



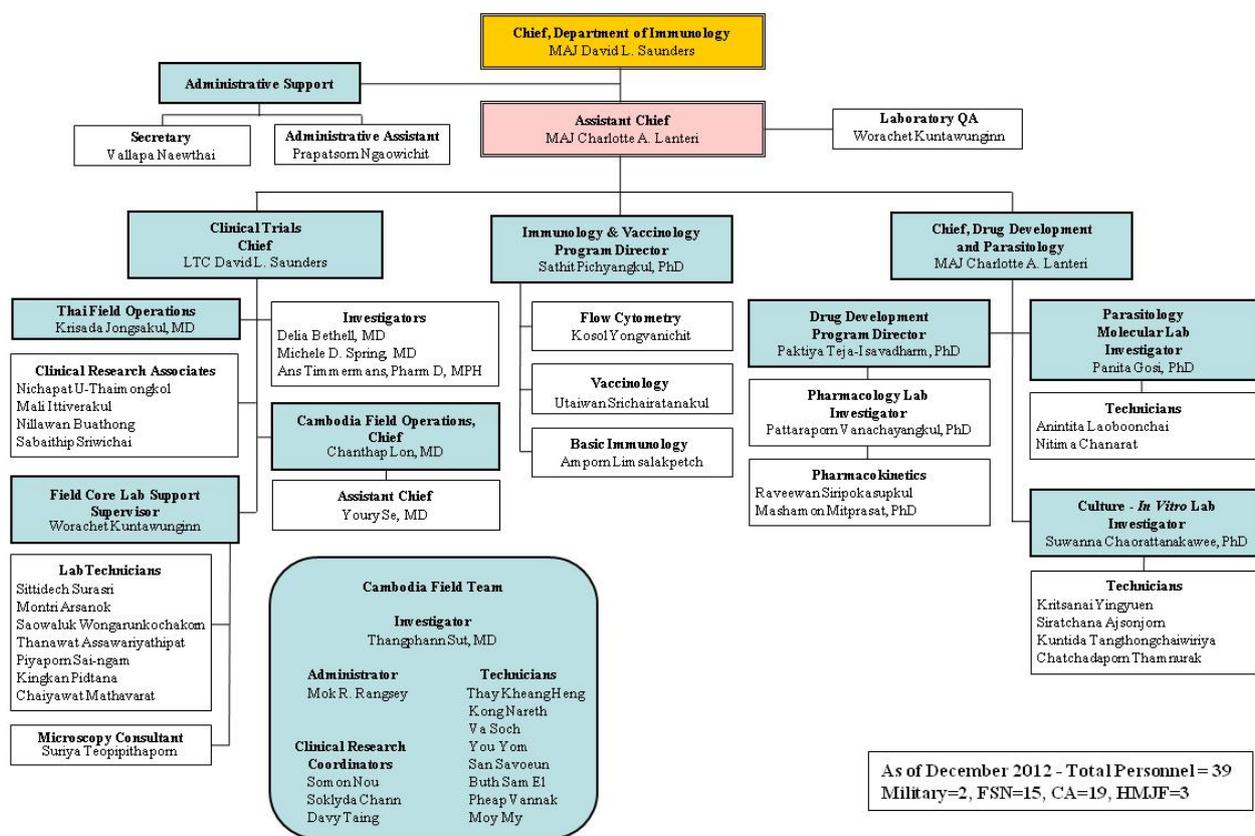
DEPARTMENT OF IMMUNOLOGY AND MEDICINE

DEPARTMENT MISSION

To support medical product development to protect the war fighter and host nation citizens, **and** conduct surveillance of diseases of military importance in SE Asia for our Sponsors:

1. U.S. Army Medical Research and Materiel Command
2. Other Department of Defense and U.S. Government Entities (GEIS)
3. Extramural Collaborators

PERSONNEL



Investigators:

- COL Douglas S. Walsh, MD (out-going Department Chief)
- LTC David L. Saunders, MD (in-coming Department Chief)
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- Dr. Thangphann Sut, MD

IN-HOUSE TRAINING PROGRAMS AND OUTSIDE TRAINING OF PERSONNEL

In-House Training of Personnel

- Validation and Analysis Influenza Data by Using Pivottable and Pivotchart Training. AFRIMS, Bangkok. 9 August 2012
- Weapons of Mass Destruction Training. AFRIMS, Bangkok. 31 August 2012
- Mosquito Dissection and Preservation, Department of Entomology. AFRIMS, Bangkok. 9-12 October 2012

