function of the core organ of the mosquito circulatory system and examine its role in sporozoite migration. Analysis of the contraction mechanics of the dorsal vessel demonstrated that the heart is a dynamic organ that contracts an average of 82 times per minute and pumps hemolymph in both anterograde (toward the head) and retrograde (toward the tip of the abdomen) directions, with greater than two thirds of the contractions occurring in the anterograde direction. Hemolymph enters the heart through paired ostia located in the anterior portion of each abdominal segment and flow is accomplished by the contraction of muscle fibers wrapped spirally around the vessel. It is the direction of the sequential contraction of these fibers, supported by alary muscles, that propels hemolymph from one end of the organism to the other. Binucleate pericardial cells laterally flank the heart and fluorescence labeling of acidic organelles suggests that they may be involved in immune surveillance. Experiments on *Plasmodium*-infected mosquitoes showed that, similar to the flow of endogenous and inoculated particles, sporozoites enter the dorsal vessel through the ostia and flow to the head at speeds much greater than can be accounted for by active motility alone. This migration event does not alter heart contraction rates or directionality, suggesting that sporozoites do not influence the normal circulatory physiology of the insect. Overall, these data expand our knowledge of the general mechanics of the mosquito circulatory system and show that the mosquito heart facilitates the transport of sporozoites from the abdomen to the salivary glands.

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GENE DUPLICATION AND GENOME EVOLUTION IN THE *IXODIDAE*

Janice Van Zee[1], Jason Meyer[1], Shannon Schlueter[1], Jessica Schlueter[1], Phil Dixon[2], Catherine Hill[1]

[1]Purdue University, West Lafayette, IN, United States, [2]Iowa State University, Ames, IA, United States

Hard ticks (Ixodidae) are obligatory hematophagous ectoparasites of worldwide medical and veterinary importance. Ixodid ticks have relatively large genomes (1 to >7 Gbp) compared to those characterized in other arthropods, but relatively little is known about their genome structure and organization. We hypothesize that the evolution of genome size and chromosome number in Ixodid ticks is associated with a vast accumulation of DNA through major duplication events and a proliferation of repetitive sequences. To begin to address this, a bioinformatics approach was used to identify putatively duplicated genes (paralogs) using expressed sequence tagged (EST) data from four species of hard ticks, namely the prostriate tick *Ixodes scapularis*, and the metastriate ticks *Amblyomma variegatum* (tropical bont tick), *Rhipicephalus microplus* (southern cattle tick) and *R. appendiculatus* (brown ear tick). Between 2-10% of EST sequences represent putatively duplicated genes in these ticks, and approximately one fourth of duplicated genes are under positive selection pressure based on ratios of non-synonymous to synonymous nucleotide substitution rates. Statistical analysis of synonymous substitution rates for duplicated ESTs suggests that two and three large gene duplication events occurred in the prostriate and metatstriate tick lineages, respectively. Ongoing research involves estimating when gene duplication events occurred using a molecular clock and determining the extent to which duplications have contributed to genome size in Ixodid ticks. All duplicated ESTs were assigned putative functions by searching a Gene Ontology (GO) database with Blast2Go software. The GO analysis identified groups of associated genes under positive selection pressure that may be evolving new functions important for tick biology, such as blood feeding. This research marks the first genome-wide analysis of gene duplication in ticks and provides insight towards an understanding of genome evolution within the Ixodidae.

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EFFICIENCY AND RELIABILITY OF RBCL AND $CRCL_3$ IN MARKING *TRIATOMA BRASILIENSIS* NYMPHS: PERSISTENCE AND INFLUENCE OF TRACE ELEMENTS ON INSECT BIOLOGY

Rafael M. Freitas, Otilia Sarquis, Livia S. Oliveira, Jailey M. Gonçalves, Reginalo L. Rego, Marli M. Lima

Oswaldo Cruz Foundation, Rio de Janeiro, Brazil

In order to mark *Triatoma brasiliensis*, vector of Chagas disease in northeastern Brazil, two chemical compounds, chloride of rubidium (RbCl) and chloride of chromo ($CrCl_3$), were tested. Former, 199 nymphs (N2=77, N3=40, N4=32, N5=50) were fed on blood with RbCl 0.025M. Overall, 70% of N2, 2.5% of N3, 12.5% of N4 and 42% of N5 were marked. Highest RbCl persistence was observed in N2, when 98 days post blood meal (pbm) five insects were still positive. Later, 265 nymphs were fed on blood with $CrCl_3$ 0.0015M (N2=65, N3=85, N4=65, N5=50), where 61.5% of N2, 73% of N3, 83% of N4 and 93% of N5 were positive; $CrCl_3$ marking persisted up to 119 days pbm. $CrCl_3$ detection was achieved after some insects have mounted to adult, suggesting this marking may persist over *T. brasiliensis* adult life. A second step of this report was to investigate if $CrCl_3$ altered marked insects biology, such as survival rates, stages developmental time and fecundity. For that we blood-fed 182 *T. brasiliensis* nymphs at two distinct life stages: N1 (Control: n = 50, Experimental: n = 50) and N4 (Control: n = 41, Experimental: n=41). Survival rates of control and experimental groups of N1 and N4 was statistically similar according to Log-rank test (N1: Log-rank = 1.44, g.l. = 1, p = 0.23; N4: Log-rank = 3.48, g.l. = 1, p = 0.062). Developmental time of blood fed individuals with $CrCl_3$ was from N4 to N5 and from N5 to adult stage was 64.8 ± 14.2 and 74.9 ± 17.5 days, respectively. Meanwhile, N4 individuals from control group lasted 73.2 ± 28.5 days to become N5 and another 72.5 ± 17.8 to become adults. Differences of developmental time between control and experimental groups were not significant. At last, the mean number of eggs laid by adult females of control (n = 77.3) and experimental group (n = 64.3) was statistically similar between groups. Finally, RbCl and especially $CrCl_3$ seem to be reliable methods to mark *T. brasiliensis* nymphs, since element trace detection was persistent and did not influence insect survival rates, developmental time and fecundity.

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FEED-THROUGH CONTROL FOR LARVAL SAND FLIES USING HOST-TARGETED INSECTICIDE

Gideon Wasserberg[1], Richard Poche[2], Larisa A. Polyakova[2], Michelle V. Chenault[1], Gabriela Zollner[1], Edgar D. Rowton[1], David Miller[2]

[1]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]Genesis Laboratories, Inc., Wellington, CO, United States

In some species of Phlebotomine sand flies larvae are known to feed on the feces of rodents. This behavior can be exploited as a potential route for sand fly control. Here, we describe the results of a study aimed at evaluating the feed-through effectiveness of the insecticide Imidaclorpid on larval sand flies. First, we conducted a pilot study using 6 laboratory rats, followed by a larger study using 22 sand rats. Rodents were exposed for 7 days to diet containing different doses of the insecticide Imidoclorpid (0, 100 and 250 ppm for rats; 0, 50, 100, 250 ppm for sand rats). Fecal pellets collected from rats at days 2 and 4 and from sand rats at day 7. These samples were used for the sand fly larvae feeding assay. Crushed

fecal pellets and standard feed samples were applied to different wells (3.5 cm diameter) containing 1st or 2nd/3rd instar larvae of *Phlebotomus papatasi* (Israeli strain) or *Lutzomyia longipalpis* (Jacobina strain) and larvae survival was monitored for 7 days. All rats and sand rats accepted diets containing imidacloprid without any apparent health abnormalities. In the rat pellets experiment, *L. longipalpis* was less sensitive to the insecticide. For both sand fly species feeding on pellets from both rodent species, 1st instar larvae were more sensitive than the older stages. For larvae feeding on rat pellets, larvae feeding on pellets collected on day 4 had higher mortality then those feeding on pellets collected at day 2. In all cases sand fly species demonstrated a typical dose-response with strongest lethal effect for the 250 ppm samples. For example, for 1st instar *P. papatasi* larvae feeding on sand rat feces, all doses higher than 50 ppm passed the larvicidal threshold of 90% mortality whereas for the older stages 90% mortality was obtained only with the highest dose of 250 ppm. In summary, these results are encouraging for the feasibility of developing a novel environment-friendly, focused, control system that would take advantage of the tight ecological association between the reservoir rodent host and the sand fly vector.

