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CREATION OF A CHIMERIC WEST NILE VIRUS CONTAINING DENGUE-2 PRE-MEMBRANE AND ENVELOPE GENES

Eric T. Beck, Betty Luy, Claire Y.H. Huang

Centers for Disease Control and Prevention, Fort Collins, CO, United States

We recently engineered a chimeric flavivirus, designated as WN/D2 virus, containing the prM/E genes of dengue-2 virus (DENV-2) 16681 in a West Nile virus (WNV) NY99 backbone. Plaque titration reveals that plaques of the chimera are visible within 2-4 days post infection, which is comparable to WNV (within 2 days) and much faster than DENV-2 (8 days). Initial growth curves showed that this chimera replicates efficiently in C6/36 cells, but grows poorly in Vero cells. However, we have identified 2 amino acid mutations in the NS2A protein, and/or 1 mutation in the E protein that may enable the chimeric virus to replicate efficiently in Vero cells. We have orally infected *Aedes* and *Culex* mosquitoes with the chimeric virus, its reverse chimera D2/WN (WNV prM/E in DENV-2 backbone; engineered previously), and clone-derived wild-type (wt) DENV-2 and WNV. DENV-2 and chimeric WN/D2 virus containing prM/E of DENV-2 are able to infect midguts of *Ae. aegypti* mosquitoes, the natural vector of DENV-2, at higher ratios than WNV and D2/WN virus containing the prM/E of WNV. In contrast, midgut infection in *Culex quinquefasciatus* mosquitoes, a natural vector of WNV, appears to be mainly controlled by the capsid (C) and/or non-structural (NS) genes. WN/D2 virus containing the WNV C and NS genes infected midguts at high rates similar to wt WNV. On the other hand, both DENV-2 and D2/WN virus which contain the DENV-2 C and NS genes fail to infect *Cx. quinquefasciatus* midguts via infectious bloodmeal. The chimeric WN/D2 virus engineered in this report can be useful for studying the biological roles of the WNV NS genes in mosquito infection, pathogenesis, and immunogenicity. In addition, this fast-growing chimera expressing DENV-2 surface structure can be used as a DENV-2 like virus in immunological assays (such as DENV-2 neutralizing antibody assay), and for more rapid DENV-2 antigen/virus productions for diagnostics and other DENV-2 research.

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YEARLY VARIATION IN WEST NILE VIRUS ANTIBODIES IN AMERICAN KESTRELS (*FALCO SPARVERIUS*) IN PENNSYLVANIA

Darcy L. Medica

Pennsylvania State University, Schuylkill Haven, PA, United States

The numbers of American Kestrels (*Falco sparverius*) nesting near Hawk Mountain Sanctuary (HMS; Kempton, PA) have decreased significantly in recent years and West Nile virus (WNV) may have contributed to this decline. This study examined the dynamics of WNV infection in kestrels nesting near HMS. We trapped kestrels during the breeding seasons (June-July) in 2004, 2005 and 2006. The birds were tested for active infection by measuring viral RNA in saliva using oral swabs analyzed by RT-PCR. All of the kestrels were negative for infection. PRNT was used to measure WNV antibodies in serum. In 2004, 22 adult kestrels were tested for WNV antibodies and most (95%) were positive (blood samples were not obtained from nestlings). In 2005, blood samples from 6 adults and 7 nestlings were tested. WNV seroprevalence in this group of birds was much lower - 33% of the adults and 14% of the nestlings were positive. In 2006, 8 adults and 8 nestlings were sampled and only 25% of the adults were positive. These results suggest that WNV antibody levels in kestrels vary between years and have been decreasing over time since 2004. WNV was widespread in birds, mosquitoes and humans in Pennsylvania in 2003, and has declined since that time. We compared the prevalence of WNV antibodies in this population of kestrels to data available for mosquitoes and dead birds tested as part of the WNV surveillance program in Pennsylvania. The percentage of seropositive kestrels was compared to the number of infected mosquito pools and infected dead birds in the state during the same year and also during the previous year (2004 seroprevalence was compared to 2003 bird and mosquito data, etc.). We found that WNV antibody prevalence in kestrels is significantly correlated (correlation coefficient 0.999; $P<0.05$) to WNV infection in birds from the previous year, but not to mosquitoes from the previous year or to or to birds or mosquitoes from the same year. These results suggest that the antibody levels observed in the kestrel population are from infections that occurred during the previous year.

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PREDICTORS FOR EPIDEMIC WEST NILE VIRUS TRANSMISSION IN EAST BATON ROUGE PARISH, LOUISIANA, 2003-2007

Sarah R. Michaels[1], Matthew Yates[2], Wayne Kramer[3], Dawn Wesson[1], Ivo Foppa[1]

[1]Tulane University, New Orleans, LA, United States, [2]East Baton Rouge Mosquito and Rodent Control, Baton Rouge, LA, United States, [3]Louisiana State University, Baton Rouge, LA, United States

East Baton Rouge Mosquito and Rodent Control (EBRMRC) conducts a comprehensive arbovirus surveillance and control program serving more than 400,000 people in the greater Baton Rouge area. In 2002, during the first season of West Nile virus (WNV) transmission, the rate of human cases of neuro-invasive disease (WNNID) was 8.96 per 100,000. From 2003-2007, both epidemic and non-epidemic years of human transmission occurred. While human cases generally occur in the summer months, EBRMRC conducts surveillance throughout the year with increased efforts during the peak transmission season. Mosquito surveillance targets the local primary West Nile virus (WNV) vector, *Culex quinquefasciatus*, through a variety of trapping methods. In addition to mosquitoes, EBRMRC also collects information on and tests for the presence of virus in dead birds, evidence of infection in live birds by a catch and release program, and the seroconversion of sentinel chickens. We will use surveillance data from 2003 through 2007 to determine the utility of this effort in predicting epidemic WNV transmission. Hierarchical statistical models of WNNID incidence are to be constructed, based on the surveillance data as well as complementary information (e.g. sociodemographic and climatic variables). These models will be used to identify spatial and temporal predictors of epidemic WNV transmission. We will discuss results from this analysis and implications for WNV prevention and control.

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REGIONAL INCREASE IN WEST NILE NEUROINVASIVE DISEASE AFTER HURRICANE KATRINA

Kevin A. Caillouët, Sarah R. Michaels, Xu Xiong, Ivo Foppa, Dawn M. Wesson

Tulane University, New Orleans, LA, United States

Hurricane Katrina was the first major tropical cyclone to make landfall in a large metropolitan area since the 1999 introduction of West Nile virus to the United States. The objective of this study was to determine whether cases of West Nile neuroinvasive disease (WNND) increased regionally after Hurricane Katrina. After the storm, the number of reported cases of WNND sharply increased in the hurricane-affected regions of Louisiana and Mississippi. In 2006, a >2-fold increase in WNND incidence was observed in the hurricane-affected areas than in previous years. WNND incidence in 2006 equaled or exceeded the incidence rates in both states during the 2002 epidemic. The increase in WNND might have been due to increased human-mosquito exposure as a result of damaged housing, increased outdoor reconstruction activities, and eventually (in 2006) due to more mosquitoes as a result of storm-related habitat creation (root ball voids from fallen trees, and flooded abandoned swimming pools).

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LIVE ATTENUATED WEST NILE VACCINE BASED ON DEN-2 PDK-53 VECTOR PROTECTS HAMSTERS FROM WILD-TYPE WEST NILE VIRUS CHALLENGE

Claire Y. Huang[1], Richard M. Kinney[2], Betty E. Luy[1], Kristin Jones[3], Nicole Marlenee[3], Dan T. Stinchcomb[2], Richard Bowen[3]

[1]Centers for Disease Control and Prevention, Fort Collins, CO, United States, [2]Inviragen, Fort Collins, CO, United States, [3]Colorado State University, Fort Collins, CO, United States

West Nile virus (WNV) continues to impact public health worldwide. We have developed a live attenuated chimeric vaccine expressing the prM/E gene of WNV (strain NY99) in a genetically characterized, clinically safe attenuated DEN-2 virus backbone (strain PDK-53). This candidate vaccine maintained the phenotypic attenuation markers of the DEN-2 PDK-53 virus and protected mice against lethal WNV challenge, as previously reported. We have improved the replication of the vaccine candidates in Vero cells by engineering additional amino acid substitutions in the genome of the chimeric vaccine virus. The golden hamster model has been successfully used for WNV infection. In this report, we tested the immunogenecity, protective efficacy, and toxicology of the WN vaccine candidate in the hamster. All 8 hamsters received 2 doses of the vaccine are seroconverted. No viremia (<100 pfu/ml) was detected after immunization. WNV viremia after wild-type WNV challenge was either undetectable or with shorter duration compared to the viremia results of non-immunized control group. Our results indicated that this vaccine candidate is safe and immunogenic in hamsters, and it protects hamsters from wild-type WNV challenge.

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DUPLEX MICROSPHERE-BASED ASSAY FOR THE DETECTION OF IGG ANTIBODIES TO WEST NILE VIRUS AND ST. LOUIS ENCEPHALITIS VIRUS

Janeen J. Laven, Mark J. Delorey, Olga L. Kosoy, Amanda J. Panella, Robert S. Lanciotti, Alison J. Johnson

Centers for Disease Control and Prevention, Fort Collins, CO, United States

Since its introduction into the United States in 1999, West Nile virus (WNV) has been a source of significant morbidity and mortality. The virus has spread throughout North America and has established itself as a virus endemic to many regions. Another similar virus from the genus *Flaviviridae*, St Louis encephalitis virus (SLEV), circulates in many of the same regions and is associated with many of the same symptoms in human infections, making diagnosis difficult. A MAC-ELISA for detection of immunoglobulin M (IgM), followed by confirmation by plaque-reducing neutralization test (PRNT) and/or detection of immunoglobulin G (IgG) by ELISA is the normal course of testing in serodiagnosis of WNV or SLEV infection. Due to cross-reactivity among flaviviruses, differentiation between WNV and SLEV based on an IgG ELISA assay is nearly impossible. A mechanism does not exist to directly compare results between the two IgG ELISAs. We developed a rapid duplex microsphere-based assay to detect IgG antibodies to these viruses, as well as a classification system based on quadratic discriminant analysis. A total of 234 samples were tested. Of the 64 confirmed negative samples, 62 were classified as negative and 2 were classified as SLEV for an error rate of 3.2%. Of 53 confirmed SLEV positive samples, 45 were classified as SLEV and 8 were classified as WNV, for an error rate of 15%. WNV was correctly classified in 103 of 117 confirmed positives, with 5 being assigned as negative and 9 as SLEV, for an error rate of 12%. This microsphere-based assay not only has the advantage of being faster to perform, but a direct comparison in a duplex arrangement and the wider dynamic range of the assay allow for distinction between WNV and SLEV in >80% of samples tested. Further analysis of a broader range of samples as well as specificity and sensitivity data will be presented.

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NONVIREMIC (NON-REPLICATIVE) TRANSMISSION OF WEST NILE VIRUS ON SPECIFIC IMMUNE RODENT HOSTS

Charles E. McGee[1], Bradley S. Schenider[2], Dana L. Vanlandingham[1], Konstantin Tsetsarkin[1], **Stephen Higgs**[1]

[1]University of Texas Medical Branch, Galveston, TX, United States, [2]Institute Pasteur, Paris, France

Nonviremic transmission (NVT) of West Nile virus (WNV) has recently been reported to occur between *Culex spp.* mosquitoes co-feeding on naive mammalian and avian hosts. Furthermore it has been suggested that this so-called “nonviremic” transmission occurs via a systemic mechanism driven by inoculation of virus directly into the circulatory system thus allowing for infection of naïve arthropod recipient mosquitoes feeding at sites distal relative to the site of infected donor mosquito feeding. One factor that likely serves to limit the potential for mosquitoes to become infected via this mechanism is the temporal span during which virions remain available for mosquito infection. This “infectious period” is influenced by the temporal kinetics of receptor mediated cellular infection and vertebrate immune status. Here we investigate the effects of preexisting immune memory to WNV and/or mosquito feeding on the potential for NVT to occur. Mice were immunized via inoculation twice with ~10^3 plaque forming units of an attenuated WNV or exposure to four weekly feedings of uninfected mosquitoes singly or in combination. Following immunizations anesthetized mice were co-fed upon by WNV infected and naïve mosquitoes for ~1 hour. NVT was observed to occur despite prior exposure to WNV (1/199) or naïve *Culex* mosquito feeding (1/72). Quantitative reverse transcriptase polymerase chain reaction analysis of paired serum samples pre and post co-feed verified the absence of circulating virus prior to feeding whilst WNV genomes were detected in all mice immediately following donor feeding. Additionally WNV specific antibody was detected by hemagglutination inhibition in serum samples from all WNV immunized animals. Taken together these data further support a blood-borne non-replicative mechanism for NVT of WNV and suggest that vertebrate immune sensitization does not necessarily preclude vector infection.

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MULTIPLEX PROTEOMICS COMPARISON OF MALE AND FEMALE *BRUGIA MALAYI*

Daojun Jiang

Washington University, St. Louis, MO, United States

Differential proteomics analysis of male and female filarial worms may provide insight into major pathways and processes involved in reproduction. We used advanced multiplex proteomics methods to compare protein profiles in male and female *Brugia malayi.* One male and two female protein samples were individually labeled with one of three fluorescent dyes (Cy1, Cy3, and Cy5) and separated on the same 2-D electrophoresis gel (pH 3-11). Protein patterns were quantified using image analysis software (DeCyder, DIA, BVA and EDA modules), and 90 protein features were selected for mass spectrometry based on the following criteria: baseline resolution from other features, reproducible spot volumes for the two female samples, and at least a 2-fold difference between spot volumes in male and female samples. Selected spots were cut from gels, digested with endoproteases, and analyzed by MALDI-TOF/TOF. Tandem mass spectra were obtained from 80 of the 90 excised gel features and were searched using MASCOT software against the updated *B. malayi* protein database (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=6279). Criteria used for protein identification included: two or more peptide matches, MASCOT score > 60, and $P < 0.05$. Fifty-five spots contained a single dominant protein, 14 contained two proteins, and six had three or more proteins. Five spots had no significant protein matches, and some proteins were present in multiple spots. Among the 55 spots with single protein matches, 25 were increased in males (20 proteins) and 30 were increased in females (27 proteins).

Some of these protein differences were expected (e.g., Major Sperm Protein 2 and troponin family protein in males and transglutaminase and several HSP's in females), but others were unexpected or novel. The protein results were compared to gender-linked gene expression data from mRNA microarray experiments. Five of 20 male abundant proteins and 12 of 27 female abundant proteins had concordant gender-biased gene expression by microarray. Thus, transcriptional and translational changes were more consistent for female than for male-biased proteins. This may be due to active embryo production and development in female worms. This integrated analysis of protein and gene expression data has increased our understanding of the molecular correlates of gender differences and reproduction in filarial parasites.

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APTAMER TECHNOLOGY FOR THE IDENTIFICATION OF NOVEL INHIBITORS OF WOLBACHIA ENZYMES FOR ANTIFILARIAL THERAPY

Smitha Pillai[1], Bo Wu[2], Sylvine Raverdy[2], Gunter Mayer[3], Barton Slatko[2], Clotilde Carlow[2], Michael Famulok[3], Achim Hoerauf[1], Kenneth Pfarr[1]

[1]Institute of Med. Microbiology, Immunology and Parasitology, Bonn, Germany, [2]Division of Parasitology, New England Biolabs, Massachusetts, MA, United States, [3]LIMES Institute, Program Unit Chemical Biology and Medicinal Chemistry, Bonn, Germany

Lymphatic filariasis and onchocerciasis are diseases caused by filarial nematodes and afflict approximately 150 million people worldwide. The anti-helmentic drugs diethylcarbamazine and ivermectin, used for mass drug administration programmes to eliminate filarial diseases as a public health concern, are effective at reducing transmission. However, both drugs must be given annually for 15 to 35 years depending on the worm species and transmission intensity. These long treatment times are logistically challenging and may also drive selection of resistant nematodes. Therefore, a drug that will sterilize or kill adult worms in less time is required. Most filarial nematodes that infect humans contain Wolbachia, an obligate bacterial endosymbiont essential for worm development, fertility and survival making it a target for antifilarial drugs. Genomic filtering of the genome of Wolbachia of *Brugia malayi* (wBm) led to the discovery of the heme synthesis enzymes, including Amino Levulenic Acid Dehydratase (ALAD), and Pyruvate Phosphate Dikinase (PPDK) involved in gluconeogenesis as potential drug targets. Using Systemic Evolution of Ligands by Exponential enrichment (SELEX) we have identified pools of RNA aptamers (small ribonucleic acid ligands that specifically bind to proteins with high affinity) against ALAD and PPDK that bind specifically and with high affinity. Studies are in progress to analyse the individual aptamers and their ability to inhibit enzyme activity *in vitro*. The aptamers which inhibit protein activity will then be used to identify inhibitory small molecules by screening fluorescent labelled aptamers against a small molecule library.

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WOLBACHIA HEME BIOSYNTHESIS AS A POTENTIAL ANTI-FILARIASIS TARGET SET

Bo Wu, Leslie Conway, Jacopo Novelli, Jeremy Foster, Barton Slatko

New England Biolabs, Inc., Ipswich, MA, United States

Filarial parasites (*Brugia malayi*, *Onchocerca volvulus*, *Wuchereria bancrofi*) are causative agents of elephantiasis and African river blindness, among the most disabling tropical diseases. Current anti-filarial chemotherapy can interrupt transmission by killing larvae, but is less effective on adult worms, which can live 10-15 years in humans. There is an urgent need to develop adulticidal drugs. Over the last several years, *Wolbachia* has been recognized as a potential target for filarial nematode life cycle intervention. The strongest line of evidence of this is the loss of worm fertility and viability upon antibiotic treatment, both *in vitro* and *in vivo*, including human trials. However, the current drug treatments are not practical due to the dosages and length of treatments which are required. Nevertheless, anti-*Wolbachia* targeting appears promising for filariasis control. Heme biosynthesis was identified as a potential target set due to potential roles in worm molting and reproduction, the absence of this biosynthetic pathway in the host *B. malayi* genome sequence but presence in the *Wolbachia* genome sequence. Preliminary *in vitro* worm viability assays suggest that both female and male *B. malayi* adult worms are killed by succinyl acetone (SA), which specifically targets the 2nd step in the heme biosynthetic pathway - 5'-aminolevulinic acid dehydratase (ALAD). However, SA does not seem to have any effect on endosymbiont-free nematode *C. elegans*, which has to salvage heme. We have therefore undertaken the cloning, over-expression and analysis of the enzymes of the heme biosynthetic pathway and will present an update of our results in preparing proteins for drug targeting. A progress report will be presented describing our phylogenetic analyses of potential drug target enzymes, recombinant enzyme assays and the use of functional complementation of *E. coli* deletion mutants as another targeting strategy.

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QUANTITATIVE ANALYSIS OF MOLTING-REGULATED GENE TRANSCRIPTS IN *BRUGIA PAHANGI* INFECTIVE LARVAE

Yuefang Huang[1], Ramakrishna U. Rao[1], Thomas R. Klei[2], Gary J. Weil[1]

[1]Washington University School of Medicine, St. Louis, MO, United States, [2]Louisiana State University, Baton Rouge, LA, United States

Filarial nematodes undergo four molts in their development from newly hatched larvae to adult worms. The L3-L4 transition, which occurs about 1 week after infection, is the first molt in the mammalian host. Nematode molting is a complex developmental process which includes secretion of a new cuticle by the hypodermis and sloughing of the old cuticle. The *Brugia malayi* genome contains close homologues for at least 56 of 159 genes that have been shown to be essential for molting in *Caenorhabditis elegans* by RNAi. The purpose of this study was to compare expression profiles of putative molting genes in pre- and post-infective infective larvae (L3) of *B. pahangi*. L3 were obtained from mosquitoes, and two types of post-infective L3 were recovered from jirds three days after injection: idL3 were recovered from popliteal lymph nodes of id-injected jirds, and ipL3 were recovered from the peritoneum of ip-injected jirds. These were compared to L3 freshly dissected from mosquitoes (vL3) and to L3 that were cultured for three days *in vitro* (cL3). We used qRT-PCR to assess relative expression levels of 56 putative molting genes in different L3 types, and results were obtained for 55 genes. 71, 58, and 65% of these genes were differentially expressed by ipL3, idL3, and cL3 relative to vL3, respectively. 19 genes (34%) had parallel changes in expression in all three post-vector L3 types. These genes encode homologues of proteases, protease inhibitors (including *mlt*-11 which encodes a protein that is essential for processing cuticular collagens), DNA binding and signaling molecules, and genes involved in cuticle remodeling (*noah-1, noah-2* and *fbn-1)*. Concordance of changes in gene expression was higher for idL3 and ipL3 (75%) than for either of these *in vivo* L3 types with cL3 (56 and 54%, respectively). Thus, some gene products involved in molting may be also important for survival *in vivo*, and others may be associated with particular locations *in vivo*. Our results suggest that many genes involved in molting are highly conserved across Nematoda. Additional studies are needed to confirm the role of these genes in filarial molting and to determine whether such conserved genes might lead to new, broad spectrum treatments for these nematode parasites.

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LATERAL TRANSFER OF THE FERROCHELATASE GENE IN THE HUMAN PARASITE *BRUGIA MALAYI*

Bo Wu, Jacopo Novelli, Jeremy Foster, Barton Slatko

New England Biolabs, Inc., Ipswich, MA, United States

A peculiar feature of the phylum Nematoda is the total absence of the heme biosynthetic pathway and the metabolic dependency for extraneous heme. We report that filarial nematodes may be unique among nematodes in possessing the terminal step of the heme biosynthesis as a consequence of a lateral gene transfer (LGT) event. The sequencing of the genome of the filarial nematode *Brugia malayi* has revealed the presence of an open reading frame encoding a putative ferrochelatase (FC) enzyme, the last step in heme biosynthesis which catalyzes the addition of ferrous iron to the protoporphyrin ring. The predicted protein sequence is most similar to α-proteobacterial FCs; however, phylogenetic analysis shows that the gene is not likely to originate from the α-proteobacterial endosymbiont *Wolbachia*, which possesses a fully functional heme biosynthetic pathway, but rather from Rhizobiales proteobacteria. Homologs of this gene are also present in the filarial nematodes *Onchocerca volvulus, Dirofilaria immitis* as well as the *Wolbachia*-free *Acanthocheilonema viteae* as indicated by EST sequence and PCR/RT-PCR analyses. The full-length *B. malayi* FC contains 9 exons spanning across a ~3.3kb genomic region. A full-length transcript can be amplified from adult worm RNA preparations. The signature residues required in bacteria for catalysis are strictly conserved, supporting the hypothesis that the protein is a functional FC. Interestingly, the first exon encodes a 36 aa N-terminal pre-sequence, which is generally not present in bacterial FC. The pre-sequence contains a putative mitochondrion-targeting signal, as predicted by TargetP program. *E. coli* complementation tests confirmed that the *B. malayi* FC is fully functional and the N-terminal extension is not required for function. Heterologous expression in *C. elegans* demonstrated that the N-terminal pre-sequence is required for mitochondrial targeting; in all non-plant eukaryotes possessing the heme biosynthetic pathway, FCs are mitochondrial-resident enzymes, and hence this result agrees with a role for the *B. malayi* FC in heme biosynthesis. The possible implications of the existence of the last step of the heme biosynthetic pathway on filarial nematode survival will be discussed.

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USE OF HETEROLOGOUS MICROARRAY HYBRIDIZATION TO IDENTIFY GENES INVOLVED IN MOSQUITO INFECTIVITY FOR *BRUGIA PAHANGI* MICROFILARIAE

Kathryn Griffiths[1], George Mayhew[2], Rebecca Zink[1], Sara Erickson[2], Jeremy Fuchs[2], Bruce Christensen[2], Colleen McDermott[1], Michelle Michalski[1]

[1]University of Wisconsin Oshkosh, Oshkosh, WI, United States, [2]University of Wisconsin Madison, Madison, WI, United States

Brugia pahangi microfilariae (mf) require a maturation period of at least 7 days before they can infect the black-eyed Liverpool strain of *Aedes aegypti* (LVP), and the aging process apparently involves changes in microfilarial surface composition. In an effort to characterize parasite molecules that are instrumental for mosquito infectivity, we repeated the earlier experiments from Denham's laboratory using immature (<7 days) and mature (1-3 months) mf, derived from the peritoneal cavity of jirds. After verifying differential infectivity, we compared transcript abundance profiles of three biological replicates via heterologous hybridization of cDNAs to the *B. malayi* oligonucleotide microarray. Using Volcano plots, we identified 93 transcripts that were differentially abundant in immature mf, and 64 in mature mf. Of these identified genes, 109 have significant predicted protein similarity to sequences present in Uniprot, and 31 have Gene Ontology assignments suggesting molecular function. Cross-hybridizing B. malayi array oligonucleotides abundantly associated with immature B. pahangi mf had sequence similarity to protein kinases/phosphatases (6), membrane lipid synthesis enzymes (1), major facilitator superfamily membrane transporter (1), molecules involved in signal transduction (5) and metabolism (3), cytoskeletal elements (3), transcription factors/histone modifiers (4), ion channels for neurotransmission (2), and a filarial excretory/secretory protein (1). Cross-hybridizing transcripts in mature mf had sequence similarity to proteins involved in signal transduction (5), cytoskeleton (2) lipid content/ metabolism (3) collagens and major sheath protein (4), DNA binding/ transcription modification (4), proteases/kinases (4), membrane transport (2), and cellular metabolism (3). We will discuss verification of differential transcript abundance in *B. pahangi* by quantitative RT-PCR, and future hypothesis testing of gene function.

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ANNOTATION AND EVALUATION VERSION 2 *BRUGIA MALAYI* OLIGONUCLEOTIDE ARRAY

Ben-Wen Li[1], Makedonka Mitreva[2], Yong Yi[2], Amy C Rush[1], Jay DePasse[3], Elodie Ghedin[3], David Spiro[4], Gary J. Weil[1]

[1]Infectious Diseases Division, Washington University School of Medicine, St. Louis, MO, USA, [2]Genome Sequencing Center, Department of Genetics, Washington University School of Medicine, St. Louis, MO, USA, [3]Division of Infectious Diseases, University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA, [4]The J. Craig Venter Institute

The *Brugia malayi* Version 2 oligonucleotide array (BmV2 array was constructed in 2005 as a joint effort by the *Brugia* Microarray Consortium. This presentation will summarize information about the array and review progress in annotation that has greatly expanded the value of this tool for post-genomic research. The slide arrays contain 18,104 65-mer oligonucleotide from *Brugia malayi* (15,412), *Onchocerca volvulus* (1,016), *Wuchereria bancrofti* (872), and the *Wolbachia* endosymbiont wBm (804). *B. malayi* oligos were based on consensus sequences of EST clusters, EST singletons, and gene models. This information was not completely integrated and annotated when the array was constructed. The work performed to update annotation for the array including: 1) Correlation of old oligo IDs to the recently released stable Pub Locus ID; 2) homology of oligo source sequences to the NCBI non-redundant (NR) database; 3) RNAi phenotypes of *C. elegans* genes with homologues on the array; 4) Protein domain matches (InterPro); 5) KEGG pathway analysis; 6) Gene Ontology (GO) annotation.11,975 of 15,412 *Brugia* elements correspond to 9,758 unique annotated gene models (~85.5% currently annotated gene models). 69% of the sequences represented on the array have homology to known or predicted proteins from other species (cutoff e-05). 9,824 *malayi* elements had best matches to 8,038 *C. elegans* genes with RNAi information; 37% of these *C. elegans* genes have RNAi phenotypes. 48% of the genes represented on the array encode proteins that map to one or more GO terms, and 22% of the genes encode proteins that map into KEGG pathways. This wealth of information (which will all be posted on public databases) will help researchers to interpret expression profiles obtained with the BmV2 array and to plan new experiments.

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DEVELOPING *BRUGIA MALAYI/BRUGIA PAHANGI* HYBRIDS AS A TOOL FOR MOSQUITO INFECTIVITY STUDIES

Rebecca Zink[1], Kathryn Griffiths[1], Sara Erickson[2], Jeremy Fuchs[2], Bruce Christensen[2], Michelle Michalski[1]

[1]University of Wisconsin Oshkosh, Oshkosh, WI, United States, [2]University of Wisconsin Madison, Madison, WI, United States

The differential infectivity of *Brugia malayi* and *B. pahangi* for *Armigeres subalbatus* has been demonstrated, and has generated many questions about the basis of mosquito immunity for filarial nematodes. This project entails a Mendelian approach to identify genetic determinants in filarial nematodes that coincide with the ability to infect *Ar. subalbatus*. We generated F1 progeny from the *B. pahangi* female x *B. malayi* male cross, and membrane-fed them to black-eyed, Liverpool strain of *Aedes aegypti* (LVP; susceptible to both parental species) and to *Ar. subalbatus*

(melanize all B. malayi, susceptible to *B. pahangi*). We examined hybrid larvae for melanotic encapsulation at days 1 and 5 post infection, and found they retained the infective phenotype for LVP but that 50% were fully melanized in *Ar. subalbatus*. This suggests that inheritance may be sex-linked or mitochondrial in nature. We will report the results of the reciprocal genetic cross, and on our efforts to develop a molecular screen designed to determine the gender and genotype of melanized vs. nonmelanized larvae.

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EVALUATION OF DIFFERENT ANTIBODIES FOR IMMUNOSTAINING OF WOLBACHIA IN *BRUGIA MALAYI* AND OTHER FILARIAL PARASITES

Kerstin Fischer[1], Gary J. Weil[1], Claudio Bandi[2], Michelle L. Michalski[3], Jefferson A. Vaughan[4], Peter Fischer[1]

[1]Washington University School of Medicine, St. Louis, MO, United States, [2]Università degli Studi di Milano, Milano, Italy, [3]University of Wisconsin, Oshkosh, WI, United States, [4]University of North Dakota, Grand Forks, ND, United States

Most filarial parasite species live in obligatory symbiosis with intracellular Wolbachia α-proteobacteria. These bacteria are necessary for filarial development and reproduction; tetracycline class antibiotics active against Wolbachia have been reported to kill adult worms of some filarial species. While Wolbachia DNA can be detected and quantified by PCR, microscopy provides important information on morphology and localization of bacteria in parasite tissues. Immunohistochemistry has been used for years to visualize Wolbachia. However, different antibodies (abs) produce different results with different filarial species. The purpose of this study was to systematically evaluate a panel of antibodies for immunohistochemical labeling of Wolbachia in filarial worms. We tested 5 different antibodies (mab B. malayi Wolbachia surface protein [WSP]; pab B. pahangi WSP; pab D. immitis WSP; mab HSP 60 LK2 [Sigma]; pab GroEL [Sigma]) using the APAAP technique. The WSP abs were better than the HSP 60/GroEL abs, because they could be used at higher dilutions, produced less background, and were more specific for Wolbachia (did not stain mitochondria). While pab Di WSP stained Wolbachia in *Onchocerca volvulus, Dirofilaria immitis,* and filarial parasites of bears (*D. ursi*) and crickets (*Gryllus bimaculatus*), this ab did not stain Wolbachia in *Brugia malayi* well (ethanol or formalin fixed). In contrast, mab Bm WSP and pab Bp WSP strongly stained Wolbachia in *B. malayi*. Mab Bm WSP is apparently directed against a conserved epitope of Wolbachia found in at least three Wolbachia supergroups (B, C, D). No Wolbachia were detected by immunohistology with any antibody in the deer filaria *O. flexuosa* or the bird filaria *Chandlerella quiscali*, and we confirmed the absence of Wolbachia in these species by PCR. Immunohistology is very useful for visualizing filarial Wolbachia in situ. Our results show that antibodies to WSP are more specific than commercial antibodies to HSP 60/GroEL and that mab Bm WSP appears to be the antibody of choice for labeling Wolbachia in *B. malayi*.

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IDENTIFICATION OF INHIBITORS OF COFACTOR-INDEPENDENT PHOSPHOGLYCERATE MUTASE (IPGM) FOR POTENTIAL TREATMENT OF LYMPHATIC FILARIASIS

Michael L. Booker[1], Amy M. Fife[1], Sylvine Raverdy[2], Martin L. Kramer[1], Alexei Belenky[1], Clotilde K.S. Carlow[2]

[1]Genzyme Corporation, Waltham, MA, United States, [2]New England Biolabs, Division of Parasitology, Ipswich, MA, United States

Lymphatic filariasis is an endemic disease in tropical and subtropical regions, characterized by a number of devastating clinical manifestations. There are about 120 million current infections with an additional one billion people at risk Current treatments are unsatisfactory in that while they are effective in eliminating the circulating microfilariae, they fail to adequately eliminate the adult macrofilariae, resulting in an inability to achieve complete cure of the disease. *Caenorhabditis elegans* has been studied extensively as a surrogate organism for the parasitic nematodes, with RNAi studies suggesting a variety of potential targets. One such target of particular interest is cofactor-independent phosphoglycerate mutase (iPGM), an enzyme catalyzing the isomerization of 2-phosphoglycerate and 3-phosphoglycerate in the glycolytic and gluconeogenetic pathways. The knockdown of this enzyme in *C. elegans* results in lethality, and the 70% identity with the corresponding enzyme in the parasitic organism *Brugia malayi* makes it a potentially attractive target. A high-throughput screen was undertaken to identify inhibitors of iPGM from *C. elegans*, with the discovery of several chemical classes possessing potency in the single digit micromolar range. Work is currently underway to determine the specificity of these inhibitors for the nematode enzymes as compared to the human cofactor-dependent form (dPGM). Studies are also ongoing to measure the ability of the compounds to kill *B. malayi* in culture, with lead compounds ultimately to be tested in the jird disease model. It is hoped that inhibition of iPGM will represent a viable approach for the prevention and treatment of lymphatic filariasis.

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ALLEVIATING THE BURDEN OF LYMPHEDEMA IN TARABA STATE, NIGERIA VIA COMMUNITY-BASED REHABILITATION (CBR)

Lola E. Adigun[1], Stanley O. Foster[1], Henry B. Perry III[2], Oladele Akogun[3]

[1]Emory University, Atlanta, GA, United States, [2]Future Generations, Franklin, WV, United States, [3]Common Heritage Foundation, Yola, Nigeria

The problem of filarial lymphedema dates back in history, and there is no known cure for this debilitating condition. Unlike most other infectious disease eradication programs, which focus only on interrupting transmission, two essential program components were envisioned for lymphatic filariasis (LF) elimination: mass drug administration to interrupt parasite transmission, and care for those who already suffer from lymphedema. While mass drug administration has gained a foothold in LF elimination programs, the management of morbidity/disability for those with filarial lymphedema is still in its infancy. The purpose of this study was to introduce and assess community-based rehabilitation (CBR) techniques (therapeutic exercises, lymphatic drainage massage, compression bandaging and remedial home procedures) as a morbidity management strategy for people with lymphedema. The study was carried out in Zing Local Government Area (LGA), Taraba state, Nigeria, over 9 weeks, and these techniques built upon existing hygiene programs for people with lymphedema. Limb girth and walking gait measurements (stride length and walking speed) were recorded for 121 adult subjects (lymphedema= 51, non-lymphedema= 70). At baseline, lymphedema subjects walked an average of 39% slower than non-lymphedema subjects, when we controlled for age and sex. Two sample t-tests showed stride length at baseline to be significantly different between both groups ($p < 0.0001$). The lymphedema group that received CBR management showed a steady reduction in mean limb girth during each 2 weeks period from week 2 through week 8 of management ($p< 0.0001$). A test of trend for mean stride lengths in the lymphedma group showed a significant increase from weeks 2 to 8 ($p < 0.0001$). Trend lines also showed increased walking speed over weeks 2 to 8 ($p < 0.0001$). When stratified by age and sex, subjects 30-39 years had a more notable response, and females had a more marked response than males. We conclude that CBR for the management of lymphedema in resource-poor settings is highly effective in improving walking gait and reducing swelling. To the extent that these measures relate to increased ability to engage in society and participate in family activities, CBR can potentially be life changing. These CBR techniques can serve as adjuncts to existing hygiene programs.

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ASSESSMENT OF KNOWLEDGE AND PERCEPTIONS ON ELEPHANTIASIS AND HYDROCELE AMONG RESIDENTS OF DAR ES SALAAM, TANZANIA

Upendo J. Mwingira[1], Christina L. Makene[1], William J. Kisoka[1], Mtango Myombo[1], Prince Mutalemwa[1], Kesheni Senkoro[1], Vivian K. Barongo[1], Edwin Michael[2], Kazuhiko Moji[3], Charles D. Mackenzie[4], Mwele Malecela[1]

[1]National Institute for Medical Research, Dar-es-salaam, United Republic of Tanzania, [2]Imperial College School of Medicine, London, United Kingdom, [3]Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan, [4]Filarial Diseases Unit, Michigan State University, East Lansing, MI, United States

Lymphatic filariasis (LF) is one of the major tropical diseases in Africa, including Tanzania. LF is endemic to all the three (3) districts of Dar es Salaam, which qualifies each district for Mass Drug Administration (MDA). The regional microfilaremia prevalence in the area is 22.6%. This descriptive study was conducted to determine the knowledge and perception of hydrocele and elephantiasis among residents of two municipal councils of Dar es Salaam region, prior to the launching of the MDA programmme. The specific aims of the research were to determine the knowledge of study participants on the modes of hydrocele and elephantiasis transmission, consequences of these disease symptoms, their perceptions of cure and treatment methods, as well as the socio-demographic factors underlying disease. Household interviews were administered to 308 household members from Kinondoni and Ilala districts. Hydrocele and elephantiasis were among the top six important diseases in the study communities. The results showed that most of the periurban residents perceived both conditions to be more of a problem than urban residents, with 71% periurban, and 45% urban residents considering elephantiasis to be a major health problem compared to 55% periurban and 37% urban residents considering hydrocele as a significant health problem respectively. The role of mosquitoes in filariasis transmission was poorly understood by most of the households. On the issue of treatment, 278 (91%) of the households in the community believed that hydrocele was curable and out of them 214 (77%) said that surgery was the primary treatment method. 156 (51%) of the study population believed that elephantiasis was curable and 135 (87%) of them believed conventional treatment (drugs) as the treatment choice though they did not know the specific drug. Only 15% of study participants perceived poverty to have a significant relationship to either hydrocele or elephantiasis. Radio, village and religious leaders were found to be the preferred and popular information sources on the disease and LF programme. These results reveal that people's knowledge of LF transmission in urban areas of Tanzania was poor and that target communities require to be educated and sensitized about the disease as well as the MDA program prior to initiation of interventions. We suggest that this will be crucial to attainment of the optimal drug coverage's required for LF elimination in the country.

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ASSOCIATION OF TOLL-LIKE RECEPTOR 2 (*TLR2*) GENE POLYMORPHISMS WITH BANCROFTIAN FILARIASIS

Alisa Junpee, Vivornpun Sanprasert, Surang Nuchprayoon

Lymphatic Filariasis Research Unit, Department of Parasitology, and Chulalongkorn Medical Research Center (Chula MRC), Chulalongkorn University, Bangkok, Thailand

Lymphatic filariasis (LF), a mosquito-borne disease, is mainly caused by the filarial nematodes *Wuchereria bancrofti* and *Brugia malayi*. The Toll-like receptor 2 (*TLR2*) mediates recognition of *Wolbachia*, the endosymbiont in filarial parasites, and induces the innate immune response. We investigated the association between the polymorphisms of the *TLR2* gene and the susceptibility to *W. bancrofti* infection. In collaboration with the Ministry of Public Health, Thailand, total of 128 asymptomatic microfilaremic/antigenemic individuals, and 138 endemic normals in Tak province were recruited for the study. The +28 to +50 insertion/deletion (ins/del) polymorphisms in the 5' untranslated region (UTR) of *TLR2* gene were investigated using the allele-specific polymerase chain reaction. Two single nucleotide polymorphisms (SNPs), +597T/C and +1350T/C, in the exon 3 of the *TLR2* gene, were identified using the polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP). The genotype frequencies of three polymorphisms tested displayed Hardy-Weinberg Equilibrium (HWE) ($p>0.05$). The presence of the +28 to +50del allele was associated with a higher risk of bancroftian filariasis ([$p = 0.005$], OR= 2.21 [95% CI = 1.25-3.92], assuming a dominant model). Under a dominant model, the C allele at both *TLR2* position +597 and +1350 strongly increased the risk of lymphatic filariasis ([$p = 0.001$], OR= 2.58 [95% CI = 1.40-4.75], and [$p = 0.0121$], OR= 2.37 [95% CI = 1.19-4.77], respectively). The *TLR2* haplotype +28 to +50del/+597C/+1350C (delCC) significantly higher in lymphatic filariasis patients (12.5%) than in the endemic normals (4.3%) ([$p = 0.0011$], OR = 3.14 [95% CI = 1.52-6.63]). Our preliminary genetic epidemiology study showed that *TLR2* +28 to +50ins/del, +597T/C, and +1350T/C polymorphisms were associated with asymptomatic bancroftian filariasis.

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IMPACT OF INCREASED NUMBERS OF COMMUNITY DIRECTED DISTRIBUTORS ON SUCCESSFUL DISTRIBUTION OF IVERMECTIN IN ETHIOPIA, 2007

Aryc W. Mosher[1], Moses Katabarwa[1], Teshome Gebre[2], Estifanos Biru Shargie[2], Patricia Graves[1], Frank O. Richards[1]

[1]The Carter Center, Atlanta, GA, United States, [2]The Carter Center, Addis Abba, Ethiopia

The collaboration between the Ethiopian Ministry of Health, the Carter Center and the African Program for Onchocerciasis Control (APOC) on ivermectin (Mectizan ™ (donated by Merck and Co.) distribution in onchocerciasis endemic communities in southwestern Ethiopia has been on-going since 2001. The joint effort utilizes the community-directed treatment with ivermectin (CDTI) approach which allows communities or kinship groups to identify community members (Community Directed Distributors or CDD) as the key players in coordinating and distributing the treatment once per year. During 2007, approximately 32,270 CDDs provided ivermectin treatment to more than 2.6 million persons in 52 woredas (districts) within the two regions of Oromiya and SNNPR. The number treated represents coverage of nearly 92% of the 2.9 million who were eligible. To assess if the number of persons for whom a CDD is responsible has an influence on the treatment coverage of those eligible, the reporting forms from the 52 woredas were reviewed and the calculated ratios of persons per CDD to percent eligible persons treated were compared. Regression analysis showed a significant negative relationship (-.0001798, p value: 0.013) between the two. As the number of persons per CDD increases, the level of coverage achieved for eligible persons decreases. In order for the Carter Center assisted CDTI project areas to increase its abilities to achieve 100% coverage, and to meet the APOC recommended coverage of 1 CDD/ 100 persons, an additional 9,240 CDDs would be needed.

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EFFECT OF NTD INTEGRATION ON RESOURCE AVAILABILITY FOR LYMPHATIC FILARIASIS

PJ Hooper[1], Maggie Baker[2], Roland Bougma[3], Nana-Kwadwo Biritwum[4], Massitan Dembélé[5], Ibrahim Komblo[6], Dominique Kyelem[1], Mary Linehan[2], Eric Ottesen[1], Jean Shaikh[2]

[1]Task Force for Child Survival and Development, Decatur, GA, United States, [2]NTD Control Program, RTI International, Washington, DC, United States, [3]Ministry of Health, Ouagadougou, Burkina Faso, [4]Ghana Health Service, Accra, Ghana, [5]Ministry of Health, Bamako, Mali, [6]Ministry of Health, Niamey, Niger

The shift in focus from disease-specific Neglected Tropical Disease (NTD) programs towards *integration* of program planning, implementation, advocacy and fundraising reflects a belief that by working together individual programs can achieve or exceed their defined targets with greater cost-effectiveness. It is thus important to document how these programs have fared by being integrated in an NTD package (including lymphatic filariasis [LF], onchocerciasis, schistosomiasis, trachoma and soil-transmitted helminths). Working with Ministries of Health, this study focused on 4 countries whose integration efforts were supported by the USAID-funded NTD Control Program (NTDCP): Ghana, Burkina Faso, Mali, Niger. To determine what effect increased resources available for *integrated* NTD control had on disease-specific programs, available funds and program achievements for LF programs were compared for the 2 years prior to integrated NTD control funding and for FY07 when these funds became available. In Niger, there were no LF treatments prior to the NTDCP due to lack of funds, thus the 2.2 million LF treatments in 2007 can be attributed to availability of new integrated program funds. In Mali, NTD control funding enabled LF treatments to double between 2006 and 2007 and coverage remained high and stable. Burkina Faso's LF program, already at national scale, continued to be primarily funded by the government even with the start of the NTDCP, allowing the scale up of treatments for other NTDs. In Ghana, NTDCP funds filled the gap from decreasing external funds to support the national-scale LF program, emphasizing the importance of mobilizing new donors. Our findings give preliminary support to the notion that each disease program will maintain focus and achievements even while funding is no longer focused on individual diseases but on an NTD package. As the NTDCP expands to include additional countries, lessons learned from the initial countries can lead to strategies to ensure maximal benefit to individual disease programs with increased efficiency in using available resources.