Outside Training of Personnel

- FSN Supervisory Skills Workshop. U.S. Embassy, Bangkok. 6-10 February 2012
- Malaria Prophylaxis Cambodia Protocol WR#1849 Study Specific Procedures and CITI Training. Anlong Veng. 16-18 February 2012
- Malaria Prophylaxis Cambodia Protocol WR#1849 SSP Training. Siem Reap, Cambodia. 26-28 March 2012
- Malaria Prophylaxis Cambodia Protocol WR#1849 Medic and Nurse Refresher Training. Anlong Veng. 13-14 March 2012
- Malaria Prophylaxis Cambodia Protocol WR#1849 Laboratory Study Specific Procedures WR#1849 Training. Anlong Veng, Cambodia. 13-15 March 2012
- Malaria Prophylaxis Cambodia Protocol WR#1849 Dry Run. Anlong Veng, Cambodia. 29-30 March 2012
- Malaria Prophylaxis Cambodia Protocol WR#1849, Medic Clinical Guidelines Training WR#1849. Anlong Veng, Cambodia. 12-20 April 2012
- Malaria Prophylaxis Cambodia Protocol WR#1849 Protocol Refresher Training and Wet Run. Siem Reap, Cambodia. 30 April-2 May 2012
- First Aid Training. Phnom Penh, Cambodia. 5-7 March 2012, 8-10 March 2012
- First Aid Training. Anlong Veng, Cambodia. 18-19 April 2012
- GIS for Beginners. Geo-Informatics and Space Technology Development Agency (Public Organization), Bangkok. 12-16 March 2012
- Influenza Sentinel Surveillance Protocol WRAIR#1630 Refresher Training (Data Collection). Pailin/Battambang/Anlong Veng, Cambodia. 14-16 March 2012
- Influenza Sentinel Surveillance Protocol WRAIR#1630 Refresher Training. Pailin/Battambang/Anlong Veng, Cambodia. 10-13 July 2012
- Influenza Sentinel Surveillance Protocol WRAIR#1630 Site Initiation and Biosafety Training. Banteay Mancheay Province, Cambodia. 1-5 October 2012
- Influenza Sentinel Surveillance Protocol WRAIR#1630 Self-Training SOP. Anlong Veng, Cambodia. 20 December 2012
- Influenza Sentinel Surveillance Protocol WRAIR#1630 Informed Consent Refresher Training. Pailin, Cambodia. 27 December 2012
- Entomology Mosquito Trap Collection Training 2. Anlong Veng. 18-22 March 2012
- Entomology Mosquito Trap Collection Training 1. Anlong Veng. 17 December 2012
- Biosafety Training. Phnom Penh, Cambodia. 4-8 April 2012



- Biosafety Training. Anlong Veng, Cambodia. 25-26 April 2012
- Laboratory and Safety Training for WR#1795A, KRCH. Sangkhlaburi, Thailand. 14-16 May 2012
- General Laboratory Knowledge Training 1. AFRIMS-CNM, Battambang, Cambodia. 1-30 July 2012
- General Laboratory Knowledge Training 2. AFRIMS-CNM, Battambang, Cambodia, 27 August-2 November 2012
- Good Clinical Practice Training. Ministry of Public Health, Thailand. 6-8 August 2012
- Biorisk Conference. Biosafety and Biosecurity Network (Thailand) and AFRIMS, at Century Park Hotel, Bangkok. 7-8 August 2012
- The Medical Effects of Ionizing Radiation (MEIR) Course. Military Medical Operations, Armed Forces Radiobiology Research Institute at Yokota Air Base, Yokota, Japan. 17-19 September 2012
- Abbas's Advanced Course in Basic and Clinical Immunology. Allergy, Asthma and Immunology Association of Thailand (AAIAT), Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. 19-20 October 2012
- qPCR Expert Seminar. Pathumwan Princess Hotel, Bangkok. 24 October 2012
- Transmission Blocking Protocol WR#1877 Nurse Training. Anlong Veng, Cambodia. 21 November 2012
- Transmission Blocking Protocol WR#1877 Nurse Training. Anlong Veng, Cambodia. 10 December 2012
- Transmission Blocking Protocol WR#1877 SSP Training. Anlong Veng, Cambodia. 31 October-3 November 2012
- Transmission Blocking Protocol WR#1877 SSP Training. Anlong Veng, Cambodia. 4-5 November 2012
- Malaria Microscopy: Inter-country Refresher Training. Saraburi, Bangkok. 27 November-1 December 2012
- Basic Malaria Microscopy Training for Clinicians from USUHS by field microscopy team
- Hosting of U.S. military physicians pursuing a career in tropical medicine and research for a clinical rotation at Kwai River Christian Hospital in Sangkhlaburi, Thailand and at AFRIMS field sites in Cambodia

AWARDS

Non-applicable

ACCOMPLISHMENTS

Methods:

The Department of Immunology and Medicine has applied as many kinds of classical and state-of-the-art technologies as possible to the above multi-faceted research. Clinical research included mobile epidemiology team able to work in adverse conditions where malaria is present, including field sample collection and processing screening, reference microscopy, assessment of rapid diagnostics for various tropical infectious diseases, and a staff well-versed in conduct of clinical trails to GCP and ICH standards. The animal research teams are all trained in laboratory animal research and regulations, current AAALAC requirements, and laboratory animal test and observation methods. State-of-the art methodologies are available for the study of vaccine and

drugs to include advanced molecular biology methods such as sequencing, SNP analysis, and real-time PCR. Cellular immunology techniques are available which include flow cytometry and sorting technologies, ELISPOT, and molecular methods. Pharmacology assays include HPLC, LC-MS, a unique malaria bioassay to measure the *in vivo* anti-malarial bioactivity of potential new anti-malarial medications, sustained malaria cell culture and radio-isotopic uptake, and antibody based methods for measuring *in vitro* drug sensitivity patterns of malaria strains against standard malaria drugs.