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ASSORTATIVE MATING IN THE DENGUE VECTOR MOSQUITO, *AEDES AEGYPTI*

Laura C. Harrington, Katherine J. Connors, Lauren J. Cator, Michelle E. Helinski

Cornell University, Ithaca, NY, United States

Understanding the mating system of mosquitoes is critically important for implementation of genetic or sterile male programs. Assortative mate choice has been demonstrated in many insect mating systems. In mosquitoes, mating itself may be a dynamic process strongly affected by assortative mating in a positive or negative direction, although little experimental data exists. We examined the potential for assortative mating in the dengue vector mosquito, *Aedes aegypti*. Virgin females and males were simultaneously presented with mates representing a range of parameters. Mating was observed and mate status was recorded. Our results indicate an assortative mating effect that is influenced strongly by several different parameters including body size, strain and other traits. The implications of these results will be discussed within the context of sterile/transgenic vector control programs.

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THE EFFECT OF MALE MATING HISTORY AND BODY SIZE ON FEMALE FECUNDITY AND LONGEVITY IN THE DENGUE VECTOR *AEDES AEGYPTI*

Michelle E. Helinski, Laura Sirot, Mariana F. Wolfner, Laura C. Harrington

Cornell, Ithaca, NY, United States

Male reproductive success is dependent on insemination success (the number of females he can mate with) and reproductive output (the total number of progeny he sires). Production of semen components (i.e. accessory gland proteins and/ or sperm) can be costly in insects and it is predicted that male reproductive success is resource-limited. In this study we investigated the role of body size and mating history on male reproductive success in *Aedes aegypti*, the principal vector of dengue viruses. Males were reared in the larval stage under two different crowding regimes to obtain large or small body sized mosquitoes. Males were mated in rapid succession to up to four females. Copulation duration and female lifetime reproductive success (i.e. life-time fecundity) and longevity after mating with large or small males were determined. Our results suggest that male body size is a strong determinant of male ability to inseminate multiple females. We found a dramatic reduction in female fecundity for females that were the fourth to mate with small males in comparison to females that had mated earlier in the sequence. We did not find a similar reduction in fecundity with mating order for the females mated to large males. The reduction in lifetime reproductive success seen for females mated to small males suggest that small males are more rapidly depleted in semen components than large males. The results of this study will contribute towards a better understanding of male mating biology, an essential component in the successful implementation of genetic control strategies for mosquitoes.

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DIAGNOSTIC TOOLS FOR LYMPHATIC FILARIASIS ELIMINATION PROGRAM: RESULTS OF A COMMUNITY AND SCHOOLCHILDREN SURVEY IN AN ISLAND OF FRENCH POLYNESIA

Catherine Plichart[1], Lam Ngoc Nguyen[1], Sylviane Teururai[1], Clemence Gatti[1], Herve Bossin[1], Jerome Marie[1], Marc Faaruia[1], Albert Tetuanui[1], Tuterarii Paoaafaite[1], Ralph Pawlowiez[1], Sandra J. Laney[2], Makoto Itoh[3], Anne-Marie Legrand[1]

[1]Institut Louis Malarde, Papeete-Tahiti, French Polynesia, [2]Smith College, Northampon, MA, United States, [3]Aichi Medical University, Aichi-ken, Japan

The Diagnostic Study Group of the Global Programme to Eliminate Lymphatic Filariasis, is testing various surveillance tools in 8 lymphatic filariasis endemic areas including French Polynesia,. This report summarizes results of the diagnostic assays conducted in Moorea, an island of the Society archipelago. Blood and urine samples were collected from 1,018 and 935 residents, and 365 and 337 schoolchildren, respectively. *Aedes polynesiensis (Ap)* and *Culex quinquefasciatus* (*Cq*) specimens were also collected in the area using attractive and gravid traps. Blood samples were tested for microfilaremia (Mf) by microscopic examination of blood smears and a PCR technique for *Wuchereria bancrofti (Wb)* larvae DNA detection. Filarial antigenemia (Ag) was evaluated by a rapid test (ICT) and an ELISA test (Og4C3). Antibodies (Ab) to recombinant filarial antigens were detected with a rapid test (PanLF) and two ELISA tests, one in blood (Bm14) and one in urine (U SXP1). Mosquito specimens sampled in pools were also tested by a PCR technique for *Wb* larvae DNA detection. Raw data in the community group and schoolchildren group were: Mf positive rates 3.8% and 0% by microscopy and 2.2% and 0% by PCR; Ag positive rates 11.7% and 1.7% by ICT and 6.9% and 0.6% by Og4C3; Ab positive rates 17.0% and 5.9% by PanLF, 48.4% and 42.5% by Bm14, and 29.2% and 3.6% by U SXP1, respectively. Mosquito infection rates were 1.7% (estimated from 3,705 specimens spread across 292 pools) for *Ap* and 0.1% (estimated from 2,780 specimens spread across 208 pools) for *Cq*. Parasitism, evaluated by positive Ag in blood, lead to non fully concordant results using ICT and Og4C3 kit. Careful examination of positive samples indicates that the two tests can be considered as complementary. Ab detection data allow to compare three groups of people: non infected, adult worm-infected with and without microfilaria. The results obtained with the 3 Ab detection tests will be discussed. Mf data and mosquito infection rates indicate that significant *Wb* larvae transmission still occurs despite 8 years of drug distribution.

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INCREASED ADHERENCE TO MASS DRUG ADMINISTRATION FOR LYMPHATIC FILARIASIS - ORISSA STATE, INDIA, 2009

Paul T. Cantey[1], Grace Rao[2], Jonathan Rout[2], Anna Jolly[1], John Williamson[1], LeAnne Fox[1]

[1]Division of Parasitic Diseases, NCZVED, Centers for Disease Control and Prevention, Atlanta, GA, United States, [2]Church's Auxiliary for Social Action, Bhubaneswar, India