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REDUCED PLASMA VEGF-C AND INCREASED SOLUBLE VEGFR3 ARE ASSOCIATED WITH THE PRESENCE OF HYDROCELE IN MEN WITH LYMPHATIC FILARIASIS

Kim Brustoski, Amy G. Hise, Daniel J. Tisch, Moses J. Bockarie, James W. Kazura

Case Western Reserve University, Cleveland, OH, United States

Lymphatic filariasis caused by the parasite *Wuchereria bancrofti* can lead to severe lymphedema affecting the legs arms, breasts and scrotum (hydrocele). In Papua New Guinea, an estimated 39% of its residents are infected with *W. bancrofti*. Filarial worms, and the endosymbiont bacterium *Wolbachia,* stimulate pro-inflammatory cytokines such as TNF-α, which up-regulates the expression of Vascular Endothelial Growth Factors (VEGFs). VEGF-C and its receptor VEGFR3 are central players in the growth of new lymphatic vessels (lymphangiogenesis). VEGF-C levels in tissue affect edema severity, and VEGFR3 mutations are known to cause congenital hereditary lymphedema. Promoting lymphangiogenesis is an attractive approach to treat lymphedema and therefore identifying the role that VEGFs play in LF-associated hydrocele is of great importance. In this study we aimed to identify the relationship between filarial infection, hydrocele and plasma levels of VEGF-C and soluble receptor VEGFR3. A retrospective cohort of 65 microfilaremia (Mf) Ag positive men was selected from a database from Papua New Guinea during a period of Mass Drug Administration (MDA) from 1993-1998. Plasma VEGF-C and sVEGFR3 were quantified by ELISA. Data was analyzed according to groups who were hydrocele+ (n=23, mean age 53, mean Mf Ag U/ml=1171) and hydrocele- (n=42, mean age 37, mean Mf Ag u/ml-792). VEGF-C levels for the hydrocele+ group in the pretreatment year were significantly lower than the hydrocele- group (median=7.1 and 8.3 ng/ml respectively, p=0.017 by Mann-Whitney test). Soluble VEGFR3 levels for the hydrocele+ group were significantly higher than the hydrocele- group (median=55.1 and 34.5 ng/ml respectively, p=0.047). Both VEGF-C and VEGFR3 levels (all individuals) significantly dropped from the pretreatment year to the last treatment year. Fourteen men who were hydrocele+ in the pretreatment were evaluated in the last treatment year: eight of these men experienced resolution of hydrocele, while six did not. Men with resolved hydrocele showed a trend toward higher levels of VEGF-C in the last treatment year compared to those whose edema persisted (median=4.1 and 2.3 ng/ml respectively, p=0.102), although all individuals in both groups were Mf-. These data show that in men exposed to *W. bancrofti*, those with lower plasma VEGF-C and higher sVEGFR3 levels have a significantly higher risk of LF-associated hydrocele.

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AN UNUSUAL CASE OF *STRONGYLOIDES STERCORALIS* COLITIS MIMICKING CROHN'S DISEASE

Shuchita Gupta, Shweta Gupta

Albert Einstein Medical Center, Philadelphia, PA, United States

Strongyloides stercoralis is an intestinal nematode that infects a large proportion of tropical populations. Infection is acquired when worms penetrate the skin, enter the lungs via the bloodstream, ascend the respiratory tract, are swallowed and grow into adult worms in the small intestinal mucosa. The well-described hyperinfection syndrome in immunocompromised hosts is characterized by severe gastrointestinal and respiratory tract involvement, skin rash, Gram-negative bacteremia and cerebral signs. We describe an unusual presentation of *Strongyloides* hyperinfection syndrome in an immunocompetent patient. A 75 year old male from Puerto Rico with a history of recent hospitalization for pneumonia presented with complaints of nausea, vomiting, abdominal distension and anorexia. He had no history to suggest an immunocompromised state, and his HIV (Human Immunodeficiency Virus) test was negative. A computed tomographic (CT) scan of the abdomen showed thickened large bowel in the region of the hepatic flexure. He subsequently developed hypotension requiring vasopressor support. Multiple sets of blood cultures grew *Klebsiella pneumoniae*, but no source of infection was identified. Following a drop in his hematocrit, he underwent a colonoscopy which revealed right-sided colitis and aphthous ulceration. Colonic biopsy showed acute cryptitis and submucosal granulomata negative for acid-fast bacilli. The patient expired due to overwhelming sepsis. His sputum Gram stain reported after death revealed worm-like organisms suggestive of *S. stercoralis*. In contrast to the usual *Strongyloides* hyperinfection syndrome with small intestinal and pulmonary manifestations in immunocompromised patients, our patient presented only with submucosal granulomatous inflammation affecting the colonic wall and clinically mimicking Crohn's disease. This form of *Strongyloides* enterocolitis apparently results from a florid immune response by eosinophils, histiocytes and giant cells with formation of granulomas that destroy the larvae, which is possible only in an immunocompetent host. Thus this case illustrates that *Strongyloides* hyperinfection can occur in immunocompetent persons and should be kept in mind in patients presenting with vague abdominal complaints and Gram-negative sepsis. Early initiation of ivermectin therapy in such cases can be life saving.

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GASTROINTESTINAL PARASITE COMMUNITIES OF NON-HUMAN PRIMATES FROM CAMEROON

Diffo J. Le Doux[1], Arouna Ndassa[2], Charles Bilong Bilong[2], Matthew LeBreton[1], Yves-Xavier Pourrut[3], Flobert Njiokou[2], Nathan Wolfe[4], Roger Moyou Somo[2]

[1]Johns Hopkins Cameroon Program, Yaounde, Cameroon, [2]University of Yaounde I, Yaounde, Cameroon, [3]IRD, Paris, France, [4]University of California, Los Angeles, CA, United States

Intestinal parasites were identified from 125 non-human primates (NHPs) (representing 15 species) among which 78 were wild animals and 47 were captive animals living in urban areas in Cameroon. Coprological analysis revealed that 82.4% of the animals studied harboured at least one species of intestinal parasite; 77% and 36% were infected with helminths and protozoa respectively. Three protozoan parasite species are reported as well as seven nematodes, one trematode (*Schistosoma mansoni*) and one cestode (*Bertiella* sp.). We compared infection rate relative to host species, sex, host age and whether the animal was captive or hunted in the wild. The proportions of captive animals infected with nematodes were relatively low (range 8.5%-31.9%) compared to that from the wild. Wild NHP populations harboured a wider diversity of parasites than captive populations. No significant difference was observed for the infection rates between males and females (χ^2=0.267, df=1; P>0.50). Our results show that *Cercopithecus cephus, C. neglectus, C. nictitans*, *C. pogonias* and *Cercocebus agilis* have the highest species richness with the number ranging from 6 to 8. *Mandrillus sphinx* and *Pan troglodytes* harboured less, with 3 parasite species each, while *Cercocebus torquatus* and *Papio anubis* harbored 2 parasite species each. This study contributes to our knowledge of the protozoa and the helminth communities from NHPs in Cameroon, and the presence of human parasites in NHPs - especially those living in close proximity to humans - and it highlights the importance of understanding wildlife disease ecology for management of conservation priorities and human health.

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OPTIMIZATION OF AN ELISA ASSAY FOR THE DETECTION OF *STRONGYLOIDES STERCORALIS* INFECTION IN HUMANS

I. McAuliffe[1], S. Handali[2], Y.M. Lee[1], J. Noh[1], E. Gleim[2], P. Wilkins[1]

[1]Parasitic Diseases Branch, Division of Parasitic Diseases, National Center for Zoonotic, Vectorborne and Enteric Diseases, Coordinating Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA, [2]Atlanta Research and Educational Foundation, Atlanta, GA, USA

Strongyloides stercoralis is an intestinal nematode that inhabits the human small intestine. It is a common parasite in many countries and it is estimated to infect 100 million people worldwide. Simple stool screening techniques for *S. stercoralis* tend to be insensitive for diagnosis, therefore we developed a quantitative enzyme linked immunoassay (ELISA) for detection of parasite specific antibodies in humans, using a crude extract from *S. stercoralis* third stage larvae (L3). We introduced a standard curve to measure arbitrary Ig units/ml. We analyzed a panel of 126 sera: 42 sera from parasitologically confirmed cases, sera known to contain *S. stercoralis* reactive antibodies and 84 from persons with no documented illnesses (normal sera). We optimized assay parameters, including antigen and conjugate concentrations and incubation times and temperatures. We calculated the positive negative cutoff using the J-index. The optimized assay has a sensitivity of 92.9% and specificity of 97.6%. When sera from persons with other parasitic infections (102 samples) were tested the specificity dropped to 78.5%. The lack of specificity is probably due to undetected/ unreported *Strongyloides* infection in patients with other parasites. Introduction of a standard curve makes the assay more reproducible and robust than assays that employ a positive to negative OD ratio using a single specimen.

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ACUTE CENTRAL NERVOUS SYSTEM INFECTION BY *TRYPANOSOMA CRUZI* IN PREGNANCY RATS

Ana Lugo-Yarbuh, Johan Rondon, Maritza Alarcon, Elio Moreno, Sonia Araujo

Universidad de los Andes, Merida, Venezuela

This study considers the effect of gestation on brain (B) and spinal cord (SC) of Wistar rats with acute Chagas infection, inoculated by the intraperitoneal route with 5x10^4 bloodstream trypomastigotes M/HOM/Bra/53/Y *Trypanosoma cruzi* strain. Quantitative analysis and immunohistochemistry assay were employed. The research was carried out in unpregnancy rats infected with *T. cruzi*, healthy gestanting rats, and infected/pregnancy rats at 10 days post-inoculation (pi). Levels of highest patent parasitemia were between 10 ± 0.8 and 27 ± 4.0 Trips./mm^3 blood in infected/pregnant rats, with 16 and 22 days pi and 6 and 12 days of gestation respectively and significantly different than the 1% between infected/pregnant and unpregnancy rats infected with *T. cruzi*. At 30 days pi the rats were anesthetized and lobes left and right of brain and cervical (CR), thoracic (TR), lumbar (LR) and sacra (SR) regions of SC were fixed in 10% formalin, dehydrated, embedded in Paraplast and 7µm sections were staining with Hematoxilin-Eosin. Inflammatory reactions of mononuclear and polymorphonuclear cells in brain were greater in infected/pregnancy rats, with lymphocytes and plasmocytes in contact with neurons. To compared the number of lymphocytes between the brain lobes and CR, TR, LR and SR of the SC of infected/ pregnancy rats and unpregnancy rats infected with *T. cruzi* was statistically significant (P<0.05). Indirect Immunofluorescence together to nuclear marker Propidium Iodide on tissue sections of B and SC of infected/pregnancy rats, revealed amastigotes and *T. cruzi* antigenic reaction in neuronal cells. These finding coincided with loss motoneurons (MN) in the SC of infected/pregnancy rats at 10 days pi. The values found were 16 ± 1.7, 21 ± 3.0, 10 ± 1.8 and 29 ± 2.5 MN compared with 53 ± 4.9, 38 ± 2.7, 33 ± 3.5 and 37 ± 3.3 MN in CR, TR, LR and SR of healthy rats respectively. Comparing MN numbers in the SC of the infected/pregnancy rats with unpregnancy infected rats revealed significant differences values (P< 0.05). Thus, motoneurons cells on sacra region of the rats, were also significantly different (P < 0.05). Of special interest was to know the effects of gestation on the nervous system central (CNS) of infected/pregnancy rats. The possible route of *T. cruzi* to reach the CNS of infected gestating rats could be possible by transvascular migration of parasited lymphocytes through the blood-cerebrospinal barrier.

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ECG ALTERATIONS IN FIRST AND SECOND STAGE HUMAN AFRICAN TRYPANOSOMIASIS BEFORE AND AFTER TREATMENT

Johannes A. Blum

Swiss Tropical Institute, Basel, Switzerland

There is evidence that the frequency and character of ECG alterations in early Human African Trypanosomiasis is higher than previously reported. This study aims to assess the situation among 406 patients with first stage HAT in the Democratic Republic of Congo, Sudan and Angola (2002-2007) and the differences of the ECG changes before and after treatment with DB 289 or pentamidine. These ECGs were compared to the ECGs of the healthy volunteers (n=61) and those of late stage patients (n=59), treated with melarsoprol or eflornithine. The QTc interval increased with the progression of the disease from early (421.6 msec) to late stage (423.2 msec) and was significantly longer than in healthy controls (403.1 msec), but did not increase during treatment. In early stage HAT, repolarisation changes (35%) and low voltage (20%) were significantly more frequent than in healthy controls (6.6% and 6.7% respectively). Repolarisation changes were similar in early and late stage (35% versus 32%) which is consistent with an inflammatory process early in the disease evolution. The lower proportion of low voltage (20% versus 30.5%, p=0.062)

in the first stage suggests that pericardial involvement becomes more relevant later in the disease. Conduction problems such as bundle brunch blocks, AV blocks and signs of necrosis were not found more frequently than in healthy controls. Treatment was associated with repolarisation changes in both, the DB 289 and the pentamidine group to a similar extent (5.7% versus 4.5%), but not with a QTc prolongation. No relevant conduction problems were observed. In conclusion, cardiac involvement, demonstrated by ECG alterations, appears early in the evolution of the disease and often precedes CNS involvement. During treatment ECG changes such as repolarisation alterations occur frequently, but are rather associated with the stage of the disease than a specific drug used for treatment.

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EVALUATION OF THE IMMUNOBLOT WITH TESA FROM THREE DIFFERENT *TRYPANOSOMA CRUZI* STRAINS FOR THE SEROLOGICAL DIAGNOSIS OF CHAGAS DISEASE IN THE USA

Debora R. Casandra[1], Eufrosina S. Umezawa[2], Charles W. Todd[1]

[1]Centers for Disease Control and Prevention, Atlanta, GA, United States, [2]Instituto de Medicina Tropical de São Paulo, São Paulo, Brazil

Chagas disease, caused by the protozoan hemoflagellate *Trypanosoma cruzi*, is an increasing public health concern in the United States as a consequence of the migration of infected persons from endemic areas to this country. Serodiagnostic assays for Chagas disease utilize several formats, for example, enzyme immuno assay (ELISA), immunofluorescence (IFA) and radio-immunoassay (RIPA). However, no method is considered the Gold Standard, and disparities in the serological results occur which complicate both individual patient care and epidemiological investigations. An immunoblot technique using trypomastigote excreted/secreted antigens (TESA) has shown a high degree of sensitivity and specificity in other studies. Assay specificity is conferred by reactivity to a 150 - 160 kDa band of proteins from the trans-sialidase family. However, because *T. cruzi* has a high degree of genetic diversity across its geographic range, we evaluated TESA from three strains (CL and Y strains from Brazil and a strain isolated in 2007 from a Mexican patient with reactivation Chagas disease) to determine the most suitable for use to diagnose Chagasic persons in the US. Initial studies indicated that in this immunoblot format, TESA from all 3 strains gave identical results with a panel of 25 positive and negative samples. Additionally, all three TESAs gave negative results with 5/5 high titer leishmaniasis sera which can give false-positive reactions in some serologic assays for Chagas disease. Validation studies of this method are in progress. We will also present TESA-immunoblot data on sera with discordant results by ELISA and IFA.

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APPLICATION OF A 384 WELL *TRYPANOSOMA BRUCEI BRUCEI* BS 427 WHOLE CELL VIABILITY ASSAY TO THE HTS OF A NATURAL PRODUCT MARINE FRACTIONATED LIBRARY

Melissa L. Sykes, Vicky M. Avery

Eskitis Institute for Cell and Molecular Therapies, Brisbane, Australia

African human trypanosomiasis (HAT) is caused by two species of trypanosomes; *Trypanosoma brucei rhodiense* and *T.b. gambiense*. The disease occurs in sub Saharan Africa with *T.b. rhodiense* prevalent in the western and central areas and *T.b. gambiense* in eastern and southern Africa. There are approximately 25,000 new cases per annum as reported by WHO. There have not have been any recent developments in the drugs available for HAT and most of the current drugs were discovered pre 1950. The drugs used in treatment either have side effects associated or are difficult to administer, with limited effectiveness across species. There is a need for new compounds for drug development for potential disease treatment of HAT. HTS is one method of early drug discovery that has been investigated for some neglected disease areas. There are currently no reports in the literature for 384 well HTS whole cell screening of *brucei* trypanosome species. We have developed and applied an HTS assay for whole cells whole cells using the Alamar Blue viability assay. The assay was applied to the HTS of 140,000 fractions from 13,000 marine biota to identify active fractions for further chemical and biological analysis. The use of a 384 well format allowed for a fast turn around time and screening was completed in 6 days (with a total incubation time of 96 hours). The HTS screening campaign controls were reproducible with a Z' of 0.75 and the standard deviation of library samples was 12%. The activity cut off for selection of active fractions applied to the screening library was 3 times the standard deviation (40%) and the hit rate of library fractions was 0.9%. Fractions were selected for further biological screening against a mammalian cell line before prioritisation for chemical analysis. Outcomes of the HTS campaign will be discussed.

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IDENTIFICATION AND EARLY HIT-TO-LEAD OPTIMIZATION OF NOVEL DRUG CANDIDATES FOR THE TREATMENT OF HUMAN AFRICAN TRYPANOSOMIASIS

Matthew D. Orr[1], Daitao Chen[1], Joe B. Perales[1], Kyle Bolduc[1], Jennifer C. Freeman[1], Tien Nguyen[1], Robert A. Noe[1], Cindy Rewerts[1], Mohanish Shrestha[1], Jessica M. Sligar[1], Bakela Nare[1], Stephen A. Wring[1], Robert Jacobs[1], Robert Don[2]

[1]SCYNEXIS, Inc., Durham, NC, United States, [2]Drugs for Neglected Disease Initiative, Geneva, Switzerland

The discovery of novel small molecule drugs for the treatment of Human African trypanosomiasis (HAT) is urgently needed. This disease is a significant public health problem in sub-Saharan Africa, and is poorly managed due to the significant toxicity, ineffectiveness and difficulty of administration of current treatments. We have initiated an integrated drug discovery program to identify clinical candidates for the treatment of HAT, beginning with an inexpensive cell-based HTS assay using *Trypanosoma brucei brucei* as a surrogate for clinical strains. Upon completion of screening a library of ~50,000 SCYNEXIS proprietary compounds representing 25 unique chemical classes, several chemotypes have been identified which exhibit good trypanocidal potency (IC_{50} ~ 1 μM), low mammalian cell cytotoxicity, and acceptable calculated physicochemical properties for progression to Hit-to-Lead optimization. We report here our initial efforts to develop structure-activity relationships (SAR) for several of these novel chemotypes, including SAR for trypanocidal activity, cytotoxicity, and *in vitro* ADME and physicochemical properties. Based on these studies, progression of selected compounds to *in vivo* models of HAT has started, providing good starting points for more extensive Lead Optimization.

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A WHOLE CELL HTS ASSAY PLATFORM TO IDENTIFY AND SUPPORT HIT-TO-LEAD PROGRESSION OF SELECTIVE INHIBITORS OF *TRYPANOSOMA BRUCEI*

Bakela Nare[1], Tana Bowling[1], Luke Mercer[1], Mari Vogel[1], Robert Don[2], Robert Jacobs[1]

[1]SCYNEXIS, INC, Research Triangle Park, NC, United States, [2]Drugs for Neglected Diseases initiative (DNDi), Geneva, Switzerland

Human African trypanosomiasis (HAT) is a tropical neglected disease caused by the trypanosomatid protozoan parasite *Trypanosoma brucei* and is a significant public health problem in sub-Saharan Africa. Current treatments for HAT are either toxic, very difficult to administer or ineffective. Few drug discovery and development programs for HAT have been initiated in the past ten years, with slow progress made towards the delivery of novel pre-clinical drug candidates. Our work is focused on the discovery and development of new and effective drug candidates for the treatment of HAT through an integrated biology-chemistry approach. The first step in this approach requires screening of large numbers of compounds to identify hits for progression into lead optimization and clinical proof of concept. To facilitate primary screening and support hit-to-lead activities for identification of trypanocidal compounds, a simple and

inexpensive cell-based HTS assay has been developed using *T.b brucei* as a surrogate for clinical strains. The assay is a fluorometric microtiter plate procedure, which measures the ability of *T. brucei* parasites to metabolize resazurin after 72 hr incubation in the presence of test compounds. Using this assay, a library of ~50,000 SCYNEXIS proprietary compounds, representing 25 unique chemical classes, was screened, and ~1000 actives (>75% inhibition at 2μg/mL) were identified. IC_{50} values for all the actives were determined through a triplicate 10-point titration procedure and preliminary SAR for trypanocidal activity within each series was examined. Compounds with an IC_{50} <1 μg/mL were evaluated for selectivity by determining IC_{50} values on the mammalian L929 cell line. Compounds showing a selectivity index for parasites versus mammalian cells of ≥50 were progressed into additional secondary assays assessing time-dose-response kinetics, reversibility and effects of serum on trypanocidal activity. Data from primary and secondary trypanocidal assays was used to drive selection of compounds to enter Hit-to-Lead chemistry programs in order to develop early SAR and progress hits into *in vitro* ADME and physicochemical assays.

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REAL-TIME PCR ASSAY FOR *TRYPANOSOMA BRUCEI* DETECTION

Elaine B. Bohórquez[1], Jonathan J. Juliano[1], Philippe Büscher[2], Reto Brun[3], Steven R. Meshnick[1]

[1]University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [2]Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium, [3]Swiss Tropical Institute, Basel, Switzerland

Surveillance for Human African trypanosomiasis (HAT) is difficult. We have developed a real-time PCR method for detection of HAT DNA in human samples. Primers were designed to amplify the abundant and highly conserved 177 base-pair satellite repeat that is present in all *Trypanosoma brucei* subspecies. A gene-specific FAM-tagged probe was used in a TaqMan® assay and compared to a SYBR Green assay. Live *T. b. brucei* were mixed with whole human blood in serial dilutions to mimic clinical samples. Trypanosome DNA was reproducibly detected at 10 trypanosomes per mL. Amplification occurred for all isolates of *T. b. brucei*, *T. b. rhodesiense*, *T. b. gambiense*, *T. congolese*, and *T. evansi* tested. Assays were then performed on blood (BL) and cerebrospinal fluid (CSF) samples from 48 microscopically confirmed HAT patients and 17 uninfected controls from the Democratic Republic of the Congo. For peripheral blood, the assay had low sensitivity (45%) but a high specificity (90%) and positive predictive value (88%). Using SYBR Green, the sensitivity was higher (81%) but both specificity and positive predictive value were much lower (21% and 63%, respectively). Further development of real-time PCR assays can facilitate surveillance for HAT.

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REVERSED AMIDINES AS ANTILEISHMANIAL CANDIDATES

Xiaohua Zhu[1], Chad Stephens[2], Arvind Kumar[2], Mark Sweat[3], Trupti Pandharkar[1], Dennis Kyle[3], David Boykin[2], Karl Werbovetz[1]

[1]The Ohio State University, Columbus, OH, United States, [2]Georgia State University, Atlanta, GA, United States, [3]University of South Florida, Tampa, FL, United States

Reversed amidines are synthetic compounds that were inspired by the diamidine antimicrobial agents, but possess significantly higher log $D_{7.4}$ values and lower pK_a values than typical diamidines. Although several classes of diamidines have shown potent activity against axenic *Leishmania donovani in vitro*, the reversed amidines are unique in that they also possess mid-nanomolar IC_{50} values against intracellular *L. donovani in vitro*. We have recently begun to re-examine this class of compounds for its activity against other *Leishmania* species and in animal models of leishmaniasis. The reversed amidines also possess potent activity against *Leishmania* species that cause cutaneous leishmaniasis. Against *L. amazonensis*, several of these compounds display IC_{50} values (0.53 to 0.042 μM) comparable to that of the clinical antileishmanial compound amphotericin B (IC_{50} = 0.13 μM) and far superior to those of the antileishmanial drugs miltefosine (IC_{50} = 15 μM) and paromomycin (IC_{50} > 50 μM). Thus far, the most potent of these compounds is DB766, which possesses an IC_{50} value of 0.042 μM against intracellular *L. amazonensis*. DB766 also displays activity against *L. donovani*-infected BALB/c mice. When given at an intraperitoneal dose of 30 mg/kg/day for five days, DB766 reduced the parasite burden in the liver by 63 ± 11% (mean ± standard deviation, $p < 0.05$) compared to the control group. DB766 also displays activity in the hamster model of visceral leishmaniasis. The synthesis, antileishmanial testing, and mechanistic evaluation of the reversed amidines are ongoing in an attempt to identify members of this compound class as useful antileishmanial drug candidates.

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A MULTIPLEX APPROACH FOR SIMULTANEOUS IDENTIFICATION OF SIX DISTINCT *LEISHMANIA* SPP

Kakali Bandyopadhyay[1], Marcos de Almeida[1], Francis Steurer[2], Ozgur Koru[1], Barbara L. Herwaldt[2], Alexandre J. da Silva[2]

[1]Centers for Disease Control and Prevention, Division of Parasitic Diseases, NCZVED and Atlanta Research and Education Foundation, Atlanta, GA, United States, [2]Centers for Disease Control and Prevention, Division of Parasitic Diseases, NCZVED, Atlanta, GA, United States

Human cases of leishmaniasis are caused by approximately 20 *Leishmania* spp., some of which are found in the same geographic region. *Leishmania* parasites are morphologically indistinguishable and identification at the species level is important for clinical management and treatment. Isoenzyme analysis is considered the gold standard technique for diagnostic identification of *Leishmania* at species level. However, this requires *in vitro* cultivation of the organism and can take weeks before a final identification is made. Hence, molecular tests offer an attractive alternative for species-specific identification of *Leishmania* spp. In this study, we developed and evaluated a microsphere assay based on the Luminex platform for multiplexed identification of *Leishmania* spp. Genomic DNA was extracted from 25 clinical (n=8) and culture (n=17) samples that contained different *Leishmania* spp. identified at species level by isoenzyme analysis. The samples were amplified by PCR with biotinylated primers that produced amplicons varying from 385bp to 450bp of the ITS 2 region. Species-specific probes were designed based on single nucleotide polymorphism (SNP) that could differentiate *Leishmania* spp. Each probe was conjugated to Luminex microsphere and hybridized in a multiplexed format to the biotinylated amplicons. Using this method, we correctly identified 6 species (16 specimens), including *L. (V.) braziliensis* (n=5), *L. mexicana* (n=4), *L. amazonensis* (n=3), *L. tropica* (n=2), *L. major* (n=1), and *L. donovani* (n=1). Although it was not able to correctly identify *L. guyanensis* (n=5) and *L. panamensis* (n=4), this SNP-based assay is a promising diagnostic tool for simultaneous identification and differentiation of *Leishmania* spp.

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PHASE 3 TRIAL OF PAFURAMIDINE MALEATE (DB289), A NOVEL, ORAL DRUG, FOR TREATMENT OF FIRST STAGE SLEEPING SICKNESS: SAFETY AND EFFICACY

Gabriele Pohlig[1], Sonja Bernhard[1], Johannes Blum[1], Christian Burri[1], Alain Mpanya Kabeya[2], Jean-Pierre Fina Lubaki[3], Alfred Mpoo Mpoto[3], Blaise Fungala Munungu[3], Gratias Kambau Manesa Deo[4], Pierre Nsele Mutantu[4], Florent Mbo Kuikumbi[2], Alaine Fukinsia Mintwo[2], Auguy Kayeye Munungi[2], Amadeu Dala[5], Stephen Macharia[6], Constantin Miaka Mia Bilenge[2], Victor Kande Betu Ku Mesu[2], Jose Ramon Franco[6], Ndinga Dieyi Dituvanga[5], **Carol A. Olson**[7]

[1]Swiss Tropical Institute, Basel, Switzerland, [2]Programme Nationale de Lutte contre la Trypanosomiase Humaine Africaine, Kinshasa, The Democratic Republic of the Congo, [3]Evangelic Hospital, Vanga, The Democratic Republic of the Congo, [4]Evangelic Hospital, Kikongo, The Democratic Republic of the Congo, [5]Instituto de Combate e de Controlo das Tripanossomíases, Luanda, Angola, [6]Malteser International, Yei, Sudan, [7]Immtech Pharmaceuticals, Inc., Vernon Hills, IL, United States

Only a limited number of drugs are available for treatment of sleeping sickness and none is applicable by the oral route. The oral prodrug pafuramidine (DB289) was selected in 2000 by the consortium for parasitic drug discovery, led by the University of North Carolina, Chapel Hill, for clinical development against first stage sleeping sickness caused by *Trypanosoma brucei gambiense*. A pivotal Phase 3 trial was initiated in August 2005 and will continue through mid-2009. 273 patients were enrolled in four centers in the Democratic Republic of Congo and in one center each in Angola and South Sudan. All patients completed the assigned study drug regimen, DB289 100 mg BID oral for 10 days or pentamidine 4 mg/kg IM for 7 days, by March 2007. The DSMB completed the interim analysis in August 2007 and recommended the study to continue as planned. In October 2007, a study to provide supportive safety data in healthy volunteers for the registration of DB289 for first stage sleeping sickness and Pneumocystis pneumonia was initiated. Subjects received DB289 100 mg BID for 14 days. In December 2007, the development program was placed on Clinical Hold when liver toxicity was observed post treatment in this study. The Phase 3 sleeping sickness trial data were then unblinded and reviewed by study sponsors and advisors. Liver toxicity was significantly less for the DB289 group than the pentamidine group. In February 2008, 5 subjects in the healthy volunteer study developed renal insufficiency approximately 8 weeks post treatment that required medical intervention. Review of the Phase 3 sleeping sickness data identified 3 subjects who developed glomerulonephritis/nephropathy post DB289 treatment; 2 of these events may now be considered possibly related to DB289. No patient in the pentamidine group was reported to have renal disease. The clinical development program for DB289 was discontinued at that time. The Phase 3 two year sleeping sickness trial follow up is continuing and the 12-month primary efficacy endpoint was reached in March 2008. Data from the primary efficacy endpoint and analysis of the safety data will be presented.

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IN-VITRO EFFICACY OF HYPERBARIC OXYGEN AGAINST *LEISHMANIA TROPICA* PROMASTIGOTES AND AMASTIGOTES

Bulent A. Besirbellioglu[1], Hanefi C. Gul[1], Hakan Ay[1], Ozgur Koru[1], Soykan Ozkoc[2], Seray Ozensoy Toz[3], Mehmet Tanyuksel[1], Omer Coskun[1], Kemal Simsek[1]

[1]Gulhane Military Medical Academy, Ankara, Turkey, [2]Dokuz Eylul University, Faculty of Medicine, Izmir, Turkey, [3]Ege University Medical School, Izmir, Turkey

In this study, we determined the toxic effect of hyperbaric oxygen (HBO) on *Leishmania tropica* promastigote and amastigote forms. The HBO treatment protocol was at a level that can be tolerated by humans. We examined the cytotoxic effect of HBO on promastigotes via the trypan blue exclusion test and a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye-reduction assay (MTT). With both methods we observed a cytotoxic effect that emerged after two hours and reached a maximum at 6 hours. To investigate the effects of the HBO treatment on the growth of the promastigotes, HBO-treated and HBO-non-treated (control) cell culture flasks were incubated at 26° C for 3 days. Numbers of dead and live promastigotes were counted microscopically via the trypan blue exclusion test, and percentage death rates were calculated each day. The number of live promastigotes was observed for 24 hours after treatment. There was a slight elevation with 2 hours of treatment, but significant suppression after 4 and 6 hours of treatment ($p=0.004$). The number of live promastigotes interacting with N-acetylcysteine (NAC) was not increased and NAC did not prevent the inhibitory effect of HBO on the growth of promastigotes. We also detected the effect of HBO on *L. tropica* amastigote forms via acridine orange staining which stains apoptotic cells (indicating the ratio of affected amastigotes). Apoptotic cells were found to be increased by 3.3%, 17.2% and 38.5% after 2, 4 and 6 hours of treatment, respectively. These results suggest that HBO may be useful in the treatment of cutaneous leishmaniasis.

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SERUM NITRIC OXIDE (NO) LEVELS IN CUTANEOUS LEISHMANIASIS (CL): CORRELATIONS WITH TREATMENT OUTCOME AND THE ADVERSE EVENT OF PANCREATITIS

Louis-Patrick Haraoui[1], Nancy Koles[2], Robin S. Howard[3], Glenn W. Wortmann[3], Mark Polhemus[3], Naomi E. Aronson[2]

[1]Internal Medicine Residency Training Program, Department of Medicine, McGill University, Montreal, QC, Canada, [2]Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [3]Walter Reed Army Medical Center, Washington, DC, United States

NO production by macrophages is essential to the human response to CL and is enhanced by treatment with pentavalent antimonials. We correlated serum NO levels during sodium stibogluconate (SSG) therapy with clinical outcome and the occurrence of pancreatitis, a side-effect of treatment. Sera from 32 parasitologically confirmed CL cases treated with 20 days of SSG at a dose of 20mg/kg/day and 8 matched controls with no exposure to *Leishmania* were assayed at baseline and days 7, 14, and 20. NO was measured using the surrogate nitrite with the Griess reaction. Pancreatitis was defined as an abnormal lipase along with nausea, vomiting or abdominal pain. Clinical outcome was characterized as healed or failed at 2 and 6 months after treatment. We observed no significant difference in NO levels between cases and controls ($p > 0.2$). There was also no correlation between clinical outcome at 2 or 6 months and NO levels at all time-points ($p > 0.120$ and $p > 0.525$, respectively). However, a longer duration of lesion(s) was significantly correlated with lower NO levels prior to starting treatment ($p < 0.05$) and greater rises in NO levels at days 14 and 20 ($p = 0.02$ and $p < 0.05$, respectively). Lipase levels peaked at day 7 and abnormally elevated lipase at day 7 was correlated with higher NO levels at day 20 ($p = 0.05$). In conclusion, our study found significant correlations in NO levels among patients with longer duration of disease prior to starting and during therapy with SSG. As well, elevated lipase levels early on during treatment significantly correlated with higher NO levels at the end of treatment.

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MINIEXON PCR-RFLP FOR *LEISHMANIA* SPECIES IDENTIFICATION IN NEW WORLD LEISHMANIASIS

Clemencia Ovalle Bracho, Yira R. Díaz Toro, Daniel Velandia, Sandra Muvdi

Centro Dermatológico Federico Lleras Acosta, Bogotá, Colombia

Leishmania species identification is relevant due to differences in pathogenesis and treatment response. Widely used methods such as Monoclonal Antibodies (MoAb)and isoenzymes require parasite culture

and are highly demanding in time and costs. We believe a technique that allows parasite detection as well as species identification would be desirable.Our main goal was to determine if PCR-RFLP using the miniexon gene as molecular target would allow *Leishmania* species identification in New World Leishmaniasis. Reference strains were used for *L. chagasi, L. mexicana, L. amazonensis, L. panamensis, L. braziliensis and L. guyanensis*. Biopsy tissue from 39 CL patients, 2 from ML (mucosal leishmaniasis) cases as well as isolates from 36 CL cases, some previously identified by MoAb and/or isoenzymes were used. Miniexon gene amplification was made with Fme/Rme primers and the amplified sequence was digested with HaeIII enzyme.Restriction profiles obtained *in silico* and *in vitro* for the reference strains and *in vitro* for the isolates were analyzed and compared using the Quantity One program. We were able to differentiate species belonging to the *Leishmania* subgenus when we amplified miniexon gene. This molecular target let us identify *Viannia* subgenus as a group. In order to identify species belonging to *Viannia* subgenus we used HaeIII enzyme which is able to distinguish *L braziliensis* from *L panamensis*/*L. guyanensis.* 9 isolates were identified as *L. amazonensis* and the remaining 26 were classified as belonging to Viannia subgenus. When tissue samples were used 100% of species were identified as members of Viannia subgenus, when HaeIII enzyme was used 21 were identified as *L. panamensis/ L. guyanensis* and 12 as *L. braziliensis.* There was absolute agreement in the complex level with MoAb for evaluated isolates. In conclusion, Miniexon gene amplification allows species differentiation from *Leishmania* subgenus and HaeIII enzyme restriction further distinguishes *L. braziliensis* from *L .panamensis*/*L. guyanensis*. Miniexon PCR-RFLP is a useful technique for leishmaniasis diagnosis and New World species identification with timely results suitable for clinical practice.

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EVALUATION OF THE INFECTIVE PROCESS BY *LEISHMANIA PANAMENSIS* IN A CELL LINE DERIVED FROM *AEDES AEGYPTI*, WITH BASE IN PHYSICOCHEMICAL AND ENVIROMENTAL VARIABLES

Felio J. Bello[1], Angela Cristina Zapata[2], Víctor Manuel Acero[2], Andrés Ramírez[2]

[1]*Universidad del Rosario, Bogotá, Colombia,* [2]*Universidad de La Salle, Bogotá, Colombia*

The parasite-host cell interactions of *Leishmania* are not well understood and merit *in vitro* studies in alternative cell models. The aim of this study was to evaluate the *in vitro* infection by *Leishmania (V) panamensis* in a cell line derived from *Aedes aegypti* embryonic tissue, with varying physicochemical and enviromental variables. The physicochemical and environmental variables evaluated for the experimental tests of the cell infection were: pH (5.0, 5.5, 6.0, 6.8, and 7.2), temperature (26, 32 and 37ºC), and presence or absence of $C0_2$. As substrate for the infected cells, glass coverslips of 12 mm diameter in 24 well plates were used. The J774 cell line was used as positive control of the infection. A morphometric study of cells was carried out, before and after infection. The *L. panamensis* MHOM/87/CL412 strain was used for infection assays and parasites were added to adherent cells in 10:1 ratio. Experimental observations indicated that the highest level of infection (53% with a 111.3 index of infection) was achieved by day 6 post-infection at 5% $C0_2$, 26ºC and pH 6.8. Establishing the optimum physicochemical and environmental conditions for *A. aegypti* cells to be infected by *L. panamensis* parasites allows obtaining relatively higher levels of infection, which could be useful in basic and applied studies in the perspective to contribute to the control of tegumentary leishmaniasis caused by these parasites.

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QUANTIFICATION OF PARASITEMIA IN *LEISHMANIA DONOVANI*-INFECTED HAMSTERS BY REAL-TIME PCR

Brian Vesely, Azliyati Azizan, J. Mark Sweat, **Dennis E. Kyle**

University of South Florida, Tampa, FL, United States

Visceral leishmaniasis affects over a half million individuals in 88 countries each year. New drugs are urgently required to treat this devastating disease caused by the intracellular parasite *Leishmania donovani*. For preclinical evaluation of new drug candidates we have adopted the hamster model of infection. The endpoint of the assay requires an estimate of parasite burden, which historically involves laborious preparation of tissue, staining, and reading slides to determine Leishman-Donovan units (LDUs). In the current study quantification of the parasite load in infected-hamster livers was analyzed by a SYBR Green-based PCR assay and compared to microscopic and growth dilution techniques. Reverse and forward primers were used that amplify a 120-bp target template of the kinetoplast DNA. Standard curves were prepared by serial ten-fold dilutions of parasites or parasites spiked into un-infected liver homogenates. We also elucidated parasite burden in liver tissue from *L. donovani*-infected hamsters that were treated with standard (Glucantime and Miltefosine) and experimental drugs. *L. donovani* DNA was readily detected in spiked homogenates and no PCR products were observed with un-infected tissue and non-template control samples. The real time PCR assay detected as few as 2 parasites in 10 mg of liver. In addition quantitative differences in the parasite load of parasite-infected liver tissue were detected at low levels of infection where the serial dilution method and microscopic confirmation was difficult or not possible. In summary the real time PCR method is sensitive and specific for the quantification of the parasite load within *L. donovani*-infected hamsters and the method will accelerate the evaluation of new drugs in this definitive preclinical model of disease.

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EVALUATION OF A RAPID IMMUNOCHROMATOGRAPHIC ASSAY FOR DETECTION OF *TRYPANOSOMA CRUZI* ANTIBODIES IN WILDLIFE RESERVOIRS

Michael J. Yabsley, Emily L. Brown, Dawn M. Roellig

University of Georgia, Athens, GA, United States

An immunochromatographic assay (Chagas Stat-Pak) was evaluated for the detection of *Trypanosoma cruzi* antibodies in four species of wildlife reservoirs. Antibodies to *T. cruzi* were detected in both raccoons (*Procyon lotor*) (wild and experimental) and degus (*Octodon degu*) using the Chagas Stat-Pak. In naturally-exposed wild raccoons, the Chagas Stat-Pak had a sensitivity and specificity of 66.7-80% and 96.3%, respectively, for raccoons. Seroconversion by Chagas Stat-Pak compared with IFA seroconversion was delayed for experimentally-infected raccoons, but occurred sooner in experimentally-infected degus. The Chagas Stat-Pak did not detect antibodies in wild or experimentally-infected Virginia opossum (*Didelphis virginiana*) nor in experimentally-infected short-tailed opossums (*Monodelpis domestica*). These data suggest that the Chagas Stat-Pak might be useful in field studies of raccoons and degus when samples would not be available for more conventional serologic assays. Because this assay did not work on either species of marsupial, the applicability of the assay should be examined before it is used in other wild species.

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GENETICALLY DISTINCT *L.DONOVANI* CAUSING CUTANOUES LEISHMANIASIS IN SRI LANKA: A STUDY ON *LEISHMANIA* SPECIES/STRAIN VARIATION

Yamuna D. Siriwardana[1], NJ Beeching[2], HA Noyes[3], ML Chance[2], P. Bates[2], Nadira Karunaweera[1]

[1]Department of Parasitology, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka, [2]Liverpool School of Tropical Medicine and Hygiene, University of Liverpool, Liverpool, United Kingdom, [3]School of Biological Sciences, University of Liverpool, Liverpool, Liverpool, United Kingdom

Cutaneous leishmaniasis is as an established disease in Sri Lanka. Iso-enzyme characterization revealed that the local species belong to zymodeme MON 37 of *Leishmania donovani* which is different from predominant Indian strain MON 2. This study was undertaken to 1) identify the species of *Leishmania* parasites in Sri Lanka by molecular methods, 2) make genetic analysis of the local *L. donovani* together with *L. donovani* of different geographical origin, and 3) explain the strain difference between Sri Lankan and Indian *L. donovani*. The study was carried out at the Department of Parasitology, Faculty of Medicine, Colombo and at the Liverpool School of Tropical Medicine and Hygiene, UK in year 2003-2004. Lesion aspirates and punch biopsies were cultured/directly used for polymerase chain reaction (PCR). *Leishmania* genus specific primers were designed (6 PGDH-F, 6 PGDH R) for a 997 bp amplicon to include partial nucleotide sequence. PCR, gene cloning and sequencing were performed on 11 local, 2 Indian and 3 known *L. donovani* isolates. Phylogenetic tree was developed using obtained and known *Leishmania* reference (10) sequences. Previously described *L. donovani* specific microsatellite loci were studied in 5 local *Leishmania* isolates. Obtained 822-nt sequences were translated to identify amino acid sequences. 14/17 *L. donovani* and *L. infantum* sequences were >99% identical and clustered together with 58% bootstrap support. This group was quite distinct from the *L. major* and *L. tropica* which are closely related to *L.donovani* but result in CL in Africa and Asia. Sri Lankan, Indian/Bangladesh/Nepal group, Sudanese, East African and *L.infantum* isolates formed distinct groups in microsatellite analysis. Protein translation showed 1 amino acid change that was consistent with the results of MLEE. Strain difference between MON2 and MON 37 was due to a single nucleotide difference at position 976 (uncharged asparagine in MON-2 and negatively charged aspartic acid MON-37 sequences) and did not have a true genetic basis. In conclusion, leishmaniasis in Sri Lanka are caused by *L. donovani* of a distinct genetic group. Ability of the local *Leishmania* parasite to visceralize, self heal or develop drug resistance is debatable. Treatment options need careful selection and follow up together with the monitoring of the clinical course in each patient.

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LEISHMANIASIS IN SRI LANKA: STUDY OF CLINICAL DISEASE

Yamuna D. Siriwardana[1], Ganga Sirimanna[2], Chandani Udagedara[3], PH Chandrawansa[4], Nadira D. Karunaweera[5]

[1]Department of Parasitology, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka, [2]National Hospital, Colombo, Sri Lanka, [3]General Hospital, Matale, Sri Lanka, [4]General Hospital, Matara, Sri Lanka, [5]Department of Parasitology, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka

Sri Lanka is a new focus with established cutaneous leishmaniasis, caused by *Leishmania donovani* belonging to a distinct genetic group. Over 1500 cases have been detected since year 2001 by our institution alone. The objective of this study was to describe the detailed clinical profile of leishmaniasis in Sri Lanka. A descriptive study of 401 cases was performed. Patients were clinically evaluated and microscopy and/or PCR and formol gel test (FGT) was performed on lesion material and blood respectively, after informed written consent. Four 0% (4%) had systemic features and 0% had positive FGT. Parasitological diagnosis was confirmed in 70.82% (n=284) cases. Among the parasite positive (PP) group, majority were between 21-40 years (64.4%), soldiers (57.4%) and males (79%). Most lesions were single (73.9%), less than 2cm (72.5%), on exposed areas (forearms/hands- 44.1%, face/head neck/pinna region-30.1%) and had well defined (58%), and regular (70%) edges. Papules (23.4%),nodules (25.4%), ulcerating nodules (19.6%), ulcers (23.7%), plaques (6.4%) and other types (i.e., DL,DCL,L.residivance) were seen. Majority of PPs were smaller lesions (P=0.004), <8 months (P=0.005), were well defined (P=0.005) and regular (P=0.029) compared to parasite negative lesions. Nodules (n= 100, 75.5%) and lesions of 5-8 months duration (77.5%, P=0.005) were most likely to harbor parasites. Also, females compared to males had more single lesions (86.7%:70.5%, P=0.011) mainly on head and neck region (P=0.00045) with marked inflammation (P=0.000) and less sporotrichoid spread (P=0.03) irrespective of similar duration (P=0.144), type (P=0.934), size(P=0.263) and parasite detection rate (P=0.549) in both sexes. No differences observed in the mean duration (5 months) and parasite detection rates (P=0.641) between the main lesion types. Non-ulcerative lesions (NUL) were the commonest type observed up to 6 months and during 6-9 months ulcerating lesions were almost twice as that of NUL (60.8% vs. 31.4%). After 1 year majority remained as complete ulcers (44.1%) or remained non-ulcerative (57.0%) and small (55.9%,P=0.047). In conclusion, local *L. donovani* results in simple CL in Sri Lanka. Most lesions completely ulcerate within one year or remain non-ulcerative. There is individual variation in this course. Parasitological investigations preferably performed early are more likely to yield positive results.