Results:

Accomplishments during the period of January-December 2012:

1. Malaria Drug Development

Managed the implementation of departmental quality practices for the execution of studies in agreement with MRMC policies and U.S. FDA standards in support of IV AS drug development program. Work involved upkeep of personnel training and qualification records; space utilization for LCMS lab, sample repository, and field clinical lab; establishment of a controlled sample tracking and inventory system; qualification of equipment used for regulated studies; and continued interaction with Medical Maintenance and service contractors. Helped integrate departmental QA/QC efforts with those of the subsequently established QA units at the AFRIMS, WRAIR and MRMC, participated in the IPT teleconferences, providing metabolism and pharmacokinetics insight.

Parenteral anti-malarial drugs are indicated for the treatment severe malaria and when oral therapy cannot be given. The goals of treatment are prevention of death and reduction of morbidity. Even when treated with appropriate anti-malarial drugs, severe malaria in austere or resource-limited settings in the developing world may be associated with high mortality rates because of complications for which treatment may not be available, such as acute renal failure and acute respiratory distress syndrome. Little has been reported in the peer reviewed literature about the burden of severe malaria in the government referral hospital in Battambang (BRH), Western Cambodia's second largest city. However, data from the Cambodian National Malaria Center (CNM) in 2007 indicates that Battambang Province had the second highest mortality rate for probable and confirmed malaria in Cambodia. We conducted a retrospective epidemiological survey to establish the burden of severe malaria in this hospital and to assess the potential for conducting clinical trials in the future. All cases of malaria admitted to the BRH from January 2006 to December 2008 with a discharge diagnosis of severe malaria were reviewed for demography, mortality, and referral patterns. There were 2,648 reported cases of severe malaria among 59,848 confirmed malaria cases in 2007 nationally, with a case fatality rate of 8.3%. There were 132 cases and 23 deaths (17.4% mortality) from severe malaria in 2007 reported from BRH, out of 4,105 confirmed malaria cases reported in Battambang Province that year. A manuscript is close to submission.

Mirincamycin is a lincosamide antibiotic structurally related to Clindamycin. Prior work in the 70s and 80s led to discovery of anti-malarial properties in primates, but the drug was never tested in humans. Recently, interest in this compound has resurfaced. A formal oral bioavailability study of this drug was conducted in non-human primates in 2008, and was found to be roughly 10-13% compared to intravenous administration. The drug was reasonably well tolerated. In 2009, an abstract was presented at ASTMH on the comparison of the absolute oral bioavailability (F) and *ex-vivo* anti-malarial activity against *P. falciparum* (as a W2 clone of *P. falciparum*) of cis-mirincamycin (c-MC) and trans-mirincamycin (t-MC) in 4 groups of healthy rhesus monkeys at a dose of 4mg/kg IV or 20mg/kg PO. No significant differences were observed between single dose c-MC and t-MC in PK or PD parameters by the IV or oral route



in non-human primates. Higher ratios of *ex vivo* activity to concentration in the oral dose groups for the first 90 minutes suggests first pass metabolism with formation of an active metabolite. Further PK-PD analysis in infected primates determined that the compound was safe, though ineffective for treatment of relapsing *P. cynomolgi* malaria. This data supported an IPT decision at the WRAIR to suspend further development of the lincosamide class, with consideration for pursuit of analog synthesis in the future. Timely data provided by the Department was instrumental in achieving a 'quick kill' - an important objective of the Army Drug Development Program; thereby, conserving resources to pursue other more promising leads. A manuscript (Khemawoot *et al*) was published in AAC in December 2011.

AFRIMS Immunology and Medicine supported the WRAIR in obtaining a new Investigational New Drug (IND) application from FDA for a safe, effective human *Plasmodium vivax* challenge model. The department implemented *P. vivax* infected blood donor screening processes to FDA standards to ensure challenge subject safety. In collaboration with the Department of Entomology, we supported the first human challenge in healthy volunteers at the WRAIR using *P. vivax* infected mosquitoes produced under regulated IND from donors in Thailand. We now seek, in collaboration with investigators from the Royal Thai Army, to replicate this model at AFRIMS in Thailand to demonstrate that volunteers can be safely and reproducibly infected with *Plasmodium vivax* (*P. vivax*) by the bites of experimentally infected *Anopheles dirus* (*An. dirus*) mosquitoes carrying *P. vivax* sporozoites in their salivary glands. The study protocol was finalized by the end of 2010, yet was put on hold by the institutional review board due to relapse concerns in primaquine treated patients.