Around 590 million Indians live at risk of infection with lymphatic filariasis (LF). Drug coverage - the percentage of the total population that consumes diethylcarbamazine (DEC) during mass drug administration (MDA) for LF - has remained low in many areas and will delay the interruption of LF transmission in India. Coverage in 2007 in Khurda district of Orissa was 56%. It is postulated that both improved MDA

education and the presence of LF morbidity control programs can increase MDA coverage. In 2008, an Indian NGO, the Church's Auxiliary for Social Action (CASA), implemented a pre-MDA education campaign, in consultation with Centers for Disease Control and Prevention, which targeted known predictors of adherence with DEC. In addition, CASA was implementing a lymphedema management program in one part of Khurda district. We examined the effectiveness of the pre-MDA education campaign on DEC coverage. A 90-village, 15-household (HH) per village, stratified, cluster survey was performed to assess drug coverage in 3 areas of Khurda district, each receiving different intervention strategies, using a HH survey for all HH members and a knowledge, attitudes, and practices (KAP) survey for one randomly selected adult per HH. The study areas were 1) Area A, which received the CDC-modified CASA pre-MDA campaign and CASA's lymphedema management program; 2) Area B, which received the CDC-modified CASA pre-MDA campaign; and 3) Area C, which received the Indian Ministry of Health (MOH) campaign. Survey questions asked about DEC compliance, side effects, and knowledge of LF and the MDA. There were 8155 persons included in the HH survey. DEC coverage was 90.2% (95% CI, 87.8-92.6%) in Area A, 75.1% (95% CI, 69.7-80.6%) in Area B, and 52.8% (95% CI, 45.8-60.3%) in Area C ($p<0.0001$). Significant predictors included knowing about the MDA in advance, knowing that the MDA was for LF, knowing that mosquitoes transmit LF, and knowing that everyone was at risk for LF. Identifying and targeting predictors of adherence to DEC in the pre-MDA educational message resulted in a substantial increase in DEC coverage. The difference between Area A and Area B in DEC coverage suggests that lymphedema management programs may synergistically augment coverage. The Indian MOH should strongly consider making use of available partnerships to develop and evaluate pre-MDA educational campaigns and implement LF disability prevention programs, particularly in areas of low DEC coverage.

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LONG LASTING INSECTICIDAL NETS ALONE CAN REDUCE TRANSMISSION OF LYMPHATIC FILARIASIS IN SOUTH EAST NIGERIA

Emmanuel Emukah[1], **Patricia M. Graves**[2], Aryc W. Mosher[2], Lindsay Rakers[2], Emmanuel Miri[3], Njoku Chidiebere[1], Nwodu Kenrick[1], Obiezu Josephine[1], Okpala T. Njideka[1], Frank O. Richards[2]

[1]The Carter Center, Owerri, Nigeria, [2]The Carter Center, Atlanta, GA, United States, [3]The Carter Center, Jos, Nigeria

In south east Nigeria, *Loa loa* co-endemicity restricts mass drug administration for lymphatic filariasis (LF) but long lasting insecticidal nets (LLIN) alone may reduce LF transmission. From July 2007, mosquitoes were collected monthly in 3 sentinel sites in each of four local government areas (LGAs). The LGAs were randomized into 2 groups and free PermaNet 2 (Vestergaard Frandsen) LLIN (total 200,000) were distributed in April 2008 by village volunteers: 2 LGAs in group A received nets according to the national policy at that time (pregnant women and children under five years) and 2 LGAs in group B were targeted for 'full coverage' i.e. one LLIN per sleeping space. Baseline and follow-up household cluster surveys performed in 2007 and 2008 showed that 43.1 [95% CI 38.9 to 47.4]% and 91.7 [88.9 to 94.0]% of households owned at least one net (up from 6.8 [4.7 to 9.4]% and 4.3 [2.6 to 6.5]% in 2007) in groups A and B respectively. The average nets per household was 1.33 [1.28 to 1.40] and 2.03 [1.95 to 2.12] respectively. The night prior to survey 11.4 [10.2 to 12.7]% of persons in group A and 62.4 [60.4 to 64.4]% in group B slept under a net (23.1% and 61.3% in under five year olds in groups A and B respectively). In sentinel sites, the average number of mosquitoes caught per site per month in group A declined significantly by 60% from 170.6 [117.5 to 223.7] in July - November 2007 (before net distribution) to 68.5 [43.4 to 93.6] in the same months of 2008. In the full coverage group B, average mosquitoes per site per month declined by 90% from 124.6 [99.9 to 149.3] to 12.3 [9.6 to 15.0]. The percent of mosquitoes infected (all larval LF stages) also declined significantly from 2.9 [2.4 to 3.4]% to 1.1 [0.7 to 1.6]% in group A ($p<0.001$) and from 1.2 [0.8 to 1.6]% to 0.0 [0.0 to 1.0]% in group B ($p=0.027$). Targeting full coverage of all sleeping spaces with LLIN achieves much higher net utilization (in children under five as well as all age groups) and may be more effective in reducing LF transmission than targeting nets only to vulnerable groups.

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TRANSMISSION INTENSITY AND BIOMARKERS OF *WUCHERERIA BANCROFTI* INFECTION 10 YEARS AFTER CESSATION OF MASS DRUG ADMINISTRATION TO ELIMINATE LYMPHATIC FILARIASIS IN PAPUA NEW GUINEA

Daniel J. Tisch[1], Will Kastens[1], Bockarie J. Moses[1], Moses Baisor[2], Melinda Susapa[2], Daphne Sepe[2], Kay Baia[2], Manasseh Baia[2], John Reeder[3], Edwin Michael[4], Peter Siba[5], James W. Kazura[1]

[1]Case Western Reserve University, Cleveland, OH, United States, [2]Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea, [3]Burnet Institute, Melbourne, Australia, [4]Imperial College, London, United Kingdom, [5]Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea

The current WHO strategy to eradicate lymphatic filariasis (LF) is based on the concept that reduction of blood microfilaria (mf) by 4 to 6 years of annual mass administration of anti-filarial drugs (MDA) will decrease the reservoir of mf below a threshold required to continue transmission of infective third stage larvae (L3) by mosquitoes. This goal may be achievable when pre-MDA mf+ rates are low (<2-3%) and Culex mosquitoes transmit LF (Lancet 2006;367:992) but the feasibility of infection extinction in areas where mf+ rates are high and Anopheles mosquitoes transmit LF has not been established. We investigated the possible resurgence of LF in Papua New Guinea where An. punctulatus transmits LF 10 years after 5 annual rounds of MDA were given from 1994 to 1998. Community mf+ rates before MDA (1993) were 42-77%; transmission intensities were such that mosquitoes harboring L3 were observed for 6-12 months/yr. After the 4th MDA (1998), mf+ rates decreased to 1-5% and transmission of L3 to 1-6 months/yr (NEJM 2002;347:1841). Ten years after cessation of MDA (2008) with no additional interventions, mf+ rates were 0-38% (filarial antigen card test+ rate 7-69%), and mosquitoes bearing L3 were observed for 1-4 months/yr. Among children born after the end of the final MDA (age <10 years) mf+ rates ranged from 0 to 12% and card positive rates ranged from 0 to 50%. The long-term effect of MDA was inversely related to pre-MDA transmission intensity. These data will be discussed in terms of their implications for the global strategy to eradicate LF.

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DOES TRANSMISSION TAKE PLACE IN HYPOENDEMIC AREAS FOR ONCHOCERCIASIS? A STUDY IN NORTH REGION OF CAMEROON

Moses N. Katabarwa[1], Albert Eyamba[2], Mouhamadou Souaibou[2], Peter Enyong[3], Thomas Kuete[4], Yaya Souleymanou[5], Abudoulaye Yougoude[5], Gervais O. Andze[6], Marceline Ntep[6], Lindsay Rakers[1], Donald Hopkins[1], Frank Richards[1]

[1]Emory University/The Carter Center, Atlanta, GA, United States, [2]The Carter Center, Yaounde, Cameroon, [3]Research Foundation for Tropical Disease and Environment, Buea, Cameroon, [4]University of Doula, Doula, Cameroon, [5]Ministry of Health, Garoua, Cameroon, [6]Ministry of Health, Yaounde, Cameroon