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SEROPREVALENCE OF *TRYPANOSOMA CRUZI* IN RACCOONS IN TENNESSEE

Jenny G. Maloney[1], Anthony Newsome[1], Junjun Huang[2], Jordona Kirby[3], Brett Dunlap[3], Michael Yabsley[4], John R. Dunn[2], L. Rand Carpenter[2], Timothy F. Jones[2], Abelardo C. Moncayo[2]

[1]Middle Tennessee State University, Murfreesboro, TN, United States, [2]Tennessee Department of Health, Nashville, TN, United States, [3]United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, Nashville, TN, United States, [4]Southeastern Cooperative of Wildlife Disease Study, University of Georgia, Athens, GA, United States

Trypanosoma cruzi is the causative agent of Chagas disease which affects nearly 20 million people in Latin America. *T. cruzi* causes infection in variety of wild and domestic mammals and is transmitted by several species of blood feeding reduviid insects commonly known as kissing bugs. Autochthonous infection of humans in the United States is rare with only six cases reported from California, Texas, Tennessee, and Louisiana since 1955. However, *T. cruzi* has been detected in a number of wild and domestic mammals in the southeastern U.S. Although the parasite is known to be present in Tennessee, little is known about the ecology and the risk of transmission of *T. cruzi* among humans and animals. We tested raccoon serum for the presence of antibodies to *T. cruzi* using the indirect fluorescent antibody (IFA) assay to better understand the presence, transmission dynamics, and infection risk of *T. cruzi* in Tennessee. More than 600 raccoon serum samples from 10 eastern counties were collected from 2005 to 2007 by the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services program as part of the Oral Rabies Vaccination Program and sent to the Tennessee Department of Health Vector-Borne Disease Laboratory. To date, 128 of these samples have been tested representing 6 counties. Twenty-nine (22.6%) samples were seropositive. Of the six counties tested so far, five have yielded raccoon serum that is positive for antibodies to *T. cruzi*, ranging from 15.2-38.5% seroprevalence per county. Peridomestic raccoons may serve as a reservoir for *T. cruzi* and potentially be a source of exposure risk for humans and domestic mammals.

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AMERICAN VISCERAL LEISHMANIASIS: II DIVERSITY OF WILD ANIMALS ASSOCIATE IN VISCERAL LEISHMANIASIS FOCUS IN TRUJILLO STATE VENEZUELA

Milagros Oviedo[1], Antonio Mendez[1], Adalberto Gonzalez[1], Jorge Suarez[1], Herminia Bendezu[1], Eliades Ledezma[1], Laura Vasquez[2]

[1]Universidad de Los Andes, Trujillo, Venezuela, [2]Universidad de Los Andes, Valera, Venezuela

Visceral Leishmaniasis is a zoonotic disease in which wild and domestic mammals act as reservoir host. In Venezuela, there is scantly information available regarding wild animals in visceral leishmaniasis focus. In this report summarizes the information obtained about wild animal associated a leishmaniasis visceral focus. Wild animals were systematically captured in the rural area of Montañas de Peraza (MP), Trujillo State (9°27"N; 70º31"W), north-west Venezuela, during one year. MP is a small town of about 300 people, in this area, both cutaneous and visceral leishmaniasis are present. The captures were in National brand live traps, baited with bananas or corn, were placed around human dwellings in the area, 10 traps per 4 nights per months throughout the study period. Wild animals were identified, anesthetized and killed; samples of blood, extracted by means of cardiac puncture and samples of liver, spleen and skin, an impression smear was prepared and a portion of the organ was frozen for PCR. Twenty two wild mammals were collected, pertaining to two families: Didelphidae y Muridae. Didhlephide Family were collections 6 *Didelphis marsupialis* and one specimen of *Marmosa robinsoni*. Diversity of Muridae was collected: *Oryzomys albigularis* (3), *Sigmodon hispidus* (2), *Sigmodon alstoni* (7), *Oecomys flavicans* (2) and *Acodon urichi* (1). Liver, spleen and skin samples from these animals were negative. Although both tegumentary and visceral leishmaniasis are endemic in the area, sampling of wild mammals failed to demonstrate natural infections with *Leishmania* spp, perhaps by the low number of collected units, since *L. infantum* y L. *braziliensis* have been isolated in three the this species in other areas of Venezuela, Brazil and Colombia

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TRYPANOSOMA EVANSI ANTIBODY LEVELS IN THE GOATS FROM SLAUGHTER HOUSES OF KOLKATA, INDIA

Satadal Das[1], Payel Bhattacharya[2], Sejuti Naha Biswas[2], Tanima Bose[2], Debrup Sen[2], Debanjana Das[2], Sajal Roy[2], **Pulak Lahiri**[2]

[1]Peerless Hospital and B. K. Roy Research Centre, Kolkata, India, [2]University of Calcutta, Kolkata, India

Trypanosoma evansi infection in caprine animals is well known. Consumption of goat meat is quite common in many parts of India. Recently, one human case report of infection by this parasite has been reported from Maharashtra state of India. Therefore, a study on *T. evansi* - antibody levels in goats is important from human health aspect. After collection of blood from goats to be slaughtered for meat shops, *T. evansi* antibody levels were detected by CATT/*T.evansi* kit obtained from King Leopold Institute, Belgium. 42.10% of the tested blood samples taken from the goat population was found to be positive for *T. evansi* antibody,out of which 25.0% showed strongly positive results. In conclusion, a significant percentage of goat population in the slaughter houses of Kolkata is positive for *T. evansi* antibody, raising concern about zoonotic and its possible human health impact.

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STUDY THE ABILITY OF MONOCYTES CORD BLOOD OF NEWBORNS NOT INFECTED THE MOTHERS INFECTED BY *TRYPANOSOMA CRUZI* TO CONTROL INFECTION

Amilcar Alejandro Flores Leon

University Mayor of San Simon, Cochabamba, Bolivia

Trypanosoma cruzi is the causative agent of Chagas disease is transmitted mainly by the insect vector (*Triatoma infestans*), and other alternative routes such as blood transfusions and congenital transmission. The absence of infection in a newborn can be due to the fact, that the parasite has not crossed the placenta, or to assume that sometime the parasite crossed the placenta, but it is destroyed by the immune system of the fetus. Previous studies have shown that blood samples of the group M+B- had a lower percentage of infected cells compared with the control group M-B- when they were infected with Tripomastigotes *T. cruzi*. In this work, monocytes M+B- and M-B- were infected with a parasitic burden the 1:5, 1:20 and 1:100 (parasite/monocytes), incubated by 22 and 96 hours and see if by decreasing the burden parasitic, these monocytes control better the infection, on the other hand, we determine the role the maternal antibodies anti *T. cruzi* transferred from the mother, in infection control. The results of the infection of the groups M+B- and M-B-, showed that the percentage of infected cells in both groups decreases when the burden parasite is lowest, however this percentage of infected cells was lower in group M+B-, also observed that when the parasitic load it is lower (1:100) the number of amastigotes in monocytes M+B- decreases, reaching a level of zero on a sample M+B-, which indicates that the infection was controlled entirely, different behavior was observed in the group M-B-, hence the parasite index was lower in group M+B-. With regard to the role of the maternal antibodies in controlling the infection, we see its essential role in controlling infection, when monocytes M+B- incubated in presence of autologous plasma which was eliminated anti *T. cruzi* antibody, showed high percentages of infected cells, and a greater number of amastigotes present, which determines a high index parasite. In conclusion this study shows that monocytes of uninfected infants of mothers infected with *T. cruzi* have an increased ability to control the infection, especially when the parasitemia is low. It also was determined that antibodies passed from mother to fetus, play a very important role in controlling the infection.

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CHARACTERIZATION OF A RARE EQUINE LEISHMANIA IN PUERTO RICO; NATIVE OR IMPORTED?

Juan Mendez[1], Ioana Brasov[1], Ghosh Kashinath[1], Peter Weina[1], Victor Melendez[1], Adeline Chan[1], Bernardino Ortiz[2], Rossana Morales[2], Elias Algarin[2], Adelfa E. Serrano[2]

[1]Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD, United States, [2]Department of Microbiology and Medical Zoology, Medical Sciences Campus, University of Puerto Rico School of Medicine, San Juan, PR, United States

Leishmania parasites obtained from biopsies of a lesion found on a horse, born and reared on the island of Puerto Rico (a non-endemic area) were evaluated for speciation and molecular characterization. The donor horse never left the island and its mother, which was also infected, was born and reared in the same geographical area. The parasites were obtained in 1996 and adapted to culture at the University of Puerto Rico, however, full speciation was not performed at that time. Recently at the Walter Reed Army Institute of Research, growth conditions were optimized, and the parasite culture expanded and differentiated. Acrylamide Electrophoresis identified a *Leishmania* genus of unknown species. Cellulose Acetate Electrophoresis was done on four isoenzymes (6PDGH, GPI, MPI, LP) used for speciation. The genus identified was consistent with *L. braziliensis*, *L. infantum,* and *L. colombiensis*. Side-by-side contrast against 15 WHO control standards and four different *L. colombiensis* strains ruled out the later one. Cloning studies of the parasite yielded a single *Leishmania* strain with *L. braziliensis* and *L. infantum* attributes. Similar observations were

made when 32 fragments of *Leishmania* parasite DNA from the databank were sequenced and compared to the DNA of the parasite found in the horse's lesion. In addition, histological comparisons of stained smears showed a cell morphology that was clearly distinguishable from the three other *Leishmania* and trypanosome genus. Combined, the data confirm the presence of a unique equine *Leishmania* species in an area adjacent to El Yunque National Rain Forest in Puerto Rico and for the first time provide sufficient characterization for its identification as a new species; a hybrid of *L. braziliensis* and *L. infantum*. The possible reappearance of the vector in the island after more the 50 years, and the risk of unreported cases in the equine industry and other mammalian hostesses merit serious attention.

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GENE ORGANIZATION AND SEQUENCE ANALYSIS OF TRANSFER RNA GENES IN TRYPANOSOMATID PARASITES

Norma E. Padilla-Mejia, Luis E. Florencio-Martinez, Elisa Figueroa-Angulo, Claudia M. Gomez-Hurtado, Juan C. Vizuet-de-Rueda, Santiago Martinez-Calvillo

National University of Mexico, FES Iztacala, Tlalnepantla, Mexico

The protozoan pathogens *Leishmania major*, *Trypanosoma brucei* and *T. cruzi,* referred together as TriTryps, are trypanosomatid parasites that produce devastating human diseases known as leishmaniasis, African sleeping sickness and Chagas disease, respectively. These organisms show very unusual mechanisms of gene expression, such as polycistronic transcription and trans-splicing. We are interested in the study of transcription by RNA polymerase III (Pol III), which produces small essential RNA molecules, like tRNA, 5S rRNA and some snRNAs. The complete sequence of the genomes of the TriTryps has been reported recently. To analyze the sequences and genomic organization of Pol III-transcribed genes, we have performed an *in silico* analysis of the tRNA genes in the TryTryps databases. First, we used a tRNA-search algorithm to confirm the identity of all the annotated tRNA genes. Our analysis revealed the presence of 82, 64 and 114 genes for *L. major, T. brucei* and *T. cruzi,* respectively. Moreover, from 1 to 4 selenocysteine tRNA genes were found in each species. Thus, the number of tRNA genes in the TriTryps is relatively low. In *L. major*, the 82 tRNA genes are distributed among 31 loci, on 18 different chromosomes. Most tRNA genes are organized into clusters of 2 to 10 genes that may contain other Pol III-transcribed genes. The distribution of genes in the *L. major* genome does not seem to be totally random, like in most organisms, since half of the chromosomes do not contain even a single tRNA gene. Additionally, 60 tRNA genes (72%) are located in only 7 chromosomes, which represent 26% of the genome. While the majority of the tRNA clusters do not show synteny (conservation of gene order), a few of them do show conservation; like a cluster of 13 Pol III genes that is highly syntenic among the three species. Analyses of the putative promoter regions (boxes A and B) and transcription termination signals (clusters of Ts) of the tRNAs will be presented. We have identified several tRNA isodecoder genes (having the same anticodon but different sequences elsewhere in the tRNA body) in the TriTryps. Interestingly, some of the differences between isodecoder genes occur in (or very close to) the internal promoter elements, which suggests that differential control of the expression of some isoacceptor tRNA genes in TriTryps is feasible.

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EVALUATION OF ALKANEDIAMIDE-LINKED BISBENZAMIDINES AS NOVEL AND POTENT ANTITRYPANOSOMAL AGENTS

Tien L. Huang[1], Jean Jacques Vanden Eynde[1], Annie Mayence[1], Cyrus J. Bacchi[2], Nigel Yartlet[2], Donna Rattendi[2], Aixa Rodriguez[2], Lakshman Mazumder[2]

[1]Xavier University of Louisiana, New Orleans, LA, United States, [2]Pace University, Haskins Laboratories, New York, NY, United States

Pentamidine, a prototype of the bisbenzamidine class, is approved in the chemotherapy of human African trpanosomiasis. However, its clinical use is limited because of severe side effects and poor oral bioavailability. As part of our research program to develop more effective and safer antiprotozoal agents, a series of novel alkanediamide-linked bisbenzamidines based on the structure of pentamidine was synthesized and evaluated for their antitrypanosomal activities. The compounds were tested *in vitro* against a drug-sensitive strain of *Trypanosoma brucei brucei* LAB 110 EATRO and a drug-resistant strain of *T.b. rhodesiense* KETRI 243. The tested compounds generally showed similar potencies against both strains. The most potent compounds were bisbenzamidines linked with a hexanediamide, heptanediamide or octanediamide group (inhibitory concentration for 50% (IC_{50}) = 1.4-2.8 nM). Replacing the terminal basic amidines with non-basic amide or cyano groups resulted in inactive compounds. Moving the amidines from the para-positions into the meta- positions or para, meta-positions of the hexanediamide derivative resulted in less active compounds. Several of the most potent compounds were effective in curing mice infected with the drug-sensitive (*T. b. brucei* Lab 110 EATRO) or drug-resistant strains of *T. b. rhodesiense* KETRI 2002 and KETRI 2538. Curative doses were $\leq$ 15 mg/kg/day for 3 days given by intraperitoneal injection in the mouse model of infections. The details of the design, synthesis and structure-activity relationships of these compounds will be presented.

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MAGNETIC RESONANCE IMAGING INVESTIGATION OF MEGASYNDROME OF THE GASTROINTESTINAL TRACT IN EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION

Herbert B. Tanowitz[1], Lars NY[2], Hua Li[1], Shankar Mukherjee[1], Stephen M. Factor[1], Linda A. Jelicks[1]

[1]Albert Einstein College of Medicine, Bronx, NY, United States, [2]Sahlgrenska University Hospital, Gothenburg, Sweden

Human infection with *Trypanosoma cruzi* causes megasyndromes of the gastrointestinal (GI) tract. Mice exhibit many of the functional, pathological and immunological alterations observed in human infection including mega-organ syndromes. Previously, we demonstrated that CD1 mice, acutely infected with the Brazil strain of *T. cruzi*, exhibited increased expression of NOS2, decreased expression of NOS1, reduced Ca^{2+}-dependent (NOS1 and NOS3) activity, and increased NOS2 activity in the gut. To further investigate the role of the NOS and NO in *T. cruzi* infection we used magnetic resonance imaging (MRI) to non-invasively monitor alterations in the GI tract of infected NOS1, NOS2 and NOS3 null and wild type C57BL/6mice. WT mice infected with the Brazil strain exhibited dilatation of the intestines as early as 30 days post-infection. Average intestine lumen diameter increased by 72%. Levels of intestinal NOS2 and NOS3 were significantly elevated in infected WT mice. Uninfected NOS1 null mice exhibited enlarged stomachs and dilatation of the intestinal tract that was exacerbated by infection. Infected NOS1 null mice exhibited increased expression of NOS3 along with high parasitemia and mortality. Although infected NOS2 null mice exhibited decreased mortality, the degree of intestinal dilatation was not different from that observed in WT mice. These mice also exhibited increased expression of NOS1 and NOS3. NOS3 null mice showed little change in expression of NOS1 or NOS2 upon infection, and they exhibited dilated intestines and high mortality even though parasitemia was comparable to that of infected WT and NOS2 null

mice. Dilation of the intestines was observed in all infected mice and was accompanied by inflammation and ganglionitis. To our knowledge this is the first study demonstrating the utility of MRI to monitor intestinal tract enlargement in *T. cruz*i-infected mice. Our observations strongly suggest that intestinal inflammation and dilation is not exclusively dependent on NO. Interestingly, our observations also indicate that *T. cruzi* associated megasyndromes of the GI tract appear much earlier that previously appreciated.

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ANALYSIS OF GENE EXPRESSION AND EVOLUTIONARY PROCESS IN *LEISHMANIA (VIANNIA) BRAZILIENSIS* AND *LEISHMANIA (VIANNIA) PERUVIANA* MODEL

Dionicia Gamboa

Instituto de Medicina Tropical, Lima, Peru

Leishmania (V.) braziliensis and *L. (V.) peruviana* are the main causative agents of Leishmaniasis in Peru. Even if these two species are very close related at genomic level, they present different clinical outcomes. *L. (V.) baziliensis* is mainly associated with cutaneous lesions and *L. (V.) peruviana*, produces only cutaneous lesions ('uta'). The objective of this work was to explore the contribution of parasite factors to the development of the pathology by analysis of genomic make up and life stage characteristics. We built up a hypothesis about the evolutionary history of both species; using a rapidly evolving genetic marker (chromosomal size of different strains from different regions of Peru was processed with a specific algorithm). In order to verify if both species showed phenotypic differences in terms of virulence, we developed *in vitro* and *in vivo* infection models, using 2 representative strains of *L. (V.) braziliensis* and 2 representative strains of *L. (V.) peruviana*. We also applied differential display to screen transcripts originating from different biological stages of a reference *L. (V.) braziliensis* strain (most virulent) and we followed the expression of four genes identified during the *in vitro* life cycle by real-time quantitative PCR. By DD-PCR, we identified a series of candidate markers of infective stages of *L. (V.) braziliensis* and validated two of them: *meta 1* and *opb*. These markers were then further explored in the two species. Metacyclogenesis can be detected in both species thanks to the expression of the *'meta 1'* gene, and interestingly, the highest level of *meta 1* expression were measured in the *L. (V.) braziliensis* strain isolated from a mucosal patient. We identified a new marker of amastigote stages, the *'opb'* gene that is more expressed in amastigotes of *L. (V.) braziliensis* than in the promastigotes stages of the same species. Interestingly, expression of this gene was lower in the two strains of *L. (V.) peruviana*. In conclusion, we hypothesise that *L. (V.) peruviana* would have evolved from *L. (V.) braziliensis* and that during the North South colonisation of the Pacific slopes of the Andes, it would have lost some of the *L. (V.) braziliensis* characters: being more and more different from a karyotype point of view, and decreasing its pathogenicity.

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INNATE IMMUNITY IN THE CONTROL OF *LEISHMANIA AMAZONENSIS* INFECTION: A ROLE FOR TYPE I IFN RECEPTOR AND NEUTROPHIL

Lijun Xin, Diego A. Vargas-Inchaustegui, Jiaren Sun, **Lynn Soong**

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

Type I IFNs exert diverse effector/regulatory functions in innate and adaptive immune responses to viruses and bacteria; however, their roles in parasitic infections are less clear. In murine models of *Leishmania* infection, it has been reported that parasite-induced type I IFNs are critical for NO-dependent disease control, and that administration of IFN-β has a dose-dependent protective effect in *L. major*-infected mice. Surprisingly, we found that following infection with *L. amazonensis* (*La*) parasites, $IFNAR^{-/-}$ mice developed significantly smaller lesions than did the wild-type controls, while mice receiving exogenous IFN-α showed an enhanced disease progression. Since $IFNAR^{-/-}$ mice produced low levels of IFN-γ and IL-10 when their draining lymph node cells were re-stimulated with parasite antigens *in vitro* and generated low levels of antigen-specific IgG in sera, we speculated that the attenuated disease in these knockouts was not due to an enhanced adaptive immunity. Given the marked reduction in parasite loads even at 3 days of infection in $IFNAR^{-/-}$ mice, we examined the possibility of neutrophil-mediated parasite clearance. Using a peritonitis model, we found that while $IFNAR^{-/-}$ mice were highly efficient in recruiting peritoneal neutrophils at 6 h post-injection of parasites, these mice displayed unique phenotypic changes in $Gr1^-CD11b^+$ macrophages and $Gr1^+CD11b^-$ neutrophils at 48 h. Both *in vitro* and *in vivo* studies revealed that $IFNAR^{-/-}$ neutrophils had significantly higher rates of spontaneous and infection-induced apoptosis than did their wild-type controls. While macrophages from both sources responded comparably to parasites, the interactions between macrophages and $IFNAR^{-/-}$ neutrophils, but not wild-type neutrophils, greatly enhanced parasite killing. These results suggested a linkage between neutrophil recruitment/apoptosis and parasite clearance. Additional studies are ongoing to examine the function of neutrophils at early stages of infection and the molecular basis underlying this attenuated cutaneous leishmaniasis. This study will provide new information on innate immunity to protozoan parasites.

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DECREASE OF PARASITIC LOAD AND LESION SIZE IN MURINE CUTANEOUS LEISHMANIASIS INDUCED BY *LEISHMANIA AMAZONENSIS* AFTER TREATMENT WITH MESOIONIC COMPOUNDS

Raquel F. Rodrigues[1], Karen S. Charret[1], Aurea Echevarria[2], Veronica Amaral[3], Leonor L. Leon[1], Marilene M. Canto-Cavalheiro[1]

[1]Oswaldo Cruz Foundation - Brazil, Rio de Janeiro, Brazil, [2]Universidade Federal Rural do Rio de Janeiro - Brazil, Rio de Janeiro, Brazil, [3]Universidade Federal Fluminense - Brazil, Niterói, Brazil

Leishmaniasis is one of the major parasitic diseases of research priority by the World Health Organization. The urgency for more selective and less toxic drugs has led us to search for others chemical therapies. The mesoionic compound (MI) of the 1,3,4-thiadiazolium-2-aminide class, have a wide-ranging of biological activities. In this study, it was investigated the therapeutic efficacy, cytotoxic and immunomodulatory effects of these compounds using a murine model of experimental infection by *Leishmania amazonensis*. Susceptible CBA/J mice were infected in the footpad with 10^6 *L. amazonensis* promastigotes. Controls and infected mice were treated subcutaneously with MI-4-OCH_3 and MI-H-H diluted in saline, during 4 weeks. Glucantime, a classical antileishmanial drug, was used as reference. While complete cure did not occur, animals treated with the MI-4-OCH_3 derivative had small lesions in the infected footpad up to 12 weeks post-infection and delayed surface ulceration. The treatment with MI-4-OCH_3 and MI-H-H was capable to reduce parasitic load in lymph node and spleen. Toxicological studies involving evaluation of transaminases (AST and ALT) and creatinine concentration, showed no apparent hepatic or renal toxicity after the treatment with mesoionic compounds. The results with specific IgG anti-*L. amazonensis* in treated mice with MI-4-OCH_3 showed lower levels than the control ones. It was also observed an important increase of IgG2a during MI-H-H and MI-4-OCH_3 treatment, and reduction in the serum levels of IgG1 at 12ª wpi of MI-4-OCH_3 and Glucantime treated mice. In addition an increase of NO in infected mice lymph node cell culture supernatants, after treatment with MI-4-OCH_3 was detected. Cytokines (IFN-γ and IL-4) are being evaluated. These results provide new perspectives for the development of drugs with less toxicity and capable to modify the immunological response to combat *Leishmania* infection.

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CHARACTERIZATION OF INTERMEDIATE DEVELOPMENTAL FORMS OBTAINED DURING *IN VITRO* DIFFERENTIATION OF *TRYPANOSOMA CRUZI* FROM TRYPOMASTIGOTES TO AMASTIGOTES

Luis A. Hernández-Osorio[1], Claudia Márquez-Dueñas[2], Luis E. Florencio-Martínez[3], Gilberto Ballesteros-Rodea[4], Santiago Martinez-Calvillo[5], **Rebeca G. Manning-Cela**[2]

[1]Centro de Investigación y de Estudios Avanzados del IPN and Facultad de Ciencias Químicas, Universidad Autónoma Benito Juárez de Oaxaca, México, México D.F. and Oaxaca, México, Mexico, [2]Centro de Investigación y de Estudios Avanzados del IPN, México D.F., Mexico, [3]Centro de Investigación y de Estudios Avanzados del IPN and FES Iztacala, UBIMED. UNAM. Edo. de México., México D.F., Mexico, [4]Centro de Investigación y de Estudios Avanzados del IPN and Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autonoma de Mexico, México D.F., Mexico, [5]FES Iztacala, UBIMED. UNAM. Edo. de México, México, México D.F., Mexico

Trypanosoma cruzi undergoes a biphasic life cycle in which alternate four developmental stages that survive a wide range of environmental conditions, experiencing complex morphological and physiological changes. Although the differentiation process is crucial for parasite survival, to continue its life cycle and to propagate the infection, the molecular mechanisms involved are unknown. *In vitro* conditions to obtain a synchronic transformation and efficient rates of pure IFs, indispensables for further biochemical, biological and molecular studies, have not been reported. We obtained and characterized different IFs resulted by the differentiation from tissue-derived trypomastigotes to amastigotes. The IFs showed a highly synchronous genotypic and phenotypic gradual differentiation. During the kinetics of transformation it was observed a progressive decrease of the size of the parasite body, undulating membrane and flagellum, concomitant with the reduction of the parasite movement and the gradual acquisition of the specific surface antigen Ssp4 of amastigotes. Also, it was observed the gradual remodeling of the nucleus, together with the displacement of the kinetoplast from the posterior to the anterior side of the parasite. All the results together showed that in the *in vitro* conditions established, it is possible to obtain large quantities of highly synchronous and pure IFs, which are clearly distinguished by morphometrical analysis.

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EVIDENCE OF GENETIC EXCHANGE IN NEW WORLD *LEISHMANIA* POPULATIONS FROM THE SEQUENCE ANALYSIS OF THREE ISOENZYME MARKERS

Pablo Tsukayama, Carmen M. Lucas, Benjamin Espinosa, David J. Bacon

U.S. Naval Medical Research Center Detachment, Lima, Peru

Population genetics analyses applied to various protozoan parasites, including species from the *Toxoplasma, Plasmodium*, *Trypanosoma*, and *Leishmania* genera; suggest that these pathogens present a largely clonal population structure. However, sexual recombination events may have important effects on parasite fitness and in their population structure dynamics. The common theme that arises is that limiting sex to generate clonal populations is advantageous, but retaining the ability for sexual reproduction is also advantageous. Attempts to demonstrate *in vitro* sexual recombination in *Leishmania* have failed. For the related kinetoplastids *Trypanosoma brucei* and *Trypanosoma cruzi*, genetic crosses have been obtained in the laboratory and their mechanism of recombination have been determined. *Leishmania braziliensis* and *Leishmania peruviana* are the two most common agents of leishmaniasis in Peru. Despite being highly similar at the DNA level, they differ markedly in their clinical and epidemiological features. Several cases of phenotypic hybrids between the two species have been reported in regions where they occur sympatrically. We analyzed the coding sequences of the mannose phosphate isomerase, malate dehydrogenase, and 6-phosphogluconate dehydrogenase genes for 28 isolates previously classified as *L. braziliensis*, *L. peruviana* or *L. braziliensis* / *L. peruviana* hybrids and identified 15 isolates that showed heterozigosity at one or several discriminative loci. These heterozygous states strongly suggest past events of sexual recombination between *L. braziliensis* and *L. peruviana*. When these sequences were cloned into *E. coli*, they revealed several different populations, including the two putative parental genotypes, but also novel marker combinations that indicate the occurrence of homologous recombination and gene shuffling. Added to previous evidence, our results support the notion that genetic recombination in *Leishmania* parasites might occur in their intracellular stage following a mechanism similar to that of *Trypanosoma cruzi*: cellular and nuclear fusion followed by the formation of transient tetraploid states, homologous recombination and loss of alleles to return to diploidy/ aneuploidy.

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CONSTRUCTION OF POLY-PROTEIN VACCINE ANTIGENS FOR LEISHMANIASIS

Yasuyuki Goto, Ajay Bhatia, Thomas S. Vedvick, Randall F. Howard, Steven G. Reed

Infectious Disease Research Institute, Seattle, WA, United States

It is certain that a consistently effective, stable and reliable source of vaccine against leishmaniasis is unlikely to arise from whole-parasite approaches. The manufacturing, quality control, stability and potency assay system of defined vaccines seem to be a key for the consistent effectiveness. There is progress in characterization of defined antigens showing protective efficacy as a single antigen in animal models of leishmaniasis. However, when diversity in human immunity like MHC molecules is concerned, a vaccine composed of not a single antigen but multiple antigens seems to be more protective against leishmaniasis in humans. Here, we present production of poly-protein vaccine antigens composed of four defined vaccine candidate antigens. Vaccination with these fusion proteins, in formulation with MPL®-SE, induced Th-1 dominant antigen-specific responses in both BALB/c and C57BL/6 mice. Four weeks after challenge with *Leishmania infantum*, a causative agent of visceral leishmaniasis, the vaccinated mice showed significant lower parasite burdens in the liver compared with non-immunized mice. The vaccines also induced significant protection against intradermal challenge with *L. major*, a causative agent of cutaneous leishmaniasis. Taken together, these new poly-protein antigens are promising as vaccine candidates for multiple forms of leishmaniasis.

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TRYPANOSOMA CRUZI-INDUCED ERECTILE DYSFUNCTION IN MICE

Moses Tar, Rowena Chua, Arnold Melman, Dazhi Zhao, Stephen M. Factor, Herbert B. Tanowitz, Michael E. DiSanto

Albert Einstein College of Medicine, Bronx, NY, United States

Infection with *Trypanosoma cruzi* is associated with dysfunction of the cardiovascular system and the urinary tract. Erectile function was assessed in anesthetized Brazil strain infected CD1male mice 35 days post infection (dpi) (n=4) and control mice (n=7) via intracavernous pressure (ICP) measurement. ICP and carotid artery blood pressure (BP) were measured. The cavernous nerve (CN) was stimulated with a platinum subdermal electrode connected to an S88 stimulator at amplitudes of 0.75, 1, 2, 4, 6 and 10 mA. Another cohort of mice 35 dpi (n=7) and controls (n=7) were sacrificed for molecular analyses and histology. Each penis was dissected free at its base and the glans penis and the connective tissue surrounding the shaft removed. The remaining corpus cavernosum (CC) tissue was frozen, total RNA extracted and real-time PCR performed. The ICP/BP ratios were significantly depressed in infected mice at 4, 6 and 10 mA stimulation ($p<0.05$) with no changes at lower stimulations. Real- time PCR revealed a significant reduction in the expressions of NOS1, and

NOS3 (p<0.0005) in the penis of infected mice compared with controls. NOS2 expression was increased consistent with the intense inflammatory response. In addition, the expression of smooth muscle (SM) myosin and α-actin were significantly reduced (p<0.0003) in the penis of infected mice as was the expression of myocardin a regulator of the SM phenotype. CN-stimulation in the setting of acute *T. cruzi* infection revealed a significant deterioration in erectile function. The decreased expression of NOS1 and NOS3 which, respectively mediate the initial and sustained nitric oxide (NO)-induced penile erection, suggest a defect in the NO/cGMP-mediated CC-SM relaxation pathway. These data taken together with a decrease in SM content provides a potential mechanism for erectile dysfunction in acute Chagas disease.

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PILOT STUDY OF A THERAPEUTIC DNA VACCINE AGAINST *TRYPANOSOMA CRUZI* IN NON-HUMAN PRIMATES

Eric Dumonteil[1], Meredith Hunter[2], Hiatzy Zapata-Estrella[1], Patricia Dorn[3], Preston Marx[2]

[1]Universidad Autonoma de Yucatan, Merida, Yucatan, Mexico, [2]Tulane University, New Orleans, LA, United States, [3]Loyola University, New Orleans, LA, United States

Previous studies showed that a DNA vaccine expressing *Trypanosoma cruzi* antigens TSA1 and Tc24, administered during the acute or chronic phase of the infection with *T. cruzi* could control at least partially the development of the disease in several strains of mice. In this pilot study, we aimed at evaluating the safety and efficacy of this DNA vaccine in rhesus monkeys. Nine animals were infected via IV with 500,000 *T. cruzi* parasites/kg (Y strain), and five were treated with three IM injections of 500 µg of DNA vaccine encosing TSA1 and Tc24 with aluminium phosphate as an adjuvant at 3, 4, and 5 months post-infection. Four control animals received the same doses of empty plasmid. Safety of the DNA vaccine treatment was assessed by monthly monitoring of blood counts and chemistry, all of which did not show any alteration 12 months post-infection. Two months after infection, all the animals were seroposivive for *T. cruzi* and/or presented a positive *T. cruzi* PCR in blood samples. Electrocardiographic reccordings at 4 and 6 months post-infection revealed that QT interval appeared longer in untreated animals compared to that of treated animals. Necropsies of some of the animals at 6 months post-infection indicated that all organs had a normal appearance, confirming the safety of the vaccine treatment. Histopathologic analysis of tissue sections indicated that there was minimal inflammation in the heart of monkeys from both groups. One treated monkey also presented minimal inflammation in the liver, and an another one mild inflammation in the stomach. On the other hand, all the untreated animals presented minimal to mild inflammation in several tissues, such as colon, stomach, liver, lung, and skeletal muscle, suggesting a more severe disease in these animals. These results suggest that the therapeutic vaccine was safe and had some therapeutic effect on the control of disease progression in non-human primates.

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EXTENDED HIGH EFFICACY (90 DAYS FOLLOW UP) OF THE COMBINATION SULPHADOXINE-PYRIMETHAMINE WITH ARTESUNATE IN CHILDREN WITH UNCOMPLICATED FALCIPARUM MALARIA ON THE BENIN COAST, WEST AFRICA

Alain M. Nahum[1], Annette Erhart[2], Daniel Ahounou[1], Chantal Van Overmeir[2], Joris Menten[2], Martin Akogbeto[1], Marc Coosemans[2], Achille Massougbodji[3], Umberto D'Alessandro[2]

[1]Centre de Recherche Entomologique de Cotonou, Cotonou, Benin, [2]Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium, [3]Laboratoire de Parasitologie, Faculté des Sciences de la Santé, Université Nationale du Bénin, Cotonou, Benin

Combining artesunate to sulfadoxine-pyrimethamine (SP) should only marginally improve the treatment efficacy where SP resistance is widespread. However, in the lagoon costal area of southern Benin treatment failure at day 28 to SP alone was more than 40%, while that of SP combined with artesunate (SP-AS) was only 5.3%. Such large difference could have been dependant on the length of the follow up period and it was suspected that a substantial number of recrudescences would occur after day 28. We report the efficacy of AS added to SP beyond 28 days of follow-up. Children treated with either chloroquine alone (CQ), SP alone or SP-AS, after a follow up of 28 days, were visited at home twice a week to detect any late clinical failure until day 90 after treatment.The PCR-corrected clinical failure risks on day 90 were 2.7% for SP-AS, 39.8% for SP alone and 42.2% for CQ alone (Log-Rank p<0,001). The hazard of failure estimated by Cox regression was significantly higher for both CQ (HR = 20.4; p<0.001) and SP (HR= 18.2, p<0.001) as compared to SP-AS. In conclusion, despite high SP resistance, the efficacy of the combination SP- AS remains high after 90 days of follow up. Experiences from several African countries indicate that the deployment of a new drug policy during the transition period suffers multiple constraints, including unavailability of the new treatment(s) due to stock-outs. Both SP and artesunate are easily available in Benin and their combination offers a good alternative whenever the two recommended antimalarial treatments (artemether-lumefantrine and amodiaquine-artesunate) are not available.

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EARLY DEVELOPMENT OF THE NEW ARTEMETHER-LUMEFANTRINE DISPERSIBLE TABLET: PALATABILITY AND PHARMACOKINETICS IN HEALTHY SUBJECTS

Gilbert Lefèvre[1], Salim Abdulla[2], John Lyimo[2], Alex Agyemang[3], Christine Reynolds[4], Steve Pascoe[3], Serge Fitoussi[5], Ching-Ming Yeh[4], Marja Nuortti[1], Gilles-Jacques Rivière[6], Romain Séchaud[1]

[1]Novartis Pharma AG, Basel, Switzerland, [2]Ifakara Health Research and Development Centre, Dar es Salaam, United Republic of Tanzania, [3]Novartis Pharma Ltd, Horsham, United Kingdom, [4]Novartis Pharma Corporation, East Hanover, NJ, United States, [5]Mediscis, Lagord, France, [6]Novartis Pharma SAS, Rueil-Malmaison, France

A dispersible tablet (DT) of artemether-lumefantrine (A-L) was developed to enable easier administration of the drug to pediatric patients. So far, in young children A-L has been usually administered as crushed commercial tablet (CT) which may be associated with drug loss and reduced intake of A-L. During early development of A-L DT, 2 studies were performed in healthy subjects to evaluate the palatability of 3 formulations with different flavors (study 1) and to assess the relative bioavailability of DT compared to CT (study 2). Study 1 was a single-blind crossover trial in 48 healthy children from Tanzania (24 girls, 24 boys; mean age: 8.6±0.7 yrs). Subjects were randomized to receive A-L (for 10 sec without swallowing) together with flavors (strawberry, orange or cherry) in 1 day followed by a rating of the palatability using a visual analogue scale (VAS). VAS scores were statistically analyzed. Study 2 was an open, randomized crossover trial in 48 healthy adults of mainly Caucasian ethnicity (22 females, 26 males; mean age: 33.1±7.8 yrs). They received singles doses of A-L (4 tablets each) corresponding to 80 mg artemether + 480 mg lumefantrine under fed conditions. Rich plasma sampling was performed for evaluation of lumefantrine, artemether and dihydroartemisinin (DHA) pharmacokinetics. The primary objective of the trial was the comparison between DT and CT. Pharmacokinetic parameters were compared using standard bioequivalence tests. Study 1 showed no statistically significant difference in VAS scores between the 3 flavors but cherry had the highest score in many questions and was overall preferred. Study 2 demonstrated that DT delivered bioequivalent lumefantrine, artemether and DHA exposure (area under the curve) to CT. Bioequivalence criteria were also met for peak concentrations (C_{max}) of lumefantrine and DHA. Thus, the cherry flavored A-L DT was selected for further clinical development in patients.

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PHARMACOKINETIC AND PHARMACODYNAMIC CHARACTERISTICS OF A NEW DISPERSIBLE TABLET FORMULATION OF ARTEMETHER-LUMEFANTRINE COMPARED TO THE CRUSHED COMMERCIAL TABLET IN AFRICAN CHILDREN WITH *PLASMODIUM FALCIPARUM* MALARIA

Abdoulaye Djimdé[1], Steffen Borrmann[2], Salim Abdulla[3], Gilbert Lefèvre[4], Kim Andriano[5]

[1]Malaria Research and Training Center, University of Bamako, Bamako, Mali, [2]Kenya Medical Research Institute, Kifili, Kenya, [3]Ifakara Health Research and Development Centre, Dar es Salaam, United Republic of Tanzania, [4]Novartis Pharma AG, Basel, Switzerland, [5]Novartis Pharma Corporation, East Hanover, NJ, United States

In an attempt to allow convenient administration of artemether-lumefantrine (A-L) to pediatric patients, a dispersible tablet (DT) was developed. A single-blind, randomized, non-inferiority study compared the efficacy and safety of DT and crushed commercial tablet (CT) in African infants and children with uncomplicated *Plasmodium falciparum* malaria (N = 899). Patients were randomized to 3 different dosing groups (5-<15 kg, 15-<25 kg, 25-<35 kg) and treatment was administered twice daily over 3 days. The study showed that the new DT is as efficacious and safe as the currently used CT. Within that trial, pharmacokinetic assessments were performed in large subgroups of patients. Plasma concentrations of artemether and dihydroartemisinin (DHA) were determined at 1 and 2 h post first dose of DT (N = 91) and CT (N = 93). Based on 310 (DT) and 315 (CT) lumefantrine plasma concentrations, collected at 6 different times across patients, a full pharmacokinetic profile was reconstituted. Comparisons between DT and CT showed no difference in artemether and DHA C_{max} across body weight groups, with an overall mean of 175 ± 168 and 211 ± 262 ng/mL for artemether, respectively; for DHA these values were 68.0 ± 64.4 and 63.7 ± 64.9 ng/mL. For lumefantrine, the mean population C_{max} amounted to 6.3 ± 4.6 (DT) and 7.7 ± 5.9 µg/mL (CT), whereas $AUC_{0\text{-last}}$ was 574 and 636 µg•h/mL. For both formulations, descriptive quintile analyses showed no association between artemether/DHA C_{max} and parasite clearance time, or between lumefantrine exposure and parasitological cure. Moreover, no association was found between lumefantrine C_{max} and the occurrence of adverse events or QTc changes. Based on this substudy, which provided pharmacokinetic data on A-L that far exceeded any to date from a single trial, it can be concluded that the systemic exposure to artemether, DHA and lumefantrine is similar after identical doses of DT and CT in African children with falciparum malaria. The new formulation may facilitate administration to infants and children and therefore helps maintaining the effectiveness of A-L in malaria endemic countries.

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SAFETY PROFILE OF ARTEMETHER-LUMEFANTRINE (AL; COARTEM®) COMPARED WITH SULFADOXINE-PYRIMETHAMINE (SP) IN PREGNANT WOMEN WITH SYMPTOMATIC MALARIA: PRELIMINARY RESULTS OF AN OBSERVATIONAL STUDY

Christine Manyando[1], Rhoda Mkandawire[2], Lwipa Puma[3], Moses Sinkala[4], Eric Njunju[1], Melba Gomes[5], Kim Andriano[6], Raymond Schlienger[7], Mailis Virtanen[7]

[1]Tropical Diseases Research Centre, Ndola, Zambia, [2]District Health Office, Choma, Zambia, [3]District Health Office, Ndola, Zambia, [4]District Health Office, Lusaka, Zambia, [5]World Health Organisation, Geneva, Switzerland, [6]Novartis Pharmaceuticals Corporation, East Hanover, NJ, United States, [7]Novartis Pharma AG, Basel, Switzerland

Safety data on artemisinins in pregnancy are limited. A prospective observational study was conducted in Zambia to compare the safety of AL and SP in pregnant women who received AL and SP to treat symptomatic falciparum malaria. The primary objective was to evaluate the perinatal mortality rate (i.e. stillbirth or neonatal death </=7 days after birth). Secondary objectives included birth weight adjusted for gestational age at delivery. Exploratory objectives included the assessment of spontaneous abortions (</=28 weeks gestation); preterm deliveries (<37 completed weeks); neonatal mortality (within 28 days after birth); maternal mortality; birth defects. Mothers and live newborns were followed up to 6 weeks post-delivery. Data from 1001 pregnant women (AL n=495; SP n=506) and fetuses/newborns (AL n=470; SP n=477) were analyzed. There were no clinically relevant differences in rates of perinatal mortality (AL 4.2%; SP 5.0%), neonatal mortality (both groups 3.0%), stillbirths (AL 1.8%; SP 2.5%), preterm deliveries (AL 14.1%; SP 17.4%) or gestational age-adjusted low birth weight (AL 9.0%; SP 7.7%). There were 7 spontaneous abortions in the AL group and 5 in the SP group. Birth defect rates were 4.9% for AL and 2.6% for SP, mainly umbilical hernia (3.7% and 1.5%, respectively). No major malformations were reported, except for 2 chromosomic aberrations. Of 6 maternal deaths (AL 1; SP 5), 3 were due to comorbid infections (pneumonia, viral encephalitis, sepsis; all SP). The most common adverse events were premature delivery (AL 13.7%; SP 17.2%), stillbirth (AL 1.8%; SP 2.6%), abortion (AL 1.2%; SP 1.0%) and infections (malaria [AL 3.4%; SP 6.7%], syphilis [AL 4.8%; SP 4.0%], respiratory tract infection [AL 1.8%; SP 1.0%]). This study indicates that the incidence of perinatal death, spontaneous abortion, neonatal mortality, premature delivery, stillbirth and low birth weight is similar after pregnancy exposure to AL compared to SP. The absence of major birth defects should be interpreted with caution as it differs from published data and as the study sample size was small.