Populations of military personnel in the region continue to suffer from an inordinate burden of malaria, particularly in forward deployed areas. A strategy to prevent malaria infection is regarded as a critical element of appropriate force health protection measures. Most militaries in the region do not use anti-malarial chemoprophylaxis on a routine basis, relying instead on personal protective measures to prevent mosquito bites. In the Royal Cambodian Armed Forces, this approach has had limitations with an ongoing burden of malaria in some areas. In the target study population, the incidence of malaria may be as high as 5-10% per month, with a high proportion of malaria naïve soldiers and dependents likely to benefit from anti-malarial chemoprophylaxis. The U.S. Army Medical Materiel Development Activity, in cooperation with the Walter Reed Army Institute of Research are currently establishing field sites to evaluate new products for anti-malarial chemoprophylaxis which are badly needed. While several viable prophylaxis drug candidates exist, no new studies to evaluate the efficacy for a prophylaxis indication have been conducted in more than a decade. As a response, our department conducted, in collaboration with the national Cambodian Malaria Program (CNM) an active malaria epidemiology cohort study in personnel and dependents of the Royal Cambodian Armed Forces (RCAF) in 2010 comparing safety and efficacy of 2 vs. 3 days of DHA-piperaquine for the treatment of uncomplicated malaria. Incidence of malaria was as high as 5-10% per month - even higher in some locations. Further, it was found that there were no differences in DHA-piperaquine efficacy whether the same dose was given over 2 or 3 days. The rates of malaria recurrence at 42 days were very similar in both groups with 89% per protocol efficacy for 2 days of DP (95% CI = 76-96%) and 92% for 3 days (95% CI = 80-97%) of DP. The effect on the cardiac QT interval was also studied intensively. EKGs were obtained at screening, pre-dose, daily for 3 days, and then weekly for 4 weeks if prolongations were seen during the dosing period. Overall, QTc prolongations were mild and transient in nature. The drug effect was modest in this population, and similar to what has been seen in the other large phase 3 studies. This study was the first step in determining the feasibility of conducting future malaria prophylaxis studies at this site, and characterizing the population, malaria epidemiology and effectiveness of currently prescribed anti-malarial therapy with 2 days of DHA-piperaquine. Data

about malaria burden in RCAF and baseline effectiveness of DHA-piperaquine in RCAF and dependents was analyzed and disseminated to partners in 2011 and will be used to design rigorous, carefully controlled clinical research studies.

In 2011, the department prepared, in collaboration with USAMMDA for the first prophylaxis study in the Royal Cambodian Armed Forces, which started in May 2012. The purpose of this study is to determine if a 2 day course of DHA-piperaquine taken monthly is safe and effective as a chemoprophylaxis regimen in an area of multi-drug resistant falciparum and vivax malaria. The study is currently halted pending review by the study's Cardiac Data Safety Monitoring Board.

2. Malaria Drug Resistance Surveillance

Artemisinin based combination therapies (ACTs) are the first line treatment for drug resistant *Plasmodium falciparum* malaria. The current major global investment in ACTs is threatened by the possible emergence of resistance to artemisinins, as signaled by a trend of increasing ACT treatment failure on the Thai-Cambodian border, which has historically been an epicenter of drug resistant malaria. Once it develops and spreads, resistance to the artemisinin derivatives, could very well be the most devastating event in the history of malaria control in the 21st century. There are no effective alternatives to artemisinins for the treatment of malaria either on the market or nearing the end of the drug development process.

Strategies for containing artemisinin resistance require the ability to detect it rapidly and accurately, both in humans (*in vivo*) and in collected parasite isolates (*in vitro*). AFRIMS' proven ability to monitor artemisinin resistance with a consistent regionally applied method and standards for its *in vitro* drug sensitivity testing and *in vivo* efficacy trials is critical in this regard.

Artesunate in combination with mefloquine has been the first-line drug for uncomplicated falciparum malaria on the Thai side of the border since 1995 and in Cambodia since 2000. Therapeutic efficacy monitoring is regularly conducted by both the Thai and Cambodian malaria control programs. Both progressively increased parasite clearance times and unusually high failure rates with artesunate-mefloquine have been reported recently on both sides of the border.

2.1 Thailand

AFRIMS began working in collaboration with the Thai MOPH in Trat Province, Thailand, to try and determine why the treatment failures described by the Thai National Malaria Program (Vijaykadjia, 2006) were occurring. An integrated *in vivo-in vitro* approach was adopted using existing protocols. This approach comprised anti-malarial treatment in accordance with MOPH guidelines (directly observed treatment with AS (6mg/kg daily for 2 days), MQ (25mg/kg split into 2 doses) and PQ (0.5mg/kg single dose on Day 2) with all doses given as DOT), and *in vitro* culture of parasites with drug sensitivity assays at admission to the study and subsequently if treatment failure occurred. Parasite growth inhibition was used as a measure for drug sensitivity of fresh samples in a HRP2 double-site antigen capture ELISA. Follow-up had previously been to Day 28 in accordance with WHO guidelines (WHO 2003) but was extended to 42 days when AFRIMS became involved since this is the preferred duration of follow-up following MQ therapy. We found that the PCR-corrected ACPR (cure rate) at 42 days for Trat in 2005 was 81% (7 out of 42 enrolled patients failed therapy and 5 were reinfected). The second (and currently on-going) Trat study, (WRAIR #1327) started in September 2007. The study also uses an *in vivo/in vitro* approach yet incorporates a more detailed human use (*in vivo*) study, with plasma drug level measurements and a comparison of 2 and 3 days AS treatment. AFRIMS and the Thai MOPH will continue to work in collaboration during the forthcoming study.