In Africa most mass drug distribution (MDA) with ivermectin for onchocerciasis is targeted to meso- and hyperendemic areas (≥ 20% onchocercal nodule and ≥ 40% microfilariae prevalence). Where onchocerciasis is below that threshold, communities are considered hypoendemic or nonendemic, and mass treatment is not recommended. The rationale for this policy is that most disease due to onchocerciasis occurs in meso/hyper endemic zones. As policy begins to shift from control toward transmission interruption, the role that hypoendemic areas play in maintaining *Onchocerca volvulus* needs to be reexamined. If independent transmission exists in hypoendemic areas then onchocerciasis

recrudescence could be 'reseeded' into adjacent meso/hyper areas should the decision be made to halt MDA. The aim of the study was to determine whether independent transmission occurs in a hypoendemic area not targeted for mass treatment in northern Cameroon. The study took place in Ngong, a hypoendemic onchocerciasis area 30 km from the nearest meso/hyper area where MDA has been implemented for over 15 years: 649 adults and 561 children (age 3 to 10 years) resident in ten communities were examined for nodules and microfilaria, and 334 adults for ocular morbidity. Also 255 *Simulium* flies from four fly collection points inside the Ngong were captured over 3 months and examined for larval stages of *O. volvulus.* Nodule and mf prevalence among adults was 12.3% and 2.9% respectively and among children 9.2% and 0.5%. Mf in the anterior chamber was observed in 0.3% and specific onchocercal punctate keratitis in 0.6% in 334 a sample of persons examined. Four (0.16 percent) out of 255 flies were infected with L3 larvae, yielding a 3 month transmission potential of 3.35. The results showed that Ngong is a hypoendemic area and yet has likely low grade autotocthonous transmission that has persisted despite years of treatment in the hyper/meso areas of North Province. If elimination of transmission becomes the goal, hypoendemic transmission zones areas such as Ngong need to be identified and treated.

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PROGRESS ON THE ELIMINATION OF ONCHOCERCIASIS (RIVER BLINDNESS) FROM THE AMERICAS

Frank O. Richards[1], Mauricio Sauerbrey[2], Guillermo Zea Flores[2], Alfredo Dominguez Vazquez[2], Alba Lucia Morales[2], Ed Cupp[3]

[1]Carter Center, Atlanta, GA, United States, [2]Onchocerciasis Elimination Program for the Americas, Guatemala City, Guatemala, [3]Auburn University, Auburn, AL, United States

Onchocerciasis in the Americas affects six countries where it is limited to 13 foci. A regional initiative called the Onchocerciasis Elimination Program for the Americas (OEPA) promotes twice-per-year ivermectin mass drug administration (MDA) to exceed 85% of the eligible population of endemic communities. OEPA was formed in 1992 after a 1991 PAHO resolution (CD35R14) to eliminate new onchocerciasis eye disease from the Americas by 2007. In September 2008 OEPA presented a report to the meeting of the PAHO Directing Counsel (DC) on progress toward this goal, which included regional wide achievement of MDA goals in the six endemic countries (Brazil, Colombia, Ecuador, Guatemala, Mexico, and Venezuela), the elimination of eye disease attributable to onchocerciais in 9 of the 13 foci, and interruption of transmission (with concommitant halt of mass treatment) in 4 of the 13 foci. No new cases of complete blindness from river blindness has been recorded in the region in the last decade. The DC issued a new resolution (CD48R12) in 2008 calling on countries and their partners to complete the elimination of onchocercal eye disease and interrupt onchocerciasis transmission in the Americas by the end of 2012. Later in 2008, at the eighteenth annual Inter-American Conference on Onchocerciasis held in Oaxaca, Mexico, two other foci announced that interruption of transmission had been achieved. At the beginning of 2009, the entire population at risk of onchocerciasis in the Americas requiring mass treatment was 360,189, a decrease of 31% from 525,543 in 2006. The six foci now off treatment are in three countries: Guatemala (Santa Rosa, Escuintla, and Huehuetenango foci), Mexico (Oaxaca and North Chiapas foci) and Colombia (the Lopez de Micay focus). In addition a riverine subfocus (Rio Santiago) in Ecuador also has halted treatment. Post MDA surveillance activities have been launched in all these areas to monitor for at least three years for potential transmission recrudescence, in accord with WHO guidelines. Brazil and Venezuela have all the remaining eye disease and the most active onchocerciasis transmission areas in the region. The difficult to access endemic area shared by these two countries on their frontiers in the Amazon region is the greatest hurdle to complete onchocerciasis elimination in the Americas.

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POSSIBLE INTERRUPTION OF MALARIA TRANSMISSION IN TWO HIGHLAND AREAS OF KENYA

Chandy C. John[1], Melissa A. Riedesel[1], Ng'wena G. Magak[2], David M. Menge[1], Kim A. Lindblade[3], John M. Vulule[4], James A. Hodges[1], Willis Akhwale[5]

[1]University of Minnesota, Minneapolis, MN, United States, [2]Moi University, Eldoret, Kenya, [3]Centers for Disease Control and Prevention, Atlanta, GA, United States, [4]Kenya Medical Research Institute, Kisumu, Kenya, [5]Ministry of Health, Nairobi, Kenya

Highland areas of unstable transmission are attractive targets for malaria elimination because malaria transmission decreases to very low levels during the dry season. Methods: Clinical visits for malaria at the local dispensaries in the highland areas of Kipsamoite and Kapsisiywa, Kenya (population ~7,400 individuals) were recorded from 2003-2008. The Ministry of Health performed household indoor residual spraying (IRS) with a synthetic pyrethroid annually in 2005, 2006 and 2007 (78% household coverage in 2007). Artemether-lumefantrine was implemented as first line malaria treatment in Kapisisiywa in October 2006 and Kipsamoite in February 2007. Insecticide treated net ownership was estimated at 13.0% in 2007. From April 2003 to March 2007, average annual malaria incidence was 36.1/1000 persons across the two sites. From April 2007 to March 2008, no microscopy positive cases of malaria were seen in 416 symptomatic individuals (0%). Polymerase chain reaction (PCR) testing for *P. falciparum* was positive in 17 of 231 of symptomatic individuals tested during this time period (7.3%), but only 2 PCR positive samples occurred after June 2007. Among asymptomatic individuals assessed in a cross-sectional survey in May 2007 and a cohort in August and November 2007 and April 2008, 0.1%, 0%, 0% and 0.2% of the population were positive for asexual *P. falciparum* by microscopy and 0.25%, 0.25%, 0% and 0% by PCR, respectively. Over the study period, there was no consistent pattern of changes in rainfall or temperature, but indoor resting vector density decreased significantly. In conclusion, in areas of unstable malaria transmission, interruption and eventual elimination of malaria may be achievable with widespread annual IRS treatment of households and the use of artemisinin combination therapy.

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PREVALENCE OF PCR-DETECTABLE *PLASMODIUM* PARASITEMIA IN PARTICIPANTS IN THE DEMOCRATIC REPUBLIC OF THE CONGO, 2007 DEMOGRAPHIC HEALTH SURVEY

Steve M. Taylor[1], Jonathan J. Juliano[2], Jeremie Muwonga[3], Augustin Okenge[3], Antoinette Tshefu[4], Janey Messina[5], Ann A. Way[6], Mohamed Ayad[6], Michael Emch[5], Steven R. Meshnick[1]

[1]Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC, United States, [2]Division of Infectious Diseases, University of North Carolina, Chapel Hill, NC, United States, [3]Programme National de Lutte Contre le Sida, Kinshasa, The Democratic Republic of the Congo, [4]Kinshasa School of Public Health, Kinshasa, The Democratic Republic of the Congo, [5]Department of Geography, University of North Carolina, Chapel Hill, NC, United States, [6]Measure DHS, Calverton, MD, United States