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EFFICACY AND SAFETY OF ARTEMETHER-LUMEFANTRINE DISPERSIBLE TABLET ACCORDING TO BODY WEIGHT IN AFRICAN INFANTS AND CHILDREN WITH UNCOMPLICATED MALARIA

Lucas Otieno[1], John Lyimo[2], Hamma Maiga[3], Mahfudh Bashraheil[4], Alain Nahum[5], Sonia Machevo[6], Peter Ouma[7], Hamad Makame[8], Nathan Mulure[9], Obiyo Nwaiwu[10]

[1]Walter Reed Project - KEMRI, Kisumu, Kenya, [2]Ifakara Health Research and Development Centre, Dar es Salaam, United Republic of Tanzania, [3]Malaria Research and Training Center, University of Bamako, Bamako, Mali, [4]Kenya Medical Research Institute, Kilifi, Kenya, [5]Centre de Recherche Entomologique de Cotonou, Cotonou, Benin, [6]Manhiça Health Research Centre (CISM), Manhiça, Mozambique, [7]Kenya Medical Research Institute/Center for Disease Control, Kisumu, Kenya, [8]Zanzibar Malaria Research Unit of the Karolinska Institute, Zanzibar, United Republic of Tanzania, [9]Novartis Pharma AG, Nairobi, Kenya, [10]Novartis Pharma AG, Lagos, Nigeria

Young children in subSaharan Africa are particularly vulnerable to *Plasmodium falciparum* malaria. The current standard of care for children is a 6-dose regimen of crushed tablets (CT) of artemether-lumefantrine (AL), but the bitter taste means this may be spat out, risking under-dosing. A sweetened and flavored dispersible tablet (DT) formulation suitable for all ages, including infants, has been developed. A randomized, multicenter, investigator-blinded study was conducted in 5 African countries to confirm non-inferiority of DT vs CT. Children ≤12 years (body weight [BW] ≥5g and <35kg) with acute uncomplicated *P. falciparum* malaria were allocated to one of 3 body weight groups (BW1, 5 to <15kg; BW2, 15 to <25kg; or BW3, 25 to <35kg) and randomized to DT or CT within each group. Patients received 6 doses of AL over 3 days, dosed according to body weight: BW1, 1 tablet/dose; BW2, 2 tablets/dose; BW3, 3 tablets/dose. Primary endpoint was PCR-corrected cure rate at day 28. 899 children were randomized: 547 were in the BW1 group, 289 in the BW2 group and 63 in the BW3 group. Cure rates were similar between formulations across the 3 BW groups: DT 97.5%, 98.6% and 96.4%, respectively; CT 99.2%, 97.1% and 100.0%. In the BW3 group, a higher proportion of DT patients (93.1%) achieved parasite clearance within 48 hours compared to CT patients (85.3%). Median time to fever clearance

was comparable between BW groups within and between treatment groups, as indicated by overlapping 95% CI values. Other than vomiting, which was more frequent with CT in the BW2 and BW3 groups, the overall safety profile of the two formulations was similar between and within body weight groups. Incidence of pyrexia tended to decrease with increasing BW. There were 3 deaths, all in the lower BW group and none suspected to be related to study drug. In conclusion, dispersible AL was similar to crushed tablet for day 28 PCR-corrected cure rate regardless of body weight. No differences were observed between body weight groups in response to treatment or in safety profile.

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INTERVENTIONS TO IMPROVE PROMPT AND EFFECTIVE TREATMENT OF MALARIA: DO WE KNOW WHAT WORKS?

Lucy A. Smith, Jayne Webster, Caroline Jones

London School of Hygiene and Tropical Medicine, London, United Kingdom

Effective case management of uncomplicated malaria is a cornerstone of successful malaria control and global targets propose at least 60% of those suffering from malaria should access and use correct, affordable and appropriate treatment within 24 hours of onset of symptoms. With current calls for the global elimination of malaria, all strategies to control malaria, including prompt and effective treatment, need to reach the highest achievable level of effective implementation. A systematic review of published and grey literature was carried out of all interventions to improve provider- and/or user-side behaviour in the prompt and appropriate treatment of uncomplicated malaria. The original search terms found 769 published articles and ten studies from the grey literature. Papers and reports were included if they involved interventions designed to improve prompt and effective treatment of uncomplicated malaria or febrile illness through changes in provider and/or user behaviour. Appropriate evaluation design was used as a further inclusion criterion: randomised controlled trials, pre-post design with or without a control group, time series measurement, and post only with control. Only studies reporting outcomes in the Roll Back Malaria indicator format were included. In total 23 studies remained for review. The results show that the evidence base is severely limited, making any conclusive comments about effective interventions extremely difficult. Only sixteen studies involved interventions targeted at providers, nine in the public sector and seven in private sector. Once this is further broken down into formal and informal providers within each category, the number of studies providing evidence on possible interventions becomes very small. Just two interventions were conducted at national scale. These data suggest that very little is known about what interventions work in improving prompt and effective treatment for malaria. In the context of scaling up effective malaria control and malaria elimination plans and in increasing access to Artemisinin Combination Therapies (ACTs), increased research to establish an evidence base from which to build effective interventions that successfully improve prompt and effective treatment is crucial.

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MALARIA TREATMENT IN THE PRIVATE SECTOR IN TANZANIA

Angel Dillip[1], Sandra Alba[1], Manuel Hetzel[2], Brigit Obrist[2], Flora Kessy[1], Christian Lengeler[2], Iddy Mayumana[1], Alexander Schulze[3], Christopher Mshana[1], Hassan Mshinda[1], Ahmed Makemba[1]

[1]Ifakara Health Research and Development Centre, Morogoro, United Republic of Tanzania, [2]Swiss Tropical Institute, Basel, Switzerland, [3]Novartis Foundation for Sustainable Development, Basel, Switzerland

Throughout Africa, the private sector is an important source of antimalarial treatment, complementing formal health services. Accredited Drug Dispensing Outlets (ADDOs) were introduced in the Kilombero and Ulanga district in 2006 to improve the performance of drug selling shops. In August 2007 ADDOs started selling highly subsidized artemether-lumefantrine (ALu). The present study, conducted in the frame of the ACCESS Programme, aimed to assess the performance of drug selling shops before and after implementation of ADDOs. We conducted yearly cross-sectional shop surveys (2004-2007) to record drug stocks, complemented by mystery shopper studies (2004-2007) with simulated clients in retail outlets for assessing quality of advice and treatment. A drug quality testing (standard analytical techniques according to US Pharmacopeia) of SP, amodiaquine, and quinine was also conducted in 2005. These studies were carried out in a Demographic Surveillance Study area in southern Tanzania. From 2004 to 2007 the number of villages without drug shops decreased from 14 to 6. From 2004 to 2005 the number of shops increased by 64% (from 0.57 to 0.94 per 1000 people, $p<0.001$) but then stayed stable from 2005 to 2007 after the introduction of ADDOs (from 0.94 to 0.84, $p=0.504$). Overall, less antimalarials were dispensed after the introduction of ADDOs. Before ADDOs, amodiaquine and quinine were dispensed as often as sulphadoxine-pyrimethamine (SP) while after the introduction of ADDOs, SP was most sold at a time when ALu was not yet available through that channel. The percentage of customers who got correct advice and treatment increased after the introduction of ADDOs. Overall, 25% of SP, Quinine and Amodiaquine tablets were found to be sub-standard. The quality of malaria case management in the retail sector has improved since the introduction of ADDOs in the study districts. However, the overall availability of antimalarials did not increase after the introduction of ADDOs and there are still villages without commercial access to drugs.

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EFFECTS OF AMODIAQUINE, ARTESUNATE, AND ARTESUNATE-AMODIAQUINE ON *PLASMODIUM FALCIPARUM* MALARIA-ASSOCIATED ANAEMIA IN CHILDREN

Akintunde Sowunmi, Sulayman T. Balogun, Grace O. Gbotosho, Christian T. Happi

University of Ibadan, Ibadan, Nigeria, Ibadan, Nigeria

The effects of amodiaquine, artesunate and artesunate-amodiaquine on *Plasmodium falciparum* malaria-associated anaemia (PfMAA) and the recovery from PfMAA were evaluated in 328 children with uncomplicated malaria randomized to the standard dose regimens of the three drugs/combination. Overall, malaria-attributable fall in haematocrit (MAFH) before treatment was $4.8 \pm 2.8\%$, 95% confidence interval (CI) 4.4-5.2%, and was not significantly different between the treatment groups ($P = 0.31$). An age < 5 years and a history of illness > 3 d were independent predictors of MAFH before treatment > 4%. Following treatment, drug-attributable fall in haematocrit (DAFH) was significantly higher in amodiaquine-treated children (4.6 ± 2.9, 2.8 ± 1.8, $3.0 + 1.8\%$ for amodiaquine, artesunate, artesunate-amodiaquine, respectively, $P < 0.0001$). The rate of DAFH was significantly lower in artesunate-treated children (1.4 ± 0.9, 0.7 ± 0.6, $1.0 + 0.6$ % per day for amodiaquine, artesunate and artesunate-amodiaquine, respectively, $P < 0.0001$). The rate of rise in haematocrit from the nadir on day 3 to day 7 was significantly higher in amodiaquine treated children ($P = 0.045$). In anaemic children ($n = 68$), the time elapsing from treatment to the attainment of a haematocrit ≥ 30%, the anaemia resolution time, and the proportion of anaemic children with complete resolution on day 14 were similar in all treatment groups ($P = 0.17$ and 0.65, respectively). Artemisinin drugs may reduce the extent and rate of fall in PfMAA during treatment and may attenuate malaria-associated anaemia in children.

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DRUG-DRUG INTERACTIONS BETWEEN ARTEMETHER/LUMEFANTRINE AND LOPINAVIR/RITONAVIR IN HIV NEGATIVE HEALTHY VOLUNTEERS

Polina German[1], **Sunil Parikh**[1], Jody Lawrence[1], Diane Havlir[1], Philip J. Rosenthal[1], Grant Dorsey[1], Niklas Lindegardh[2], Francesca Aweeka[1]

[1]University of California-San Francisco, San Francisco, CA, United States, [2]Mahidol University, Bangkok, Thailand

Treatment of patients co-infected with malaria and HIV is complicated in that clinically important pharmacokinetic (PK) drug interactions may occur between antiretroviral (ARV) and antimalarial drugs compromising the treatment of either infection. Artemether (AR) is rapidly demethylated into its active metabolite dihydroartemisinin (DHA) by CYP3A4/A5. AR may also induce CYP3A4, and lumefantrine (LR) is desbutylated by CYP3A4 isozymes. Ritonavir is also a potent inhibitor of CYP3A enzymes. PK data evaluating interactions between ARVs and artemether/lumefantrine (AL) are scarce, and the concomitant administration of AL with lopinavir/ritonavir (LPV/r) has been avoided. Healthy HIV- negative volunteers received 6-doses of co-formulated AL 80/480mg twice daily (BID). This regimen was followed by a 26-day course of LPV/r 400/100mg BID alone followed by a combination course with AL. Intensive steady state serial PK sampling for AR/DHA and LR was conducted. Non- compartmental analysis was performed via the linear up-log down trapezoidal rule in conjunction with oral input model using WinNonlin 5.0.1. Ten subjects (6 males and 4 females) completed the study. The mean age was 31 years (21-45 years). All subjects tolerated study medications well. Slight decreases in AR Cmax, AUClast and AUC0-inf were noted in the setting of LPV/r relative to AL administration alone. Cmax decreased by 22%, whereas AUClast and AUC0-inf decreased by 39% and 34%, respectively. Co-administration of AL with LPV/r was associated with decreases in DHA Cmax (36%), AUClast(45%) and AUC0-inf (45%). Coadministration of LPV/r with AL led to 2.3-fold increases in both the LR AUC0-264 and AUC0-inf compared with the same subjects who received AL alone. The elimination half-life of LR in the setting of LPV/r was not altered. LPV/r pharmacokinetics was not altered in the presence of AL. These findings suggest coadministration of AL and LPV/r is acceptable. Although both antimalarial regimes were well tolerated by the study participants, further studies evaluating safety of combination are warranted.

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PHARMACOVIGILANCE AND ANTIMALARIAL TREATMENT IN UGANDA: A PILOT SYSTEM OF ENHANCED PASSIVE SURVEILLANCE

Hasifa Bukirwa[1], Norah Mwebaza[2], Susan Nayiga[1], Heidi Hopkins[3], Umberto D'Alessandro[4], Ambrose O. Talisuna[5], Sarah G. Staedke[6]

[1]Uganda Malaria Surveillance Project, Kampala, Uganda, [2]Makerere University, Kampala, Uganda, [3]University of California, San Francisco, CA, United States, [4]Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium, [5]Ministry of Health, Kampala, Uganda, [6]London School of Hygiene and Tropical Medicine, London, United Kingdom

Widespread deployment of new artemisinin-based combination therapies (ACTs) in Africa offers an important opportunity to assess for drug safety. However, pharmacovigilance is challenging, particularly in developing countries. To inform strategies for sustainable pharmacovigilance of antimalarial treatment in Uganda, we began a pilot study of enhanced passive surveillance in September 2007. The system was developed based on results of a qualitative study with input from stakeholders, and is designed to collect data from the community and from health facilities in the public and private sector. Health workers and key community members were trained on the importance of pharmacovigilance, recognition of adverse events, and methods of reporting. Of the expected target group (n=70), 87% were trained, including 36 health workers and 25 community members. Most health workers (72%) stationed at the participating facilities were nursing aides/assistants. Adverse event reports are collected monthly, and continuing education is provided. As of March 2008, 581 events had been reported; 324 (56%) were considered by our physician reviewers to be suspected adverse drug reactions (ADRs). Overall, the total number of events reported has decreased over time; however, the number of non-adverse events reported has decreased, and the proportion of adverse events suspected to be ADRs has increased, suggesting an improvement in the quality of reporting. Most events have been reported from either the community (62%) or the private sector (36%); only 6 (1%) events have been reported from public health facilities. Approximately 60% of event reports were associated with antimalarial treatment, with 31 cases associated with artemether-lumefantrine, including reports of weakness (10 cases), vomiting (9), pruritus (5), anorexia (4), skin rash (3), dizziness (2), and abdominal pain (1). Our results suggest that passive surveillance for adverse events may need to be extended beyond formal health centers, to lower-level health workers in the community and private sector. Motivation of health workers and under-reporting of events are challenges to successful passive reporting. Active population-based surveillance with prospective follow-up of patients after treatment may be necessary to estimate the full safety risk of ACTs.

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MASSIVE REDUCTION OF ANTIMALARIAL PRESCRIPTIONS AFTER RAPID DIAGNOSTIC TESTS IMPLEMENTATION IN DAR ES SALAAM, TANZANIA

Valérie D'Acremont[1], Judith Kahama-Maro[1], Deo Mtasiwa[2], Blaise Genton[3], Christian Lengeler[4]

[1]City Medical Office of Health, Dar es Salaam City Council, United Republic of Tanzania, [2]Ministry of Health and Social Welfare, Dar es Salaam, United Republic of Tanzania, [3]Ifakara Health Research and Development Center, Dar es Salaam, United Republic of Tanzania, [4]Swiss Tropical Institute, Basel, Switzerland

Presumptive treatment of all febrile children with antimalarials leads to an over-treatment and huge wastage of drugs, especially in areas with moderate and low endemicity. Laboratory-confirmed diagnosis should drastically reduce antimalarial consumption, provided the test result is taken into account by the clinicians. The objective of this study was to assess the effect of implementing malaria Rapid Diagnostic Tests (RDT) as first line diagnostic tool in the routine management of febrile patients living in a moderately endemic area on prescription of antimalarials [standard treatment of artemether/lumefantrine (ALu)]. After training of all health workers of 3 district hospitals, 3 health centers and 3 dispensaries, RDT were introduced as first line diagnostic method for malaria. Supervision, problem-solving and quality control of RDT performance took place every 3 months. One similar health center and one dispensary without RDT implementation were used as controls. Data on antimalarial use were extracted from ledger books of the main storage place in each health facility. When comparing the consumption of ALu during the three months prior to RDT implementation with that of the seven months post initiation, there was a mean of 8-fold (range: 1 to 26) decrease for the small children blisters (6 tabs) and 3-fold (range: 1 to 7) decrease for the adult blisters (24 tabs). The other 2 types of blisters (12 and 18 tabs) were almost not used anymore after RDT implementation. During the same period, a mean of 1.5-fold reduction for the blisters of 6 tabs and no reduction for blisters of 24 tabs were observed in the 2 control health facilities. There was no stock out of ALu during the whole study period. In conclusion, in the conditions of programmatic implementation of RDT in a moderately endemic area where microscopy is available, overtreatment of febrile episodes with antimalarials was drastically reduced. Clearly, properly trained clinicians with minimal support comply (much better in some health facilities than in others) to the recommendation of not treating patients with a negative result. RDTs used as first line diagnostic tool instead of microscopy have a huge potential for reducing inappropriate prescriptions of antimalarials and improve the management of patients.

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POTENT AND SELECTIVE INHIBITORS OF HISTONE DEACETYLASE IN *PLASMODIUM FALCIPARUM* AND *P. BERGHEI*

Ralph Mazitschek[1], Vishal Patel[2], Joseph Cortese[1], Liu Hanlan[3], Mandy Cromwell[3], Adelfa Serrano[4], Cokey Nguyen[3], Dyann Wirth[5], Roger Wiegand[1], **Robert H. Barker**[3]

[1]Broad Institute of Harvard and MIT, Cambridge, MA, United States, [2]Harvard Medical School, Boston, MA, United States, [3]Genzyme Corporation, Waltham, MA, United States, [4]University of Puerto Rico-School of Medicine, San Juan, PR, United States, [5]Harvard School of Public Health, Boston, MA, United States

A number of different studies have shown that inhibitors of histone deacetylase (HDAC) can prevent proliferation of *Plasmodium falciparum in vitro* and *P. berghei in vivo*. However, the vast majority of HDAC inhibitors that have been tested against *Plasmodium* were originally developed as therapeutics for oncology indications, and so are not selective for the parasite enzyme. Further, the hydroxamic acid scaffold which has received the greatest attention has a high rate of biliary clearance, making the plasma half-life very short (< 30 min). We therefore sought to develop a high throughput assay that could potentially identify alternative chemotypes through small molecule screening (HTS). A competitive binding assay was developed following synthesis of a reagent consisting of suberoylanilide hydroxamic acid (SAHA - a known inhibitor of Class 1 and Class 3 HDACs) linked to fluorescein isothiocyanate. This assay was used to screen a HDAC-biased compound library. That same library was also tested for inhibition of *P. falciparum* growth *in vitro*. "Hits" were then tested in a histone acetylation assay to identify compounds that potently and selectively inhibited *P. falciparum*, not human HDAC. Both selective and non-plasmodium-selective inhibitors such as LBH 589 were tested both *in vitro* and *in vivo* to confirm validation of HDAC as a target. In preparation for screening larger compound libraries, the pfHDAC 1 gene sequence was codon-optimized and expressed in *Drosophila melanogaster* S2 cells. The plasmodial enzyme had a kcat of 0.006. It is presently being over-expressed for running a HTS on Genzyme small molecule libraries.

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HIGH THROUGHPUT SCREENING TO IDENTIFY CHEMOTYPES AS POSSIBLE ANTIMALARIALS

Joseph Cortese[1], Cassandra Celatka[2], Baniecki Mary Lynn[1], Liu Hanlan[2], **Robert H. Barker**[2], Dyann F. Wirth[3], Roger Wiegand[1]

[1]Broad Institute of Harvard and MIT, Cambridge, MA, United States, [2]Genzyme Corporation, Waltham, MA, United States, [3]Harvard School of Public Health, Boston, MA, United States

The rapid rate of drug resistance evolution makes it extremely important to identify new chemotypes that can form the chemical basis for future antimalarial therapy. The Broad Institute of Harvard and MIT, Genzyme Corporation and Malaria Medicines Ventures have formed a broad partnership to discover and develop new drugs. As part of this effort, a screening cascade has been developed to facilitate selection of compounds for further analysis. A non-isotopic high throughput assay has been developed with high reproducibility (Z' = 0.80) and was used to screen more than 120,000 compounds against *Plasmodium falciparum* strains Dd2, HB3 and 3D7. The IC_{50} values were determined against the 3 strains for approximately 1,200 compounds that inhibited growth by ≥ 90% in the initial 6 μM screen. Of these, ~134 had IC_{50}'s of ≤ 1 μM. Compounds were then tested for cytotoxicity against mammalian cells, for solubility, permeability and *in vitro* metabolic stability. Selection criteria from these results have reduced the current number of compounds to 30. Several chemotypes have been selected based upon chemical tractability for further proof of concept testing against *P. berghei in vivo.*

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QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS (QSARS) FOR CANDIDATE ANTIMALARIALS AGAINST CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM*

Simon J. Hocart, Huayin Liu, Haiyan Deng, Frances M. Krogstad, Dibyendu De, Donald J. Krogstad

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

The single most important factor in the current global resurgence of malaria is the increasing prevalence of resistance to chloroquine (CQ) and other aminoquinoline (AQ) antimalarials. Because there is an urgent need for antimalarials which are effective against CQ-resistant *P. falciparum* and are also affordable, we have synthesized a series of AQs and examined quantitative structure-activity relationships (QSARs) for 108 AQ analogues. Compounds were examined using three-dimensional, comparative molecular similarity indices (CoMSIA) which probed their structures in three dimensions to define steric bulk, electrostatic charge, hydrophobicity, and H-bond acceptors and donors. Eleven compounds were randomly assigned to a test set and the remaining 97 were used to generate a CoMSIA model for activity against CQ-resistant *P. falciparum.* The CoMSIA model yielded r^2=0.78, SE=0.29 and r^2 prediction=0.57. Contours identified denote favored (S+) and disfavored (S-) steric interactions, favored (H+) and disfavored (H-) hydrophobic interactions and favored (P+) positive charges associated with activity against CQ-resistant parasites. The CQ side chain coincides with the disfavored steric region (S-), whereas the side chains of active long chain AQ analogues coincide with favored (S+) steric regions. The chlorine atom at position 7 corresponds with favored hydrophobic (H+) and with positive charge (P+) interactions.

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IDENTIFICATION OF MODELS TO PREDICT A NON-HEMOLYTIC 8-AMINOQUINOLINE

Aruna Sampath[1], Babu Tekwani[1], Rosemary Rochford[2], Prabhati Ray[3], Jeffery Friedman[4], David McMillan[5], Jayendra Bhonsle[3], Yarrow Rothstein[3], Alan Magill[3], Larry Walker[1], Colin Ohrt[3]

[1]University of Mississippi, University, MS, United States, [2]SUNY Upstate Medical University, Syracuse, NY, United States, [3]Walter Reed Army Institute of Research, Silverspring, MD, United States, [4]The Scripps Research Institute, La Jolla, CA, United States, [5]University of Nebraska Medical Center, Omaha, NE, United States

The 8-aminoquinoline antimalarials such as primaquine, pamaquine and tafenoquine are active against *Plasmodium vivax* hypnozoites and stage 5 *P. falciparum* gametocytes. The drugs are highly desirable for malaria prevention, control and elimination. However, it has long been known that the patients with glucose 6-phosphate dehydrogenase (G6PD) deficiency develop hemolytic anemia after the administration of 8-aminoquinolines. Until today, it has been difficult to select a member of this class that does not cause hemolysis, primarily because we are lacking predictive preclinical models. An advisory meeting with 61 individuals from 13 institutions was held in Bethesda, Maryland on January 15-16, 2008. A series of 17 presentations by experts in the field covered the following topic areas: chemistry of 8-aminoquinolines, metabolism of 8-aminoquinolines and predictive models (*in vitro* and *in vivo*) for hemolytic toxicity that are currently available. Based on these presentations and the discussions that followed, 15 potential models (*in vitro* and *in vivo*) were identified and were ranked by a steering committee. Based on priority and available funding, five were selected for funding. One *in silico*, two *in vitro,* and two mouse models were selected for detailed studies. Three additional models were of high priority, but funding was not sufficient. Proof of concept will be assessed with 3-5 known hemolytic drugs and one non-hemolytic drug (chloroquine) in each model. Models that appear promising will have at least 10 hemolytic and 10 non-hemolytic drugs evaluated, as well as a full dose-response determined for the hemolytic drugs. The details of these models and progress toward proof of concept will be presented. In conclusion, if predictive models can be identified and validated, the first

next step will be to determine if efficacy can be separated from G6PD-toxicity. If so, the 8-aminoquinoline class will be opened up for new safer analogs from this essential class of drugs for malaria elimination.

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A MEDICINAL CHEMISTRY PROGRAM FOR THE DISCOVERY OF *PLASMODIUM FALCIPARUM* DIHYDROOROTATE DEHYDROGENASE INHIBITORS WITH ANTIMALARIAL ACTIVITY

Vishal Patel[1], **Michael L. Booker**[2], Cassandra Celatka[2], Martin L. Kramer[2], Hanlan Liu[2], Joseph Cortese[3], James Lillie[2], Roger Wiegand[3], Jon Clardy[1], Dyann F. Wirth[4]

[1]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, United States, [2]Genzyme Corporation, Waltham, MA, United States, [3]Infectious Diseases Initiative, Broad Institute of Harvard and the Massachusetts Institute of Technology, Cambridge, MA, United States, [4]Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA, United States

Dihydroorotate dehydrogenase (DHODH), an enzyme of the de novo pyrimidine biosynthetic pathway, catalyzes the formation of dihydroorotate through a coupled redox reaction utilizing a mitochondrial respiratory chain ubiquinone. *Plasmodium falciparum* is unable to salvage pyrimidines and must rely on *de novo* biosynthesis for survival. The anti-malarial agent atovaquone indirectly inhibits DHODH activity by disrupting the electron transport chain. A chemical library of approximately 208,000 compounds was screened for inhibitors of *P. falciparum* DHODH (pfDHODH) activity. Fifty potent, species-selective inhibitors of pfDHODH were identified. These compounds represented a variety of chemical classes from which six displayed submicromolar *in vitro* efficacy against *P. falciparum* 3D7, Dd2, and HB3. The mechanism of action of these compounds was confirmed using a transgenic *P. falciparum* strain expressing a divergent homolog of DHODH, this parasite strain being resistant to four of the six compounds afforded from the high-throughput screen. These compounds were further examined for physical properties, cytotoxicity to human cells, and metabolic stability. On the basis of these results a single chemical class was identified around which analogue synthesis is actively being pursued. Fifteen analogues have been synthesized to date, with several demonstrating activity equivalent to the parent compound and improved secondary characteristics. Scale-up of relevant compounds for *in vivo* tolerability studies has been initiated and an iterative optimization process is underway to identify a lead compound.

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ANTIMALARIAL ACTIVITY OF ARYL-SUBSTITUTED 2-ETHOXYACETAMIDES

Clare E. Gutteridge[1], Joshua W. Major[1], Daniel A. Nin[1], Apurba K. Bhattacharjee[2], Lucia Gerena[2]

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

The chalcone class of compounds appears to possess antimalarial activity by a mechanism distinct from those employed by other antimalarial agents. We have developed a pharmacophore from the structure-activity relations of the chalcones, and with it determined that 2-ethoxy-2,*N*-diphenylacetamides should also mediate antimalarial effects by this novel mechanism. In contrast to the chalcones, which we have shown to be susceptible to degradation by human liver microsome preparations, such acetamides appear to be stable. We will describe the synthesis of a series of bisarylated 2-ethoxyacetamides, their *in vitro* efficacy against *Plasmodium falciparum*, the emerging *in vitro* structure-activity relations of these compounds and results from *in vivo* efficacy testing of select compound(s).

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CHLORPROGUANIL-DAPSONE-ARTESUNATE VS. CHLORPROGUANIL-DAPSONE: A RANDOMISED, DOUBLE-BLIND PHASE III TRIAL FOR THE TREATMENT OF ACUTE UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA

Alfred Tiono[1], Alassane Dicko[2], Dennis A. Ndububa[3], Tsiri Abenyega[4], Simon Pitmang[5], Jacob Awobusuyi[6], Allan Pamba[7], Stephan Duparc[8], Li-Ean Goh[7], Emma Harrell[7], Nick Carter[7], Stephen A. Ward[9], Brian Greenwood[10], Peter A. Winstanley[11]

[1]Centre National de Recherche et de Formation sur le Paludisme, Ouagadougou, Burkina Faso, [2]Malaria Research and Training Centre, Faculty of Medicine, Pharmacy and Dentistry, University of Bamako, Bamako, Mali, [3]Obafemi Awolowo University Teaching Hospital, Department of Medicine, Ile Ife, Nigeria, [4]Komfo Anokye Teaching Hospital, Kumasi, Ghana, [5]Department of Medicine, Plateau State Specialist Hospital, Jos, Plateau State, Nigeria, [6]Lagos State University Teaching Hospital, Department of Medicine, Ikeja, Lagos, Nigeria, [7]GlaxoSmithKline, Greenford, Middlesex, United Kingdom, [8]Formerly at GlaxoSmithKline, Greenford, United Kingdom, now at Medicines for Malaria Venture, Geneva, Switzerland, [9]Liverpool School of Tropical Medicine, Liverpool, United Kingdom, [10]Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom, [11]School of Clinical Sciences, University of Liverpool, Liverpool, United Kingdom

This study was undertaken to compare chlorproguanil-dapsone-artesunate (CDA) and chlorproguanil-dapsone (CD) efficacy and safety in acute uncomplicated *Plasmodium falciparum* malaria. Haematological safety in glucose-6-phosphate dehydrogenase (G6PD)-deficient patients was studied carefully. Non-inferiority of CDA to CD for efficacy was tested in a randomised parallel-group, double-blind, double-dummy, study conducted at 7 sites in 4 African countries (Burkina Faso, Ghana, Mali, Nigeria). Patients (>=1 year) received CDA 2.0/2.5/4.0 mg/kg or CD 2.0/2.5 mg/kg od x 3 days. G6PD genotype and phenotype were determined. A haematological safety composite endpoint was defined as haemoglobin (Hb) drop of >=40 g/L or >=40% vs. baseline or Hb <50 g/L or blood transfusion. 892 subjects were randomised; 600 to CDA, 292 to CD. Baseline demographic/clinical characteristics were similar between treatment groups: mean age 7.3 (1-72) years, weight 20.8 (8-112) kg, 49% male. Parasitological cure rate (polymerase chain reaction-corrected) at Day 28 for the per-protocol population (primary efficacy comparison) for CDA was 89.1% (416/467), statistically superior to CD, 83.0% (176/212) (treatment difference 6.1% 95%CI 0.3, 11.9%). Significantly fewer patients had parasites at 24-h after treatment start with CDA (48.0%) vs. CD (88.7%, $p<0.001$). The adjusted mean decrease in Hb at Day 7 vs. baseline was significantly greater with CDA (14.1 g/L) vs. CD (12.0 g/L 95%CI 0.4, 3.9 $p=0.016$). Mean Hb reached its minimum value at Day 3 for CD (91.89 g/L) and Day 7 for CDA (93.21 g/L). 175/844 (21%) patients were G6PD A-: 68 males/homozygous females, 107 heterozygous females. For CDA and CD, occurrences of the composite haematological safety endpoint were more frequent in G6PD-deficient vs. normal patients. No deaths were reported. There were no other remarkable safety observations. In conclusion, CDA was more efficacious than CD; though cure rates were lower than expected for both agents. The haematological safety risk in G6PD-deficient patients does not support further CDA development or CD use in Africa.

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TRAINING ON PHARMACOVIGILANCE IN AFRICAN RURAL AREAS: THE EXPERIENCE OF ALIVE

Abdunoor M. Kabanywanyi[1], Nathan Mulure[2], Aggrey Malila[3], Christian Lengeler[4], Blaise Genton[5]

[1]Ifakara Health Research and Development Centre, Dar es Salaam, United Republic of Tanzania, [2]Novartis Pharma (EACA), Nairobi, Kenya, [3]Ifakara Health Research and Development Centre, Ifakara, United Republic of Tanzania, [4]Swiss Tropical Institute, Basel, Switzerland, [5]Ifakara Health Research and Development Centre and Swiss Tropical Institute, Basel, Switzerland

The ALIVE (Artemether/Lumefantrine (ALu) In Vulnerable patients: Exploring health impact) project is a community-based observational study to assess the impact of ALu on mortality and morbidity when used as a national policy for treatment of uncomplicated malaria. To ensure safety reporting compliance with the Tanzanian Food and Drug Administration (TFDA) and Novartis, a capacity building workshop was performed at the start of the study. About 40 health workers were offered a 1-day training conducted jointly by the Ifakara Health Research and Development Center and the Swiss Tropical Institute investigators, Novartis (sponsor) and TFDA, on reporting and recording of serious adverse events (SAEs) and adverse drug reactions (ADRs). SAEs, ADRs as well as pregnancy forms from TFDA and Novartis were provided and participants taken through each item step by step. Breakout sessions in small groups with case studies on real life situations gave participants an experience of how to handle SAEs and ADRs. Participants got training materials, handouts, reporting forms for further reference. Study coordinator was tasked to ensure timely reporting. Reporting was through fax, email and reminders were done through SMS alerts. Surveillance has been ongoing for the last 6 months. So far, 17 adverse events (AEs) have been reported (14 after ALu) of which 7 were SAEs (3 after ALu). Two deaths occurred due to progression to severe malaria (reported within 24 hrs) and 1 death resulting from pneumonia complicating malaria (report delayed by 1 week). The most frequent AEs after ALu intake were vomiting (5 cases) and itching or rash (4 cases). Causality assessment was a challenge, since it is subjective and depends on level of knowledge. Such dedicated trainings, set-up in collaboration between research institutions, health authorities, industry and health care workers in the context of observational studies could be valuable to help in emphasizing the need and the importance of ADR/SAE reporting in Africa.

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THERAPEUTIC EFFICACY OF GSK932121, A 4(1H)-PYRIDONE CANDIDATE FOR CLINICAL DEVELOPMENT AGAINST *PLASMODIUM YOELII* AND *P. FALCIPARUM*

Belén Jiménez-Díaz, Teresa Mulet, Sara Viera, Vanesa Gómez, Helen Garuti, Angela Alvarez, Iñigo Angulo-Barturen, Javier Ibáñez, Elena Jiménez, Pablo Castañeda, Isabel Camino, Magdalena Jiménez, Domingo Gargallo-Viola

GlaxoSmithKline, Tres Cantos, Spain

(1H)-pyridones are selective inhibitors of the bc1 complex of the mitochondrial respiratory chain of *Plasmodium spp*. The compound GSK932121 was selected for further progression. The therapeutic efficacy of GSK932121 against *P. yoelii* 17XNL or the strain *P. falciparum Pf*3D7$^{0087/N9}$ was evaluated as the effective doses that reduced parasitemia by 50 (ED_{50}) or 90 % (ED_{90}) 24 h after the last dose and the minimum dose for cure (non-recrudescence dose, NRD). In preliminary experiments to assess the safety of GSK932121, no significant adverse effects were observed in mice after oral treatment at 500 mg/Kg once a day for four days. In a standard *yoelii* model, groups of n = 5 mice were inoculated intravenously with 6.4×10^6 infected erythrocytes. One hour after infection the mice were treated p.o. once a day for 1 day (1-day test) or 4 days (4-day test). In these assays GSK932121 showed potent antimalarial efficacy with $ED_{50\,=}$ 0.58, ED_{90} = 1.26 mg/Kg (1-day test) and $ED_{50\,=}$ 0.3, ED_{90} = 0.6 and NRD = >32 mg/Kg (4-day test). However, treatment at 4 mg/Kg for 8 days was able to eradicate infection from mice. These results are not inconsistent with the 4-day test and are probably related to the short half life of GSK932121 in mice. In the murine model of *falciparum* malaria, groups of mice n=3 were infected with 20×10^6 infected erythrocytes i.v. (day 0). The treatment (p.o., once a day for 1 or 4 days) started at day 3 after infection. GSK932121 showed potent antimalarial efficacy with $ED_{50\,=}$ <0.9, ED_{90} = 0.9 mg/Kg (1-day test) and $ED_{50\,=}$ 0.47, ED_{90} = 0.64 and NRD = >30 mg/Kg (4-day test). The microscopic and flow cytometry analysis suggested that the trophozoite stage was the most sensitive to GSK932121. A study in our *falciparum* murine model aiming to provide estimates of the relevant pharmacodynamic parameters for helping the selection of target doses in Phase I and Phase II studies is in progress.

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NOD-*SCID IL2R$^{-/-}$* MICE ENGRAFTED WITH HUMAN ERYTHROCYTES SUPPORT HIGHER *PLASMODIUM FALCIPARUM*-PARASITEMIAS THAN NOD-*SCID* β *MICROGLOBULIN$^{-/-}$* ENGRAFTED MICE

Belén Jiménez-Díaz[1], Teresa Mulet[1], Sara Viera[1], Vanesa Gómez[1], Helen Garuti[1], Angela Alvarez[1], Javier Ibáñez[1], Leonard Shultz[2], Domingo Gargallo-Viola[1], Iñigo Angulo-Barturen[1]

[1]GlaxoSmithKline, Tres Cantos, Spain, [2]The Jackson Laboratory, Bar Harbor, ME, United States

Plasmodium falciparum causes a high number of clinical cases of malaria with a relatively high fatality rate. Recently, we have described a reproducible murine model of *P. falciparum* malaria after i.v. infection of non-myelodepleted NOD-*scid* β*2 microglobulin$^{-/-}$* mice engrafted with human erythrocytes (~ 50 % of total erythrocytes in peripheral blood of mice) with *Pf*3D7$^{0087/N9}$. This strain of *P. falciparum* was obtained by *in vivo* selection of isolates of *P. falciparum* 3D7 able to grow reproducibly in peripheral blood of engrafted NOD-*scid* β*2 microglobulin$^{-/-}$* mice. However, this mouse strain shows a high incidence of thymic lymphomas that limit their lifespan and constrains long term studies in these mice. Hence, we tested whether NOD-*scid IL2r*γ$^{-/-}$ mice, which do not develop lymphomas, were able to support the growth of *Pf*3D7$^{0087/N9}$. Interestingly, the kinetics of engraftment before infection was similar in both murine strains. Although infection with *Pf*3D7$^{0087/N9}$ resulted in exponential growth of the parasite in both strains, the peak level of parasitemia (day 7 after infection) was three times higher in NOD-*scid IL2r*γ$^{-/-}$ than in NOD-*scid* β*2 microglobulin$^{-/-}$* mice and the overall concentration of infected erythrocytes (×10^6 ml^{-1}) *vs* time (days) Area Under the Curve was 8 times higher in NOD-*scid IL2r*γ$^{-/-}$mice (up to day 35 after infection). The enhanced ability of NOD-*scid IL2r*γ$^{-/-}$ mice to support the growth of *Pf*3D7$^{0087/N9}$ was not explained by a lower clearance of human erythrocytes because the concentration of human erythrocytes (×10^6 ml^{-1}) *vs* time (days) was similar in both strains. Thus, our data suggest that NOD-*scid IL2r*γ$^{-/-}$ mice are able to support higher parasite burdens due to a lower capacity to clear infected erythrocytes than NOD-*scid* β*2 microglobulin$^{-/-}$* mice. Accordingly, the NOD-*scid IL2r*γ$^{-/-}$ strain has been validated for standardized therapeutic efficacy assays using chloroquine, pyrimethamine and GSK932121, a (1H)-pyridone candidate for clinical development.

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DEVELOPMENT OF A HIGH-THROUGHPUT *IN-VITRO* SCREEN TO IDENTIFY INHIBITORS OF THE *PLASMODIUM FALCIPARUM* HEAT SHOCK PROTEIN 90 BINDING ACTIVITY

Martin L. Kramer[1], Vishal Patel[2], Jon Clardy[2], Dyann F. Wirth[3]

[1]Genzyme Corporation, Waltham, MA, United States, [2]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, United States, [3]Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA, United States

The rate of drug resistance in *Plasmodium falciparum* is increasing as a result of significant phenotypic variability among strains to currently available therapeutics. This has prompted a broader search for novel drug targets, with the Heat shock proteins (HSP) being identified as a potentially interesting class. The HSP90 chaperone protein is responsible for essential cellular functions, such as the activation of many protein kinases and transcription factors in addition to thermotolerance. The recruitment of co-chaperones and client proteins is regulated by conformational cycling driven by ATP hydrolysis at an N-terminal ATP binding domain. Inhibition of the ATPase activity of human HSP90 (hsHSP90) by geldanamycin (GA) or radicicol dampens the chaperoning capacity of the protein causing deregulation of the proteome and subsequent cell death. It has been determined that *P. falciparum* HSP90 (pfHSP90) is the most highly expressed of these vital chaperone proteins and is therefore a valuable target for anti-malarial drug development. An *in vitro* high-throughput ligand screen (HTS) was developed using full-length recombinant pfHSP90. The use of GA labeled with fluorescein-5-isothiocyanate (FITC) dye at the C17 position allows for real-time monitoring of the GA/HSP90 complex formation. GA-FITC displayed a comparable affinity for hs- and pfHSP90. Displacement studies were conducted using unlabeled GA or derivatives and a HTS of >250,000 compounds is planned. Despite the fact that hsHSP90 shares a high homology (73%) with pfHSP90, demonstration of 100-fold specificity of radicicol for the human homolog suggests that the discovery of a species-specific inhibitor of pfHSP90 is feasible.

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PHARMACOKINETIC-PHARMACODYNAMIC RELATIONSHIPS OF IMIDAZOLIDINEDIONE DERIVATIVES

David Saunders, Michael Kozar, Melendez Victor, Qiqui Li, Paktiya Teja-Isavadharm, Gettayacamin Montip, Michael O'Neill, Colin Ohrt, Alan Magill, Ai J. Lin

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

Novel 2-guanidinoimidazolidinediones (IZ) have demonstrated excellent *in vivo* antimalarial activity against murine and primate Plasmodial species. Potent oral causal prophylactic activity against the liver stages of *Plasmodium yoelli* in mice was not reproduced orally in primates, though intramuscular administration was causally prophylactic, and led to long delays (up to 91 days post treatment) in hyponozoite relapse in a relapsing *P. cynomolgi* rhesus model. Oral activity in primates has correlated more closely with the sporozoite-induced mouse *P. berghei* model. Pharmacokinetic oral plasma levels appear to correlate closely between primates and mice, but not rats where observed levels were much lower. While it was initially postulated that s-triazene metabolites were responsible for the observed activity, recent primate pharmacokinetic data indicates that activity resides in the parent compounds. Furthermore, carboxamide and carbamate IZ analogs have a natural long-lasting intramuscular depot effect with measurable compound levels beyond 7 days, and no observed injection site toxicity when administered in a 3 day series at 30 mg/kg. Oral pharmacodynamic activity in primates appears to be low at 60 mg/kg for 3 days, despite good measured oral bioavailability. This suggests an antimetabolic mechanism of action, and raises the possibility that the compound class could require a saturable active transport mechanism to achieve effective concentrations at the site of action. Efforts to overcome limitations of oral administration are ongoing. While currently being developed for the radical cure of *P. vivax* malaria, lead optimization of this compound class has led to several potential novel applications including an injectable causally prophylactic antimalarial for short term travel to malarious areas up to 60 days.

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PHARMACOKINETIC CHARACTERIZATION STUDIES IN MICE AND BEAGLE DOGS OF 4(1H)-PYRIDONE DERIVATIVE GSK932121

Adolfo García, María Martínez, Hugo Salgado, Laura Alameda, Ciriaco Maraschiello, Domingo Gargallo-Viola, Santiago Ferrer

GlaxoSmithKline, Tres Cantos, Spain

4(1H)-Pyridones are a novel class of antimalarials acting as selective inhibitors of *Plasmodium* mitochondrial function with proven activity and selectivity both *in vitro* and in animal models. After an exhaustive evaluation program including more than one hundred derivatives, compound GSK932121 was one of the most promising compounds and was selected to enter full pre-clinical development. Preliminary pharmacokinetics (PK) studies for GSK932121 were performed in mice and beagle dogs to determine oral bioavailability and to gain insight about initial PK properties of the compound. GSK932121 was administered as single dose by intravenous and oral route to mice at 0.2 and 10 mg/Kg respectively and to dogs at 0.2 and 3 mg/Kg. For intravenous administration compound was prepared as solution whereas for oral dosing suspensions, (solid formulations) were used. Preliminary PK profiling in the mouse was completed by determining the *in vivo* Blood to Plasma (B/P) compound distribution ratio (index of particular interest for antimalarials acting against *Plasmodium* erythrocytic stages). The possible impact of active infection over the pharmacokinetic profile was also studied using two animal models, the standard murine parasites (*P. yoelii*) and in a model of *P. falciparum* infection in mice. In the mouse, GSK932121 displayed PK properties defined by its low clearance (Cl) (3.3% of liver blood flow) volume of distribution (Vd) around 1.5folds total body water and an estimated half-life (t1/2) of 3.8 hours. Oral bioavailability after solid dosage administration as suspension in 1% methyl cellulose reached 49.9%. Regarding B/P ratio, GSK932121 appeared to be mainly located in plasma with ratio values around 0.5. On their turn, PK profile of GSK932121 was almost conserved in infected animals compared with uninfected subjects with only minor increases in systemic exposure. Main PK features determined in mice were maintained in dog such as low Cl (2.5% of liver blood flow), volume of distribution above total body water (around 4.6folds) and notably long t1/2 of 42.1 hours. Although lower than in mice, solid dosage bioavailability of 16% in the dog was considered promising and with margin for improvement during full development process.