The *in vivo* component aims to compare the efficacy and tolerability of artesunate



(12mg/kg) and mefloquine (25mg/kg) given over 2 or 3 days for the treatment of uncomplicated *P. falciparum* malaria in Trat Province, Thailand. This has important public health implications as it may influence future treatment policy. Due to the changed local epidemiology of malaria in Trat and the malaria containment efforts in border districts in Trat, this site will not generate a sufficient number of enrolled volunteers with *P. falciparum* malaria before the end of the trial in 2012. The protocol is currently kept open for data analysis.

Concerns with regard to artemisinin resistance in *P. falciparum* parasites are now extending to the Thai-Myanmar border where cases of poor response to standard antimalarial regimens are beginning to emerge. Furthermore, *P. vivax* parasite resistance to chloroquine (CQ), the recommended first-line drug for treatment of vivax malaria, appears to be spreading towards Thailand: Elevated IC₅₀ levels to CQ have been detected *in vitro* in *P. vivax* parasites from Thai patients, and a recent case report described a case of high-grade CQ resistance in a vivax malaria patient from western Thailand.

In collaboration with our colleagues at the Royal Thai Army Medical Department, we plan from 2013, to expand our anti-malarial drug resistance work to include malaria patients presenting to RTA facilities along Thailand's other international borders.

In vitro drug sensitivity and molecular data will be obtained using standardized departmental methodologies and will augment the harmonized data currently being generated by our other anti-malarial drug resistance projects in Cambodia. Furthermore, we wish to expand our current role to increase host nation capacity by establishing labs for analysis of known molecular markers of anti-malarial drug resistance within Thailand. Selected RTA field staff were trained in malaria microscopy in 2010 and the study protocol received ethical review board approval in 2012. An integrated *in vivo-in vitro* therapeutic efficacy surveillance study is also scheduled to start at AFRIMS' malaria research center at the Kwai River Christian Hospital (KRCH) in Sangkhlaburi, close to the Thai-Myanmar border.

2.2 Cambodia

Data from AFRIMS' earlier ARC1 study conducted in Western-Cambodia in 2006 suggest that along parts of the Cambodian-Thai border there are individual *P. falciparum* isolates, which are highly resistant to artemisinins. Although the prevalence of these isolates was low, the overall sensitivity of the parasite isolates was significantly reduced as compared to Western Thailand. In ARC1 some individual isolates were associated with greatly increased parasite clearance times, treatment failures despite 7 days of artesunate monotherapy (4mg/kg), and very high inhibitory concentrations for artemisinins *in vitro*. Reports from the Ministries of Public Health on both sides of the Thai-Cambodian border indicate increasing numbers of treatment failures with artemisinin-based combination therapies.

In 2009, the Department of Immunology and Medicine completed the ARC2-trial, a follow-up study to ARC1. The aim was to determine whether regimens with increased artesunate doses could overcome the problem of reduced drug sensitivity to artemisinins and to determine whether these experimental regimens, particularly the high-dose regimen, were safe and well tolerated. Similarly like in ARC1, the study was conducted in a purpose-built AFRIMS study ward at Tasanh Health Center in Western Cambodia, due south of Pailin and close to the border with Thailand. Tasanh Health Center and its referral health clinics stand in the middle of the crucial area of the growing reports of emergence of artemisinin resistance. The study was conducted in a designated study ward and staffed by a team of Cambodian and Thai nurses, physicians, microscopists and laboratory technicians, in close collaboration with the National Center for Parasitology, Entomology and Malaria Control (CNM) in Cambodia.

The study determined that increasing doses of artesunate monotherapy given for

7 days did not improve clinical or parasitological outcomes in Cambodian patients with uncomplicated *P. falciparum* malaria. Even with high-dose treatment (6mg/kg/day for 7 days) cure rate was 88%, comparable to previous AS monotherapy studies in terms of efficacy. However, when patients receiving AS 4 mg/kg/day in this study were compared to those treated with exactly the same regimen in our previous 2006 study at the same site, the proportion of patients still parasitemic at 72 hours had almost doubled from 29 to 56%. This finding confirms the emergence over the last 3 years of parasite strains that are more resistant to AS *in vivo*, and underscores the importance of current containment strategies.

The pharmacokinetics and pharmacodynamics of oral artesunate monotherapy were also explored as part of the ARC2 trial. Despite weight-based dosing, a wide variability in artesunate concentrations were observed. There were significant reductions in plasma concentrations between day 1 and day 7 of dosing, suggesting auto-induction of metabolic clearance pathways. Dose limiting hematologic toxicity with neutropenia in 5 of 26 subjects occurred at the 6mg/kg dose level.