Most global malaria data are based on sentinel surveillance systems, not nationally representative population-based surveys. The Democratic Republic of the Congo (DRC) is a country of 61 million people with an estimated 23 million cases of clinical malaria each year. However, reliable estimates of malaria prevalence are lacking. We are measuring the prevalence and distribution of malaria in the DRC using dried blood spots obtained during the 2007 Demographic and Health Survey (DHS), a nationally-representative household survey of over 12,000 people conducted in 2007. Dried blood spots, collected for HIV seroprevalence determination, are now being used to measure malaria prevalence by a high throughput pooling algorithm using real-time polymerase chain

reaction (PCR). Thus far, we have extracted gDNA from 499 patients, of which 141 were positive by real-time PCR for Plasmodia (28%), using a real-time PCR assay that targets a sequence of the Plasmodia 18s ribosomal subunit (rDNA) that is conserved between species. Among 115 subjects infected with the human immunodeficiency virus (HIV), 22 were parasitemic (19%), whereas 119 of 384 HIV-negative patients were parasitemic (31%; p=0.013 by Fisher's exact test for comparison). Samples demonstrating amplification were subsequently amplified in a speciation real-time PCR assay that targets species-specific sequences of the rDNA in *P. falciparum*, *P. ovale*, and *P. malariae*. Speciation revealed that *P. falciparum* alone accounts for >99% of infections. Our study demonstrates that the prevalence of asymptomatic parasitemia among community-dwelling citizens of the DRC is high, and that nationally-representative epidemiologic surveys can offer critical information on the burden of infectious diseases. Processing of the remaining samples is ongoing.

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DOES INDOOR RESIDUAL SPRAYING PROVIDE ADDED PROTECTION TO INSECTICIDE TREATED NETS IN PREVENTING MALARIA - PRELIMINARY RESULTS OF AN INCIDENCE COHORT

Mary J. Hamel[1], Peter Otieno[1], Nabie Bayoh[1], Simon Kariuki[1], Kayla Laserson[1], Willis Akhwale[2], John Williamson[3], Laurence Slutsker[3], John Gimnig[3]

[1]Centers for Disease Control and Prevention/Kenya Medical Research Institute Research Station, Kisumu, Kenya, [2]Kenya Ministry of Health, Nairobi, Kenya, [3]Centers for Disease Control and Prevention, Atlanta, GA, United States

Prior to wide-spread introduction of insecticide-treated bednets (ITN), indoor residual spraying (IRS) was shown to reduce malaria in areas of high malaria transmission. However, the added benefit of IRS in areas with high ITN use is unknown. In August-September, the Kenya Division of Malaria Control implemented IRS in Rachuonyo (IRS-District), but not Nyando (no-IRS-District). In December 2008, we randomly selected compounds within 1 Km of 6 health facilities (3 per district, matched for catchment size and health-facility type) and enrolled subjects into a malaria parasitemia incidence cohort to determine the added benefit of IRS plus ITNs vs. ITNs alone. All non-pregnant household members over 6 months of age were eligible for inclusion. Consented subjects were interviewed, provided blood samples for malaria smears, and were presumptively treated with artemether-lumefantrine to clear any malaria infection. ITNs were provided for every sleeping space in included compounds. Subjects were visited at home monthly and blood smears made. Subjects were requested to present to study facilities when ill for examination and blood smear. Subjects have been followed for 4 of 6 planned months. A total of 1807 subjects were enrolled in the incidence cohort (921 in IRS-District, 886 in no-IRS-District). Preliminary data indicate malaria parasite prevalence was similar in the two district at enrollment (5.6% in IRS-District, 5.5% in no-IRS-District; p=0.94). Malaria incidence in the IRS-District and no-IRS-District is 3.5% and 9.5% respectively (Rate Ratio (RR) 0.37; Protective Efficacy (PE) 63%, p<0.001). The PE was especially pronounced among children <5 years of age (RR=0.18; PE=82%, p<0.001). Reported ITN use was higher in the IRS-District compared with no-IRS-District at enrollment (32% and 27% respectively, p=0.01,) and lower at month-4 (77% and 99% respectively, p<0.001). In this preliminary analysis, IRS + ITN provided significant added protection against malaria infection compared to ITN alone. The combination of IRS and ITN could be considered for use to further reduce transmission in selected areas of high malaria transmission.

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THIRTY YEARS OF *PLASMODIUM FALCIPARUM* TRANSMISSION IN KENYA: TIME-SPACE MODELLING OF PARASITE PREVALENCE

Abdisalan M. Noor[1], Peter W. Gething[2], Victor A. Alegana[1], Eric Muchiri[3], Robert W. Snow[1]

[1]Kenya Medical Research Institute/Wellcome Trust/University of Oxford Collaborative Research Programme, Nairobi, Kenya, [2]Spatial Ecology and Epidemiology Group, Tinbergen Building, Department of Zoology, University of Oxford, Oxford, United Kingdom, [3]Division of Vector Borne and Neglected Disease, Ministry of Health, Nairobi, Kenya

Examining temporal and spatial changes parasite prevalence provides useful insight into changing malaria epidemiology and resource needs. Methods: *Plasmodium falciparum* parasite prevalence data for the period 1975 - 2009 were assembled from unpublished random community and school surveys, national reports, and peer-reviewed articles. Presence of parasites was examined using either rapid diagnostic tests or microscopy. Spatial-temporal Bayesian geostatistical models were used with environmental and survey covariates to predict continuous maps of malaria prevalence in Kenya, taking into account uneven distributions of surveys in space and time. Time-series plots of the parasite prevalence data were constructed showing the median distributions for each year from 1975 - 2009 to identify spatial and temporal changes in infection rates. Results: There have been overall declines in *P. falciparum* infection rates across Kenya. The time-series plots showed peaks in parasite prevalence coinciding with the periods of large-scale CQ (1985 - 1994) and SP (1995 - 2004) failures. A significant drop of parasite prevalence was registered in the periods following the national-scale of ITN (2005 - 2009). Conclusions: Spatial-temporal maps of malaria prevalence provide useful tools to track the evolution of malaria epidemiology. These maps should be used in determining target areas for malaria control and forecasting changes in disease epidemiology.

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SPATIAL RISK PROFILING OF *PLASMODIUM FALCIPARUM* PARASITEMIA IN WESTERN CÔTE D'IVOIRE

Giovanna Raso[1], Kigbafori D. Silué[1], Penelope Vounatsou[2], Burton H. Singer[3], Marcel Tanner[2], Juerg Utzinger[2], Eliézer K. N'Goran[4]

[1]Centre Suisse de Recherches Scientifiques, Abidjan, Cote d'Ivoire, [2]Swiss Tropical Institute, Basel, Switzerland, [3]Princeton University, Princeton, NJ, United States, [4]University of Cocody-Abidjan, Abidjan, Cote d'Ivoire

The objective of this study was to identify demographic, environmental and socioeconomic risk factors and spatial patterns of *Plasmodium falciparum* parasitemia in a highly endemic area, and to specify how this information can facilitate improved malaria control at the district level. A questionnaire was administered to ~4,000 schoolchildren in 55 schools in western Côte d'Ivoire to determine children's socioeconomic status and their habit of sleeping under a bednet. Blood samples were collected and examined for *P. falciparum* parasitemia using standardized, quality-controlled methods. Environmental data were obtained from satellite images, digitized ground maps and questionnaires aimed at schooldirectors. Bayesian variogram models were utilized for spatial risk modeling and mapping of *P. falciparum* parasitemia at non-sampled locations, assuming for stationary and non-stationary underlying spatial dependence. Two-third of the children were infected with *P. falciparum* and the mean parasitemia among infected children was 959 parasites/µl of blood. Age, socioeconomic status, not sleeping under a bednet, coverage rate with bednets and environmental factors (e.g., normalized difference vegetation index, rainfall, land surface temperature, and living in close proximity to standing water) were significantly associated with the risk of *P. falciparum* parasitemia. After accounting for spatial correlation, age, bednet coverage, rainfall during the transmission season and distance to rivers remained significant covariates. Our results point out that a massive

increase in bednet coverage, particularly in villages in close proximity to rivers and integrated with other control measures, is necessary to reduce malaria parasitemia in this setting.