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COMPLEXITY OF *PLASMODIUM FALCIPARUM* CLINICAL SAMPLES FROM UGANDA DURING SHORT-TERM CULTURE

Samuel L. Nsobya[1], Moses Kiggundu[1], Moses Joloba[1], Grant Dorsey[2], **Philip J. Rosenthal**[2]

[1]Makerere University, Kampala, Uganda, [2]University of California, San Francisco, San Francisco, CA, United States

Plasmodium falciparum infections are commonly polyclonal. Not all isolated parasites successfully grow in culture, and it is unclear how well *in vitro* culture represents the complexity of clinical infections. We characterized changes in complexity of infection (COI) during short-term culture of parasites from children in Kampala, Uganda with uncomplicated malaria. Of 211 available samples, 98 were successfully cultured for at least 9 days. Parasite density at diagnosis was greater in samples that successfully grew for 9 days (mean 30,400/μl) than in those that did not (11,500/μl). COI was assessed daily for 9 days based on analysis of polymorphic regions of the msp-2 gene in parasite DNA extracted from dried blood spots. For 53 samples, only a single genotype was detected at

culture initiation (Day 0). The mean COI at was 1.73 at Day 0, remained stable for the first 4 days of culture, and then decreased gradually to 1.56 on Day 8. To gain insight into the sensitivity of single time point assessment of COI, we followed the appearance of new strains after Day 0. Strains not present on Day 0 were first identified on Days 1-5 in 20 cultures; half of these appeared on Day 1. Thus, the Day 0 genotype understated the true COI in 20% of samples. We were also interested in the loss of strains after Day 0. Strains disappeared after Day 0 in 25 (56%) of 45 cultures that were initially mixed. Upon analysis by PCR and restriction endonuclease digestion, persisting strains more commonly had drug-sensitive wild type dhfr (C59) and dhps (K540), and mutant pfmdr-1 (86Y) sequences. Thus, initial genotypes offer an imperfect representation of clinical COI, and loss of strains in culture may be due to diminished fitness of some drug resistant strains. Our study highlights potential limitations both in the use of single time point assessment of COI as a baseline for drug efficacy determination and the reliability of associations between clinical outcomes and genotypes of stably cultured parasites.

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MOLECULAR EVIDENCE FOR CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM* IN HAITI

Berlin L. Londono[1], Thomas P. Eisele[1], Joseph A. Keating[1], Adam Benett[1], Chandon Chattopadhyay[2], Brian Mack[1], Jean Francois Vely[3], Olbeg Disinor[4], Donald J. Krogstad[1]

[1]Tulane University, New Orleans, LA, United States, [2]Swiss Tropical Institute, Basel, Switzerland, [3]Hôpital Albert Schweitzer, Deschapelles, Haiti, [4]Ministry of Health and United States Agency for Agency for International Development, Port-au-Prince, Haiti

For more than 30 years, Haiti has been remarkable as a *Plasmodium falciparum* malaria-endemic country without evidence for chloroquine (CQ) resistance. After obtaining informed consent, we prepared filter paper blots from Haitian adults with positive thick smears for asexual *P. falciparum* parasites using active and passive case detection during the high transmission seasons (November-December) in 2006 and 2007. After extraction of those filter paper blots, parasite DNA was amplified using PCR with primers for the *P. falciparum* pfcrt gene. Of the 82 specimens studied, 10 were resistant to cleavage by ApoI. After ligation into the TOPO TA cloning vector, and transfection into E. coli, these 10 amplicons were subjected to dideoxy sequencing. Six had the CQ-resistant (ACA, Thr=T) sequence at position 76, and 4 had the CQ-susceptible, wild-type (AAA, Lys=K) sequence. In terms of the position 72-76 haplotype, the CQ-resistant parasites were CVIET and CVMNT; the CQ-susceptible parasites were CVMNK. However, no SVMNT (South American) sequences were identified. These results suggest that *P. falciparum* parasites with the mutation K76T in the pfcrt gene are now circulating in Haiti. These results also suggest that studies of children and other non-immune subjects should be performed to clarify the prevalence of CQ resistance and whether the current reliance on chloroquine in Haiti should continue for both chemoprophylaxis and treatment.

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ASSESSMENT OF THE ORIGINS AND SPREAD OF PUTATIVE RESISTANCE-CONFERRING MUTATIONS IN *PLASMODIUM VIVAX* DIHYDROPTEROATE SYNTHASE

Vivian N. Hawkins[1], Stephanie Suzuki[1], Kanchana Rungsihirunrat[2], Hapuarachchige C. Hapuarachchi[3], Amanda Maestre[4], Kesara Na-Bangchang[5], Carol Hopkins Sibley[1]

[1]University of Washington, Seattle, WA, United States, [2]Chulalongkorn University, Bangkok, Thailand, [3]University of Kelaniya, Ragama, Sri Lanka, [4]Universidad de Antioquia, Medellín, Colombia, [5]Thammasat University, Pathumthanee, Thailand

Plasmodium vivax is a serious health concern in many regions; inadvertent treatment with sulfadoxine/pyrimethamine is common. Sulfadoxine is a competitive inhibitor of dihydropteroate synthase (DHPS). While it has been commonly assumed that *P. vivax* is less susceptible to sulfadoxine than is *P. falciparum*, mutations in Pvdhps may contribute to sulfadoxine resistance in a manner similar to that observed for *P. falciparum*. Based on homology to the *P. falciparum* enzyme, it has been proposed that non-synonymous mutations in Pvdhps codons 382, 383, 512 and 553 may contribute to sulfadoxine resistance in *P. vivax*. Single nucleotide polymorphisms and variable repeat regions both within and flanking Pvdhps provide information about the manner in which putative resistance-associated alleles have spread through natural populations. We amplified *P. vivax* pppk-dhps and its entire flanking intergenic region (497 bp upstream/631 bp downstream) from 92 contemporary global isolates. We found a range of polymorphisms within both introns and exons of pppk-dhps, as well as in the flanking intergenic regions. A variable repeat region was identified in the dhps coding region. Notably, all of the isolates in our dataset carried a valine at dhps codon 585; this valine has been implicated in the presumed lower susceptibility of *P. vivax* to sulfadoxine. Eighteen haplotypes were associated with alleles wildtype at all codons of interest, while 9 haplotypes were associated with the 383G allele. One isolate carried the 382A/383G allele. 12 isolates carried the 383G/553G allele, with 2 distinct haplotypes observed. Two isolates carried the 382C/383G/553G allele, with only one haplotype observed. Six isolates, all from Thailand, carried the 382A/383G/553G allele, with 3 haplotypes observed; two of these haplotypes were closely related and one was quite divergent. In all, putative resistance-associated mutations in Pvdhps are associated with a variety of haplotypes, lending credibility to the hypothesis that these mutations may be important for resistance to sulfa drugs.

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PLASMODIUM FALCIPARUM HEME DETOXIFICATION PROTEIN (HDP) IS NOT LINKED TO CHLOROQUINE RESISTANCE GENOTYPE

Sumiti Vinayak[1], Dharmendar Rathore[2], Simon Kariuki[3], Laurence Slutsker[4], Ya Ping Shi[4], Leopoldo Villegas[5], Ananias Escalante[6], Venkatachalam Udhayakumar[7]

[1]Atlanta Research and Education Foundation, Atlanta, GA, United States; Centers for Disease Control and Prevention, Division of Parasitic Diseases, Malaria Branch, Atlanta, GA, United States, [2]Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, VA, United States, [3]Center for Vector Biology and Control Research, Kenya Medical Research Institute, Kisumu, Kenya, [4]Centers for Disease Control and Prevention, Division of Parasitic Diseases, Malaria Branch, Atlanta, GA, United States, [5]Asociación Civil Impacto Social, Tumeremo, Venezuela, [6]Arizona State University, Tempe, AZ, United States, [7]Centers for Disease Control and Prevention, Division of Parasitic Diseases, Malaria Branch, Atlanta, GA, United States; Atlanta Research and Education Foundation, Atlanta, GA, United States

Malaria parasites infecting host red blood cells (RBCs) degrade hemoglobin, detoxifying heme into hemozoin. This conversion of heme to hemozoin is performed by a potent protein-Heme Detoxification Protein (HDP), thus making HDP an attractive target for antimalarial drug development. Since chloroquine (CQ) also acts by disrupting the heme polymerization process, we hypothesized that HDP may be a potential target for CQ resistance. To test this hypothesis, we sequenced HDP from *Plasmodium falciparum* laboratory strains with known CQ sensitivity and field isolates from Venezuela and Kenya, with high levels of CQ resistance. The complete HDP gene sequence encompassing 3 exons and 2 introns (AT and ATTT repeat-rich regions in intron 1 and only AT rich repeat region in intron 2) was determined in 5 laboratory strains and 50 field isolates. Sequencing of HDP revealed two nonsynonymous mutations, C41F and F91L in exon 1 and exon 2 respectively. Only the HB3 laboratory strain harbored the mutant 41F allele while all the other strains and field isolates were wild type (C41) at this position. However, sequencing analysis for the other mutation (at 91 amino acid) revealed that all field isolates and laboratory strains harbored the mutant (91L) allele. The sequencing of the intron 2 region of HDP revealed no variation in the number of AT repeats as all the isolates exhibited 7 conserved AT repeats. However,

there was wide variation in the intron 1 region of HDP; 13 out of 26 Venezuelan *P. falciparum* isolates exhibited 16 AT repeats in this region while the rest of the isolates revealed 15 and 17 repeats. Among Kenyan isolates (n=24), there was even greater intron 1 AT repeat variation; isolates had repeats ranging from 6 to 26. The ATTT repeats in intron 1 were less diverse than the AT repeats, with repeat numbers ranging from 3 to 7. Both Venezuelan and Kenyan isolates revealed 3, 4 or 6 repeats with the exception of one Kenyan isolate that exhibited 7 ATTT repeats. Furthermore, to test our hypothesis, we also genotyped the 72-76 amino acids of the *pfcrt* gene conferring chloroquine resistance in all these field isolates as well as laboratory strains with known CQ sensitivity profile. Collectively, the results indicate that HDP is not under any potential drug selection pressure. Also, the 91L mutation in HDP was not linked to CQ resistant SVMNT or CVIET *pfcrt* genotype. In conclusion, we did not find any evidence for involvement of HDP in CQ resistance.

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RANDOMIZED CONTROLLED CLINICAL TRIAL OF ARTESUNTE/MEFLOQUINE PAEDIATRIC FORMULATION *VERSUS* ARTEMETHER/LUMEFANTRINE FOR UNCOMPLICATED CHILDHOOD *FALCIPARUM* MALARIA IN IVORY COAST

Toure A. Offianan[1], Kouame G. Menan[1], Yapi J. Didier[1], Ako A. Aristide[1], Khali D. Djerea[2], Gomez G. Opportune[3], Penali K. Louis[1]

[1]Pasteur Institute, Abidjan, Cote d'Ivoire, [2]Anonkoua-koute Hospital, Abidjan, Cote d'Ivoire, [3]Anonkoua-koute hospital, Abidjan, Cote d'Ivoire

Artemisinin-based combination therapy (ACT) is being widely promoted as a strategy to counteract the increase in *Plasmodium falciparum* antimalarial drug resistance. We undertook a randomised clinical trial of antimalarial drug combinations for children (aged 6-59 months) with uncomplicated malaria in Anonkoua kouté (Abidjan-Ivory Coast), an area with a high prevalence of resistance to sulfadoxine-pyrimethamine and chloroquine. Children were randomly allocated 3 days of artesunate-mefloquine (n=77) and artemether-lumefantrine (n= 79). Drugs were taken orally, under supervision by medical staff. The Primary endpoints included day 28 follow-up and gametocyte carriage. Analysis was by intention to treat. In june to September 2007, a total of 156 patients with slide confirmed uncomplicated *P. falciparum* malaria were randomly assigned to receive artesunate-mefloquine (n = 77) or artemether-lumefantrine (n = 79) and were followed for 28 days. Adequate Clinical and Parasitological Response (ACPR) was 73 (97.33%) and 70(93.33%) for artesunate-mefloquine and artemether-lumefantrine respectively. One (1.33%) patient in the artesunate-mefloquine group and 5 (6.67%) in the group artemether-umefantrine had Late Clinical Failure. One (1.33%) patient in artesunate-mefloquine group and none in artemether-lumefantrine had Early Treatment Failure. Both regimens were very well tolerated with no serious adverse events observed attributable to either combination. In conclusion, artesunate-mefloquine and artemether-lumùefantrine have high and comparable cure rates and tolerability among under-five children in Ivory Coast.

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MOLECULAR GENOTYPING AND DRUG RESISTANCE ANALYSES OF *PLASMODIUM FALCIPARUM* RECURRENT PARASITEMIAS IN A CLINICAL TRIAL IN THE PERUVIAN AMAZON REGION

Christopher Delgado

Instituto de Medicina Tropical, Lima, Peru

In the present study, we standardized a strategy of molecular genotyping of *Plasmodium falciparum* in order to distinguish between recrudescence and new infection (or reinfection) in recurrent parasitemias in patient samples from a clinical trial study in the Peruvian Amazon. Furthermore, we characterized the *pf*mdr1 gene (multidrug resistant gene) and *pf*atp6 gene (calcium-transporting ATPase) of recurrent parasitemias in order to monitor mutations related to drug susceptibility and resistance. The study involved 522 patients in two therapy groups: mefloquine-artesunate and dihydroartemisyn-piperaquine. Fourteen patients presented recurrent parasitemias. In order to discriminate recrudescence or new infection (or reinfection), these recurrent parasitemias were genotyped on day zero (D0, before treatment) and on the day of recurrent parasitemia using the genes *msp1/msp2* (merozoite surface proteins) and *glurp* (glutamate-rich protein). Besides, all the 522 baseline samples (D0) were genotyped to calculate the probability of a second infection with a particular multilocus genotype occurring purely by chance *(P(match))* and to asses the performance (polymorphism) of the molecular markers. Moreover, the recurrent parasitemias were analyzed using *pf*mdr1 gene by sequencing (single point mutations S1034C, N1042D and D1246Y), *pf*atp6 gene (S769N and A623E) and by qPCR (gene amplification). 9/14 recurrent parasitemias were new infections, 4 recrudescences and 1 unresolved sample. Using *P(match),* just 1 recurrent parasitemia resulted in a "true" recrudescence (*P<0,011*) while the rest of the samples were considered as reinfections (new infection with the same haplotype of the first infection). The best strategy for sequential genotyping was starting with the most polymorphic gene for this study area, MSP1 (Heterogozigocity index=0,86); followed by GLURP and MSP2. The *pf*mdr mutations associated to drug sensibility were found in all the recurrent samples. The *pf*atp6 mutations associated to drug resistance were not found in all recurrent samples**.** In conclusion, in the Peruvian Amazon, the molecular analyses used to determine recrudescence and monitor drug resistance are useful tools to evaluate the efficacy of antimalarial drugs particularly in this area where the rise and spread of drug resistance occurred too fast in the last decade.

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SELECTION OF *PLASMODIUM FALCIPARUM* MULTIDRUG RESISTANCE GENE 1 ALLELE IN ASEXUAL STAGES AND GAMETOCYTES BY ARTEMETHER-LUMEFANTRINE IN NIGERIAN CHILDREN WITH *FALCIPARUM* MALARIA

Onikepe A. Folarin[1], Christian T. Happi[1], Grace O. Gbotosho[1], Akin Sowunmi[1], Tunde Balogun[1], Dyann F. Wirth[2], A. M. Oduola[3]

[1]University Of Ibadan, Ibadan, Nigeria, Ibadan, Nigeria, [2]Harvard School of Public Health, Boston, MA, United States, [3]Special Programme for Research and Training in Tropical Diseases, WHO/TDR, Geneva, Switzerland

We assessed *Plasmodium falciparum mdr1 (pfmdr1)* polymorphisms and copy numbers as well as *P. falciparum* Ca^{2+} ATPase (*pfATPase 6)* gene polymorphisms in 96 Nigerian children presenting with uncomplicated *falciparum* malaria and enrolled in an artemether-lumefantrine (AL) efficacy study. The nested PCR-RFLP and the quantitative real-time PCR methodologies were used to determine alleles of *pfmdr1* and copy numbers respectively in samples collected from all patients prior to treatment and in all recurrent parasites during a 42 day follow-up. The *pfmdr1* haplotype 86N-184F-1246D was significantly associated (p=0.000; OR=0.1; 95%CI-0.001-0.107) with treatment failures and was also strongly selected among post treatment samples obtained from patients with newly acquired or recrudescing infections (p=0.000; X^2=36.5) and in gametocytes (Log rank statistics=5; P=0.0253) after treatment with AL. All pre- and post treatment samples as well as gametocytes harboured a single copy of the *pfmdr1* gene and the wild type allele (L89) *pfATPase6* at codon 89 of this gene. These findings suggest that polymorphisms in *Plasmodium falciparum pfmdr1* gene are under directional AL selection. *Pfmdr1* polymorphisms may result in reducing the therapeutics efficacy of this newly adopted combination for uncomplicated *falciparum* malaria in sub-Saharan countries of Africa.

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SELECTION OF *PLASMODIUM FALCIPARUM* WITH DIMINISHED RESPONSE TO AMODIAQUINE FOLLOWING TREATMENT WITH COMBINATION THERAPY IN UGANDA

Fatima Nawaz[1], Samuel L. Nsobya[2], Moses Kiggundu[2], Moses Joloba[2], Bryan Greenhouse[3], Grant Dorsey[3], **Philip Rosenthal**[3]

[1]University of California, Berkeley, Berkeley, CA, United States, [2]Makerere University, Kampala, Uganda, [3]University of California, San Francisco, San Francisco, CA, United States

Despite some known resistance, amodiaquine (AQ) remains a key component of antimalarial therapy, particularly when paired with artesunate (AS) or sulfadoxine-pyrimethamine (SP). It is unclear how readily resistance will develop to AQ when it is used in combination chemotherapy. We studied the impact of AQ-containing therapies on the sensitivity of parasites causing recurrent infections soon after prior therapy. We collected data for 61 *Plasmodium falciparum* samples from patients who were treated during 2006-2007 with AQ/SP, AQ/AS, or artemether-lumefantrine (AL) for uncomplicated malaria. These samples were placed in culture using standard methods, and *in vitro* sensitivity to the principal AQ metabolite desethylamodiaquine was measured by comparing growth to that of untreated controls with a histidine rich protein-2-based ELISA. Resistance-mediating polymorphisms were also evaluated from parasite DNA extracted from dried blood spots. Parasites from subjects who were previously treated with AQ/SP or AQ/AS within 12 weeks were less sensitive to AQ (n=16; mean IC_{50} 63.7 nM; range 12.7-158.3 nM) than were parasites from those not treated with an AQ-containing regimen within 12 weeks (n=45; mean IC_{50} 38.3 nM; p=0.095 (Kruskal-Wallis rank test); range 6.3-184.7 nM) or only those in a treatment arm that did not contain AQ (AL subjects; n=22; mean IC_{50} 31.3 nM; p=0.017; range 6.3-121.8 nM). The proportion of strains with polymorphisms expected to mediate diminished response to AQ (pfmdr-1 N86Y and D1246Y) increased after prior AQ therapy, although differences between treatment groups were not significant. Our findings show that therapy with AQ rapidly selects for diminished response to this drug, suggesting that AQ-containing combination regimens may rapidly lose efficacy in Africa. However, the mechanism of diminished AQ response is probably not fully explained by known mutations in pfmdr-1, and additional studies to identify mechanisms of resistance to AQ are needed.

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FAILURE OF ARTESUNATE-MEFLOQUINE COMBINATION THERAPY FOR UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA IN SOUTHERN CAMBODIA

William O. Rogers[1], **Rithy Sem**[2], D'thong Tero[3], Phektra Chim[4], Parath Lim[4], Sinuon Muth[2], Duong Socheat[2], Frederic Ariey[4], Chansuda Wongsrichanalai[1]

[1]Naval Medical Research Unit #2, Jakarta, Indonesia, [2]National Malaria Centre, Phnom Penh, Cambodia, [3]Ministry of Health, Phnom Penh, Cambodia, [4]Institut Pasteur du Cambodge, Phnom Penh, Cambodia

We assessed the efficacy of standard anti-malarial therapies for uncomplicated *P. falciparum* and *P. vivax* malaria in Chumkiri, Kampot Province, Cambodia. One hundred fifty-one subjects with uncomplicated *P. falciparum* malaria received directly observed therapy with 12 mg/kg artesunate (over three days) and 25 mg/kg mefloquine, up to a maximum dose of 600 mg artesunate/1000 mg mefloquine. Forty-seven per cent of subjects were still parasitemic on day 2 and 11.3% on day 3. We followed the subjects for 42 days for recurrent parasitemia and used PCR genotyping of *msp1*, *msp2*, and *glurp* to distinguish treatment failure from new infections. The PCR corrected treatment failure rates determined by survival analysis at 28 and 42 days were 13.1% and 18.8%, respectively. Treatment failure was associated with increased *pfmdr1* copy number, higher initial parasitemia, higher mefloquine IC50, and longer time to parasite clearance. One *P. falciparum* isolate, from a treatment failure, had markedly elevated IC50 for both mefloquine (130 nM) and artesunate (6.7 nM). Subjects with uncomplicated *P. vivax* malaria received a total of 25 mg/kg chloroquine, up to a maximum dose of 1500 mg, as directly observed therapy over three days. Of 109 subjects 45 (41.3%) suffered recurrent *P. vivax* parasitemia. None acquired new *P. falciparum* infection. The results suggest that artesunate-mefloquine combination therapy is beginning to fail in Cambodia and that resistance has spread beyond the provinces at the Thai-Cambodian border. It is unclear whether the treatment failures are due solely to mefloquine resistance or to artesunate resistance as well. The findings of delayed clearance times and elevated artesunate IC50 suggest that artesunate resistance may be emerging on a background of mefloquine resistance.

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STABILITY OF *PFMDR1* AMPLIFICATION IN *PLASMODIUM FALCIPARUM IN VITRO*

Nanhua Chen[1], Marina Chavchich[1], Jennifer Peters[2], Michelle Gatton[2], Dennis E. Kyle[3], **Qin Cheng**[1]

[1]Australian Army Malaria Institute, Brisbane, Australia, [2]Queensland Institute of Medical Research, Brisbane, Australia, [3]University of South Florida, Tampa, FL, United States

Pgh glycoprotein encoded by *pfmdr1* is implicated in multi-drug resistance in *Plasmodium falciparum*. Both sequence polymorphisms and gene amplification have been identified and correlate with resistance to various antimalarial drugs. In our previous studies we observed amplification of *pfmdr1* from 1 copy to 3 copies when parasites were under artelinic acid selection pressure *in vitro*. This amplification was associated with a reduction of parasite susceptibility to artelinic acid, artemisinin, mefloquine, quinine, halofantrine and lumefantrine. However when the drug selection pressure was withdrawn, the *pfmdr1* copy number decreased to 1 or 2 copies in the parasite population within several months indicating that the higher copy *pfmdr1* was not a favourable state for the parasite. To elucidate whether the reduction of *pfmdr1* copy number was due to deamplification of the gene or to a loss of fitness associated with the amplification of the chromosomal fragment containing *pfmdr1*, we investigated the stability and fitness of the parasites with 3 and 1 copies of *pfmdr1*. The findings to be presented are of practical importance as the stability of *pfmdr1* amplification may influence the development and spread of multi-drug resistance in malaria parasites, especially resistance to mefloquine and artemisinin derivatives.

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A DECLINING BURDEN OF MALARIA IN NORTHEASTERN TANZANIA

John P. Lusingu[1], Lasse Vestergaard[2], Bruno P. Mmbando[1], Samuel Gesase[1], Deus Ishengoma[1], Chris Drakeley[3], Pamela Magistrado[2], Andrew Y. Kitua[4], Martha M. Lemnge[1], Thor G. Theander[2], Thor G. Theander[2]

[1]National Institute for Medical Research, Tanga, United Republic of Tanzania, [2]University of Copenhagen, Copenhagen, Denmark, [3]London School of Tropical Medicine and Hygiene, London, United Kingdom, [4]National Institute for Medical Research, Dar es Salaam, United Republic of Tanzania

The planning and assessment of malaria interventions is complicated due to fluctuation of the burden of malaria in societies over time. Recently, the burden of malaria in Africa has been widely informally reported to decline. Evidence-based longitudinal monitoring data are sparse and the reasons for the apparent decline are not well understood. Malaria prevalence and morbidity in two different villages in north-eastern Tanzania; a lowland village and a highland village have been monitored from 2003 to 2007. Trained village health workers treated presumptively uncomplicated fever with the Tanzanian first-line antimalarial drug and collected blood smears that were examined later. The prevalence of malaria parasitaemia was monitored through biannual surveys. The prevalence of malaria parasitaemia in the lowland village decreased from 78.4 % in 2003 to

50.9% and 24.0 % in 2006 and 2007, respectively. In the highland village, the malaria prevalence dropped from 24.7% to 7.4% and 6.5% in the same period. Similarly, the incidence of febrile malaria episodes in the two villages dropped by almost 85% in the same period. The average rainfall in the area was low in 2003 and 2005, and increased in 2006 and 2007 compared to 2003, but there was no correlation between rainfall and malaria incidence. There has been a marked decline in malaria in the study villages across years. The decline in malaria might be due to combination of factors that include improved access to malaria treatment provided by the trained village helpers, protection from mosquitoes by increased availability of bed nets and reduced vector density.

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KNOWLEDGE AND UTILIZATION OF MALARIA PREVENTION STRATEGIES IN PREGNANCY IN TWO STATES OF INDIA

Blair J. Wylie[1], Neeru Singh[2], Ahmar H. Hashmi[3], Mrigendra P. Singh[2], Jordan Tuchman[3], Lora Sabin[3], Kojo Yeboah-Antwi[3], Mobassir Hussain[2], Camellia Banerjee[3], Aditya P. Dash[4], Davidson H. Hamer[3]

[1]Department of Obstetrics, Massachusetts General Hospital, Boston, MA, United States, [2]National Institute For Malaria Research Field Station, Jabalpur, India, [3]Center for International Health and Development, Boston, MA, United States, [4]National Institute For Malaria Research, Delhi, India

Malaria transmission in India is both stable and unstable. Current government policy recommends chloroquine (CQ) prophylaxis for pregnant women (PW) living in endemic areas. It is neither known whether this policy or other malaria in pregnancy (MIP) prevention strategies are widely utilized nor whether effective methods of diagnosis/treatment are available to PW in India. This cross-sectional study was conducted during 2006-2008 in 2 states of India, Jharkhand (JH) and Chhattisgarh (CH), at 7 facilities representing a range of rural and urban populations and areas of more vs less stable transmission. Detailed facility assessments were performed at each site to determine availability of antimalarials, lab supplies and bednets.280 antenatal visits (ANVs)(40/site) were observed by study personnel coupled with PW interviews to assess availability and utilization of MIP prevention measures. 23 in-depth interviews with HWs were also conducted to further clarify MIP knowledge and practices. CQ was stocked regularly at all facilities although the consistency of stock varied, particularly at rural sites. 46% of HWs interviewed had never heard of chemoprophylaxis for PW. HWs recommended CQ antimalarial prophylaxis only 3 times among the 280 ANVs. SP was stocked regularly in only 2 of 7 facilities. In JH, none of the antenatal facilities had bednets available for distribution and no HW worker recommended use of a bednet. 90.8% of the PW had bednets in their household and 81.7% slept under the bednet most nights. Only 3.3% of households had insecticide-treated nets. In CH, bednets were available at all facilities but only 14.4% of HWs recommended sleeping under the net in pregnancy during the observed ANV. 40% of PW had bednets in their households. Only 20% slept under the net most nights. None were insecticide-treated. For diagnosis, all facilities were well equipped to perform blood smears but none were using rapid diagnostic tests. In addition to CQ, all facilities routinely stocked artemether for parenteral use. In conclusion, a disconnect remains between MIP government policy and practice in India. Prevention strategies, in particular chemoprophylaxis and use of insecticide-treated bednets, appear underutilized. Gaps highlighted by this study combined with recent estimates of MIP prevalence in these areas should be used to revise governmental policy and target increased educational efforts among HWs and PW.

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SPATIAL DISTRIBUTION AND TEMPORAL DYNAMICS OF CLINICAL MALARIA CASES IN A WESTERN KENYA HIGHLAND SITE

Yaw A. Afrane[1], Andrew Karanja Githeko[1], Guiyun Yan[2]

[1]Kenya Medical Research Institute, Kisumu, Kenya, [2]University of California at Irvine, Irvine, CA, United States

One importance issue for evaluating the efficiency of new malaria control measures on febrile malaria is the accuracy of hospital-based malaria case data, including underreporting, misdiagnosis or over-reporting. Further, the distribution pattern of febrile malaria may be used to infer environmental risk factors. This study investigated the spatial distribution and temporal dynamics of clinical malaria cases through active and passive case surveillance in a western Kenya highland site. Active case surveillance was done with a cohort of over 1,800 participants selected randomly from 400 houses in Kakamega District. These houses were stratified by topography. Participants were visited every two weeks and screened for clinical malaria whilst passive case surveillance was done from the local health facility in the study area. A clinical malaria case is defined as an individual with malaria-related symptoms (fever [axillary temperature ≥ 37.5°C], chills, severe malaise, headache or vomiting) at the time of examination or 1-2 days prior to the examination and a presence of a *Plasmodium falciparum* positive blood smear. Topography was associated with increased malaria risk. Clinical malaria cases from active case surveillance were clustered along valley bottoms with a 2 to 2.5-fold increase during the rainy season. Children between the ages of 6-10 years had 45% of clinical cases followed by children under the age of 5 years (40%) , then people older than 10 years had the least (15%). Bednet coverage was over 60% among households. However, households with bednets still had clinical malaria cases. There was no significant correlation between case numbers obtained from active clinical cases and passive hospital hospital-based case surveillance, suggesting some level of unreliability of hospital-based clinical case data. Hospital cases seem to be the same throughout the year with no pattern of seasonality compared to the active clinical cases. We are investigating whether misdiagnosis and over treatment from the hospitals account for the ambiguity in the passive cases.

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MICROSATELLITE ANALYSIS OF MULTIPLE-CLONE *PLASMODIUM VIVAX* INFECTIONS

Tatiana Havryliuk[1], Pamela Orjuela-Sánchez[2], Marcelo Urbano Ferreira[3]

[1]Mount Sinai School of Medicine, New York, NY, United States, [2]Institute of Biomedical Sciences, Unversity of Sao Paulo, Sao Paulo, Brazil, [3]Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil

Microsatellite typing has greatly advanced the field of population genetics of malaria parasites. Although its use is widespread, this technique has not been validated in multiple-clone infection analysis. Because it remains unknown whether the proportions of clones co-infecting the same host are maintained after PCR amplification of microsatellite alleles, the estimation of multiple clone infection rates and haplotype abundance may be unreliable. Here we analyze 12 highly polymorphic microsatellite markers in five different artificial mixtures of two single-clone *Plasmodium vivax* isolates. Opposite to our expectations, only two of the twelve initial 1:1 isolate mixtures yielded nearly equal peaks. The rest had varying ratios, suggesting differential PCR amplification of particular microsatellite alleles from genomic DNA, leading to error in the determination of the predominant haplotype based on the most abundant alleles at each locus. However, when results were corrected for the amplification biases found in the analysis of 1:1 mixtures, we obtained quite accurate estimates of relative allele abundance in the four different mixtures of genomic DNA. These estimates remained reliable even when whole-genome amplification (WGA) was applied to genomic DNA mixtures prior to PCR. While testing some of the most cited criteria for minor allele detection

in mixed-clone infections, we discovered that many multiple alleles were missed depending on criteria used, and that no multiple alleles at any one locus were found in our 1:0.25 isolate dilution when 1/3 the predominant peak height was taken as a cut-off value for minimal minor peak height. We conclude that microsatellite typing conserves relative isolate proportions and is thus a valid technique in the study of multiple clone malaria infections if used carefully. Likewise, whole-genome amplification (WGA) of DNA prior to PCR did not alter isolate proportions and remains a promising method for *P. vivax* DNA pre-amplification for large-scale genotyping.

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A COMMUNITY EFFECTIVENESS TRIAL ON STRATEGIES PROMOTING INTERMITTENT PREVENTIVE ANTIMALARIAL TREATMENT IN PREGNANT WOMEN IN RURAL BURKINA FASO

Sabine Gies[1], Sheick O. Coulibaly[2], Florence T. Ouattara[3], Clotilde Ky[4], Bernard J. Brabin[5], Umberto D'Alessandro[1]

[1]Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium, [2]UFR Sciences de la Santé, Université de Ouagadougou, Ouagadougou, Burkina Faso, [3]District Sanitaire Boromo, Boromo, Burkina Faso, [4]Laboratoire National de Santé Publique, Ouagadougou, Burkina Faso, [5]Child and Reproductive Health Group, Liverpool, United Kingdom

Intermittent preventive treatment with sulfadoxine-pyrimethamine for pregnant women (IPTp/SP) is currently being scaled up in many countries in sub-Saharan Africa. However, despite high ANC attendance, coverage with the required 2 doses of SP remains low. We investigated whether a targeted community-based promotion campaign to increase ANC attendance and SP uptake could effectively improve pregnancy outcomes in the community. Twelve health centres in rural Burkina Faso were randomly assigned to the intervention (IPTp/SP and promotion) or one of two control arms (IPTp/SP without promotion or weekly chloroquine). Primi- and secundigravidae were identified at village level and haematocrit and peripheral parasitaemia assessed at 32 weeks gestation and at delivery. Placental parasitaemia and birth weight were also assessed. 2,288 primi- and secundigravidae were recruited and followed up. SP-uptake (≥2 doses) was higher in the intervention (70%) than in the SP-control arm (49%) (p=0.014). Peripheral and placental parasite rates were significantly higher in the chloroquine-control arm (peripheral: 33.3%; placental: 30.3%) [(CQ vs SP (intervention+SP-control): p<0.001] but no difference between the intervention (17.4%; 18.1%) and the SP-control (20.1; 20.5%) arm was found (p>0.3). Mean haematocrit [intervention: 34.7 (SE 0.59); SP-control: 34.7 (0.59); CQ-control: 33.8 (0.47)] and birth weight [2845g (13.4); 2815g (25.4); 2808g (41.5)] and prevalence of anaemia (31.1%; 32.8%; 40.2%) and low birth weight (16.2%; 18.6%; 22.3%) did not differ significantly between study arms (p>0.1). In conclusion, the promotional campaign targeted to pregnant women resulted in a major increase in coverage, with two thirds of women at delivery having received at least two doses of SP. Despite lower prevalence of malaria infection this did not translate into a significant difference in maternal anaemia or birth weight. A much greater coverage with IPTp/SP is needed to achieve a significant impact at community level.

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IMPROVING UPTAKE OF INTERMITTENT PREVENTIVE ANTIMALARIAL TREATMENT IN ANTENATAL CLINICS THROUGH COMMUNITY BASED PROMOTION IN RURAL BURKINA FASO: A HEALTH CENTRE RANDOMIZED TRIAL

Sabine Gies[1], Sheick O. Coulibaly[2], Clotilde Ky[3], Florence T. Ouattara[4], Bernard J. Brabin[5], Umberto D'Alessandro[1]

[1]Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium, [2]UFR Sciences de la Santé, Université de Ouagadougou, Ouagadougou, Burkina Faso, [3]Laboratoire National de Santé Publique, Ouagadougou, Burkina Faso, [4]District Sanitaire Boromo, Boromo, Burkina Faso, [5]Child and Reproductive Health Group, Liverpool, United Kingdom

The objective of this study was to assess the additional benefit of a community based promotional campaign on antenatal attendance and coverage and uptake of intermittent preventive antimalarial treatment (IPTp) in pregnant women. The impact of a community-based promotion campaign aiming at improving the coverage of intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP) given at antenatal clinics (ANC) was assessed within a health centre randomized trial. Outcome measures were number of antenatal visits, timing of booking visit, proportion of women with ≥3 ANC visits, proportion of women with complete IPTp-SP uptake (≥2doses). Factors associated with ≥3 ANC and ≥2 SP were analysed in multivariable logistic regression. 463 (64.2%) of 721 women in promotion villages attended ≥3 ANC compared to 679 (44.7%) of 1,519 in non-promotion villages (p=0.05). During the rainy season, the effect of promotion on ANC was modified by age (< 20: AOR 1.2 [0.3-2.5], p=0.647; ≥ 20: AOR 2.4 [0.9-6.4], 0.085). Uptake of IPTp-SP (≥2 doses) was 71.8% (518/721) in promotion and 49.1% (389/793) in non-promotion villages (p=0.008). IPTp-SP uptake was lowest for adolescents delivering in the rainy season with (29%) or without promotion (30%). The effect of the promotion on SP uptake was greater during the dry season (AOR 3.15 [1.8-5.5] p=0.001) than during the rainy season (AOR 1.82 [1.1-3.1] p=0.036). In conclusion, raising community awareness about the risks of malaria in pregnancy and the benefits of antenatal care and malaria preventive measures can effectively increase ANC attendance and IPTp-SP uptake in a rural community. However, adolescent mothers are the most difficult to reach, particularly during the rainy season when malaria transmission is the highest. Specific approaches designed to reach this high risk group are urgently needed. IPTp-SP will not be able to have a major impact on malaria in pregnancy unless this high risk group is protected.

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PREVALENCE AND DISTRIBUTION OF *PLASMODIUM VIVAX* CIRCUMSPOROZOITE PROTEIN, VK210 AND VK247 VARIANTS, IN PAPUA NEW GUINEA

Cara N. Henry[1], Daphne Sepe[2], Melinda Suspau[2], David T. McNamara[1], Moses Bockarie[1], Christopher King[1], Peter A. Zimmerman[1]

[1]Case Western Reserve University, Cleveland, OH, United States, [2]Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea

Plasmodium vivax (Pv; inclusive of VK210 and VK247 strains), endemic in lowland and coastal regions of Papua New Guinea (PNG), is responsible for a third of malaria cases. VK210 and VK247 differ in the circumsporozoite protein (*Pvcsp*) sequence. Since *Pvcsp* is a leading vaccine development candidate, it is important to understand the prevalence and distribution of the Pv strains. We created a post-PCR diagnostic assay based upon polymorphisms in the *Pvcsp* gene to distinguish the Pv strains. Using this assay along with a published method for detecting the four human *Plasmodium* species, we analyzed human blood samples from the Wosera (WS n=703) and Mugil (MG n=986) regions of PNG to evaluate the prevalence and distribution of *Plasmodium* species and Pv strains. Contrary to past findings we observed VK210 to be the most prevalent Pv strain present in WS (64%) and MG (57%). Single strain VK210 infections were

most commonly observed in both populations (WS-13.5%, MG-14.5%). VK247 single infections and mixed strain infections were observed less often (WS-7.1%, 11.9%; MG-3.95%, 9.7% respectively). Strain distribution between sites was not significantly different. Single strain infections were observed 30% less often while mixed strain infections were observed 250% more often than expected (X^2 p<0.0098). VK210 and mixed strain are the primary infection types in PNG. These assays make it possible to monitor distribution of *Plasmodium* species and strains between geographic regions, different collection times and within vector mosquitoes. This assay improves capacity for monitoring the effectiveness of Pv control strategies and evaluating Pv vaccine development. Additionally, *Pvcsp* was sequenced from 18 isolates and used along with GenBank *Pvcsp* sequences for amino acid comparison and phylogenetic analysis. Phylogenetic comparison of the *Pvcsp* repeat region of PNG isolates and global sequences did not reveal strong phylogeographic clustering; suggesting Pv strains are widely distributed among different geographic regions.

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EPIDEMIOLOGY OF MALARIA IN AN AREA PREPARED FOR CLINICAL TRIALS IN KOROGWE, NORTHEASTERN TANZANIA

Bruno P. Mmbando[1], Method D. Segeja[2], Hamis H. Msangeni[2], Samwel H. Sembuche[2], Deus Ishengoma[2], Seth Misago[2], Ezekiel Malecela[2], Rashid Madebe[2], Acleus Rutta[2], Mathias L. Kamugisha[2], Martha M. Lemnge[2]

[1]University of Copenhagen, Copenhagen, Denmark, [2]National Institute for Medical Research, Tanga Medical Research Centre, Tanzania, Tanga, United Republic of Tanzania

Twelve villages in lowlands and highlands of Korogwe, Tanzania, have been prepared for malaria vaccines testing. In this area, malaria transmission varies across a short distance and is generally lower in the highlands. Lowlands comprises semi-urban and rural villages. Four of the 12 villages are under passive case detection (PCD) of fevers, using community owned resource persons (CORPs), who treat uncomplicated malaria cases using first line antimalarial drug. Four cross-sectional malaria surveys were conducted during short (November/December) and long (May) rains between November 2005 and May 2007, A total of 10971 consenting individuals aged 0-19 years were examined in the four surveys and they were screened for malarial parasites, anaemia and splenomegaly. On average, malaria parasite prevalence was 11.5%, 13.4%, and 39.4 % in highland, semi-urban lowland, and lowland rural respectively. Adjusting for covariates, risk of *Plasmodium falciparum* infection was significantly lower in semi-urban (by 65.8%, p<0.001) and highlands (by 78.4%, p<0.001) compared to lowland rural. Overall, bed-net coverage was lowest in highlands (38.4%) whilst coverage was higher (64.0% to 70.7%) among children aged <5years in semi-urban strata; and was significantly associated with reduced malaria parasite prevalence (OR=0.60, p<0.001). Highest parasite prevalence was in children aged 5-14 years in all strata whilst highest parasite density was in children aged 0-5 years. Gametocyte prevalence was highest in lowland rural (4.2%). Spleen rate was highest in lowland rural and was significantly lower in May 2007 (OR=0.772, p<0.013)**.** Risk of anaemia (Hb<11g/dl) was significantly lower in semi-urban compared to rural villages (OR=0.72, p<0.001), and availability of PCD of fever (OR=0.55, p<0.001). Factors associated with low mean Hb were: presence of *P. falciparum* (by 0.456g/dl, p<0.001), village without PCD (by 0.243g/dl, p<0.001) and not using bed-nets (by 0.1g/dl, p<0.001). Lower spleen rate in May 2007 might be indicative of declining malaria transmission; whilst low malaria parasite prevalence and anaemia in semi-urban lowland might be due to better socio-economic status and/or high bed-net coverage. The site has now reached GCP level and is currently conducting AMANET sponsored MSP3 phase 1b malaria vaccine trial in children.

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MAPPING THE NUMBER OF PREGNANT WOMEN AT RISK OF MALARIA GLOBALLY

Stephanie Dellicour[1], Feiko ter Kuile[1]

[1]Liverpool School of Tropical Medicine, Liverpool, United Kingdom, [2]Department of Infectious Diseases, Tropical Medicine and AIDS, Academic Medical Centre, University of Amsterdam, Amsterdam, Netherlands

Comprehensive estimates of the number of women at risk of malaria during pregnancy are not available, particularly for endemic areas outside Africa. The most commonly cited number is 50 million pregnancies annually of which 25-30 million are thought to occur in Africa. We derived global estimates of the projected number of pregnancies at risk of *Plasmodium falciparum* and *P. vivax* malaria in 2008. We utilised country specific data of the total population at risk of *P. falciparum*, *P. vivax* or mixed infections in 2005 and 2007 from the Malaria Atlas Project (MAP). The population at risk was then multiplied by the fraction of the population estimated to be women of child bearing age (15-49 years) with a live birth in 2005 or 2007 using published total fertility rates and the sex distribution by age of the population for each country, obtained from the international database of the US Census Bureau. The total number of pregnancies was then estimated by multiplying the total number of annual live-births with 1.22 (1/(1-0.18)) to allow for pregnancy loss due to miscarriages (15%) and stillbirths (3%). Lastly, the 2005/2007 figures were adjusted for population growth to obtain 2008 estimates. In 2008, 85.5 million pregnant women are estimated to be at risk of *P. falciparum* and/or *P. vivax* infections; 29.4 million in Africa (34.3%); 52.8 in the Asia-Pacific region (61.8%); 3.1 in the Americas (3.6%), and 0.3 in Europe (0.3%), respectively. In conclusion, the number of pregnancies at risk of predominantly *P. falciparum* malaria in 2008 in Africa was consistent with previous estimates. By contrast the number at risk of *P. vivax* and/or mixed infections in the Americas represents fewer than 4%, but the number in the Asia-Pacific region is much higher than previously estimated and represents almost 2/3 of the global number of women potentially exposed to malaria during pregnancy each year.