In vitro drug sensitivity assays have been used as a tool to characterize the drug susceptibility phenotype of clinical *Plasmodium falciparum* isolates and to screen new candidate drugs in development. Variability in *in vitro* drug sensitivity testing throughout the malaria research world makes comparison between different data sets, different labs, and different time periods difficult. In order to develop a testable model system for generating IC50 values with patients' specimens, we finalized the evaluation of dynamics of W2 standard clones as a mechanism to establish a validated control in 2009.

After these stringent method validations, the ARC 2 study has successfully managed to culture malaria parasites and generate IC50 values for a range of anti-malarial drugs (AS, DHA, chloroquine, mefloquine, lumefantrine, quinine) from 136 fresh patient samples, the largest number of fresh parasite isolates from a single clinical study in the region. IC50 values for DHA (major artemisinin metabolite) were higher in isolates of patients with delayed parasite clearance times, indicating that prior exposure to AS and its metabolites may select for development of resistance.

The *in vitro* methodology used in the ARC2 trial was used to initiate a dedicated *in vitro* survey (#WR1576) in Cambodia in 2009 for the purposes of measuring the distribution of resistant phenotypes, as defined in ongoing clinical trials of artemisinins, and obtaining adequate numbers of samples for planned genome-wide association studies.

In order to further characterize malaria parasites collected in surrounding areas at risk, we established a reference lab at the Battambang Referral Hospital (BRH) which was put into operation in September 2009 and inaugurated by the U.S. Ambassador and the Cambodian Ministry of Health in 2010. The lab in Battambang is unique to Western Cambodia, as it is the first fully functional and permanent molecular diagnostics facility in this part of the country and has provided useful data to the CNM to help identify communities at risk for drug resistance. The lab is fully equipped to support advanced malaria culture and drug efficacy studies, as well as, the analytic support for influenza molecular testing.

The major outcome of the Cambodia *in vitro* study in 2010 was to demonstrate that parasites collected in Battambang Province in Western Cambodia had significantly different IC50 values for the artemisinins than parasites collected in Northern Cambodia with a preliminary indication that the spread of the artemisinin resistance phenotype has not moved very far within Cambodia. More than 200 parasite samples were collected. However, in order to identify genetic signatures conferring clinical artemisinin resistance, larger numbers of parasites will need to be collected to fully characterize the underlying parasite population structure. Expansion into more provinces will provide a more robust sample set for large scale analyses.

In 2011, the study expanded to cover more areas of Cambodia including provinces



in the North (Oddar Meanchey, Preah Vihear), West (Battambang, Pailin), and East (Kampong Cham, Kampong Speu) to provide comparisons between the more resistant parasites in the West, and less resistance parasites in the East. More systematic parasite collections were performed from patients with uncomplicated *P. falciparum* in an effort to improve representativeness of the sample population. To date (31 December 2011 update), 748 specimens have been collected of which 420 (or 56.14%) were positive for *P. falciparum*, 291 (or 38.90%) for *P. vivax* and 37 (or 4.94%) were mixed *P. falciparum/P. vivax* infections. Approximately 13.5% of the subjects tested G6PD-deficient. Data analysis is planned for 2012.

Late 2012, the department integrated 2 new components in their anti-malarial surveillance studies that, U.S. force protection issues aside, will also answer key questions in Cambodia's goal of malaria elimination by 2025. Firstly, and in collaboration with the AFRIMS Department of Entomology, an entomological vector surveillance component was added to compare parasites from human hosts to those in circulation within vectors, including genomic signatures of selection for resistance, and the multiplicity of infection. Secondly, Immunology and Medicine added a transmission-blocking component (sexual stage) to the therapeutic efficacy monitoring of standard 3-day DHA/piperazine treatment by randomizing volunteers in arms that do or do not receive a single oral dose of primaquine 45mg given on day 3.

Isolates of Thai and Cambodian malaria patients with treatment failures will be further screened for molecular markers of artemisinin resistance, in collaboration with the University of Maryland. Once identified and validated as predictors of clinical outcomes, molecular markers for artemisinin resistance will be used in a surveillance network (such as the World Wide Anti-Malarial Resistance Network-WWARN) employing molecular, *in vitro*, and clinical tools to measure the present extent of resistance and then guide rational containment strategies to deter its further spread. AFRIMS also conducted drug sensitivity assays in 2009 of new and unknown anti-malarial drug candidates (on blood samples containing malaria parasites, both from patients that have successfully completed and that have failed artemisinin treatment) developed by the MMV, a non-profit foundation created to discover, develop and deliver new, affordable anti-malarial drugs through effective public-private partnerships.

Funding for anti-malarial drug resistance work is sourced from DoD-GEIS, WHO, MMV and the Bill and Melinda Gates Foundation.

Future Plan: Through *in vitro/in vivo* studies with a range of currently used anti-malarial therapies, and in collaboration with other key anti-malarial surveillance research institutions in the region and the U.S., we will continue to monitor drug efficacy, measure the distribution of resistant phenotypes, and collect adequate numbers of samples for population genetics studies that aim to identify molecular markers for artemisinin resistance. In addition, we will use GIS and geographically mapping techniques for all clinical, *in vitro*, and molecular mutation data associated with malaria drug resistance, especially artemisinins, collected by AFRIMS in Thailand and in Cambodia. GIS will permit to track spatially and temporally the emerging trend of anti-malarial resistance to inform public health policy.