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SUBMICROSCOPIC *PLASMODIUM FALCIPARUM* INFECTION IN ENDEMIC POPULATION SURVEYS: A SYSTEMATIC REVIEW AND META-ANALYSIS

Lucy C. Okell[1], Azra C. Ghani[2], Emily Lyons[2], Chris Drakeley[1]

[1]London School of Hygiene and Tropical Medicine, London, United Kingdom, [2]Imperial College London, London, United Kingdom

Light microscopy of blood slides is the main method of detecting malaria infection, however it has limited sensitivity. Low density infections are most likely to be missed but contribute significantly to the infectious reservoir. Quantifying these submicroscopic infections is therefore key to understanding transmission dynamics and reducing parasite transmission. We conducted a systematic review of endemic population surveys in which *Plasmodium falciparum* prevalence was measured both by microscopy and a more sensitive PCR-based technique. The combined microscopy:PCR prevalence ratio was estimated by random-effects meta-analysis and the impact of covariates by meta-regression. Seventy two pairs of prevalence measures were included. The prevalence of infection measured by microscopy was on average 50.2% (95% CI 44.8-56.3%) of the prevalence measured by PCR. For gametocyte-specific detection it was 8.7% (95% CI 2.8-26.6%). A significantly higher proportion of infections were detected by microscopy in areas of high compared to low transmission (69% where PCR prevalence>75%, vs 12% where PCR prevalence<10%). Microscopy can miss a substantial proportion of *P. falciparum* infections in endemic population surveys. The extent of the submicroscopic reservoir needs to be taken into account for effective surveillance and control.

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BIOLOGICAL AND CLINICAL IMMUNITY TO MALARIA INCREASE WITH AGE IN KENYAN HIGHLAND AREAS WITH UNSTABLE TRANSMISSION

Melissa A. Riedesel[1], Matthew McCarra[1], Ng'wena G. Magak[2], Kacey C. Ernst[3], Chandy C. John[1]

[1]University of Minnesota, Minneapolis, MN, United States, [2]Moi University, Eldoret, Kenya, [3]University of Arizona, Tucson, AZ, United States

In highland areas of Africa with unstable transmission, retrospective studies have produced conflicting results regarding the risk of clinical malaria in adults as compared to children. We conducted a prospective study to better define the development of biological and clinical immunity to malaria in two highland areas of Kenya. Active surveillance for clinical malaria was conducted in cohorts of randomly selected individuals in the highland areas of Kapsisiywa (n=571, 2003-2005) and Kipsamoite (n=279, 2001-2003; n=542, 2004-2005) in Nandi Hills District, Kenya. Clinical malaria was defined as *Plasmodium falciparum* on blood smear microscopy in individuals with a history of fever, chills, severe malaise, or headache. Temperature and parasite density were measured in symptomatic individuals. Data was analyzed according to age group (0-5 years, 6-15, 16-40, and ≥41 years) and adjusted for year of observation. In both sites, individuals >5 years of age had significantly lower malaria incidence than those <5 years of age. Using children <5 years of age as the reference group, incidence rate ratios (95% confidence intervals) for individuals 6-15 years, 16-40 years and ≥41 years of age were 0.49 (0.25-0.95), 0.28 (0.14-0.56), and 0.22 (0.10-0.46) in Kapsisiywa, and 0.59 (0.34-1.05), 0.45 (0.26-0.78) and 0.52 (0.29-0.94) in Kipsamoite, respectively. In both areas, parasite density at the time of clinical symptoms decreased only after 15 years of age, while measured temperature started to decrease after 5 years of age in Kapsisiywa and after 15 years of age in Kipsamoite. Intermittent malaria exposure leads to a degree of age-related biological and clinical immunity to malaria even in areas of unstable, low transmission. Clinical immunity to malaria may precede reduction of the level of parasitemia.

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INDUCTION OF VACCINE-SPECIFIC T CELL AND ANTIBODY RESPONSES IN RHESUS MACAQUES IMMUNIZED WITH TWO *PLASMODIUM VIVAX* CIRCUMSPOROZOITE-BASED VACCINE ANTIGEN CANDIDATES FORMULATED IN AS01$_B$

Yannick Vanloubbeeck[1], Sathit Pichyangkul[2], Babak Bayat[1], Kosol Yongvanitchit[2], Utaiwan Srichairatanakul[2], Amporn Chluaydumrong[2], Marie-Noelle Donner[1], Virginie Garze[1], Aram Afsar[1], Lisa Ware[3], Marie-Claude Dubois[1], Pascal Mettens[1], Laurence Lemiale[4], Mark Polhemus[3], Brent House[3], Jetsumon Sattabongkot[2], Kurt Schaecher[3], Christian Ockenhouse[3], Joe Cohen[1], Anjali Yadava[3]

[1]GlaxoSmithKline Biologicals, Rixensart, Belgium, [2]Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, [3]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [4]The PATH Malaria Vaccine Initiative, Bethesda, MD, United States

A challenging call for the worldwide eradication of malaria has recently been adopted as a long-term goal within the malaria community. A vaccine against *P. vivax* malaria will be an important tool in order to reach this goal. We have designed a pre-erythrocytic vaccine candidate based on the *P. vivax* CS (CSV)-protein, which includes the N- and C-terminal parts of the CSV protein and a truncated repeat region that contains repeat sequences from both the VK210 (type 1) and the VK247 (type 2) parasites to address the polymorphism encountered in the repeat region. We have made two versions of this vaccine candidate: 1) a recombinant protein expressed in *E. coli*, designated VMP001 and 2) a particulate antigen designated CSV-S,S, resulting from the co-expression in yeast of CSV-S, a fusion protein between VMP001 and Hepatitis B surface antigen (HBsAg), and free HBsAg that self assemble into mixed particles.The immunogenicity of both vaccine candidates formulated in GSK proprietary Adjuvant System AS01$_B$ was evaluated in rhesus monkeys following 3 intramuscular immunizations. Both vaccine candidates induced antibodies recognizing the different regions of the CS molecule *in vitro* (ELISA) and the native CS molecules expressed on sporozoites *ex vivo* (IFA). Both also elicited vaccine-specific T cell responses, mainly CD4 T cells producing IL-2 and/or IFN-γ and/or TNF-α. The data support further clinical testing of these vaccine candidates.