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PEDIATRIC MALARIA IN THE NATION'S CAPITAL AND VICINITY: CHILDREN'S NATIONAL MEDICAL CENTER 1999-2006

Patrick W. Hickey[1], Joseph Campos[2], Edward Wong[2], Nalini Singh[2]

[1]Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [2]Children's National Medical Center, Washington, DC, United States

Travel associated malaria affects more than 1000 persons annually in the U.S. but the burden of disease in children is not well documented. Our experience at Children's National Medical Center (CNMC) in Washington D.C. suggested that the number of cases was on the rise. Recently published cases from US hospitals generally amalgamate adult and pediatric patients or represent point source clusters. The most recent study exclusively of pediatric malaria in the US dates from 1999. The goal of this study was to define the burden of pediatric malaria in the D.C. region identifying high risk groups to allow for targeted outreach of future education and prevention efforts. A retrospective cohort of pediatric malaria cases diagnosed and treated between 1999-2006 at CNMC was identified through electronic query of the CNMC labartory database and confirmed with review of medical records. Demographic, clinical, epidemiological, and health care cost data were collected. Ninety-eight cases of malaria were identified during the study period. Country of exposure was identifiable in 92 cases, coming from 22 countries. Nigeria, accounted for 39% of exposures while West Africa, in general, accounted for 66% of cases. Manifestations ranged from asymptomatic parasitemia to cerebral malaria with hyperparasitemia of 45%. Mean peak parasitemia was 5.3%. There were no deaths. The average cost of

an admission was $19,522 for four hospital days. 57% came from a small geographic zone in the western D.C. suburbs which correlates with U.S. Census data showing where people claiming sub-Saharan ancestry reside. In conclusion, the absolute number of cases of pediatric malaria in the D.C. region is increasing, placing a significant burden in terms of disease and cost upon at-risk communities. Better knowledge of high risk groups, local residence patterns, and supplemental information such as Census Reports, may allow for prevention efforts directly targeting the highest risk communities through educational campaigns and outreach to improve use of prophylactic measures.

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EVOLUTIONARY FITNESS OF MINORITY-VARIANT CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM* IN MADAGASCAR

Jonathan J. Juliano[1], Milijaona Randrianarivelojosia[2], Frederic Ariey[3], Steven R. Meshnick[1]

[1]University of North Carolina, Chapel Hill, NC, United States, [2]Institut Pasteur de Madagascar, Antananarivo, Madagascar, [3]Institut Pasteur de Cambodge, Phnom Penh, Cambodia

The *Plasmodium falciparum* chloroquine (CQ) resistance transporter (pfcrt) mutation K76T appears to have been recently introduced to Madagascar. The reported prevalence of this mutation is low (0 to 3%). However, since 1982, late CQ treatment failure has been reported with a prevalence as high as 35% in 14 day follow-up. Conventional PCR is insensitive to low levels of mutant parasites present in patients with polyclonal infections. Thus, the current estimates may be an under representation of the prevalence of the mutation in the malaria population. We conducted heteroduplex tracking assay (HTA) analyses on 27 paired initial and recurrent parasitemia samples from Malagasy children treated with chloroquine. Genotypically resistant parasites were documented in 4 (14.8%) infections, including one initial parasitemia and 3 recurrent parasitemias. K76T mutant variants represented 24.5% of the parasites from one patient before treatment and 2.7%, 2.2%, and 1.8% of parasites from each of three patients at recurrence. In the one patient with the pfcrt mutation before treatment, all recurrent parasites were wild type. While in all the recurrent samples, the pfcrt-bearing parasites represented minority variants. Thus, >97% of recurrent parasites did not bear pfcrt K76T mutation. These findings suggest that, while the pfcrt K76T mutation is present in Madagascar, strains bearing this mutation do not appear to be responsible for chloroquine failures. This also suggests that *in vivo* there is not a significant fitness advantage for pfcrt K76T-bearing parasites in the majority of patients in Madagascar and other factors are involved in CQ therapy failures.

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HOME MANAGEMENT OF MALARIA EPISODES AMONG THE UNDERFIVES PRIOR TO ACT IMPLEMENTATION IN AN URBAN SETTING

Dickson S. Nsagha

University of Buea, Buea, Cameroon

In Cameroonian urban settings mothers of underfives always give some sort of home treatment when their children have fever or hot body, but their actions are always not appropriate. The objective of this study was to identify the best strategy to be used in the implementation of artemisinin-based combination therapy in the home management of malaria among children below five years in an urban setting/ An exploratory study was conducted in an urban environment in a malaria high risk area. Focus group discussions and in-depth interviews were conducted among mothers children of all age groups, men, health staff, community guides, community health workers and opinion leaders. Home treatment of underfive malaria by mothers is common in urban settings. The treatment of malaria among children is guided by the way mothers perceive signs and symptoms of the disease. The frequent change of malaria first line drugs by the national health policy and financial difficulties are the main problems mothers faced in treating children with hot body at home. Pre-packaged drugs for malaria treatment in children and the availability and use of rapid diagnostic tests (RDTs) would be highly appreciated if offered at an affordable price. Cultural barriers within the urban setting and religious beliefs might hinder the delivery of these services. Though radio and television figure among the current sources of information within the community, cultural meetings, churches, schools and other public gatherings are the best venues for social mobilisation while community health workers and community leaders are the best sensitisation agents for positive behaviour change. Monetary incentives for community drug distributors was the most appreciated means , as this can be included in the combined price of pre-packed drugs and RDT. Potential challenges for motivation include getting the community to adhere through social mobilisation and sensitisation, and overcoming strong negative religious beliefs. In conclusion, before launching home management of malaria in an urban setting, there is need for proper education, sensitisation, and social mobilisation as well as continuous monitoring and evaluation of field activities to ensure that there are no logistic problems with the delivery of pre-packed drugs and RDTs within the framework of the intervention activities.

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DETECTION OF MINORITY-VARIANT CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM* BY A NON-RADIOACTIVE HETERODUPLEX TRACKING ASSAY

Jonathan J. Juliano[1], Milijaona Randrianarivelojosia[2], Steven R. Meshnick[1]

[1]University of North Carolina, Chapel Hill, NC, United States, [2]Institut Pasteur de Madagascar, Antananarivo, Madagascar

Most *Plasmodium falciparum* infections are polyclonal and conventional PCR genotyping methods are insensitive to minority variants. Previously we described a heteroduplex tracking assay (HTA) for pfcrt (*Plasmodium falciparum* chloroquine resistance transporter gene) K76T-bearing *P. falciparum* minority variants which could detect the mutation in patients even when it was undetectable by standard PCR. However, as this assay required a radiolabeled probe, it could not be used in many resource limited settings. Here we describe a digoxigenin (DIG)-labeled chemiluminescent heteroduplex tracking assay (DIG-HTA) that is sensitive to pfcrt K76T-bearing minority variant parasites that are less than 5% of the parasite population. This method also allows for accurate quantitative analysis of wild type and mutant parasite populations. We examined 31 clinical *P. falciparum* isolates from 17 Malagasy children (15 initial parasitemias and 16 recurrent parasitemias) following chloroquine treatment. The pfcrt K76T mutation was detected in 2 of 17 (11.8%) patients, a prevalence higher than previously noted by conventional PCR techniques. Both of the isolates were minority variants, representing less than 5% of the parasites in the infection, and not detected by standard PCR. These findings suggest that genotypically chloroquine resistant *P. falciparum* parasites are more common than previously thought and exist as minority variants among circulating parasites in Madagascar. In addition, these results show assays sensitive for minority variants are useful in tracking the emergence of drug resistance alleles. The use of a non-radioactive label allows for the use of minority variants sensitive HTAs in areas where they were previously prohibited.

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DYNAMICS OF MALARIA PARASITE AND ANAEMIA PREVALENCE IN RURAL TANZANIA: COMMUNITY CROSS-SECTIONAL SURVEYS, 2001-2006

Rashid A. Khatib[1], Meredith L. McMorrow[2], Elizeus Kahigwa[1], Berty F. Elling[1], Peter B. Bloland[2], Salim M. Abdulla[1], S. Patrick Kachur[2]

[1]Ifakara Health Research and Development Centre, Dar-es-Salaam, United Republic of Tanzania, [2]Centers for Disease Control and Prevention, Atlanta, GA, United States

Since 2001, there have been substantial investments in malaria control interventions in Tanzania. As part of the Interdisciplinary Monitoring Project for Antimalarial Combination Therapy in Tanzania (IMPACT-Tz), we conducted repeated household surveys between 2001 and 2006 in two demographic surveillance sites (Ifakara DSS and Rufiji DSS) to compare trends in anemia in children less than five years of age and malaria parasitemia in consenting household members. Artesunate-SP was introduced as first-line treatment in health facilities in Rufiji at the start of 2003. SP remained the first-line treatment in Ifakara. In 2001, prevalence of parasitemia was higher in Rufiji (26%) than in Ifakara (18%). However, in 2004, parasite prevalence decreased to 19% in Rufiji and increased to 25% in Ifakara. In 2006, parasite prevalence decreased in both Rufiji (15%) and Ifakara (13%). Parasite prevalence was higher among children aged 1 to 5 years and 5 to 15 years in both study sites across all surveys (Rufiji, 36% and 30%; Ifakara, 27% and 29%). In Rufiji, the prevalence of parasitemia was much higher in infants (23%) than in adults (11%) while in Ifakara, infants and adults had equal parasite prevalence (12%). Anemia prevalence (<8g/dl) for children under five in Rufiji declined from 23% in 2004 to 16% in 2006. In Ifakara, it was 12% in 2004, increased to 18% in 2005, and declined to 10% in 2006. Overall, the prevalence of anemia was significantly higher among infants than children aged 1 to 5 years in Rufiji (27% vs 17%, (p=0.027). In Ifakara there was no statistically significant difference between anemia in infants (16%) and children aged 1 to 5 years (13%). Although different malaria control interventions were introduced in Rufiji and Ifakara, malaria parasitemia and anemia decreased in both DSS sites from 2001 to 2006. Other factors that may have influenced malaria parasitemia and anemia include ITN use, climate and treatment-seeking behavior.

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KENYAN POST-ELECTION VIOLENCE 2007-2008: USE OF A DEMOGRAPHIC SURVEILLANCE SYSTEM TO DOCUMENT THE DEMOGRAPHIC AND HEALTH BURDEN OF INTERNALLY DISPLACED PERSONS

Daniel Feikin[1], Kubaje Adazu[1], David Obor[1], Peter Ofware[1], John Vulule[2], Mary Hamel[1], Kayla Laserson[1]

[1]Centers for Disease Control and Prevention, Kisumu, Kenya, [2]Kenya Medical Research Institute, Kisumu, Kenya

The disputed Kenyan presidential election of December 27, 2007 led to widespread post-election violence (PEV). An estimated 1,200 people were killed during the PEV and more than 300,000 persons were forced to flee their homes. The KEMRI/CDC Demographic Surveillance System (DSS) in rural, western Kenya, a malaria-endemic area, was the final destination for many internally displaced persons (IDP) from other parts of the country. The DSS censuses households 3 times per year and registered 213,719 residents on April 22, 2008. In response to PEV, an IDP registration form was implemented in early March 2008. The form captures basic demographics and arrival information on all IDPs. Sick visits and hospitalizations by IDP children to facilities within the DSS were also documented. As of April 22, 2008, 6,626 IDPs had been registered (in-migration rate of 31 IDPs per 1,000 permanent DSS residents.) Most arrived in January (50%) and February (40%). IDPs resided in 2,344 (6.4%) of 36,518 compounds; the median number of IDPs per compound was 4 (range 1-22). The median age of IDPs was 19 years, with 20% under 5 years. IDPs mostly came from provinces with little malaria -- Rift Valley (38%), Nairobi (32%) and Central (14%). The median number of years living in that province was 5 (range <1-69 years). To date, no deaths among IDPs have been recorded in the DSS. Among IDP children from March 8 - April 9, monthly rates of 50 outpatient sick visits and 15 hospitalizations per 1,000 IDP children were recorded, compared with 27 outpatient visits and 4.6 hospitalizations per 1000 resident children (p<0.001 for both). The most common diagnosis for IDP sick visits was malaria -- 70% of outpatient and 77% of inpatient, which are similar percentages as resident children. In conclusion, most IDPs came from non-malarious areas and are at risk for severe malaria. Although an increased rate of malaria sick visits and hospitalizations was seen among IDP children, no excess malaria deaths have yet been noted in IDP children or adults. The rapid influx of so many IDPs will undoubtedly strain the resources of an area with pre-existing economic and health-related problems.

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A HOLISTIC VIEW OF THE LONG-TERM IMPACT OF MALARIA INTERVENTION STRATEGIES

Michelle L. Gatton[1], Qin Cheng[2]

[1]Queensland Institute of Medical Research, Brisbane, Australia, [2]Australian Army Malaria Institute, Brisbane, Australia

For thousands of years *Plasmodium* parasites and humans have co-existed and evolved in regions where suitable climatic conditions and vector populations are present. There is a balance between these factors which generates different levels of endemicity and disease. In an effort to reduce the burden of the disease numerous intervention strategies and efforts have been employed over the last 70 years. These include, but are not limited to, vector control, improved disease diagnosis and treatment of clinical cases, insecticide treated bednets and intermittent preventative treatment. The variation in implementation of intervention strategies and numerous endemic settings leads to a situation where it is extremely difficult to compare the results, especially since the interactions between the human host, mosquito and parasite are complex are intimately linked. For the first time, a complete stochastic simulation model of malaria transmission in villages of 1000 people has been used to investigate the long-term outcomes of different intervention strategies. Different levels of transmission have been simulated, and the influence that seasonality has on the effect of the intervention was also investigated. As expected the simulation results indicate that interventions have different long-term outcomes, however other, more subtle changes, can also occur. The results suggest that the parasite population is very plastid to external pressure and even small changes in chemotherapy regimes can alter the population structure in endemic regions. These selective pressures have long-term effects that reduce the prevalence of clinical cases while asymptotic carriage increases.

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THE IMPACT OF ACCESS TO PRIMARY HEALTH CARE ON THE INCIDENCE OF CLINICAL MALARIA IN CHILDREN

Wendy P. O'Meara[1], Abdisalan Noor[2], Benjamin Tsofa[3], F. Ellis McKenzie[1], Kevin Marsh[4]

[1]Fogarty International Center, National Institutes of Health, Bethesda, MD, United States, [2]KEMRI/Wellcome Trust Collaborative Program, Nairobi, Kenya, [3]Ministry of Health, Kilifi, Kenya, [4]Kenya Medical Research Institute, CGMRC/Wellcome Trust Collaborative Program, Kilifi, Kenya

The convergence of malaria endemicity and poor health care infrastructure has resulted in persistently high rates of malaria morbidity and mortality in many parts of sub-Saharan Africa. Reducing the burden of disease in these countries is frequently limited by the difficulty of disseminating interventions through weak health care infrastructure. Primary care facilities are increasingly becoming the focal point for distribution of intervention strategies, but physical access to these facilities may limit the

extent to which communities can be reached. Here we investigate the impact of access to primary care on the incidence of hospitalized malaria episodes in a rural district in Kenya. Our analysis of the spatial distribution of hospitalized malaria with respect to proximity to primary care facilities demonstrates the potential of primary health care to reduce the burden of malaria disease, but also shows that lack of physical access to primary care facilities is still an important risk factor for developing life-threatening malaria, one that may be inequitably distributed to the poorest households. Children who live a short distance to the nearest public health facility experience less than half of the serious malaria episodes as those living more than an hour away. In a typical rural district in Kenya, the protective efficacy of the primary care facilities in reducing hospitalization with malaria was 33%. Improved access to primary health facilities may reduce the burden of disease by as much as 66%. The protection afforded by access to treatment and prevention delivered through imperfect health systems is equivalent to that offered by alternative interventions in carefully controlled efficacy studies. This suggests that improvements in access to primary care have tremendous potential to increase the effectiveness of existing interventions. Global targets for malaria prevention, treatment, and disease reduction are unlikely to be achieved without considerable investment in health delivery systems. The potential for reducing the burden of malaria by investing in primary health care systems is considerable and should not be neglected in the context of scaling-up of malaria control programs.

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PATTERN OF CORD, PLACENTAL AND POST-DELIVERY MATERNAL MALARIA PARASITAEMIA IN CROSS RIVER STATE, NIGERIA

Chioma M. Oringanje, Martin M. Meremikwu

Institute of Tropical Disease, Research and Prevention, Calabar, Nigeria

Malaria is responsible for about 25% of under-five mortality and 11% of maternal mortality in Nigeria. National and local efforts to reduce high malaria disease burden in these vulnerable groups have increased remarkably following the Abuja declaration on Roll Back Malaria in April 2000. Maternal, cord and placental parasitaemia are important indices of malaria in pregnancy. As part of effort to evaluate current status of malaria in pregnancy, we assessed the rates of maternal, cord and placental malaria parasitaemia in Cross River State, an area of Nigeria with perennial and high transmission of mostly *Plasmodium falciparum* infections. The objective of this study was to determine the prevalence of maternal, placental and cord malaria parasitaemia, and the relationship with season, parity and place of residence (rural or urban). A total of 626 pregnant women who delivered at two rural health facilities and two urban facilities. Deliveries were consecutively enrolled if the mothers consented. Demographic data were obtained from the women at delivery, maternal, placental, and cord blood samples were collected for microscopy. Of the 626 women examined, 120 (19.2%) were positive for malaria parasite; 69 of the 469 (14.7%) placenta samples and 62 of 459 (13.5%) cord samples were positive for malaria parasites. Parasitaemia rates in the rainy season were higher than in the dry season (20.3%, 17.4%, 16.1% vs 18.0%, 10.2%, 9.2%) for maternal, placental and cord respectively. Higher rates of parasitaemia also was recorded among the urban (21.7%, 16.3%, 15.5%) than the rural (16.3%, 13.1%, 11.5%) for maternal, placental and cord respectively. There was no significant difference between parasitaemia levels due to parity. In conclusion, the prevalence rates of cord, placental and maternal malaria parasitaemia at delivery are high in this area, and correlates with poor control indices. These rates are expected to decline with the scale up of malaria control efforts boosted by the award of grants by Global fund for control of malaria, TB and HIV.

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BOTTLENECKS FOR HIGH COVERAGE OF INTERMITTENT PREVENTIVE TREATMENT IN PREGNANCY IN A RURAL AREA IN BURKINA FASO

Koen Peeters, S. Gies, S. O. Coulibaly, C. Ky, U. D’Alessandro

ITM, Antwerp, Belgium

Intermittent preventive treatment with sulfadoxine-pyrimethamine for pregnant women (IPTp/SP) is currently being scaled up in many countries in sub-Saharan Africa. However, despite high ANC attendance, coverage with the required 2 doses of SP remains low. This is particularly true for adolescent mothers, often primigravidae, who have a higher risk of malaria infection resulting in a higher risk of anaemia and low birth weight. Reasons for lower coverage among adolescent pregnancies were investigated using a combination of qualitative and quantitative research methods. A focused ethnography evaluating obstacles for IPTp/SP was carried out in the framework of a large health centre randomised trial. Field work comprised participatory observation, focus group discussions, in-depth interviews and standardized half-open questionnaires. Qualitative data were continuously analysed and research techniques adapted until saturation of results was obtained. Data were further interpreted and compared with those obtained by the health centre randomised trial which identified and followed up 2,288 primi- and secundi-gravidae. Antenatal clinic attendance and coverage with at least 2 doses of IPTp-SP were lower in adolescent mothers (<20 years old) who were also less affected by the health promotion campaign, particularly during the rainy season. Such vulnerability could be explained by 3 main factors related the adolescents’ social position. As newcomers into their husbands’ households, adolescents have a heavier work load, particularly during the labour-intensive rainy season, limiting their mobility and access to antenatal care. Traditionally, pregnancies should be socially acknowledged before women attend the health centre, delaying their first attendance. This is further enhanced by the adolescent mothers’ characteristic shame in publicising their pregnancies in a public space such as a health centre. In conclusion, adolescent mothers need to be specifically targeted by a health promotion campaign able to overcome the structural constraints limiting their access to health interventions.

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ASSESSING THE CORRELATION BETWEEN GROWTH INHIBITION ACTIVITY AND MALARIA RISK IN A LONGITUDINAL STUDY IN MALI

Peter D. Crompton[1], Kazutoyo Miura[2], Boubacar Traore[3], Kassoum Kayentao[3], Aissata Ongoiba[3], Greta Weiss[1], Safiatou Doumbo[3], Didier Doumtabe[3], Younoussou Kone[3], Chiung-Yu Huang[4], Ogobara K. Doumbo[3], Louis H. Miller[2], Carole A. Long[5], Susan K. Pierce[1]

[1]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Immunogenetics, Bethesda, MD, United States, [2]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Malaria Vaccine Development Branch, Bethesda, MD, United States, [3]Malaria Research and Training Center, Faculty of Medicine, Pharmacy and Dentistry, University of Bamako, Bamako, Mali, [4]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Biostatistics Research Branch, Bethesda, MD, United States, [5]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Malaria and Vector Research, Bethesda, MD, United States

Immunity to the asexual blood stage of *Plasmodium falciparum* is complex and likely involves several effector mechanisms. Antibodies are thought to play a critical role, and a corresponding *in vitro* correlate has long been sought to facilitate malaria vaccine development. The Growth Inhibition (GI) assay, measures the capacity of antibodies to limit erythrocyte invasion and/or growth of *P. falciparum in vitro*. In *Aotus* monkeys, GI activity induced by MSP1 vaccination correlates with protective immunity. In humans, naturally-acquired and vaccine-induced antibodies have GI

activity, but it is unclear if this correlates with protection from malaria. In a longitudinal study in Mali, purified IgG from plasma collected before the 6 month malaria season was assayed for GI activity in 171 individuals aged 2-10 years. The number of malaria episodes was determined by passive surveillance over the subsequent malaria season. Logistic regression analysis showed that increased age and GI activity were independently associated with decreased malaria risk (OR 0.76, p=.002; OR 0.97, p=.038, respectively); however, both age and GI activity lacked the sensitivity and specificity to accurately classify individuals as protected or susceptible to malaria. Studies in other epidemiological settings are needed to fully understand the relationship of GI activity and malaria risk.

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PROFILING PROTECTIVE HUMORAL IMMUNE RESPONSES TO *PLASMODIUM FALCIPARUM* BY PROTEIN MICROARRAY IN A LONGITUDINAL STUDY IN MALI

Peter D. Crompton[1], Matt Kayala[2], Boubacar Traore[3], Greta Weiss[1], Chad Burk[4], Kassoum Kayentao[3], Aissata Ongoiba[3], Safiatou Doumbo[3], Louis H. Miller[5], Ogobara K. Doumbo[3], Denise L. Doolan[6], Pierre Baldi[2], Philip L. Felgner[4], Susan K. Pierce[1]

[1]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Immunogenetics, Bethesda, 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]Department of Medicine, Division of Infectious Diseases, University of California, Irvine, CA, United States, [5]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Malaria Vaccine Development Branch, Bethesda, MD, United States, [6]Vaccine Immunology Laboratory, Queensland Institute of Medical Research, Brisbane, Australia

Passive transfer studies in humans demonstrate that humoral immunity contributes to protection from malaria, however, seroepidemiologic studies have been limited to evaluating antibody responses to one or a few antigens at a time, and have not yielded unequivocal correlates of protection. The completion of the *Plasmodium falciparum* genome, and the advent of high-throughput cloning methods and a highly efficient *in vitro* transcription/translation system led to the development of a protein microarray representing 1200 known and hypothetical *P. falciparum* proteins (2320 ORFs). In a longitudinal study in Mali of 225 individuals aged 2 to 25 years, plasma samples collected before and after the malaria season were probed against this microarray. Analysis of antibody profiles and clinical malaria outcomes has provided insight into the repertoire of antibodies against known and hypothetical proteins that correlates with anti-disease immunity. Data also shed light on the overall gene ontology- and stage-specific humoral immune response to *P. falciparum*. Validation of this microarray in other epidemiologic settings may provide the framework for a genome-based approach to the development of a multi-antigen malaria vaccine.

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THE MEMORY B CELL RESPONSE TO AMA1-C1/ALHYDROGEL® VACCINATION IN SEMI-IMMUNE ADULTS IN MALI, WITH OR WITHOUT THE CPG 7909 OLIGODEOXYNUCLEOTIDE ADJUVANT

Boubacar Traore[1], Safiatou Doumbo[1], Didier Doumtabe[1], Younoussou Kone[1], Abdrahamane Traore[1], Marko Mircetic[2], Ruth D. Ellis[3], Laura B. Martin[3], Louis H. Miller[3], Peter D. Crompton[2], Susan K. Pierce[2], Ogobara K. Doumbo[1]

[1]Malaria Research and Training Center, Faculty of Medicine, Pharmacy and Dentistry, University of Bamako, Bamako, Mali, [2]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Immunogenetics, Bethesda, MD, United States, [3]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Malaria Vaccine Development Branch, Bethesda, MD, United States

A likely requirement for an effective blood stage malaria vaccine is the capacity to induce vaccine-specific memory B cells (MBC) and plasma cells, B cell subsets known to be necessary for the maintenance of long term humoral immunity. Little is known about the cellular basis of the humoral immune response to *Plasmocium falciparum,* and there is no published data on the MBC response to candidate malaria vaccines administered in malaria endemic areas. Recent advances permit the phenotypic and functional identification of MBC by flow cytometry and ELISPOT assay. Within a phase I study in which 24 Malian adults were randomized to receive AMA1-C1/Alhydrogel®±CpG 7909, we are investigating the total IgG and AMA1-specific MBC response. Apical membrane antigen-1 (AMA1) is a protein expressed during the asexual blood stage of *Plasmodium falciparum*, and CpG 7909 has been shown to enhance B cell responses to co-administered antigens via toll-like receptor 9. At the end of the malaria season, individuals were vaccinated on days 0 and 28 and peripheral blood mononuclear cells (PBMC) were isolated from venous blood on days 0, 3, 28, 35, 42, 90 and 210. For the ELSIPOT assay (in progress) thawed PBMC are cultured for 5 days with *S. aureus* Cowan, CpG, and pokeweed extract, polyclonal mitogens that drive the differentiation of MBC into antibody secreting cells. Total and AMA1-specific IgG secreting cells are detected on plates coated with anti-human IgG or AMA1, respectively. PBMC are also stained with fluorescently-labeled antibodies against CD19, CD27 and CD38 for phenotypic analysis. We have applied the same ELISPOT assay to a phase 1 trial of AMA1-C1/Alhydrogel®±CpG 7909 in the U.S., and demonstrated that AMA1 vaccination generates AMA1-specific MBC in malaria-naïve individuals, and that this response is significantly enhanced by CpG 7909. In light of evidence that *P. falciparum* may modulate the host immune response, it is of interest to compare the responsiveness to AMA1±CpG in malaria-naïve vs. semi—immune individuals recently exposed to malaria, since significant differences may have implications for the design and implementation of malaria vaccines.

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VACCINATION WITH MSP142-C1/ALHYDROGEL® GENERATES ANTIGEN-SPECIFIC MEMORY B CELLS IN MALARIA-NAÏVE U.S. ADULTS AND THE CPG 7909 OLIGODEOXYNUCLEOTIDE ADJUVANT ENHANCES THIS RESPONSE

Marko Mircetic[1], Greta Weiss[1], Gregory E. Mullen[2], Laura B. Martin[2], Louis H. Miller[2], Susan K. Pierce[1], Peter D. Crompton[1]

[1]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Immunogenetics, Bethesda, MD, United States, [2]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Malaria Vaccine Development Branch, Bethesda, MD, United States

Long term humoral immunity requires the generation and maintenance of memory B cells (MBC) and plasma cells, thus an effective blood-stage malaria vaccine would be expected to induce this response. Recent studies suggest that merozoite surface protein-1 (MSP1), a blood stage vaccine

candidate, interferes with this response due to multiple disulfide bonds that impede antigen processing, and T cell epitopes that down-regulate CD4+ T cells which provide B cell help. Therefore it is of interest to directly assess the human MBC response to MSP1 and to compare it to that of other blood-stage vaccine candidates. Within a phase I study we evaluated the MBC response of 20 malaria-naïve individuals randomized 1:1 to vaccination with $MSP1_{42}$-C1/Alhydrogel®±CpG 7909, a toll-like receptor 9 agonist shown to enhance B cell responses to co-administered antigens. Individuals were vaccinated on days 0, 28 and 56 and peripheral blood mononuclear cells (PBMC) were isolated on days 0, 7, 28, 31, 56, 59 and 140. Thawed PBMC were cultured for 5 days with *S. aureus* Cowan, CpG, and pokeweed extract; polyclonal mitogens that induce the differentiation of MBC into antibody secreting cells. Total and $MSP1_{42}$-specific IgG secreting cells were detected on plates coated with anti-human IgG or $MSP1_{42}$, respectively. In the CpG group, $MSP1_{42}$-specific MBC, expressed as a percentage of total IgG MBC, were increased over background 28 days after the 2nd vaccine on day 56 (0.97% [95% CI, 0.50-1.43]; p<0.001), peaked 3 days after the 3rd vaccine on day 59 (1.19% [95% CI, 0.71-1.68]; p<0.001) and declined to 0.82% on day 140 (p=0.001). In the non-CpG group, an increase over background was also observed on day 56 (0.09% [95% CI, 0.03-0.144]; p=0.005), day 59 (0.07% [95% CI, 0.01-0.13]; p=0.022), and day 140 (0.12% [95% CI, 0.07-0.18]; p<0.001), but it was lower than the CpG group (p<0.05 for days 56, 59 and 140). These data demonstrate that vaccination with $MSP1_{42}$-C1/Alhydrogel® generates antigen-specific MBC in malaria-naïve individuals and that CpG 7909 enhances this response. Interestingly, under the same experimental conditions, our analysis of a phase I trial of AMA1/Alhydrogel®±CPG 7909 in malaria-naïve individuals shows that AMA1 induces a higher percentage of vaccine-specific MBC, suggesting that *Plasmodium falciparum* antigens may indeed vary in their capacity to generate MBC.

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SOME CHILDREN THAT LACK MEROZOITE SURFACE PROTEIN-1(MSP1) SECONDARY PROCESSING-INHIBITORY ANTIBODIES STILL POSSESS $MSP1_{19}$-SPECIFIC ERYTHROCYTE INVASION-INHIBITORY ANTIBODIES

Muyideen K. Tijani[1], Munira Grainger[2], Alex A. Odaibo[3], Chiaka I. Anumudu[4], Henrieta O. Awobode[3], Olajumoke A. Morenikeji[3], Yusuf Omosun[5], Christian M. Okafor[6], Josiane Ngoundou-Landji[1], Micheal C. Asuzu[7], Adanze Asinobi[8], Brendan S. Crabb[9], Holder A. Anthony[2], Roseangela I. Nwuba[4]

[1]Cellular Parasitology Programme, Department of Zoology, University of Ibadan, Ibadan, Nigeria, [2]Parasitology Unit, National Institute For Medical Research, London, United Kingdom, [3]Parasitology Unit, Department of Zoology, University of Ibadan, Ibadan, Nigeria, [4]Cellular Parasitology Programme, Department of Zoology, University of Ibadan, Ibadan, Nigeria, [5]Division of Parasitic Diseases, Center for Disease Control and Prevention, Atlanta, GA, United States, [6]Seattle Biomedical Research Institute, Seattle, WA, United States, [7]Department of Community Medicine, College of Medicine, University of Ibadan, Ibadan, Nigeria, [8]Department of Peadiatrics, University College Hospital, Ibadan, Nigeria, [9]Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Australia

The C-terminal 19-KDa fragment of *Plasmodium falciparum* merozoite surface protein 1 elicits the production of protective antibodies against erythrocyte invasion. Previous *in vitro* studies have suggested that MSP1 processing inhibitory antibodies have erythrocyte invasion- inhibitory activity. However, the mechanism as well as the relationship between these antibodies and other potentially protective $MSP1_{19}$- specific antibodies in individuals naturally exposed to malaria remains unclear. To understand this, plasma samples from 148 Nigerian children 12 years and below, which had been previously characterized for the presence of MSP 1 secondary processing activity, were analyzed for the presence of invasion inhibitory antibodies using a functional assay based the on difference in invasion rate between wild-type *P. falciparum* D10 and its transgenic PcMEGF derivative. Out of the children studied, approximately 46% had invasion inhibitory antibodies while 6% had processing inhibitory antibodies. There was a low prevalence (2.7%) of individuals that had both the processing-inhibitory and invasion-inhibitory antibodies. The prevalence of children with invasion inhibitory antibodies increase across the age groups, 0-6 months(27%), 7months-2years(37.5%), 2.5-5 years (57.7%), and 6-12years (64%). There was a relatively low parasitemia in children with high mean invasion inhibitory antibody levels. There was no significant correlation between total anti-$MSP1_{19}$ antibodies and invasion inhibitory antibodies detected using the functional assay. This work shows that children living in malaria endemic regions develop antibody-dependent erythrocyte invasion inhibitory mechanisms independent of $MSP1_{42}$ processing-inhibitory activity. The coexistence or otherwise of these antibodies may have an implication on protection against malaria and may be used as a marker for protective immunity for individuals living in malaria endemic regions.

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TRANSPLACENTAL TRANSFER OF ANTIBODIES TO THE FETUS THAT COULD PROTECT INFANTS FROM MALARIA

Patrick T. Wilson[1], Peter Mungai[2], Indu Malhotra[2], Chris King[2], Arlene Dent[1]

[1]Rainbow Babies and Children's Hospital, Cleveland, OH, United States, [2]Case Western Center for Global Health and Diseases, Cleveland, OH, United States

Infants born in malaria endemic areas rarely develop clinical malaria until after 6 months of age. Transplacental transfer of antibodies from partially immune women during pregnancy to their fetus is thought to provide passive protection. We use a Growth Inhibitory Assay (GIA) that measures antibodies in the newborn's cord blood (CB) and at 6 months that inhibit parasite invasion and growth in host erythrocytes. Our overall hypothesis is that children who acquire high levels of GIA antibodies will be protected from malaria during infancy. Maternal infection was determined by examining samples of maternal venous, placental intervillous and CB for the presence of *Plasmodium falciparum* (Pf) in women residing in a malaria endemic area of Kenya. CB and 6 month plasma were dialyzed to retain antibodies and to remove drugs and other factors that may inhibit parasite growth and tested against 3 laboratory Pf strains (PfD10, W2mef, 3D7). GIA was determined by measuring the amount of parasite growth inhibition after 24 hours of culture in Kenyan CB (n =104) and 6 month plasma (n= 37). Control CB was obtained from 4 North American deliveries. Overall median GIA levels in CB varied with Pf strain (PfD10, 19% (0-81%); W2Mef, 27% (0-80%); 3D7, 33% (0-88%) inhibition; p<0.001), although there was good correlation of GIA levels among Pf strains (R=0.68-0.76). CB from offspring of malaria-infected women (n=8) had lower GIA levels compared to CB from uninfected women (n=85). When these 8 CB samples from mothers with malaria infection were age matched to CB samples from mothers without evidence of malaria infection, a statistically significant decrease in GIA was observed (p<0.05). Paired 6 month GIA was significantly lower than CB GIA with PfD10 (p<0.01) and 3D7 (p=0.05) strains. Kenyan CB samples display a wide range of GIA to blood stage malaria parasites. Lower CB GIA associated with maternal malaria infection could reflect consumption of protective antibodies during infection or may represent a baseline lack of functional antibodies in these mothers making them more susceptible to malaria infection. The trend of decreased GIA at 6 months compared to CB may reflect the physiological waning of maternal antibodies at this age. Further CB and 6 month samples will be analyzed. Correlating GIA levels, maternal malaria infection and infant susceptibility to malaria disease are endpoints in the ongoing study.

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IMMUNOGLOBULIN G SUBTYPE RESPONSES TO UB05, A DOMINANT *PLASMODIUM FALCIPARUM* ANTIGEN BY INDIVIDUALS LIVING IN A HIGH TRANSMISSION ENDEMIC AREA OF THE CAMEROONIAN RAINFOREST

Anong D. Nota

University of Buea, Buea, Cameroon

Differential immunoscreening of a 3D7 *Plasmodium falciparum* library using high titred sera from susceptible and immune subjects together with bioinformatics analysis, as reported previously, identified a hypothetical malaria antigen (UB05) on chromosome 10 of *P. falciparum*. This has been submitted to genbank with accession number DQ235690.This antigen which has been cloned and expressed is presently being characterized for its role in protective immunity against malaria. The aim of this study was to find out if the newly identified UB05 antigen elicites protective antibody immune responses. 180 randomly selected adults and children were studied. Demographic and clinical data were collected. Subjects were split into three age groups 1-5 (infants),10-14years(children) and ≥18 years(adults) each having equal numbers of both *P. falciparum* positive and negative individuals. Fever was defined as temperature ≥ 37.5°C and anaemia as PCV≤ 31%. Sera from each individual was analysed by ELISA for antibodies to UB05 IgG subclasses.The absorbance was measured at 405nm.The data were analysed with SPSS 11.0 IgG levels to all subclasses of UB05 increased with age and were significantly higher in adults than in infants (P<0.05). IgG1 and IgG3 were significantly higher in adults than in children (p<0.05). IgG3 responses were significantly higher than IgG1 in all age groups (p<0.0001).There was a negative correlation of IgG3 antibodies and parasite density. Individuals with fever had Lower levels of IgG3 as compared with those without fever and this was most obvious in children. IgG2 and IgG4 responses to UB05 were generally higher in infected subjects than in uninfected subjects. This data strongly suggest that immune response to UB05 in individuals residing in Bolifamba is preferentially cytophilic and is acquired with age.

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CYTOKINE PROFILE IN MURINE MODEL OF PREGNANCY-ASSOCIATED MALARIA

Rosette Megnekou[1], Trine Staalsoe[2], Lars Hviid[2]

[1]Faculty of Sciences and the Biotechnology Center, University of Yaounde I, Cameroon, Yaounde, Cameroon, [2]Center for Medical Parasitology, Department of International Health, University of Copenhagen, Copenhagen, Denmark

In areas of stable *Plasmodium falciparum* transmission, women become susceptible to malaria infection during pregnancy, despite pre-existing acquired immunity. This susceptibility however decreases with increasing parity. Pregnancy-associated malaria (PAM) is a major cause of maternal anemia, intra-uterine growth retardation, pre-term deliveries (PTDs) and low birth weight. Available evidence suggests that poor pregnancy outcome and placental pathology in humans are associated with cytokine changes. It has been shown that pregnant *P. berghei* K173-infected Balb/C mice develop higher parasitemias compared to non-pregnant mice, but the etiology is obscure. We have therefore conducted series of experiments to develop an experimental immune murine model of PAM and investigate Th1 and Th2 cytokine profile by luminex assay. We found that recrudescence rates were lower during the 2nd than during the 1st pregnancy and that pregnancy-associated recrudescences decreased with increasing parity. Hemoglobin levels and the proportion of mice with anemia correlated with parity ($P(r_s=0.40)<0.001$ and $P(\chi^2=14.5)<0.001$, respectively). Corresponding correlations were observed in parasitemic group and the proportion of mice with patent parasitemia ($P(r_s= -0.40) <0.001$ and $P(\chi^2=9.3)=0.009$, respectively). There was massive accumulation of the parasites in the kidney and the intervillous spaces of the placenta, with placental parasitemia higher (p=< 0.001) than the peripheral. Also, pregnancy status boosted cytokine production. In primigravid mice, apart from IL-10 ($P (r_s= -0.18) = 0.16$), concentrations of IL-5, IL-12p/70, IL-13, IFN-γ, TNF-α correlated with hemoglobin level ($P(r_s= \geq 0.5) =<0.001$). Correlations were observed with respect to levels of parasitemia with the same cytokines ($P(r_s= - \geq 0.7) =<0.001$), except IL-10 ($P(r_s= 0.16) =0.23$). IL-10 decreased with increasing parity ($P(r_s=- 0.25)= 0.004$) while concentrations of the other cytokines increased with parity ($P(r_s= \geq 0.35)= <0.001$). Elevated IL-10 and decreased of other cytokines significantly associated with PTDs. These data suggest that alteration in cytokine levels may strongly contribute to poor pregnancy outcome due to impairment of the cellular immune response. Taken together, our mouse model reproduces pathogenesis of women PAM and may be useful to study PAM-related research questions that cannot be pursued in studies of women with PAM.

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IDENTIFY B-CELL EPITOPES IN DUFFY BINDING PROTEIN ASSOCIATE WITH PROTECTION *PLASMODIUM VIVAX* INVASION

Patchanee Chootong[1], Kelly M. VanBuskirk[1], Francis B. Ntumngia[1], Moses Biasor[2], Christopher L King[3], John H. Adams[1]

[1]Global Health Infectious Disease Research Program, University of South Florida, Tampa, FL, USA, [2]Papua New Guinea Institute for Medical Research, Madang, Papua New Guinea, [3]Division of Geographic Medicine, Case Western Reserve University, Cleveland, OH, USA

Plasmodium vivax infects 70-80 million persons per year, causing substantial morbidityworldwide. The Duffy binding protein (DBP) plays a vital role in maintaining the erythrocytic cycle of *P. vivax* infection, making it an important candidate for inclusion in a vaccine against this parasite. The interaction of DBP with its receptor, the Duffy blood group antigen, is required for junction formation, the first irreversible step of invasion. Naturally occurring antibodies to DBP are prevalent in individuals living in areas of high malaria endemicity, but individuals show distinct quantitative and qualitative differences in their serological responses to the antigen. Polymorphisms in the *dbp* gene are clustered in its ligand domain (region II), suggesting immune selection pressure drives diversity in antibody-accessible, functionally sensitive residues. We studied the anti-DBP-specific responses in the residents of a malaria endemic region of Papua New Guinea (PNG), which is highly endemic for *P. vivax*. Measurement of antigen-specific titers to DBP demonstrated significant changes in titer over time. Surprisingly, a range of inhibition from complete to none was observed even among high responders to the DBP, indicating that some anti-DBP antibodies are considerably more effective at blocking binding. Using these immune sera we identified ten dominant B-cell epitopes on the DBP ligand domain that correlated with the protective inhibitory responses. Many of the B-cell epitopes contain polymorphic residues and most are surface exposed on the DBP ligand domain. These characteristics are consistent with strain-specific responses observed in PNG residents. A better understanding of the DBP epitopes that are targeted by protective natural immunity will aid a rationale approach to vaccine development that can prevent *P. vivax* infections.

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MALARIA RECRUDESCENCE IN MICE PREGNANCY

Rita Neres[1], Claudio R. Marinho[1], Lígia A. Gonçalves[1], Manuela B. Catarino[2], Carlos Penha-Gonçalves[1]

[1]Instituto Gulbenkian de Ciencia, Oeiras, Portugal, [2]Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal

Malaria has been shown to be associated with increased morbidity during pregnancy, leading to severe maternal anemia and low birth weight, responsible for a vast loss of life. It has been reported that primigravida women are more susceptible to malaria in pregnancy as compared to multigravida. These observations have raised the hypothesis that immunological protection is cumulative and directed towards parasite antigens specifically expressed in pregnant hosts. Early studies reported

that female mice exposed to *Plasmodium berghei* and treated to cure show in subsequent pregnancy loss of resistance and parasitemia recrudescence. We are setting up an experimental system that uses BALB/c females infected with *P. berghei* ANKA to study the malaria pathogenesis across multi-parity. Our preliminary results show that parasitemia recrudescence was not observed in all pregnant females. Nevertheless, the proportion of recrudescent females was higher among primigravidae as compared to multigravidae. In contrast, non-pregnant females never showed malaria recrudescence. These results indicate that malaria recrudescence in pregnant mice is attributable to the pregnancy status. Moreover, the results also suggest that the incidence of pregnancy malaria in mice is reduced by cumulative exposure. This experimental system constitutes an important tool to study the immunological components and mechanisms that protect multigravidae from pregnancy associated malaria.

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IGG ANTIBODIES AGAINST MSP-1 (19-KDA) IN PATIENTS INFECTED WITH DIFFERENT *PLASMODIUM FALCIPARUM* GENOTYPES IN IQUITOS, PERU

Katherine Torres

Instituto de Medicina Tropical, Lima, Peru

In order to evaluate the relationship between the genetic parasite diversity and the immune response (humoral response) generated in the host to subsequent infections, we evaluated the IgG response in 61 adults infected with *Plasmodium falciparum* by means of ELISA assay. The previous results were used to correlated with the MSP-1 block 2 genotypes (K1 and Mad-20) found in these patients in this particular area. Our aim was to compare the humoral response (IgG) and these 2 genotypes. The molecular and immunological analyses were performed using dried blood spotted on filter paper (Whatman 3). We performed PCR genotyping of *P. falciparum* samples using MSP1 family - specific primers and the ELISA assays was performed using MSP1 block 17 antigen. We report here the results from patients which were positives and negatives to IgG response for both K1 and Mad20 genotypes (K1 Negatives n=12, K1 Positives n=22, Mad20 Negatives n=13, Mad 20 Positives n=14). There was no statistical significant difference between IgG response against MSP-1 19kD fragment and the genotypes K1 and Mad-20 MSP-1 block 2 (P=0.310620) found in the Amazon region (Iquitos-Peru). We do not have data about the relationship between IgG responses against MSP-1 block2 but we suggest that the whole humoral response against *P. falciparum* is an integrated system and the protection could be related to the response to each block of MSP-1 independently, one more than other .