The Department of Entomology will continue to assist the surveillance effort in Cambodia by adding systematic vector and parasite collections.

Malaria infection rates in mosquito species/populations will be used to identify potential vectors. Seasonal trends will be analyzed through the incorporation of human data with mosquito species/infection data.

3. Vaccinology and Immunology Studies in Support of Malaria Vaccine Program and Influenza Studies

3.1 Malaria Immunology and Vaccinology

Immunologic studies have contributed to elucidate humoral and cellular immune responses to malaria, as well the immune suppression that is caused by acute falciparum malaria infections. In 2012, our team assessed whether titers of antibodies to the blood stage protein merozoite surface protein-1 (MSP1) provided protection from subsequent malaria infection and/or contributed efficacy of DHA-piperaquine given for uncomplicated *P. falciparum* malaria. A preliminary data analysis of data did not reveal that levels of antibodies to *P. falciparum* or *P. vivax* MSP1 prevented subsequent infection.

Additional multivariate analyses are planned to assess additional factors affecting antibody levels and protection from malaria. Also in October 2012, MIDRP funding was received to also develop a multiplexed bead array assay which allows for measurement of titers to multiple malaria antigens in a single well. Further testing of 1,737 samples will be conducted using antigens such as circumsporozoite protein (CSP), apical membrane antigen 1 (AMA1), and liver stage antigen-1 (LSA1).

The Vaccinology Section has pre-clinically evaluated multiple recombinant candidate vaccines against *Plasmodium vivax*. In 2012, the Vaccinology Section also completed pre-clinical testing of a Pf malaria vaccine in primates (IDRI Celtos Pf) that will be challenged with *P. knowlesii* malaria.

3.2 Influenza Research

3.2.1 Evaluation of *In Vitro* Cross-Reactivity with Avian Influenza H5N1 and Swine Flu H1N1 2009 Viruses in Healthy Volunteers Vaccinated with a Prime Boost Regimen of Seasonal Influenza Vaccine

Recent studies demonstrated that vaccination with inactivated seasonal influenza vaccine elicited heterosubtypic antibodies which neutralized avian influenza H5N1. However, the induction of heterosubtypic antibodies was observed in only a small proportion of vaccines. In order to enhance HSI and evaluate the hypothesis that a prime boost seasonal vaccine regimen would enhance its development, we administered 2 dose regimens of 2009-2010 seasonal influenza vaccine 8 weeks apart to healthy adult volunteers. Twenty-six subjects, 9 males and 17 females, were randomized to receive either 2 doses of intranasal live, attenuated influenza vaccine (LAIV) (n=6), 2 doses of intramuscular inactivated seasonal influenza vaccine (IIV) (n=6), LAIV then IIV (n=8), or IIV then LAIV (n=8).

Results: We found that a prime boost regimen of seasonal influenza vaccine did not enhance cross-reactive immunity against H5N1 and 2009 pandemic H1N1.

Update: Manuscript under review by PLoS One in December 2012 and accepted for publication early 2013.

3.2.2 Cross-Reactive Antibodies to Avian Influenza H5N1 and 2009 Pandemic H1N1 in Non-Exposed U.S. Military Personnel

In humans, the role of cross-protective immunity against influenza A viruses is unclear. Epidemiological data indicate that avian influenza H5N1 and 2009 pandemic H1N1 (pH1N1) unlike seasonal influenza, is less common in older persons (≥ 60 years). This suggests that the elderly may have pre-existing immunity against some influenza A viruses. Here we comprehensively evaluated the presence of cross-reactive antibodies against H5N1 and 2009 pH1N1 in serum samples (N = 200) collected from U.S. military personnel born between 1936 and 1977 (4 cohorts, N=50/cohort; ≤ 1949 , 1960-1965, 1966-1971, 1972-1977).

Results: Our results demonstrate that some U.S. military personnel have functional cross-reactive antibodies against H5N1 and 2009 pH1N1. These pre-existing antibodies may play a role in protection and reduce the severity of disease.

Update: Manuscript under review by EID

3.3 Influenza Surveillance (DoD-GEIS)

This DoD-GEIS-funded project will allow for ongoing surveillance of Influenza-Like-Illnesses (ILIs) and detection of influenza and highly pathogenic influenza among vulner-



able military and civilian populations in Southeast Asia who are not included in other surveillance mechanisms.

AFRIMS is part of the U.S. DoD surveillance program and participates in the collection and characterization of influenza viruses circulating within the human population in Asia. Various AFRIMS departments collect respiratory specimens from sites in Thailand, Nepal, the Philippines, Bhutan and U.S. Embassies in Southeast Asia with plans on expansion to Cambodia and Vietnam, and definitive test results are shared with the Ministries of Health and WHO Flu Net. This surveillance data gathered contributes towards the annual re-formulation of the influenza vaccine as well as early detection of novel influenza strains or existing subtypes with pandemic potential which can increase the lead time for implementation of control and prevention measures.