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EVALUATION OF A HETEROLOGOUS PRIME-BOOST VACCINE SCHEDULE BASED ON LOW SEROPREVALENT ADENOVIRUS VECTORS FOR MALARIA

Ariane Rodríguez Rodríguez, Gert Gillissen, Dennis Tax, Ratna Mintardjo, **Harmjan Kuipers**, Jerome Custers, Maria Grazia Pau, Stefan Kostense, Jenny Hendriks, Mariska ter Haak, Jaap Goudsmit, Katarina Radošević

Crucell, Leiden, The Netherlands

Heterologous prime-boost vaccinations involving adenoviral vectors of different serotypes, specifically designed to elicit both antibody and T-cell responses, represent a promising approach for protection against pre-erythrocytic malaria. Vaccination with adenoviral vectors induces a high level of antigen-specific T-cell response and, in addition, different adenoviral serotypes elicit qualitatively distinct T-cells. Accordingly, the combination of serologically distinct adenovectors in prime-boost vaccinations can elicit a potent T-cell immunity with a broad polyfunctional phenotype. In a previous study we demonstrated the potential of a heterologous prime-boost regimen that combines using the rare adenovirus serotype Ad35, encoding the circumsporozoite (CS) antigen, with the serologically distinct adenovector Ad5.CS for vaccination of paediatric populations living in malaria endemic areas. Due to the high prevalence of neutralizing antibodies against Ad5, particularly in Africa,

where the large majority of malaria cases occur, prime-boost vaccination schedules involving rare adenovirus serotypes are of particular interest. In the current study we evaluated the ability of a heterologous prime-boost schedule using two low seroprevalent adenovectors, Ad35.CS and Ad26.CS to elicit a type of immune response that is required for protection against malaria. We show that this heterologous prime-boost combination is highly efficient inducing strong CS-specific IFN-γ T-cell responses and antibodies that are able to bind *P. falciparum* sporozoites.

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A NON-ADJUVANTED POLYPEPTIDE NANOPARTICLE VACCINE TARGETING *PLASMODIUM FALCIPARUM* CSP INDUCES 100% STERILE PROTECTION AGAINST LETHAL CHALLENGE WITH LIVE SPOROZOITES

Stephen A. Kaba[1], Clara Brando[1], Christian Mittelholzer[2], Qin Guo[1], Ian McWilliams[1], Andrea Crisanti[3], Roberta Spaccapelo[4], Peter Burkhard[5], David E. Lanar[1]

[1]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]M.E. Muller Institute, Basel, Switzerland, [3]Imperial College, London, United Kingdom, [4]Universita degli Studi di Perugia, Perugia, Italy, [5]University of Connecticut, Storrs, CT, United States

We have previously shown that immunization with a Self-Assembling Polypeptide Nanoparticle (SAPN) displaying a B cell determinate from the *Plasmodium berghei* CS protein (PbCSP-SAPN) induced 100% protection against lethal challenge with *P. berghei* sporozoites in mice in the absence of adjuvant. The protection is granted by antibodies and it is transferable by serum but not cells from immunized to unimmunized mice. In this study we have investigated the immunogenicity and protective efficacy of a SAPN displaying the B cell epitope (NANP) from the repeat region of the *P. falciparum* CS protein (PfCSP-SAPN) in the mouse model. C57BL/6 mice were immunized, at 0, 14 and 28 days, with either PfCSP-SAPN (displays only Pf epitope), PvCSP-SAPN (displays only *P. vivax* epitope), or Pf/PvCSP-SAPN (displays 50:50 chimera of Pf and Pv epitopes) in PBS without adjuvant. Mice given PBS alone served as infectivity controls. Fourteen days post third immunization all animals were challenged with a lethal dose (5000) of sporozoites with a transgenic *P. berghei* parasite where the entire full length CS gene was replaced with the full length *P. falciparum* CS gene. Animals immunized with the PfCSP-SAPN or PvCSP-SAPN constructs developed high titer, high avidity antibodies against their respective CSP peptide. Mice immunized with the chimera developed lower but still significant antibody titer levels against both *Pf* and *Pv* CSP repeats. All mice immunized with PfCSP-SAPN were protected (developed no blood stage parasites) against lethal challenge with transgenic *P. berghei/P. falciparum* CSP sporozoites. All mice in the PBS control and PvCSP-SAPN groups developed blood stage parasitemia. Two of five mice in the Pf/PvCSP-SAPN group were protected against the lethal challenge, indicating the generation of some protective antibodies. These results demonstrate both the effectiveness of the SAPN model to induce a powerful, effective immune response against a lethal parasite challenge and the usefulness of the transgenic *P. berghei/ P. falciparum* CSP parasites for evaluation of CSP based vaccines in mice.

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IMAGING MURINE NALT FOLLOWING PROTECTIVE INTRANASAL IMMUNIZATION WITH *PLASMODIUM FALCIPARUM* CS PROTEIN CONJUGATED TO FLAGELLIN, A POTENT TLR5 AGONIST

Adéla Nacer[1], Daniel Carapau[1], Robert Mitchell[1], Alan Shaw[2], Ute Frevert[1], Elizabeth Nardin[1]

[1]New York University School of Medicine, New York, NY, United States, [2]VaxInnate Corporation, Cranbury, NJ, United States

Protective immunity can be elicited in a murine transgenic mouse model by intranasal (IN) immunization with a *Plasmodium falciparum* vaccine comprised of *P. falciparum* circumsporozoite (CS) conjugated to a bacterial TLR5 agonist derived from *Salmonella typhimurium* flagellin B *(STF2)*. The serum of mice immunized intranasally (IN) with STF2.CS reacted with viable transgenic sporozoites expressing *P. falciparum* CS repeats (PfPb), indicating that the antibodies recognized the CS repeats in their native configuration. Serum of the IN immunized mice neutralized >90% infectivity of transgenic PfPb sporozoites *in vitro*, as measured by inhibition of invasion of hepatoma cells when sporozoites were pre-incubated in serum from the IN immunized mice. Consistent with sporozoite neutralizing activity *in vitro*, there was >90% reduction in the levels of liver stage parasites *in vivo* when intranasally immunized mice were challenged by exposure to the bites of PfPb infected mosquitoes. To understand the immune responses functioning in induction of protective immunity, we explored the interaction of flagellin modified CS with the innate immune cells required for adaptive immunity. The STF2.CS was internalized by murine bone marrow-derived dendritic cells (DC) and human monocyte-derived DC *in vitro*, as measured by confocal microscopy and FACS. Immunohistochemical confocal microscopy was used to examine murine Nasalpharyngeal Associated Lymphoid Tissue (NALT) at various time points after IN administration of flagellin modified constructs to quantify T and B cell responses and antigen localization. These *in vitro* and *in situ* microscopic analyses advance our understanding of the innate and adaptive immune responses induced in the murine NALT following intranasal immunization with a needle-free malaria vaccine based on flagellin modified *P. falciparum* CS protein.

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FROM RODENTS TO HUMANS: DEVELOPMENT OF A P52KO BASED WHOLE ORGANISM MALARIA VACCINE

Ben C. Van Schaijk[1], Martijn Vos[1], Geert-Jan van Gemert[1], Marga van de Vegte-Bolmer[1], Audrey Gego[2], Samir Yalaoui[2], Jean-Francois Franetich[2], Ivo Ploemen[1], Dominique Mazier[2], Chris J. Janse[3], Shahid M. Khan[3], Robert W. Sauerwein[1]

[1]Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, [2]INSERM, Université Pierre et Marie Curie-Paris, Paris, France, [3]Leiden University Medical Centre, Leiden, The Netherlands

Difficulties with inducing sterile and long lasting protective immunity against malaria with subunit vaccines has renewed interest in vaccinations with attenuated *Plasmodium* parasites. Immunizations with sporozoites that are attenuated by radiation (RAS) can induce strong protective immunity both in humans and rodent models of malaria. Recently, in rodent parasites it has been shown that through the deletion of a single gene, sporozoites can also become attenuated in liver stage development and, importantly, immunization with these sporozoites results in immune responses identical to RAS. The promise of vaccination using these genetically attenuated sporozoites (GAS) depends on translating the results in rodent malaria models to human malaria. In this study, we perform the first essential step in this transition by disrupting p52 through single cross over technology in *P. falciparum*. P52 is an ortholog of the rodent parasite gene, p36p, which we had previously shown can confer long lasting protective immunity in mice. These *P. falciparum* P52 deficient sporozoites demonstrate gliding motility, cell traversal and an invasion rate into primary human hepatocytes *in vitro* that is comparable to wild type sporozoites. However, inside the host hepatocyte development is arrested very soon after invasion. This study reveals, for the first time, that disrupting the equivalent gene in both *P. falciparum* and rodent malaria Plasmodium species generates parasites that become similarly arrested during liver stage development. These results pave the way for further development of a multiply attenuated sporozoite vaccine through double cross over technology which will be safe for human use.