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MSP1 AND MSP2-BASED ESTIMATES OF GENETIC DIVERSITY IN *PLASMODIUM FALCIPARUM* FROM THE ARTIBONITE VALLEY OF HAITI, 2006-2007

Berlin L. Londono[1], Thomas Eisele[1], Joseph Keating[1], Adam Benett[1], Ian Rawson[2], Donald J. Krogstad[1]

[1]Tulane University, New Orleans, LA, United States, [2]Hôpital Albert Schweitzer, Deschapelles, Haiti

Genetic diversity (the average number of parasite genotypes per infected individual) has been used as a proxy for the intensity of malaria transmission and may correlate with disease, including the risk of severe malaria. The purpose of this study was to examine the genetic diversity of *Plasmodium falciparum* parasites from Haiti based on the 3 allotypes of Block 2 in msp1 (K1, RO33, MAD20) and the 2 allotypes of Block 3 in msp2 (IC, FC27). After obtaining informed consent, filter paper blots were prepared from: 1] Adult volunteers in a population-based survey to estimate the prevalence of *P. falciparum* infection (active case detection) and 2] Individuals seeking diagnosis and treatment for malaria at the Outpatient Clinic of the Hôpital Albert Schweitzer (passive case detection). A total of 82 malaria cases (positive smears) for which filter paper blots were available were included in this study. Nine distinct msp1 Block 2 amplicons (differentiated by size and allelic type) were detected in 2006 and 2007 although with different distribution among alleles in each year. For example, RO33 was the most frequent msp1 allele in 2006, whereas MAD20 was most frequent in 2007. RO33 was the least polymorphic allotype found, with only one genotype of 120 bp in both years. Both msp2 alleles were present, and IC was the most frequent allele in both years. To our knowledge, this study represents the first examination of genetic diversity in Haiti. These results suggest that there is little genetic diversity in Haiti, consistent with a low intensity of transmission.

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DETERMINATION OF GENETIC DIVERSITY OF VACCINE CANDIDATE ANTIGENS IN *PLASMODIUM VIVAX* ISOLATES FROM THE AMAZON BASIN OF PERU

Stella M. Chenet[1], OraLee H. Branch[2], Carmen M. Lucas[1], Benjamin J. Espinosa[1], David J. Bacon[1]

[1]Naval Medical Research Center Detachment, Lima, Peru, [2]University of Alabama at Birmingham, Birmingham, AL, United States

A major concern in malaria vaccine development is the polymorphism observed among *Plasmodium* isolates in different geographical areas across the world. *P. vivax* emerged in recent history in the Peruvian Amazon in 1991. Since 2000, there has been sustained low transmission of *P. vivax* at 1.6 infections/person/month estimated in a relatively high transmission community in years 2003-2004. In this study, we investigated the population diversity of *P. vivax* Peruvian isolates by DNA sequencing. Genetic diversity was characterized in polymorphic regions in genes encoding the circumsporozoite protein (CSP), merozoite surface protein-1 (MSP-1), apical membrane antigen (AMA-1), thrombospondin related anonymous protein (TRAP) and Duffy-binding protein (DBP) in 106 isolates Substantial genetic heterogeneity was found in dimorphic *CSP* gene especially in the repetitive region with a total of 35 different alleles with the majority (92.2%) belonging to the VK210 type. DBP was highly variable as well, with 33 alleles detected and the majority of the polymorphisms being nonsynonymous substitutions located in the ligand domain. We also identified 11 alleles in the MSP-1 high activity binding region (HARB 1) and TRAP and 9 alleles in AMA-1. Multilocus analysis of 95 infections with complete typing for these 5 loci revealed 71 different haplotypes. When considering alleles with ≥ 3% population frequency, there was only one haplotype that was observed more than once. We observed significant multilocus linkage between TRAP and AMA-1 (p=0.0002), TRAP and MSP-1 (p=0.0040) and TRAP and DBP (p=0.0081). There was no multilocus linkage in the other pairwise comparisons. These data indicate the existence of related allelic families within the vaccine candidate antigen genes; however, the presence of high genetic diversity and disequilibrium between the different loci suggests out-crossing in this region of recent and low *P. vivax* transmission.

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GENETIC ANALYSIS OF THE *DIHYDROFOLATE REDUCTASE-THYMIDYLATE SYNTHASE* GENE FROM GEOGRAPHICALLY DIVERSE ISOLATES OF *PLASMODIUM MALARIAE*

Naowarat Tanomsing[1], Mallika Imwong[1], Sasithon Pukrittayakamee[1], Kesinee Chotivanich[1], Sornchai Looareesuwan[1], Mayfong Mayxay[2], Christiane Dolecek[3], Tran Tinh Hien[3], Virgilio E do Rosario[4], Ana Paula Arez[4], Pascal Michon[5], Georges Snounou[6], Nicholas J White[1,7], Nicholas P J Day[1,7]

[1]Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, [2]Wellcome Trust-Mahosot Hospital-Oxford Tropical Medicine Research Collaboration, Microbiology Laboratory, Mahosot Hospital, Vientiane, Lao PDR, [3]Oxford University Clinical Research Unit, Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam, [4]Centro de Malária e outras Doenças Tropicais, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira, Lisboa, Portugal, [5]Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea, [6]Parasitologie Comparée et Modèles Expérimentaux USM[0307], Muséum National d'Histoire Naturelle, Paris, France, [7]Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, United Kingdom

Plasmodium malariae, the parasite responsible for quartan malaria, is transmitted in most malaria endemic areas, and is associated with significant morbidity. The sequence of the gene coding for the enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS) was obtained from field isolates of *P. malariae* and from the closely related simian parasite, *P. brasilianum*. The two sequences were nearly 100% homologous, adding weight to the notion that they represent genetically distinct lines of the same species. A survey of polymorphisms of the *dhfr* sequences in 35 isolates of *P. malariae* collected from 5 countries in Asia and Africa, revealed a low number of non-synonymous mutations in five codons. In five of the isolates collected in southeast Asia, a non-synonymous mutation was found at one of the three positions known to be associated with anti-folate resistance in other *Plasmodium* species.

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EXTENSIVE GENETIC DIVERSITY IN THE HUMAN MALARIA PARASITE *PLASMODIUM VIVAX*

Nadira D. Karunaweera[1], Marcelo U. Ferreira[2], John W. Barnwell[3], Anusha Munasinghe[4], Christopher King[5], Fumihiko Kawamoto[6], Daniel Hartl[7], Dyann F. Wirth[4]

[1]Faculty of Medicine, University of Colombo, Colmbo [8], Sri Lanka, [2]University of Sao Paulo, Sao Paulo, Brazil, [3]Center for Disease Control, Atlanta, GA, United States, [4]School of Public Health, Harvard University, Boston, MA, United States, [5]Case Western Reserve University, Cleveland, OH, United States, [6]Oita University, Oita, Japan, [7]Harvard University, Boston, MA, United States

The population structure of *Plasmodium vivax* is yet to be elucidated. The markers of choice for large-scale population genetic studies of eukaryotes, short tandem repeats known as microsatellites, have been recently reported to be little polymorphic in *P. vivax*. Here we investigate the microsatellite diversity and geographic structure in *P. vivax,* at both local and global levels, using 14 new markers consisting of tri- or tetranucleotide repeats. The local-level analysis, that involved 50 field isolates from Sri Lanka, revealed unexpectedly high diversity (average virtual heterozygosity [H_E], 0.807) and significant multilocus linkage disequilibrium in this region of low malaria endemicity. Multiple-clone infections occurred in 60% of isolates sampled in 2005. The global-level analysis of field isolates or monkey-adapted strains from four continents identified 150 unique haplotypes among 164 parasites typed. Individual *P. vivax* isolates could not be unambiguously assigned to geographic populations. Parasite relapses, that may extend the duration of *P. vivax* carriage in humans, are suggested to facilitate the spread of strains across continents, breaking down any pre-existing geographic structure.

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SEQUENCE ANALYSIS OF THE CIRCUMSPOROZOITE PROTEIN GENE OF *PLASMODIUM FALCIPARUM* POPULATIONS IN THAILAND

Somchai Jongwutiwes[1], Thongchai Hongsrimuang[1], Kriengsak Limpitikul[2], Sunee Seethamchai[3], Chaturong Putaporntip[1]

[1]Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, [2]Department of Tropical Pediatrics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, [3]Department of Biology, Faculty of Science, Naresuan University, Pitsanulok, Thailand

To survey allelic repertoire of the gene encoding circumsporozoite protein of *Plasmodium falciparum* (PfCSP), a leading pre-erythrocytic malaria vaccine candidate, we analyzed 158 isolates collected during 1996 and 165 isolates during 2006 from Tak Province. All isolates were amplified by the polymerase chain reaction using primers derived from the 5' upstream and 3' downstream to the coding region of the gene, followed by direct sequencing. Results have shown that 116 isolates collected in 1996 and 107 in 2006 harbored single PfCSP sequences. Of these, 40 alleles of the tetrapeptide repeats were observed. The number of tetrapeptide repeat units varied from 40 to 48 units that encode either NANP or NVDP. However, deduced amino acid sequences revealed 11 sequence types of the central repeat region. Frequency distribution of the repeat alleles seems to be stable between *P. falciparum* populations collected 10 years apart. Sequence variation in the 3' portion of the PfCSP gene is confined to 2 T helper epitope-encoding regions. Nine haplotypes of the 2 helper epitopes, Th2R and Th3R, were identified. The predominant haplotypes of the Th2R and Th3R remain unchanged over a decade. Evidence of purifying selection was found in the repeats of PfCSP while positive selection was reaffirmed in the T cell epitope-encoding region based on comparison of the rate of synonymous substitution per synonymous sites and the rate of nonsynonymous substitution per nonsynonymous sites. Despite the presence of selective pressure on the PfCSP gene, allelic stability of this locus in *P. falciparum* populations collected over a decade apart may not compromise incorporation of this protein in a malaria vaccine.

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PROSPECTIVE IDENTIFICATION OF MALARIA PARASITE ANTIGEN GENES UNDER BALANCING SELECTION

Kevin K. Tetteh

London School of Hygiene and Tropical Medicine, London, United Kingdom

Endemic human pathogens are subject to strong immune selection, and methods to interrogate pathogen genome variation for signatures of balancing selection should be able to identify important target antigens. Population samples of 12 previously-studied *Plasmodium falciparum* polymorphic antigen genes show a correlation between positive Tajima's D indices (excess of intermediate frequency alleles) and positive skew in MK tests (excess of coding versus non-coding polymorphisms compared with fixed differences from the closely related *P. reichenowi*), four genes having highest values in both tests. To identify additional antigens under selection, 26 genes known or predicted to encode merozoite surface-associated proteins were studied in a panel of *P. falciparum* isolates and *P. reichenowi*. These exhibited a wide range of polymorphism and substitution levels, and two genes had a significantly positive MK skew (*rhop148* and *Pf92/6-cys*). Analysis of allele frequencies in a Gambian *P. falciparum* population showed these and four other polymorphic genes not to have significantly positive Tajima's D indices, but another gene (MSP3/6-like *PF10_0348*) as well as a positive control (*ama1*) did. As only a small minority of genes encoding surface-exposed proteins show significant signatures of balancing selection, use of both codon-based and allele frequency-based approaches are recommended for investigating polymorphism throughout the genome.

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SEQUENCE DIVERSITY IN THE MEROZOITE SURFACE PROTEIN 1 GENE OF *PLASMODIUM VIVAX* AS INFERRED FROM 200 THAI ISOLATES

Chaturong Putaporntip[1], Tongchai Hongsrimuang[1], Pannatat Areekul[1], Sunee Seethamchai[2], Somchai Jongwutiwes[1]

[1]Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, [2]Department of Biology, Faculty of Science, Naresuan University, Pitsanulok, Thailand

To gain insight into the extent of variation in merozoite surface protein 1 of *Plasmodium vivax* (PvMSP1), a strong candidate for a blood stage vaccine, we explored the complete nucleotide sequences of 200 clinical isolates from Thailand. Sequence comparisons have shown that PvMSP1 can be partitioned into 13 blocks, consisting of 7 conserved and 6 variable blocks, as inferred from homology of the deduced amino acid sequences and nucleotide diversity among haplotypes. Nucleotide substitutions in conserved blocks of PvMSP1 are basically dimorphic, i.e. one or the other nucleotide at a position wherever substitutions occur, and that various combinations of these substitutions have created microheterogeneity in the regions. Four of the 6 variable blocks are characterized by repeat motifs containing 2 or more basic sequence types and several novel types, resulting in size and sequence polymorphism among haplotypes. The extensive sequence variation in PvMSP1 is partly attributable to frequent meiotic intragenic recombination as evidenced by a rapid decline in significant linkage disequilibrium tests between pairs of loci with increasing molecular distance. Several novel types were apparently generated by recombination between the basic sequence types, spanning variable blocks from the 5' to the 3' portions of the gene. The rate of synonymous substitutions per synonymous sites (d_s) significantly outnumbers that of nonsynonymous substitutions per nonsynonymous sites (d_n) in 4 segments within conserved blocks of PvMSP1. This implies that amino acid residues conserved among alleles occurred exclusively in conserved blocks of PvMSP1, suggesting purifying selection stemming probably from functional constraints in these blocks. These findings are essential for a rational design of a subunit vaccine against *P. vivax*.

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PFCRT GENETIC MUTATIONS AS MARKERS OF CHLOROQUINE RESISTANCE AMONG SEVERE MALARIA PATIENTS IN GHANA

Harry H. Abruquah

University Hospitals NHS Trust, University of Nottingham, Nottingham, United Kingdom

While shortages of resources undeniably pose significant obstacles to malaria control in many developing countries, drug resistance has been a major additional contributor to the failure of the battle against malaria in many of these countries. Of 140 children presenting with severe malaria to the Komfo Anokye Teaching Hospital, 109 (77.9%) had detectable pre-treatment chloroquine levels. A molecular study was conducted to determine the presence of mutant alleles of the chloroquine resistance gene, *Pfcrt,* among the isolates of *Plasmodium falciparum* from these children. Polymerase Chain Reaction and restriction digestion analysis of *P. falciparum* chloroquine resistant transporter gene (*Pfcrt*) indicated that 123 (87.9%) had the mutant T76 gene, and that *Pfcrt* mutant T76 gene correlated well with higher chloroquine levels (P >0.01). Sequencing analysis of these showed consistent genetic sequences for chloroquine resistant and sensitive parasites with respect to *Pfcrt* amino acid positions 72, 73, 74, 75, and 76 (P >0.01). The *Pfcrt* T76 mutation was found in 88.4% of samples that also harbored the *P. falciparum* multi-drug resistance 1 (*Pfmdr*-1) Y86 mutation (odds ratio (OR) =4.8 [95% CI: 1.7-13.3]; P=0.002). The *Pfmdr*-1 Y86 mutation was found in 67.6% of the samples having the *Pfcrt* T76 mutation. This suggests that both mutations, occurring on two different chromosomes, are independently selected by plasma chloroquine levels and that one mutation (Y86) might modify/increase the effect of the other (T76). These results are consistent with other studies, and affirm *Pfcrt* as a better chloroquine resistant marker over *Pfmdr*-1. The data show that a high proportion of children admitted at the Komfo Anokye Teaching Hospital had sub-therapeutic pretreatment chloroquine levels. This study also confirms the true picture of the much-overlooked antimalarial drug resistance situation in the area and recognizes the need for a proper treatment strategy.

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GENETIC VARIATION AMONG *PLASMODIUM VIVAX* PRIMATE ISOLATES AND THE IMPLICATION FOR VACCINE DEVELOPMENT

Francis B. Ntumngia[1], Amy M. McHenry[2], John W. Barnwell[3], Jennifer Cole-Tobian[4], Christopher L. King[4], John H. Adams[1]

[1]Global Health Infectious Disease Research, University of South Florida, Tampa, FL, United States, [2]University of Notre Dame, Notre Dame, IN, United States, [3]Center for Disease Control and Prevention, Atlanta, GA, United States, [4]Center for Global Health and Disease at Case Western Reserve University School of Medicine, Cleveland, OH, United States

The *Plasmodium vivax* Duffy binding protein (*DBP*) is vital for invasion of host erythrocytes and its DBL domain (region II) contains residues critical for receptor recognition thereby making this molecule a good vaccine candidate against vivax malaria. Development of a *P. vivax* vaccine usually requires the use of primate models prior to testing efficacy in humans. However, little is known about the allelic diversity that occurs in the DBP in these primate isolates. Our study analyzes the complete gene coding for the DBP in several *P. vivax* isolates that are used routinely for experimental primate infections and compares the heterogeneity in these sequences to the Sal1 DBP product, which is being used for vaccine development. Our results affirm that primate isolates are genetically similar to *P. vivax* circulating in humans in dispersed geographic regions. This allelic diversity includes the portion of DBP that is the receptor recognition site and potentially the target of protective antibodies. Similar to *P. falciparum* vaccine trials, analysis of the efficacy of a DBP vaccine may be complicated by heterogeneity in the vaccine target of the *P. vivax* isolates available for *in vivo* challenge. Vaccine development is further complicated by the multi-clonal nature of several of the *P. vivax* primate isolates.

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VARIATION AT -607C/A IN THE IL-18 PROMOTER IS ASSOCIATED WITH PROTECTION AGAINST MALARIAL ANEMIA IN KENYAN CHILDREN

Samuel Anyona[1], Collins Ouma[1], Gregory Davenport[2], Tom Were[1], John Vulule[3], Jeremy Martinson[2], Robert Ferrell[2], John Michael Ong'echa[1], Douglas Perkins[4]

[1]University of New Mexico/KEMRI, Kisian, Kenya, [2]University of Pittsburgh, Pittsburgh, PA, United States, [3]KEMRI, Kisian, Kenya, [4]University of New Mexico, Albuquerque, NM, United States

Interleukin (IL)-18 is an important regulator of innate and acquired immunity to infectious diseases. Polymorphisms in the IL-18 promoter are associated with a number of inflammatory and autoimmune diseases. However, the association between IL-18 promoter polymorphisms and malaria disease outcomes in holoendemic *Plasmodium falciparum* transmission areas remains unexplored. As such, the role of an IL-18 polymorphism (-607C/A) in conditioning severe malarial anemia (SMA; Hb<6.0 g/dL), malarial anemia (MA; Hb<8.0 g/dL) and high-density parasitemia (HDP >10,000 parasites/µL) was investigated. Children with acute malaria (*n*=394) were enrolled at Siaya District Hospital in western Kenya. Complete hematological and parasitological measures were performed. IL-18 -607C/A genotyping was carried out by PCR and *Tru9*I restriction enzyme digestion. Prevalence of the genotypes was: CC, 34.0%; CA, 48.0%; and AA, 18.0%, with C and A allele frequencies of 0.58 and 0.42, respectively. Multivariate logistic regression analysis controlling for age, gender, sickle-cell trait, HIV status and bacteremia

revealed that relative to the CC genotype, carriage of homozygous A allele was moderately associated with protection against MA (OR; 0.54, 95% CI 0.29-1.04, *P*=0.06). None of the other IL-18 -607 variants were associated with malaria disease outcomes. These findings suggest that variation at -607 in the IL-18 is associated with protection against malarial anemia in pediatric populations exposed to high levels of *P. falciparum* transmission.

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ASSESSMENT OF THE ABILITY OF ANTIBODY REAGENTS WITH SPECIFICITY AGAINST VAR2CSA TO RECOGNIZE THE SURFACE OF INFECTED ERYTHROCYTES FROM PREGNANT WOMEN

Pamela A. Magistrado[1], Ali Salanti[1], Davis John[2], Nicaise G. Tuikue Ndam[3], Steven B. Mwakalinga[1], Mafalda Resende[1], Madeleine Dahlbäck[1], Martha Lemnge[4], Raimos Olomi[2], John Lusingu[4], Thor G. Theander[1], Morten A. Nielsen[1]

[1]*Institute of International Health, Immunology and Microbiology, Copenhagen, Denmark,* [2]*Kilimanjaro Christian Medical Centre, Moshi, United Republic of Tanzania,* [3]*Institut de Recherche pour le Développement, Paris, France,* [4]*National Institute for Medical Research, Tanga, United Republic of Tanzania*

Pregnancy-associated malaria is a major problem in sub-Saharan Africa causing maternal aneamia, low birth weight and still births due to the accumulation of infected erythrocytes in the placenta. This accumulation is due to the interaction of VAR2CSA on the surface of the infected erythrocytes (IE) and chondroitin sulfate A (CSA) in the placenta. Placental isolates and parasite lines selected *in vitro* for CSA adhesion transcribe high levels of *var2csa* and express the VAR2CSA protein on the surface of the IE. However, data on VAR2CSA surface expression by fresh placental isolates is lacking. In this study, we have used flow cytometry and affinity-purified human and rabbit antibodies and crude rabbit sera specific to DBL1 through DBL6 of the VAR2CSA protein, to demonstrate that VAR2CSA is expressed on the surface of placental isolates in a malaria endemic area in Tanzania. Results show that polyclonal antibody preparations against recombinant VAR2CSA domains on the genetic background of the 3D7 and FCR3 clones can cross-react with parasites transmitted in endemic areas. The existence of cross-reactive anti-VAR2CSA antibodies is encouraging for the efforts to develop a vaccine to prevent pregnancy-associated malaria.

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A PHASE 1 STUDY OF THE BLOOD STAGE MALARIA VACCINE CANDIDATE AMA1-C1/ALHYDROGEL WITH CPG 7909, USING TWO DIFFERENT FORMULATIONS AND DOSING INTERVALS

Ruth D. Ellis[1], Laura B. Martin[1], Mark Pierce[1], Kazutoyo Miura[1], Gregory E. Mullen[1], Michael P. Fay[1], Carole A. Long[1], Donna Shaffer[2], Allan Saul[1], Louis H. Miller[1], Anna P. Durbin[2]

[1]*National Institutes of Health, Rockville, MD, United States,* [2]*Johns Hopkins Center for Immunization Research, Washington, DC, United States*

An effective blood stage malaria vaccine has the potential to reduce the tremendous burden of morbidity and mortality from *Plasmodium falciparum*, particularly in sub-Saharan Africa. Vaccines containing apical membrane antigen 1 (AMA1) have been shown to be protective in animal models and have advanced to Phase 2 trials in malaria-exposed children. A previous Phase 1 study showed markedly enhanced antibody induction when the immune stimulant CPG 7909 was added to the recombinant protein vaccine AMA1-C1/Alhydrogel. In order to enhance stability of the vaccine and to allow for the addition of more proteins to future formulations, the buffer of the CPG 7909 was changed from phosphate to saline. A Phase 1 study in 24 malaria naïve adults was conducted to confirm the safety and immunogenicity of the new formulation, and also explored the effect of a two month versus one month dosing schedule. Participants were randomized in a 2x2 design to receive either 80 µg AMA1-C1/Alhydrogel + 500 µg CPG 7909 (phosphate) or 80 µg AMA1-C1/Alhydrogel + 500 µg CPG 7909 (saline), at either 0, 1 month or 0, 2 months. All vaccinations were given IM and both the investigators and volunteers were blinded until after initial assessment of safety data was complete. Adverse events related to vaccinations were all mild or moderate except one severe injection site erythema. Frequency of local reactions and solicited adverse events was similar among the groups. Peak antibody responses in the saline groups were not inferior to those in the phosphate groups (p=.5586, 95% CI 0.4416, 1.6175). Peak responses in the groups vaccinated at a 2 month interval were 2.4 fold higher than those vaccinated at a 1 month interval (p=.0368, 95% CI 1.020, 4.196). Peak growth inhibition in 3D7 parasites ranged from 30-93% (average 56%) at Day 42 and from 42-96% (average 76%) at Day 70; inhibition in FVO parasites was slightly less. AMA1-C1/Alhydrogel with CPG 7909 in a saline buffer has moved forward to Phase 1 studies in a malaria endemic population, and future trials will use a two month initial dosing schedule.

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EVALUATION OF HUMORAL AND CELLULAR RESPONSES INDUCED BY *PLASMODIUM BERGHEI* CELTOS ADMINISTERED BY RECOMBINANT PROTEIN AND GENE-GUN DELIVERY

Elke S. Bergmann-Leitner, Ryan M. Mease, Kari M. Laquer, Elizabeth H. Duncan, Tatiana Savranskaya, Jack L. Williams, Christian F. Ockenhouse, **Evelina Angov**

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

The malarial protein designated CelTOS, for cell-traversal protein for ookinetes and sporozoites, from *Plasmodium berghei* has previously been shown to mediate malarial invasion of both vertebrate and mosquito host cells and is required for establishing their successful infections. In the vertebrate host, *Plasmodium* sporozoites traverse to hepatocytes via a complex passage initiating at the dermis and traversing through cellular barriers in the skin and the liver sinusoid. Induction of immunity targeted to molecules involved in sporozoite motility and migration into hepatocytes may lead to nonproductive and/or reduced hepatocytic infection. To investigate the potential of CelTOS as target antigen for a pre-erythrocytic vaccine, we generated a soluble, well expressed recombinant *P. berghei* CelTOS protein in *Escherichia coli* utilizing codon harmonization. We immunized Balb/c and ICR mice with either the recombinant protein adjuvanted with Montanide ISA-720 or with a pCI-TPA plasmid encoding the *P.berghei* CelTOS (epidermal delivery by gene-gun) to characterize their abilities to induce protective responses against a homologous *P. berghei* challenge. Humoral and cellular immune responses induced by either protein or plasmid immunizations were assessed in an effort to establish immune correlates. Results of the studies will be presented. A finding of arrested hepatocytic invasion by inducing immunity to target antigens involved in sporozoite traversal or motility may lead to further development of CelTOS as a vaccine component.

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ADVANCED GENERATION ADENO-BASED VECTORS FOR MALARIA VACCINE DEVELOPMENT

Joseph T. Bruder[1], Ping Ping Chen[1], Elena Semenova[1], Charlie Thomas[1], Melissa Hamilton[1], Noelle B. Patterson[2], Maureen E. Stefaniak[2], Keith Limbach[2], Thomas L. Richie[2], Jason Gall[1], C. R. King[1], Duncan McVey[1], Denise L. Doolan[2]

[1]*GenVec, Gaithersburg, MD, United States,* [2]*United States Military Malaria Program, Silver Spring, MD, United States*

The development of an effective malaria vaccine is a high global health priority. Adenovirus type 5 (Ad5) vectors are capable of generating robust and protective T cell and antibody responses in animal models and are considered a leading viral vector platform for vaccines. Early clinical data conclusively show that Ad5 vectors can induce potent CD8+ and CD4+ T cell and antibody responses. However, the high prevalence of neutralizing antibodies (NAb) to Ad5 in human populations, including individuals in

sub-Saharan Africa, has the potential to limit the effectiveness of an Ad5-based malaria vaccine. We are pursuing two independent approaches to develop adenovectors capable of inducing robust immune responses in populations with prevalent Ad5 NAb. The first is to develop E1/E3/E4-deleted Ad5 vectors with modifications in the major targets of Ad5 NAb, the hypervariable regions of the Ad5 hexon protein. The second approach is to develop a malaria vaccine based on adenovirus serotypes that are less prevalent such as Ad35 and Ad28. These vectors may be combined in heterologous adenovector prime-boost regimens to induce potent and protective T cell and antibody responses targeting antigens that are expressed in the pre-erythrocytic and blood stages of the *Plasmodium falciparum* life cycle. We have demonstrated that hexon-modified Ad5 vectors are not neutralized by hexon-specific Ad5 neutralizing antibodies *in vitro* using sera from mice, rabbits and human volunteers. We have further demonstrated that a hexon-modified adenovector that expresses *Py*CSP is as immunogenic in mice as an unmodified Ad5 vector and can induce robust T cell responses in mice with high levels of pre-existing anti-Ad5 NAb. We have also established that the hexon-modified vector can effectively boost Ad5 vector primed T cell and antibody responses. We are currently assessing the capacity of these vectors to protect against sporozoite challenge in the *P. yoelii* model, and are advancing this technology for evaluation of responses to *P. falciparum* antigens.

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COMPARATIVE ANALYSIS OF MALARIA VACCINE CANDIDATE AMA1-C1/ALHYDROGEL WITH THE ADDITION OF UNIQUE CPG SEQUENCES

Kelly M. Rausch, Bhanumati Ramineni, Lynn Lambert, Kazutoyo Miura, Emma K. Barnafo, Carole A. Long, Louis H. Miller, Laura B. Martin

National Institutes of Health, Rockville, MD, United States

The recombinant protein blood-stage vaccine based on *Plasmodium falciparum* apical membrane antigen 1 (AMA1) formulated on Alhydrogel has been evaluated in multiple clinical trials. The immunogenicity of this vaccine has been enhanced by the addition of a toll-like receptor 9 agonist, CPG 7909. Alternate oligodeoxynucleotide sequences have been developed for human use (Coley Pharmaceutical Group, a Pfizer Company) and were compared to CPG 7909 for their immunostimulating potential. AMA1-C1/Alhydrogel alone or with the addition of the B class sequences CPG 7909, CPG 10104 or CPG 10105 were evaluated for their biochemical stability prior to *in vivo* evaluation. Subsequently, these formulations were used to vaccinate mice and rats; sera from these animals was tested by ELISA for the presence of anti-AMA1 antibodies and the ability of these antibodies to inhibit parasite growth *in vitro*. No differences in the biochemical stability of the AMA1-C1/Alhydrogel formulations containing the different CpG sequences was noted. All point-of-injection formulations were stable for 24 hours at both 4 and 37°C. The level of the antibody responses induced by AMA1-C1/Alhydrogel was increased with the addition of CpGs in both animal species. No statistical difference in the antibody response was noted when the groups vaccinated with the three CpG formulations were compared. Each CpG-containing formulation generated antibodies in rats that inhibited parasite growth significantly more than AMA1-C1/Alhydrogel alone. These data support the further development of CPG 10104 including GLP toxicology studies in preparation for clinical evaluation.

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MULTI-FUNCTIONAL T-CELL RESPONSES INDUCED BY THE AS01 OR AS02 ADJUVANTED MALARIA VACCINE CANDIDATE APICAL MEMBRANE ANTIGEN-1 (AMA-1) ADMINISTERED TO MALARIA-NAÏVE ADULTS

Shannon McGrath[1], James Cummings[1], Mark Kortepeter[2], Robin Nielsen[1], Ev Tierney[3], Lisa Ware[1], Opokua Ofori-Anyinam[4], Sheetij Dutta[1], David Lanar[1], Martha Sedegah[1], Kent Kester[5], Elissa Malkin[3], Carter Diggs[6], Lorraine Soisson[6], Marie-Claude Dubois[4], Ripley Ballou[7], Joe Cohen[4], Urszula Krzych[1], Chris Ockenhouse[1], Gray Heppner[1], Michele Spring[1]

[1]U.S. Military Malaria Vaccine Program, Silver Spring, MD, United States, [2]U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick, MD, United States, [3]PATH Malaria Vaccine Initiative, Bethesda, MD, United States, [4]GlaxoSmithKline Biologicals, Rixensart, Belgium, [5]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [6]U.S. Agency for International Development, Washington, DC, United States, [7]Bill and Melinda Gates Foundation, Seattle, WA, United States

Apical Membrane Antigen-1 (AMA-1) is a protein located on merozoites of *Plasmodium falciparum* and is one of the leading blood stage antigen vaccine candidates currently being evaluated as a potential component of a multi-stage, multi-antigen malaria vaccine. Several preclinical and human sero-epidemiological studies from the field support the involvement of cell-mediated immune (CMI) responses to AMA-1 in promoting resistance to clinical disease, although the exact mechanisms have yet to be elucidated. A Phase 1/2a study at the Walter Reed Army Institute of Research evaluated two vaccine candidates, AMA-1/AS02 and AMA-1/AS01, administered intramuscularly to malaria-naïve adults on a 0, 1, 2-month schedule (*NCT 00385047*). Peripheral blood mononuclear cells were collected at Day 0 and Day 70 (two weeks post-third immunization) for analyses of antigen-specific T-cell responses. Using flow cytometry, we evaluated the production of IL-2, IFN-γ, and TNF-α among CD3, CD4, and/or CD8 expressing cells by intracellular cytokine staining (ICS) assay. These results will be compared to CMI responses previously assessed by IFN-γ ELISPOT assays. This is the first study to characterize multi-functional T-cells by ICS induced by the recombinant protein vaccine antigen AMA-1.

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PHASE 1A OPEN-LABEL DOSE ESCALATION STUDY TO EVALUATE THE SAFETY, REACTOGENICITY, AND IMMUNOGENICITY OF THE CANDIDATE *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN-1 (MSP-1_{42}) ADMINISTERED INTRAMUSCULARLY WITH GSK BIOLOGICALS' ADJUVANT SYSTEM AS01B IN HEALTHY MALARIA-NAÏVE ADULTS

Michele Spring[1], Elke Bergmann-Leitner[1], Cummings James[1], Brent House[1], Urszula Krzych[1], Donna Tosh[1], Lori Miller[2], Lisa Ware[1], Olivier Godeaux[3], Marie-Claude Dubois[3], Jeffrey Lyons[2], Ripley Ballou[4], Lorraine Soisson[5], Carter Diggs[5], Joe Cohen[3], Gray Heppner[1], Chris Ockenhouse[1], Evelina Angov[1]

[1]U.S. Military Malaria Vaccine Program, Silver Spring, MD, United States, [2]Walter Reed Army Institute of Research, Silver Spring, MD, United States, [3]GlaxoSmithKline Biologicals, Rixensart, Belgium, [4]Bill and Melinda Gates Foundation, Seattle, WA, United States, [5]US Agency for International Development, Washington, DC, United States

The ultimate and ideal malaria vaccine will arguably be a multi-antigen, multi-stage immunogen. Thus, many of our research efforts at the Walter Reed Army Institute of Research (WRAIR) have focused on developing blood stage antigens to combine with RTS,S/AS01, the pre-erythrocytic stage vaccine currently planned for large-scale field trials. Merozoite Surface Protein-1 (MSP-1), a protein found on the surface of invading merozoites, is one such candidate, with the 42 kDa C-terminal fragment developed as the vaccine antigen. In preclinical rabbit studies, the FVO

allele of MSP-1_{42} has been shown to have improved immunogenicity over the 3D7 allele, in terms of antibody titers as well as growth inhibitory activity of antibodies against both heterologous and homologous parasites. Based on these encouraging results, we conducted a first-in-human Phase 1a vaccine study of MSP-1_{42} (FVO) adjuvanted in AS01. The study was designed to enroll 26 adults, each receiving 3 intramuscular vaccinations spaced one-month apart: six receiving a one-fifth dose (10 µg) of MSP-1_{42} in 0.5 mL of AS01, and 20 receiving a full dose (50 µg) of MSP-1_{42} in 0.5 mL of AS01. The safety and reactogenicity of the vaccine as well as humoral immunogenicity results will be reported, including titers of antibodies to both the FVO and 3D7 MSP-1_{42} alleles by ELISA, and inhibitory activity of the antibodies against homologous and heterologous *P. falciparum* parasites by growth inhibition assay (GIA). These results will be compared to results from the previous Phase 1a vaccine trial of MSP-1_{42} (3D7) in AS02A conducted at Walter Reed Army Institute of Research.

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ADJUVANT AND CARRIER EFFECT OF SELF-ASSEMBLING POLYPEPTIDE NANOPARTICLES (SAPN)

Clara Brando[1], Stephen Kaba[1], Qin Guo[1], Molly Steele[1], Christian Mittelholzer[2], Senthilkumar Raman[2], Peter Burkhard[3] and David E. Lanar[1]

[1]Division of Malaria Vaccine Development, Walter Reed Army Institute of Research, Silver Spring, MD, USA, [2]M.E. Müller Institute, Basel, Switzerland, [3]The Institute of Materials Science, University of Connecticut, Storrs, CT, USA

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 the absence of any extraneous adjuvant. Protection was T-cell dependent and could be transferred with serum. It is our hypothesis that the SAPN core domain has at least one T cell epitope which causes clonal expansion and activation of SAPN-specific T cells which will in turn interact with the cognate PbCSP specific B cells, providing help for Ab production. Thus, we hypothesize that the SAPN acts as a carrier/adjuvant. To investigate the carrier/adjuvant effect of the SAPN mice were immunized with PbCSP-SAPN. Immune splenocytes were tested for the production of IL-2 following *in vitro* incubation with PbCSP-SAPN, CS-peptide, Con A, or medium control, in the presence or absence of anti-CD4 blocking antibody. To investigate the effect of SAPN on antigen presenting cells, mice were injected intra-peritoneal (ip) with SAPN. Six, 24 and 48 hr post injection post injection macrophages were examined for up regulation of activation markers. An increased number of IL-2 producing splenocytes could be isolated from PbCSP-SAPN immunized animals. IL-2 production in the ELISpot assay was reverted by anti-CD4 Ab. Thus immunization with PbCSP-SAPN generates PbCSP-SAPN specific $CD4^+$ T cells. PbCSP-SAPN given ip increases the number of F4/80+ cells, however does not effect expression of activation markers. Thus SAPN do not directly activate macrophages, but induce their recruitment. In vitro incubation of human dendritic with PbCSP-SAPN resulted in the up regulation of CD83, CD80, CD86, and MHC II. Thus PbCSP-SAPN induces maturation of dendritic cells, therefore enabling them to provide signal 2 to the T cell, therefore are carriers and adjuvant.

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RECOMBINANT PVS230C SPECIFICALLY RECOGNIZES GAMETE STAGE PARASITES OF *PLASMODIUM VIVAX* AND MAY BE USED TO DETECT ANTIBODIES IN HUMAN SERUM, BUT DOES NOT BLOCK OOCYST DEVELOPMENT IN EXPERIMENTAL MOSQUITO INFECTION

Victor Neyra

Instituto de Medicina Tropical, Lima, Peru

Previous studies have demonstrated that antibodies raised against block c of the *Plasmodium falciparum* protein Pfs230 significantly reduce the development of oocysts in mosquitoes experimentally infected with *P. falciparum* malaria. Based on this knowledge and the similarity reported between antigens of *Plasmodium falciparum* and *vivax*, our group generated a recombinant protein of Pvs230 block c, the vivax orthologe of Pfs230. The protein was expressed in *Escherichia coli* as a fusion protein of maltose-binding protein (MBP). Rabbit polyclonal antibodies were raised against the recombinant Pvs230c fusion and, following pre-absorption with MBP, were shown to react specifically with native protein in Western blots and indirect immunoflorescence assays using *P. vivax* parasites acquired from natural human infections around Iquitos, Peru. *Plasmodium vivax* transmission-blocking activity was assessed by mixing polyclonal antibodies with blood acquired from acutely infected patients. Antibodies were not shown to have any transmission-blocking activity and neither reduced the average number of oocysts per midgut nor the percentage of mosquitoes infected (*Anopheles darlingi*). We additionally evaluated 94 human sera from patients infected with *P. vivax* and observed that only 26% (25/94) were seropositive for antibodies against Pvs230 block c, indicating that there is a selective immune response against Pvs230 in infected patients. In conclusion, together, these results suggest that Pvs230 does not demonstrate transmission-blocking immunity in the manner of Pfs230. Evaluation of other domains of Pvs230 may be indicated as a means to locate any transmission-blocking activity present.

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IMMUNOGENICITY STUDIES OF *PLASMODIUM VIVAX* MALARIA VACCINE CANDIDATES BASED ON RECOMBINANT MODULAR CHIMERAS

Balwan Singh, Monica Cabrera-Mora, Jianlin Jiang, **Alberto Moreno**

Emory Vaccine Center at Yerkes National Primate Research Center, Atlanta, GA, United States

We have previously reported the use of synthetic peptides and recombinant subunit chimeras as effective delivery system for malaria vaccines. A distinctive feature of such constructs is the inclusion of *Plasmodium* promiscuous CD4+ T cell epitopes derived from homologous proteins. Proof of principle studies using synthetic or recombinant chimeras based on the circumsporozoite protein or the Merozoite Surface Protein-1 of rodent malaria parasites have been reported. To further assess the relevance of this approach for malaria vaccine development we designed two synthetic genes that include several *P. vivax* promiscuous T cell epitopes fused to the corresponding functional domain of two merozoite proteins. The synthetic genes were codon optimized for expression in *E. coli.* Groups of BALB/c and C57BL/6 mice were used to test the immunogenicity of the chimeric constructs. The immune responses elicited by immunization were compared with that obtained using control recombinant proteins that expressed similar domains but not autologous promiscuous T cell epitopes. A single immunization induced antibody titers several folds higher in mice immunized with the recombinant chimeras in comparison with those immunized with the control proteins. Specific antibody concentration for each IgG isotype differed in both strains of mice indicating that different population of T helper cells are induced after immunization with the recombinant constructs. Protein-specific cytokine recall responses were identified in mice after *ex vivo* stimulation of splenocytes. Sera samples obtained from rhesus monkeys experimentally infected with the simian malaria parasite *P. cynomolgi*, a parasite closely related to *P. vivax*, cross react with the chimeric proteins. The robust immune responses induced by immunization and the extensive cross-reaction exhibited with the simian malaria parasite *P. cynomolgi* open the possibility to test such vaccine constructs in rhesus macaques using hybrid molecules or antigenic cocktails.

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STABILITY OF *PLASMODIUM FALCIPARUM* MSP 1-19 HAPLOTYPES INFECTING KENYAN CHILDREN IN TWO REGIONS

Kee Thai Yeo[1], Christopher Yohn[2], Rhonda Kimmel[2], Peter Mungai[2], John Vulule[3], Indu Malhotra[2], Christopher King[2], Ann Moormann[2], James Kazura[2], Arlene Dent[1]

[1]Rainbow Babies and Children's Hospital, Cleveland, OH, United States, [2]Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, United States, [3]Kenya Medical Research Institute, Kisumu, Kenya

Antibodies directed against *Plasmodium falciparum* (Pf) Merozoite Surface Protein 1-19 (MSP 1-19) have been associated with protection against Pf disease. MSP-1 is currently being studied as a potential vaccine candidate. Four major MSP 1-19 haplotypes have been described: ETSR (PNG-MAD20 type), EKNG (Uganda-PA type), QKNG (Wellcome type), QTSR (Indo type). The recently described MSP 1-19 specific Polymerase Chain Reaction / Ligase Detection Reaction - Fluorescent Microphere Assay (PCR / LDR-FMA) technique is an inexpensive and high through-put method for detecting and quantifying the haplotypes present in Pf infections. Ascertaining the predominant Pf MSP 1-19 haplotypes within a population over time is important in assessing the efficacy of an MSP-1 vaccine and immunologic responses. Blood samples from children living in two separate malaria endemic regions, Kisumu (Western Kenya) and Msambweni (Coastal Kenya) from two separate time periods (2000-2004 and 2006-2007) were included in this study. DNA was extracted from blood samples and PCR / LDR-FMA was performed. Haplotype detection and assignments were made based on previously determined criteria. To date, 166 and 80 blood samples from the 2000-2004 and 2006-2007 time periods have been analyzed respectively. From 2000-2004, Pf infections were detected in 83 Kisumu samples and 13 Msambweni samples. In the Kisumu samples, the distribution of haplotypes were : QKNG (42%), EKNG (41%), ETSR (13%) and QTSR (4%). In Msambweni samples, the haplotype frequency distribution was very similar - QKNG (40%), EKNG (40%), ETSR (20%). From 2006-2007, 26 Pf infections have been detected thus far - 19 from Western Kenya and 7 from Coastal Kenya. EKNG and QKNG haplotypes were also most common in both areas. For both time points, QTSR haplotype was only found with mixed infections. Mixed haplotype infections were more common in Kisumu samples (64%) than Msambweni samples (8%). In conclusion, the frequency distribution of Pf MSP 1-19 haplotypes infection children was similar in two separate regions of Kenya in 2000-2004 and appears to be stable through 2006-2007.

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ANTI-APICAL MEMBRANE ANTIGEN 1 IGG IS MORE EFFECTIVE IN INHIBITING *PLASMODIUM FALCIPARUM* GROWTH AS MEASURED BY *IN VITRO* GROWTH INHIBITION ASSAY THAN ANTI-MEROZOITE SURFACE PROTEIN 1 42 IGG

Kazutoyo Miura[1], Hong Zhou[2], Ababacar Diouf[2], Samuel E. Moretz[2], Louis H. Miller[1], Laura B. Martin[1], Gregory E. Mullen[1], Carole A. Long[2]

[1]Malaria Vaccine Development Branch/NIAID/National Institutes of Health, Rockville, MD, United States, [2]Laboratory of Malaria and Vector Research/NIAID/National Institutes of Health, Rockville, MD, United States

Apical Membrane Antigen 1 (AMA1) and Merozoite Surface Protein 1 42kDa ($MSP1_{42}$) are leading malaria vaccine candidates. Several preclinical and clinical trials have been conducted and an *in vitro* parasite Growth Inhibition Assay (GIA) has been used to evaluate the biological activity of antibodies. We have shown that when the anti-AMA1 or $MSP1_{42}$ antibody units (as measured by ELISA) of total IgGs were plotted against the % inhibition (as measured by GIA), there was a strong correlation between them and the relationship followed a symmetrical sigmoid curve. In a U.S. phase 1 trial, AMA1-C1/Alhydrogel+CPG 7909 vaccine elicited anti-AMA1 IgGs which showed up to 96 % inhibition of growth against *P. falciparum* 3D7 parasites. However, antibodies induced by $MSP1_{42}$-C1/Alhydrogel+CPG 7909 vaccine in a U.S. trial showed less than 32 % inhibition *in vitro*. In this study, the amount of IgG that gives 50% inhibition of parasite growth (EC_{50}) for these two vaccine candidates were compared. Anti-AMA1-specific IgGs were purified from rabbit and human anti-AMA1-total IgGs. Both rabbit and human anti-AMA1-specific IgGs showed the same level of growth-inhibitory activity as corresponding anti-AMA1-total IgGs when the levels of IgG were normalized. The EC_{50} of rabbit and human anti-AMA1 IgGs against 3D7 parasites were 64 and 78 µg/mL, respectively. Similarly, anti-$MSP1_{42}$-specific IgGs were purified from rabbit and human anti-$MSP1_{42}$-total IgGs and the EC_{50} were 179 and 430 µg/mL, respectively. EC_{50} data against FVO parasites also demonstrated that anti-AMA1 IgGs were more effective than anti-$MSP1_{42}$ IgGs Thus considerably more anti-$MSP1_{42}$ antibodies than anti-AMA1 antibodies were required to attain comparable levels of GIA activity. Although it is unknown whether the biological activity as measured by GIA reflects protective immunity *in vivo*, the data of this study may explain a lower growth-inhibitory activity observed in the $MSP1_{42}$ clinical trial and provides a benchmark for antibody levels for future AMA1- or $MSP1_{42}$-based vaccine development in preclinical and clinical trials.