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DESIGNING A PAN-REACTIVE MALARIA VACCINE BASED ON THE CROSS-REACTIVE EPITOPES OF APICAL MEMBRANE ANTIGEN-1 OF *PLASMODIUM FALCIPARUM*

Sheetij Dutta[1], Lisa Dlugosz[1], Margaret Schwarz[1], Joshua Clayton[1], Michael Foley[2], Robin Anders[2], Adrian Batchelor[1]

[1]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]Department of Biochemistry, La Trobe University, Australia

Apical Membrane Antigen-1 (AMA1) based vaccines are under advanced clinical evaluation for efficacy at several field sites. The primary concern of vaccinologists is that the vast diversity of AMA1 will make it difficult for a mono- or biallelic AMA1 vaccine to control infection in the field. Several studies show that hundreds of unique AMA1 haplotypes exist and it would require numerous AMA1 proteins to be manufactured and mixed for a polyvalent vaccine to cover the major haplotypes. We are taking an alternative approach - an AMA1 protein will be engineered to preferentially display cross-reactive epitopes. Such a vaccine would be expected to induce higher levels of broadly inhibitory antibodies as compared to the native protein. Towards this end we are mapping the cross-reactive epitopes of AMA1 using inter-species chimeras that display various structural elements of *P. falciparum* AMA1 on an immunologically non-reactive *P. berghei* AMA1 scaffold. The chimeras that are being produced will display: the 3 domains based on the disulphide bond structure, the 3 domains based on the crystal structure, the conserved hydrophobic trough, the residues surrounding the C-terminal proteolytic site, the polymorphic face, the conserved face and the mAb 4G2 epitope that includes the conserved domain-2 loop. These chimeric proteins are being produced in *E. coli* and are used to reverse the antibody mediated growth inhibition of the parasite. The extent of reversal will be used to determine which epitopes are targeted by cross-reactive inhibitory antibodies. In a parallel set of experiments we are testing strategies to enhance or dampen the immunogenicity of AMA1 epitopes. Data on mapping cross-reactive inhibitory epitopes and immunogenicity studies with engineered AMA1 proteins will be presented. It is anticipated that this project will lead to the development of a Pan-reactive AMA1 vaccine. If successful, a similar approach can be applied to other infectious diseases where diversity remains a major hurdle to vaccine development.

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ANTIBODIES TO PLANT-PRODUCED *PLASMODIUM FALCIPARUM* SEXUAL STAGE PROTEIN PFS25 EXHIBIT TRANSMISSION BLOCKING ACTIVITY

Vidadi Yusibov[1], Jessica A. Chichester[1], Christine E. Farrance[1], Vadim Mett[1], Konstantin Musiychuk[1], Moneim Shamloul[1], Satish Sharma[1], Stephen Streatfield[1], Natalia Ugulava[1], Robert W. Sauerwein[2], Will Roeffen[2], Yimin Wu[3], Olga Muratova[3], Louis H. Miller[3], Douglas A. Holtzman[4]

[1]Fraunhofer USA, Center for Molecular Biotechnology, Newark, DE, United States, [2]Radboud University Medical Centre, Nijmegen, The Netherlands, [3]Malaria Vaccine Development Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, United States, [4]The Bill and Melinda Gates Foundation, Seattle, WA, United States

Malaria is a serious and sometimes fatal mosquito-borne disease caused by a protozoan parasite. Each year, millions of new cases of malaria occur throughout the world, and it is estimated that over one million people are killed by *Plasmodium falciparum* each year. The disease burden is especially high in Africa with devastating impact on both human lives and the economy. Yet malaria is preventable and treatable. Developing vaccines against the parasite is a critical component of the fight against malaria and can target different stages of the pathogen's life cycle. We are targeting sexual stage proteins of *P. falciparum* which are found only on the surfaces of the parasite reproductive cells which imbed themselves in the mosquito gut. Antibodies against these proteins block the progression of the parasite's life cycle in the mosquito, and thus block transmission to the next human host. We have successfully produced multiple versions of the Pfs25 antigen in our plant-based launch-vector system and evaluated these vaccine candidates in animal models. All the proteins express in plants at a high level, are soluble and most importantly, generate strong transmission blocking activity as determined by a standard membrane feeding assay. These data demonstrate the feasibility of expressing *Plasmodium* antigens in a plant-based system for the economic production of a transmission blocking vaccine.

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HEMOGLOBIN AND RED BLOOD CELL POLYMORPHISMS THAT CONFER PROTECTION AGAINST SEVERE *PLASMODIUM FALCIPARUM* MALARIA ARE EXCEEDINGLY COMMON IN MALI

Seidina A. S. Diakité[1], Michael A. Krause[2], Karim Traoré[1], Tatiana M. Lopera-Mesa[2], Saibou Doumbia[1], Drissa Konaté[1], Maria Cecilia Huaman[2], Mory Doumbia[1], Ababacar Diouf[2], Jennifer M. Anderson[2], Seydou Doumbia[1], Carole A. Long[2], Rick M. Fairhurst[2], **Mahamadou Diakité**[1]

[1]Malaria Research and Training Center, Bamako, Mali, [2]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, United States

The intense evolutionary pressure that malaria exerts on human survival has led to the selection of genetic polymorphisms affecting hemoglobin (Hb) and red blood cells (RBC). Epidemiologic studies have shown that HbC, HbS, α-thalassemia, hemizygous G6PD deficiency, and type O blood group confer protection against severe malaria in African children. As part of a longitudinal cohort study in Mali, which aims to identify novel polymorphisms protecting wildtype children from severe malaria, we have assessed 5 malaria-protective traits as covariates. In May 2008, we conducted a survey in 3 villages (Bozokin, Fourda and Kenieroba) located within 3 km of the Niger River 70 km from Bamako. We obtained a finger prick blood sample from 1270 children aged 6 months to 17 years. For each child, we typed their ABO blood group (agglutination assay), determined their Hb type (HPLC) and whether the 3.7-kb deletional determinant of α-thalassemia and the mutational determinant of the A- form of G6PD deficiency were present (PCR). We found that 40.6%, 6.3%, and 14.5% of children had type O blood group, HbC-trait, and HbS-trait, respectively. We detected hetero- and homozygous α-thalassemia in 29.6% and 2% of children, respectively. We identified hetero- and homozygous G6PD deficiency in 19.4% and 1.7% of females, respectively, and hemizygous G6PD deficiency in 10.2% of males. HbC-trait was more prevalent in Kenieroba (7.5%) than in Bozokin (2%, P=0.02) and Fourda (3%, P=0.05). The other 4 malaria-protective traits were similarly distributed in the 3 villages. Overall, 72.2% of the children in our cohort study carry at least 1 Hb or RBC polymorphism known to protect against severe malaria in Africa. Genetic polymorphisms of Hb and RBC are extremely common in Mali and can potentially confound natural history studies of severe malaria as well as future vaccine trials. The 5 commonest Hb and RBC polymorphisms are readily detected by simple laboratory methods and should be identified and analyzed as covariates in studies of severe malaria in Mali and elsewhere in Africa.