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THE STATUS OF THE PFMSP3 N-TERMINUS AS A VACCINE CANDIDATE: CROSS-REACTIVE ANTIBODIES IN HYPOENDEMIC TRANSMISSION

Stephen J. Jordan, Oralee H. Branch, Julian C. Rayner

University of Alabama at Birmingham, Birmingham, AL, United States

Although no commercially available Malaria vaccine exists, multiple subunit-based vaccines are in development including a *Plasmodium falciparum* Merozoite Surface Protein-3 (PfMSP3)-based vaccine, comprised of the highly conserved, yet poorly immunogenic, C-terminal domain. Recent work by our lab has demonstrated that the N-terminal domain of PfMSP3 is, in fact, highly stable in hypoendemic environments, casting doubt on its exclusion from vaccine development. With recent reports suggesting the N-terminal domain is actually highly immunogenic, clearly the ability of each PfMSP3 sub-domain to induce potent and protective antibody responses needs to be extensively studied before the optimal PfMSP3-based vaccine constituent can be identified. To this end, we decided to conduct a systematic study of antibody dynamics generated against each PfMSP3 domain in individuals living in a malaria-hypoendemic environment. Through collaboration with an ongoing cohort study in the Peruvian Amazon, we have access to sera samples from *P. falciparum*-infected study participants. To measure antibody responses against PfMSP3 sub-domains, we constructed 3 antigens for use in ELISA assays: both N-terminal domain alleles and the conserved C-terminal domain. These antigens were used to identify the PfMSP3 sub-domain that is the most immunogenic and assess for cross-protection between alleles. These studies were subsequently followed by determining both the antibody isotype profile of all positive responders and the ability of PfMSP3 sub-domains to induce long-lasting antibody responses. Finally, correlates of protection were investigated by comparing this data with the clinical and epidemiologic data. Our results demonstrate that the N-terminal domain of PfMSP3 is significantly more immunogenic than the C-terminal domain. There were 63% more positive responders to the N-terminus than the C-terminus, as well as a 4-fold increase in the number of individuals with particularly high IgG responses. Additionally, our data suggests, for the first time, that antibodies against the PfMSP3 N-terminus can mediate cross-protection between alleles. Thus, these data suggest that an exclusive focus on the PfMSP3 C-terminus for vaccine development is premature, and that further work is needed to investigate the possibility of generating a cross-protective vaccine using the more immunogenic PfMSP3 N-terminal domain.

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POLYMORPHISM OF *AEDES AEGYPTI* DEFENSIN A GENE

Tereza Magalhaes[1], Ieda F. Oliveira[1], Karen L. Haag[2], Constancia F. Ayres[1]

[1]Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, Recife-PE, Brazil, [2]Universidade Federal do Rio Grande do Sul, Porto Alegre-RS, Brazil

Defensins are cysteine-rich antimicrobial peptides (AMP) found in a variety of organisms. In *Aedes aegypti*, it is the most important AMP, reaching high concentrations in the hemolymph. So far, three defensin isoforms (A, B, and C) have been identified in *A. aegypti*. Isoforms A and B are predominant and produced by the fat body. However, there have been contradictions regarding defensins nomenclature. Here, we have analyzed the polymorphism of defensin A (*defA*) in *A. aegypti* infected and not infected with *Wuchereria bancrofti*, a nematode of which this mosquito is refractory to. For that, 10 *defA* clones from individual mosquitoes (infected and non-infected) were sequenced. In total, about 200 transcripts of *A. aegypti defA* were analyzed through MEGA® and DnaSP® softwares. For control, transcripts of the ribosomal gene rpl8 were also sequenced and analyzed. Results showed a high diversity of *A. aegypti defA*. At least 40 alleles were identified, with most of them being rare alleles (singletons). No specific sequences were linked to infected or non-infected mosquitoes. There were no significant differences between synonymous and non-synonymous nucleotide substitutions. The mature peptide region showed the lowest polymorphism compared to the signal peptide and pro-peptide regions. These preliminary analyses point towards a purifying selection upon *A. aegypti defA* gene.

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TRANSCRIPTION PROFILING OF FAT METABOLISM GENES IN DIAPAUSING *CULEX PIPIENS*

Cheolho Sim, David L. Denlinger

The Ohio State University, Columbus, OH, United States

Culex pipiens, the mosquito that vectors West Nile virus in North America, overwinters in an adult diapause (dormancy) that is programmed by the short day length of autumn. In response to this environmental signal, females cease feeding on blood and instead seek sources of nectar used to generate huge fat reserves that provide the energy source for winter survival. To clarify the regulatory networks that are modulated in early fat accumulation and consumption, we investigated metabolic gene expression during early and late diapause and at diapause termination. We examined 33 fat metabolism genes from nondiapausing and diapausing mosquitoes and identified 18 that were significantly down-regulated during fat accumulation in early diapause. In addition, 22 genes were significantly up-regulated during fat consumption in late diapause. Fat metabolism genes, include multiple kinetic classes and genes involved in β-oxidation, were suppressed in early diapause but highly up-regulated in late diapause and at diapause termination. We also present results suggesting that the transcript profile of the *Culex* nuclear hormone receptor 49 (cNHR49) is strongly correlated with transcripts involved in fat metabolism.

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ENERGY METABOLISM IN DIAPAUSING *CULEX PIPIENS* MOSQUITOES

Guoli Zhou, Roger L. Miesfeld

University of Arizona, Tucson, AZ, United States

The diapause process in *Culex pipiens* females plays an important role in the epidemiology of West Nile virus (WNV) transmission as the virus can survive over wintering in *Cx. pipiens*. In order to investigate the fate of carbon contained in uniformly ^{14}C-labeled glucose, we fed U-[^{14}C]-glucose to 1-day-old and 5-day-old females that had been reared under diapause or nondiapause conditions from the second instar larval stage onward. Based on the results from the 1-day-old 14C-labeled mosquitoes, we found that glucose utilization during the first 24 hours post-labeling is independent of diapause conditions as both sets of mosquitoes had the same relative pattern of ^{14}C incorporation into lipid, protein, amino acids, glycogen, and simple sugars. However, from 3-7 days post-labeling, the diapause-destined mosquitoes showed a significant increase in lipid reserves compared to nondiapause-destined females. To examine metabolic profiles during diapause, 5-day-old diapause- or nondiapause-destined females, were fed ^{14}C-glucose for 24 hours, followed by water only feeding for 49 days. These analyses showed that pools of ^{14}C-labeled simple sugars, amino acids, and protein, were rapidly depleted by the third day of starvation in diapausing mosquitoes. The glycogen reserve was maintained during the first week of starvation, and was reduced by about half after 2 weeks with complete depletion by 1 month. This was in contrast to nondiapausing mosquitoes that depleted nearly all of their glycogen and lipid reserves in the first week of starvation with a 100% mortality by 14 days. In the diapausing mosquitoes, the size of the lipid reserve reached a maximal level in 8-day-old mosquitoes and was maintained for up to 1 month. Diapause was terminated after 49 days by increased temperature, an extended light cycle, and sugar feeding. Labeling analysis showed that lipids were still the dominant energy reserve during this phase. Lastly, 14 days post-diapause, the surviving females were blood fed and the ^{14}C-protein and ^{14}C-lipid levels in the eggs and carcass were analyzed. The results revealed that at the end of gonotrophic cycle, 2.5% of the original ^{14}C label ingested by 5-day-old mosquitoes was contained in the egg lipids, whereas, 5% remained in the carcass. Taken together, these data suggest that >90% of the ^{14}C-labeled glucose fed to 5-day-old mosquitoes was used for energy conversion processes during the 2 month diapause and post-diapause phases.

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REGULATION AND FUNCTION OF MIDGUT PROTEASE GENES IN *AEDES AEGYPTI* MOSQUITOES

Brianna Kolody, Susan Kunz, Jun Isoe, Roger L. Miesfeld

University of Arizona, Tucson, AZ, United States

Blood meal digestion in mosquitoes is initiated by increased levels of protease enzymes in midgut epithelial cells that are then secreted into the midgut lumen. In *Aedes aegypti*, the level of early phase (1-6 hrs PBM) and late phase (8-30 hrs PBM) proteases is increased dramatically by feeding as a result of translational and transcriptional control mechanisms, respectively. To better understand late phase protease gene regulation, we have focused on expression of the late trypsin (LT), 5G1-14, serine collagenase-1 (SC-1), and Culex late trypsin homolog (CxLT) protease genes in the midgut of blood and protein fed mosquitoes using quantitative real-time PCR. We found that protein in the midgut is sufficient to induce the expression of all four proteases >100-fold based on results using a protein enema to deliver protein to the midguts of decapitated female mosquitoes. In order to identify transcription factors that might be required for coordinate expression of these midgut protease genes in response to feeding, we selected candidate RNAi knock-down targets in the Target of Rapamycin (TOR) kinase and phosphoinositol-3 kinase (PI-3K) signaling pathways. Injections of dsRNA into newly emerged *Ae. aegypti* females was used to decrease the expression of TOR, as well as, three TOR-associated transcription factors, Tif-1α, PGC-1, and YY1. We have also targeted several forkhead box transcription factors that have been linked to PI-3K signaling; FOXO, FOXA1/A2, FOXN1, FOXK1, and FOXK2. We have shown that we can knock-down expression of these genes in the midgut and are now analyzing LT, 5G1-14, SC-1, and CxLT gene expression in blood fed mosquitoes. Although it has been assumed that midgut proteases function together to digest the blood meal, it is not known how important each of these proteases are in providing the nutrients required for completing the gonotrophic cycle. To better understand the involvement of midgut proteases in reproduction, and to identify possible targets for vector control, we are knocking-down the expression of specific late phase protease genes using dsRNA. The efficiency of protease knock-down is being monitored with Western

blots and biochemical assays, and effects on blood meal metabolism and reproduction are being analyzed by metabolic labeling experiments and fecundity analysis. A better understanding of blood meal digestion in Ae. aegypti could lead to the development of novel strategies for vector control.

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REGULATION OF FATTY ACID METABOLISM IN *AEDES AEGYPTI* MOSQUITOES

Amy Alabaster, Jorge Zamora, Roger L. Miesfeld

University of Arizona, Tucson, AZ, United States

Female *Aedes aegypti* mosquitoes require a blood meal to obtain sufficient nutrients for egg development and to replenish maternal reserves, however, blood feeding can also lead to the transmission of pathogenic diseases. A novel approach to vector control is to develop mosquito-selective metabolic inhibitors that block biochemical pathways necessary for blood meal metabolism. To this end, we are investigating the possibility that inhibition of blood meal-induced fatty acid synthesis could lead to decreased reproductive capacity as a result of insufficient lipid stores. In order to identify key components of lipid metabolism in blood fed female mosquitoes, we have recently cloned and characterized numerous *Ae. aegypti* genes required for fatty acid synthesis, transport, and triacylglycerol metabolism in the midgut, fat body, and ovaries. These genes include orthologs of mammalian acetyl-coA carboxylase (ACC), two fatty acid synthases (FAS-1, FAS-2), fatty acid binding proteins (FABP, FABPm), the fatty acid membrane translocases (CD36A, CD36B), the lipase/transacylase isoforms (iPLA2-ε, ζ, and ǀ), and three enzymes involved in the final fatty acid acylation step in triacylglycerol synthesis (DGAT1, DGAT2A and DGAT2B). Initial studies have focused on gene expression in the midgut, fat body, and ovaries in response to blood feeding, and the effect of gene-specific RNAi knock-down on egg production. Transcript levels of most genes were found to be altered by blood-feeding in all three tissues, indicating that lipid metabolizing enzymes are transcriptionally-regulated by nutrient signaling. For example, the transcript levels of FABP and DGAT genes are initially high in midgut tissues, but decrease by up to 30-fold at 24-36 hours post blood meal feeding. The transcript levels then return to pre-feeding levels by 96 hours and decrease again at 120 hours. Preliminary analysis of gene-specific knock-downs have shown that FABP and DGAT genes play an important role in egg production based on our finding that injection of FABP or DGAT dsRNA leads to a 48-72 hour delay in oviposition. We are currently using RNAi knock-down strategies in conjunction with metabolic labeling studies with 14C-protein to investigate the role of these genes in fatty acid synthesis, transport, and storage. Our long term goal is to exploit the knowledge gained by these analyses to develop novel metabolic inhibitors for use as vector control agents.

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POSSIBLE INVOLVEMENT OF AGSGS PROTEINS DURING INVASION OF *ANOPHELES* SALIVARY GLANDS BY *PLASMODIUM SPOROZOITES*

Jonas G. King, Julián F. Hillyer

Vanderbilt University, Nashville, TN, United States

Malaria is arguably the greatest scourge of mankind and has proven resilient to monumental control efforts. The successful invasion of the female mosquito's salivary glands is a key interaction between *Plasmodium* and its mosquito host that is necessary for transmission to vertebrates. Despite the importance of this process, the molecular mechanisms of salivary gland invasion remain largely unknown. A salivary gland specific protein named aaSGS1 was recently shown to be important for the successful invasion of *Aedes aegypti* salivary glands by *Plasmodium gallinaceum.* Four homologs were later identified bioinformatically in the *Anopheles gambiae* genome and named agSGS2, 3, 4 and 5. In the current study, RT-PCR and western blot analyses showed that the expression of agSGS4 and agSGS5 is salivary gland specific and occurs only in female mosquitoes. Western blots also showed that agSGS4 and 5 are only present in adult mosquitoes more than one day old and that both proteins are upregulated after a blood meal. These data suggest a possible involvement of agSGS4 and 5 in blood feeding activity. Immunohistochemistry showed that agSGS4 and 5 are normally present on the basal surface of the distal lateral lobes of the salivary glands, the region where highest aaSGS1 localization was previously observed. However, higher levels are detected on the basal surface of the proximal lateral lobes and on the apical cellular faces of the proximal lateral and intermediate "neck" regions. Subsequent immunoblots involving disarticulated salivary glands showed that agSGS4 and 5 are normally present in the intermediate and proximal regions of the lateral lobes and are occasionally present as slightly more massive forms (~20 kDa) in the distal lateral lobes, a region of known significance during sporozoite invasion. Ongoing experiments examining the *in vivo* effect of anti-agSGS antibodies on salivary gland invasion by *P. berghei* will be discussed, along with further investigations into the dynamics of agSGS localization.

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BINDING OF THE CRY4B TOXIN OF *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS* TO THE CADHERIN RECEPTOR OF *ANOPHELES GAMBIAE* MEDIATES CELL DEATH

Mohamed Ibrahim, Natalya Griko, Xuebin Zhang, Jian Sun, Lee Bulla

Biological Targets, Inc., Pilot Point, TX, United States

The Cry4B toxin produced by *Bacillus thuringiensis* subsp. *israelensis* exerts insecticidal action upon binding to a BT-R_1 homolog (BT-R_3), a cadherin localized in the midgut epithelium of *Anopheles gambiae* larvae. The BT-R_3 transcript consists of 4911 bp that codes for 1637 amino acids. Structural motif analysis of the sequence reveals the presence of four domains: ectodomain (EC), membrane-proximal extracellular domain (MPED), transmembrane domain (TM) and a cytoplasmic domain (CYTO). The EC consists of nine cadherin repeats made of β-sheets that are structured as EC modules (EC1-EC9). The EC harbors a cell adhesion sequence (HVV) and three integrin-binding sequences (two RGDs and one LKV). RT-PCR analysis showed that high levels of BT-R_3 pre-mRNA and mRNA transcripts are present in third and fourth larval instars. The BT-R_3 coding sequence was cloned into an insect expression vector and the recombinant plasmid was used to transfect High Five (H5) cells. H5 cells producing BT-R_3 protein were susceptible to the Cry4B toxin. Furthermore, a DNA fragment encompassing EC5 to the MPED (amino acid residues 782-1476) was expressed in *E. coli* and purified to homogeneity. The purified polypeptide bound Cry4B with high affinity, indicating that it contains the Cry4B toxin-binding site. Binding of Cry4B to BT-R_3 apparently is necessary for cell death. Importantly, *Aedes aegypti* and *Culex pipiens* contain cadherin receptors that share sequence homology with BT-R_3, particularly in the toxin-binding region.

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GLYCOCONJUGATE ANALYSIS IN *ANOPHELES GAMBIAE*

Paul J. Linser, Marco V. Neira Oviedo, Kristin E. Smith

University of Florida Whitney Laboratory, Saint Augustine, FL, United States

The cell and tissue biology of mosquitoes is only poorly understood. A detailed understanding of an organism's biology and biochemistry can provide the bases for new approaches to population control. In this vein, we have been working toward a detailed picture of larval cell biology and tissue architecture. Among many biochemical features that can distinguish and highlight cell function and specialization is the nature of macromolecules synthesized by or associated with defined tissues and cells. Carbohydrate chains added to cellular proteins and lipids frequently provide distinguishable characteristics to the cells that produce or associate with the specific glycoconjugates. To expand our analyses of cell and

tissue biology of Anopheles gambiae larvae we have applied fluorescently labeled lectins (Vector Lab) to cross sections of the early 4th instar. Lectins have very specific binding characteristics and recognize sugars in the context of the carbohydrate chains that are frequently added as post translational modifications to many proteins and are also enzymatically added to some lipids. Our confocal microscopy analyses of the lectin labeling showed a remarkable diversity in tissues, cells and extracellular matrices with regard to glycoconjugate character. Among the most interesting observations was that the peritrophic matrix of the midgut and the physically similar matrix of the caecal membrane were differentially labeled. This shows that the biochemical nature of the peritrophic matrix is distinct from that of the caecal membrane. Additionally, the larval salivary glands showed remarkably intense and specific labeling for certain glycoconjugates both within the gland cells and in the lumen (saliva). This observation is consistent with the role of the salivary glands in producing a mucin-rich saliva. Other labeling specificities will be discussed in reference to specific glycoconjugate structure.

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THE RELATIONSHIP BETWEEN VITELLOGENIN EXPRESSION AND AUTOGENY IN *CULEX TARSALIS*

Katie Provost-Javier, Jason Rasgon

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

Mosquitoes require a nutrient-rich blood meal to initiate vitellogenesis and egg production. This necessity drives the persistent vector-host interactions that facilitate the transmission of pathogens. Among populations there can be different reproductive strategies: Autogenous mosquitoes are able to produce their first batch of eggs in the absence of a blood meal while anautogenous mosquitoes cannot initiate egg development without blood feeding. This difference has considerable implications for vector-borne disease, since autogeny reduces the number of blood meals it decreases the likelihood of the mosquito becoming infected with and transmitting a pathogen. In anautogenous mosquitoes, vitellogenin (Vg) (the major yolk protein precursor) is induced and expressed by fat body cells within 36 hours of a blood meal, but little is known about how vitellogenesis is governed in autogenous species. We utilize an important arboviral vector, *Culex tarsalis*, as a model to study vitellogenesis in autogeny. Autogeny in *Cx. tarsalis* is genetically determined, but its expression is plastic and varies between populations both spatially and temporally. We identified the Vg genes from autogenous *Cx. tarsalis*. The Vg gene expression profile was characterized by rt-PCR through larval and pupal development, during autogenous ovarian development as well as following a bloodmeal. We are also using RNAi to knock down Vg gene expression in *Cx. tarsalis*.

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CHARACTERIZATION OF IMMUNE PEPTIDES IN RESPONSE TO FILARIAL WORM INFECTION IN THE MOSQUITO, *ARMIGERES SUBALBATUS*

Matthew T. Aliota, Jeremy F. Fuchs, Thomas A. Rocheleau, Bruce M. Christensen

University of Wisconsin, Madison, WI, United States

Anti-microbial peptides (AMPs) are short, immune-related molecules named for their *in vitro* activity against bacteria and are detectable in the fat body, hemocytes, midgut, and epithelial tissues of mosquitoes. Although considered a primary defense mechanism against bacteria in mosquitoes, AMP transcription has been correlated with responses to Brugia infection in Armigeres and Aedes, Plasmodium infection in Anopheles, and fungal infection in other mosquito species. Despite these correlations, our knowledge of the molecular mechanisms and the true role of these peptides in mosquito innate immunity remain limited. *Ar. subalbatus* is an interesting model for immune peptide characterization, because AMP transcripts are abundant in filarial worm-*Armigeres* cDNA libraries, and because of the correlation between AMPs and certain intermediates involved in melanization immunity. As a result, we have initiated a study characterizing the transcriptional profile of three AMPs: cecropin A, defensin A, and gambicin, in Ar. subalbatus. All three of these AMPs show transcriptional activity in response to natural infection with *Brugia malayi* and *Brugia pahangi*, and preliminary studies provide evidence of dose dependency with defensin. The fact that transcriptional activity is evident with all three AMPs in response to infection with both *B. malayi* and *B. pahangi*, as well as, during mosquito development suggests that these AMPs are functioning beyond their proposed bactericidal role during an immune response. Therefore, the anti-microbial activity of AMPs might be one property of AMPs, and they may have other more important roles related to parasite infection. Furthermore, the *Armigeres-Brugia* system represents a natural mosquito-parasite system; accordingly, it could provide significant insight into the role that these peptides play in mosquito innate immunity.

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EX VIVO PROMOTER ANALYSIS OF *ANOPHELES GAMBIAE* HEAT SHOCK COGNATE (HSC70) GENE DURING O'NYONG-NYONG VIRUS INFECTION

Seokyoung Kang[1], Brian D. Byrd[2], Cheolho Sim[3], Frank H. Collins[4], Young S. Hong[1]

[1]Tulane University, New Orleans, LA, United States, [2]Western Carolina University, Cullowhee, NC, United States, [3]Ohio State University, Columbus, OH, United States, [4]University of Notre Dame, Notre Dame, IN, United States

The *Anopheles gambiae* heat shock cognate gene (Ag_hsc70) is a constitutively expressed member of the hsp70 family. Our previous studies found that expression of Ag_hsc70 is induced by o'nyong-nyong virus (ONNV) infection, suppressing ONNV replication in this mosquito. To further characterize the role of hsc70 in ONNV infection, we cloned a 2-kb region immediately 5' upstream of the starting codon of Ag_hsc70 into a luciferase reporter vector, and studied its promoter activity in transfected mosquito (C6/36) and mammalian (Vero) cells during ONNV infection. Sequence analysis of the 2-kb region revealed binding sites for heat shock elements and NF-kB as well as a TATA box. Following genomic PCR, this region was inserted into the promoterless pGL3-Basic vector for promoter assays. For both C6/36 and Vero cells, experimental infections (pGL3-hsc70 transfected cells plus ONNV) and two controls (uninfected pGL3-hsc70 transfected cells and uninfected pGL3-Basic transfected cells) were sampled in triplicate at 1, 6, 12, 24, 48, and 72 hr post infection (MOI=0.01). ONNV titers were also measured at each time point. While no difference in the hsc70 promoter activity was observed between ONNV infected and uninfected C6/36 cells, luciferase expression was significantly higher in ONNV-infected Vero cells than the uninfected controls at 48 and 72 hr post-infection when viral titer reached its peak of ~1.8 x 10^7 pfu/ml. This demonstrates that the Ag_hsc70 promoter region contains genetic elements that induce hsc70 expression in response to ONNV infection. The absence of increased Ag_hsc70 promoter activity in ONNV infected C6/36 cells may be due to endogenous genetic elements masking the Ag_hsc70 promoter activity in mosquito cells. An increase in luciferase activity that correlated with viral titer and cytopathic cell lysis in the ONNV infected Vero cells, indicates that mammalian cells lack a sufficient endogenous defense mechanism against the virus. The implications of these findings and the fine genetic structure of the Ag_hsc70 promoter are discussed.

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BLOODMEAL ANALYSIS OF *CULEX* SPECIES IN CENTRAL ILLINOIS

Nina M. Krasavin

University of Illinois, Champaign, IL, United States

Blooded Culex *pipiens* and *Culex restuans* were collected in gravid traps placed at 12-18 sites encompassing residential, commercial, animal

housing, and natural/park habitats. The samples were tested by primers specific for cytochrome b of mammals and birds and those with an adequate amount of amplicon were sequenced for species determination in GenBank. Based on the numer of bird and mammal bands on gels, we were able to determine the importance of host availability to the ratio of mammal to bird feeding. In areas with an abundance of cows and horse, the proportion of mammals increased up to about 20%. We also determined the species of hosts and number of mixed bird and mammal bloods. The main avian species was American robin followed by other common birds. The blood feeding pattern is compared to host antibody pattern and the variability in host species is discussed.

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SELECTION OF A PEPTIDE INHIBITOR OF WEST NILE VIRUS INFECTIVITY FROM A PHAGE DISPLAY LIBRARY

Young S. Hong[1], Seokyoung Kang[1], Brian D. Byrd[2]

[1]Tulane University, New Orleans, LA, United States, [2]Western Carolina University, Cullowhee, NC, United States

West Nile Virus (WNV) is transmitted from infected birds to humans via mosquito bites. Since WNV infects host cells by receptor-mediated endocytosis, it is hypothesized that blocking of WNV envelope glycoproteins may reduce infectivity of the virus in the host. To identify inhibitory peptides that bind to WNV envelope proteins, we screened a random phage display library expressing a complexity of ~1.0 x 10^9 different dodecapeptides. For the panning of the library, ~7.0 x 10^6 WNV particles were immobilized in 100 µl PBS buffer in a 96-well plate overnight at 4 °C. Following immobilization and washing, ~2.0 x 10^12 phages (input) were added to the WNV-coated well. The input phages were incubated with WNV for 3 hrs at room temperature. After the incubation with WNV, unbound phages were removed by washing with PBST buffer (PBS, pH 7.5 plus 0.5% Tween) three times. Phages bound to WNV were eluted with 100 µl of glycine buffer (pH 2.0). The eluted phages were amplified using a bacterial host, K91 and this panning procedure was repeated two more times. At the completion of three-rounds of panning, 72 individual clones were selected and amplified for WNV affinity assays using sandwich and indirect ELISAs. In the affinity assays, a phage clone (WN10.1) showed strong signals that were comparable to a positive WNV control. We tested this candidate phage for its inhibitory effect on WNV infectivity using plaque assays with Vero cells. For the plaque assay, each of 6 serial 10-fold dilution of WNV titers from 2 x 10^5 to 2 virions was mixed with 2.0 x 10^14 WN10.1 phages. As a negative control, the same WNV dilutions were also mixed with phages (f88-4) without peptides. The WN10.1 decreased both number and size of WNV plaques while the negative control had no effect. In addition, $TCID_{50}$ assays demonstrated ~92% inhibition of WNV infectivity by WN10.1. Thus, this peptide appears to be a potent inhibitor of WNV infectivity in host cells. Potential applications of WN10.1 for WNV therapeutic and mosquito vector control via transgenesis will be discussed.

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COMPARATIVE GENOMICS OF ANTI-VIRAL RNA INTERFERENCE PATHWAYS IN MOSQUITOES

Corey L. Campbell, Ann M. Hess, William C. Black, IV, Brian D. Foy

Colorado State University, Fort Collins, CO, United States

Small RNA regulatory pathways control many aspects of development and anti-viral defense in metazoans. Member(s) of the Argonaute family of catalytic enzymes degrade target RNAs in each of these pathways. These gene silencing pathways are controlled by different types of small RNAs: microRNAs (20-22nt), small interfering RNAs (siRNA) (20-22nt) or PIWI-type small RNAs (piRNAs) (24-30nt). The *Ae. aegypti, Anopheles gambiae* and *Culex pipiens* genomes encode all major orthologs required for functional miRNA, siRNA, and piRNA pathways. Mosquitoes generate viral siRNAs when infected with RNA arboviruses. However, in some mosquitoes, arboviruses survive antiviral RNA interference (RNAi), to be later transmitted via mosquito bite to a subsequent vertebrate host. Increased knowledge of these pathways and functional components should help us better understand the limitations of anti-viral defense in vector mosquitoes. Toward this goal, we compared the genomic structure of RNA regulatory pathway components across three mosquito species and all three major small RNA pathways. *Ae. aegypti* and *Cx. pipiens* have undergone expansion of Argonaute and PIWI subfamily genes. Further, a gene expansion has also occurred in a family of RNA helicases, orthologous to *D. melanogaster* Rm62. Phylogenetic analyses were performed, and cis-acting upstream regulatory motifs were identified for all small RNA pathway components. Recent functional studies in *Cx. pipiens* echo those of earlier findings in *Anopheles* and *Aedes* mosquitoes, wherein silencing of the siRNA pathway enzyme, Argonaute 2, results in increased dissemination of alphaviruses. However, *Cx. pipiens* is unique, in that a gene duplication has occurred, and multiple transcript isoforms have also been confirmed. The gene silencing experiments successfuly reduced transcripts from all three categories. Isoform-specific gene silencing experiments are underway. These data substantiate our hypothesis that the anti-viral RNA defense pathway is functional in mosquitoes and suggest that regulation or escape from this pathway is a key feature of vector competence.

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CHARACTERIZATION OF PI3K AND ITS REPRODUCTIVE ROLE IN THE MOSQUITO *AEDES AEGYPTI*

Michael A. Riehle[1], Benjamin M. Pri-Tal[2]

[1]University of Arizona, Tucson, AZ, United States, [2]Portland State University, Portland, OR, United States

Insulin and the insulin/insulin growth factor 1 signaling (IIS) cascade regulate a wide range of physiological processes in invertebrates, including lifespan, reproduction, and metabolism. We characterized one of the IIS components, the catalytic subunit of phosphatidylinositol 3-kinase (Aaegp110), in the yellow fever mosquito *Aedes aegypti*. The *Aaegp110* gene encodes five putative domains (adapter binding, ras binding, C2, helical, and PI3-kinase) that were identified by sequence homology with other p110 proteins in both vertebrates and invertebrates. The *Aaegp110* transcript was expressed during all *A. aegypti* life stages except late pupae, and particularly high levels were found in embryos. In female tissues, *Aaegp110* transcript and protein, like many other components of the IIS cascade, were strongly expressed in ovaries. Aaegp110 was also moderately expressed in midguts, fat bodies and heads. Since the IIS cascade plays a key role in mosquito reproduction we examined Aaegp110 ovarian expression during a reproductive cycle. Aaegp110 was expressed in ovaries prior to and during the first 24 hours post-bloodmeal, but was undetectable 36 to 48 h post- bloodmeal. Following oviposition Aaegp110 protein levels returned to pre-bloodmeal levels. In reproductively arrested ovaries Aaegp110 was present predominantly in the cytoplasm of follicle cells surrounding the oocyte. In vitro stimulation of the ovaries with 17µM bovine insulin resulted in translocation of Aaegp110 from the cytoplasm to cell membrane in 15 s. Lower concentrations (0.17 µM) also recruited Aaegp110 to the cell membrane.

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THE AGING MOSQUITO: INCREASED INSULIN SIGNALING IN THE MIDGUT OF *ANOPHELES STEPHENSI* REDUCES LIFESPAN

Laurel Watkins de Jong, Michael Riehle

University of Arizona, Tucson, AZ, United States

The insulin/insulin growth factor 1 signaling (IIS) cascade regulates both lifespan and reproduction in invertebrates. Studying this regulation in mosquitoes can lead to significant insights into the vector's ability to transmit disease. Akt is an important signaling molecule in the IIS cascade that can be expressed in an active form without prior activation of upstream components. Such an active variant of the mosquito

Akt, myr-AsteAkt-HA, was genetically engineered into *Anopheles stephensi* mosquitoes under the control of a midgut specific promoter (carboxypeptidase). The transgene was specifically expressed in the midgut of transgenic mosquitoes both prior to and after blood feeding, although increased transcript and protein levels were observed 2 to 6 h after the bloodmeal. As expected, a significant reduction in lifespan was observed in transgenic mosquitoes overexpressing myr-AsteAkt-HA. Transgenic mosquitoes had higher mortality rates than wild-type mosquitoes when given both sugar only or sugar supplemented with weekly bloodmeals. 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. Protein studies of individual midguts from transgenic mosquitoes revealed high levels of transgene expression in approximately half of the samples and little or no expression in remaining samples. Reproductive studies presented a similar difference amongst the transgenic adults with half laying normal quantities of eggs and half being sterile. Midgut proteins blotted with an anti-phosphoAkt antibody revealed a unique band in transgenic mosquitoes both prior to and after bloodfeeding in addition to the native phosporylated Akt observed only after bloodfeeding. Tests using Akt inhibitors on the transgenic mosquitoes and exploration of the status of other components of the IIS were conducted.

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GENE EXPRESSION IN ADULT MOSQUITOES DURING POST-EMERGENCE DEVELOPMENT

Jinsong Zhu, Jeff Busche, Xing Zhang

Virginia Tech, Blacksburg, VA, United States

Newly emerged female mosquitoes need about 2-3 days to become physiologically competent for blood feeding and egg maturation, and to develop behaviors essential for host-seeking and mating. The genetic programs that govern the post-emergence development remain largely unknown. Although insect juvenile hormone (JH) has been implicated as a decisive factor in these drastic physiological changes, it is not clear to what extent JH controls this development and whether the hormonal effects are mediated through gene regulations. We utilize a genomic approach to explore the JH responses in the adult *Aedes aegypti* mosquitoes. First, we compared gene expression between the young and mature adult mosquitoes to elucidate molecular basis of the post-emergence development. Next, abdomens collected upon eclosion from the young female adults were topically treated with JH, while the organic solvent (acetone) was used as control. Microarray analysis has led to identification of many mosquito genes that are either up-regulated or down-regulated by *in vitro* JH treatment. Finally, we are constructing a mosquito transgenic strain that ectopically expresses a juvenile hormone esterase, causing a JH deficiency in the newly emerged mosquitoes. This transformant will be used to validate the molecular targets of JH and to characterize the JH effects during this developmental stage.

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DEVELOPMENT OF A MULTIPLEXED PCR DIAGNOSTIC TO IDENTIFY COMMON MEMBERS OF THE SUBGENERA *CULEX (CULEX)* AND *CULEX (PHENACOMYIA)* IN GUATEMALA

Rebekah J. Kent, Stephen Aspen, Martin Williams, Harry Savage

Centers for Disease Control and Prevention, Fort Collins, CO, United States

There are currently 16 recognized species in the subgenus *Culex (Culex)* and two species of *Culex (Phenacomyia)* in Guatemala. Morphological differentiation of these species is extremely tedious, with reliable identification ensured only through careful examination of larval and pupal skins from reared specimens as well as male genitalia. West Nile virus (WNV) has recently been isolated from at least three species of *Culex (Culex)* in Guatemala; however, some isolates were from mosquito pools of uncertain taxanomic origin. Therefore, our objective was to develop a multiplexed PCR diagnostic which would reliably differentiate common *Culex* mosquitoes, including known arbovirus vectors, in this tropical ecosystem. *Culex* mosquitoes were individually reared from larval collections, and identification verified through examination of each life stage. Internal transcribed spacer regions of the rDNA gene array were amplified and sequenced from reared voucher specimens. A universal forward primer and species-specific reverse primers were manually selected from a multiple sequence alignment and optimized on specimens of known identity. Multiplexed primers were validated on unknown field specimens. This diagnostic will be a valuable tool for confirming mosquito species identification for entomological and arbovirus ecology studies in Guatemala.

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THE POPULATION GENETIC STRUCTURE OF A*NOPHELES GAMBIAE* IN KENYA

Janet T. Midega[1], Charles M. Mbogo[1], Guiyun Yan[2], John I. Githure[3], John Beier[4]

[1]Kenya Medical Research Institute, Kilifi, Kenya, [2]University of California, Irvine, CA, United States, [3]International Centre for Insect Physiology and Ecology, Nairobi, Kenya, [4]University of Miami, Miami, FL, United States

Anopheles gambiae Giles is a major vector of malaria in Africa, where the species exhibit an ability to adapt to a wide range of ecological settings and seasonal conditions. Along the Kenyan coast, *An.gambiae* s.s. populations undergo periodic fluctuations in population size and during the dry season, *An. gambiae* larval and adult numbers reduce considerably. We hypothesized that the seasonal rise and fall in *Anopheles* numbers and severe population reductions during the dry season might have implications for the population genetic structure of *An. gambiae* s.s. We genotyped a total of 417 adult mosquitoes from two sites (Mtepeni and Jaribuni) using 11 microsatellite primers. Results indicated insignificant differences in the number of alleles or heterozygosity within the two *An. gambiae* populations ($p>0.05$). However interpopulation comparisons indicated significant differences between the total number of alleles in the two sites ($p=0.04$). Overall, expected heterozygosity (He) was higher than observed heterozygosity (H*o*). Between season comparisons indicated departures from HWE and significant heterozygote deficiency at all loci at the Jaribuni site, while in Mtepeni, the dry season populations were in HWE. Population differentiation was not significant between sites and seasons. *An. gambiae* s.s between the two sites were panmictic.

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GENE FLOW OF *AEDES AEGYPTI* IN URBAN REGIONS BASED ON THE USE OF NEW MICROSATELLITE MARKERS

Paulo R. Melo[1], Luciano K. Silva[1], Gilmar J. Ribeiro[1], Carlos G. Santos[1], Ronald E. Blanton[2], Mitermayer G. Reis[1]

[1]Oswaldo Cruz Foundation, Salvador, Brazil, [2]Case Western Reserve University, Cleveland, OH, United States

Aedes aegypti is the main vector for dengue and other arboviruses. An important characteristic is its short flight range, which is likely to limit gene flow. Reduction of adult mosquito densities has is one control measure for this vector, however, reinvasion of treated areas reduces its efficacy. A gene flow map based on microsatellite analysis could better guide this approach. This study was undertaken to identify and standardize new microsatellite markers for A. aegypti and evaluate population structure at 3 different locations in Salvador, Bahia, Brazil. *A. aegypti* were collected from traps and breeding areas at 38 sites clustered in 3 regions of Salvador, and DNA was extracted. Potential microsatellites were identified from publicly available data bases. Genotyping of PCR products was performed via an ABI 3100 sequencer. Four polymorphic loci consisting of simple trimer repeats produced clear, easily interpretable patterns by PAGE. All repeat polymorphisms were stepwise, but there were occasional

small deletions in the flanking regions. There were no deviations from Hardy-Weinberg proportions. Heterozygote deficiency was observed in at least one marker for each population at each study site. When comparing the areas North and South of the city, the Fst was -0.022, and for the Center and the South it was -0.006, while between the North and Center the Fst was 0.051. In conclusion, for the North and South of the city or the Center and the South, there is significant gene flow, but the Center is relatively isolated from the North. Although these results are preliminary due to the small number sampled, they mirror the spread of dengue in the city's first epidemic. This also mirrors the likely flow of human traffic in the city. In future mapping efforts, we plan to take advantage of the city's periodic mosquito surveys of thousands of sites to begin to fine map the distribution and movements of *Ae. aegypti* within the city.

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EVOLUTIONARY PLASTICITY OF THE MALARIA MOSQUITO GENOME

Ai Xia, Maria V. Sharakhova, Zhijian Tu, Igor V. Sharakhov

Virginia Tech, Blacksburg, VA, United States

Anopheles gambiae, *An. funestus*, and *An. stephensi* are primary malaria vectors and are members of different series of the subgenus *Cellia*. These species are highly polymorphic for inversions which are associated with ecological adaptations and distributed non-randomly on five chromosome elements. The goal of this study was to determine the rates of inversion fixation and to identify molecular features that correlate with unequal rates of karyotype evolution. We mapped 326 *An. stephensi*, *An. funestus*, and *An. gambiae* cDNA and BAC clones to *An. stephensi* polytene chromosomes and used 231 uniquely located markers for comparative mapping with *An. gambiae* and *An. funestus*. The comparative mapping has clearly demonstrated a striking contrast among chromosome arms in length of conserved segments: small conserved blocks (< 2 Mb) on arm 2R and large conserved blocks (up to 8 Mb) on 3R and 3L of *An. gambaie*. There were significantly more synteny blocks shared among all three species than synteny blocks shared between only a pair of species. This suggests existence of functional gene clusters that constrain chromosomal breakage. The analysis using the Genome Rearrangements In Man and Mouse (GRIMM) program revealed that the X chromosome has the highest rate of inversion fixation, whereas autosomes vary in the inversion density: 2R>2L>3R>3L. Another remarkable observation was a significant positive correlation between polymorphic inversions and fixed inversion on autosomes. The analysis of the *An. gambiae* genome revealed a significant negative correlation between the number of fixed inversions and the density of Scaffold/Matrix Attachment Regions (S/MARs) suggesting a role of nuclear architecture in determining the chromosome specificity of rearrangement rates. S/MARs can potentially mediate an interaction of specific chromosome sites with a nuclear envelope and can affect intra-chromosomal interactions. In addition, we found a positive correlation between the rates of inversion fixations and the simple repeat content on five chromosomal arms. This suggests involvement of simple DNA repeats in facilitating rearrangements.

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HIGH-RESOLUTION CYTOGENETIC PHOTOMAP FOR THE MAJOR MALARIA VECTOR *ANOPHELES GAMBIAE*

Phillip George, Maria V. Sharakhova, Igor V. Sharakhov

Virginia Tech, Blacksburg, VA, United States

The availability of polytene chromosomes in Anopheline mosquitoes provides the unique opportunity for studying how differing levels of chromatin compaction affect functionality of chromosomal regions. However, current mosquito cytogenetic maps are either derived from low-resolution photo images or drawn chromosomes that in some cases vaguely represent polytene chromosomes seen under the microscope. The advent of digital imaging has given us the ability to create high-resolution images that provide a better representation of the actual chromosome structure. Using a new technique for chromosome preparations, we have developed an updated cytogenetic map of the major *Plasmodium falciparum* transmitting vector, *Anopheles gambiae*. This map incorporates an experimental high-pressure treatment that increases the clarity of structures along the chromosome. We follow the normal procedure for creating chromosome squashes, spreading ovaries evenly across the slide and submersing them in a 50% propionic acid solution to remove excess proteins and cause surrounding tissues to become transparent. The process is modified by using a mechanical vise to apply additional pressure when flattening the chromosomes. The added pressure evenly squashes the slide, causing the entire slide to be on the same plane of focus. Using coordinates of *A. gambiae* Bacterial Artificial Chromosomes (BAC) clones which were previously hybridized to the polytene chromosomes, we have also integrated a crude 0.5 Mb coordinate map for each chromosomal arm. BAC clone locations were taken from Vector Base and only include clones that hybridized to specific loci. Some regions along the chromosomal arms either contain BAC clones that did not hybridize specifically enough to provide an accurate estimate of the clone's location or did not contain BAC clones at all and require additional mapping. This new map is being used to precisely map bands, interbands, and heterochromatin - euchromatin junctions to the genome assembly.

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GENETIC STRUCTURE IN THE ARBOVIRAL VECTOR *CULEX TARSALIS*: A SPATIAL ANALYSIS OF POPULATION DIFFERENTIATION ACROSS THE WESTERN UNITED STATES

Meera Venkatesan, Jason L. Rasgon

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

Culex tarsalis is an important vector of West Nile Virus, Western Equine Encephalitis Virus and St. Louis Encephalitis Virus. Molecular tools have recently become available to study genetic variability, quantitative trait loci and population structure in *Cx. tarsalis*. In this study we present the first detailed analysis of the genetic structure of *Cx. tarsalis* across its geographic range. 20 populations in 17 states were genotyped using 14 microsatellites and a mitochondrial locus. Microsatellites reveal significant genetic differentiation and isolation by distance while ND4 mitochondrial sequences indicate panmixia and high within-population variation, corroborating earlier evidence that a range expansion during the Pleistocene period may have homogenized populations across North America. Using the recently developed Bayesian analysis tools GENELAND and BAPS, we have delineated genetic structure in a spatial context to define two-dimensional geographic population boundaries of *Cx. tarsalis*. We have also identified land use and habitat parameters associated with population boundaries and potential barriers to gene flow in this mosquito.

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RECONSTRUCTING ANCESTRAL CHROMOSOMAL ARRANGEMENTS IN THE *ANOPHELES GAMBIAE* COMPLEX

Igor V. Sharakhov, Ai Xia, Maria V. Sharakhova

Virginia Tech, Blacksburg, VA, United States

Members of the *Anopheles gambiae* complex have remarkably distinct ecological adaptations, behaviors, and degrees of vectorial capacity. Inferring phylogenetic relationships in the complex can be helpful for identifying the genomic changes associated with the origin and loss of epidemiologically important traits. However, the high level of sequence similarity, genetic introgression, and shared molecular ancestral polymorphisms makes reconstruction of the *A. gambiae* complex phylogeny difficult. Phylogenetic relationships among the members of species complexes can be inferred from the distribution of fixed chromosomal inversions if outgroup arrangements are known. The aim of this work is to test a possibility of determining ancestral autosomal arrangements in the *A. gambiae* complex using outgroup chromosomes