3.3.1 Kwai River Christian Hospital Surveillance of Influenza Like Illness

The Department of Immunology and Medicine, in collaboration with AFRIMS' Department of Virology, conducts ILI surveillance along the Thai-Burma border at the Kwai River Christian Hospital in Kanchanaburi Province.

The Department of Virology is responsible for protocol development and provides principal investigator and laboratory support. The Department of Immunology and Medicine provides clinical and administrative support, training and supervision.

Results: A new protocol was approved by the Thai MOPH IRB in December 2011. Site re-initiation was conducted in February 2012.

3.3.2 Sentinel Human Surveillance for Influenza in Western Cambodia

This 5-year study aims to characterize influenza types and subtypes and determine genetic heterogeneity and antiviral susceptibility of influenza A viruses circulating in Western Cambodia. The Department of Immunology and Medicine is responsible for protocol development, clinical support, laboratory support for rapid antigen testing and real-time RT-PCR, site supervision, training. Laboratory support for influenza-negative specimens will be provided by the Department of Virology and reference laboratory support will be provided by the Pasteur Institute in Phnom Penh, surveillance data will be collected and analyzed in collaboration with the Cambodian Communicable Disease Control Department and members of the Technical Working Group for influenza in Cambodia (U.S. CDC, Cambodian CDC, NAMRU-2, Institut Pasteur, NIPH).

Approach: Respiratory samples are collected from outpatients with influenza-like-illness (ILI) symptoms at various hospitals. Data regarding household risk factors to avian and human influenza infection and weekly number of ILI-cases will be collected as well and shared with the national ILI surveillance network on a weekly basis. After rapid diagnostic testing on-site, samples will be sent to the AFRIMS-CNM laboratory in Battambang for rapid detection and simultaneous subtyping of clinical influenza specimens. Primers include universal, A, A/H1, A/H1sw, A/H3, A/H5, B.

Since the Cambodian Ministry of Health does not allow transfer of influenza specimens out of the country, confirmation of type and subtype by multiplex PCR and viral isolation will be contracted to the Pasteur Institute in Phnom Penh, which is also the National Influenza Center (NIC) in Cambodia. HA, NA, MP complete genes will be routinely amplified and sequenced of selected isolates. Out of a subset of influenza isolates, full genome sequencing and uploading in GenBank will be conducted. In addition, oseltamivir sensitivity assays will be conducted by measurement of IC50 values using NASTar kit. Respiratory samples negative samples will be sent to AFRIMS for further characterization by MasTag PCR. Data will be shared with DoD-GEIS and key stakeholders in Cambodia through the Technical Working Group for Influenza. Study enrollment started in May 2010 at Thmor Koul Hospital, Battambang Province, AFRIMS' first influenza surveillance sentinel site in Cambodia. Additional sites were opened in Oddar Meanchey and Pailin Province in 2011, followed by Banteay Meanchey

Province in 2012. The AFRIMS-CDC influenza project now covers every border province in Western and North Western Cambodia. The influenza season in Cambodia is well-defined and usually starts in June. The AFRIMS clinical and laboratory teams continue to train and support Cambodian sentinel site and laboratory staff with study specific procedures and quality control.

Update: In October 2011, the AFRIMS Cambodia field team assisted the Royal Cambodian Armed Forces and the CDC Cambodia (MOH) within 24 hours with the laboratory investigation (malaria and influenza) of febrile disease outbreak in 400 troops (from 2 separate units) and their dependents along the Thai-Cambodia border, of which 130 were screened for malaria and/or influenza (which revealed an influenza outbreak). We presented our first preliminary influenza surveillance data collected since the start of the project (in May 2010) until October 2011 titled “Human Sentinel Influenza Surveillance in Remote Border Populations in Western Cambodia” at the annual ASTMH meeting in Philadelphia in December 2011.

Pandemic influenza A/H1N1(2009) being the most common virus identified in 2010 and influenza B being the most prevalent virus in 2011. Highly Pathogenic Avian Influenza (HPAI) virus H5N1 was not detected, and few common respiratory pathogens were isolated from influenza-negative samples. Despite the sites’ proximity to Thailand, influenza activity, seasonality, antigenicity and anti-viral susceptibility in this sample of isolates are following similar trends as observed elsewhere in Cambodia, supporting earlier recommendations from the Cambodian NIC to use Southern Hemisphere influenza vaccine for prevention and neuraminidase inhibitors as treatment and chemoprophylaxis.

During CY12, 298 subjects were enrolled from 4 sentinel sites in 3 provinces. Rapid antigen test results, RT-PCR results were shared with study volunteers (RDT results only), sentinel site staff, provincial health department, Department of Communicable Disease, DoD-GEIS. Overall influenza positivity rate of the 586 specimens collected between May 2010 and 31 December 2012 was 29% and predominant influenza subtypes detected for 2010, 2011 and 2012 respectively were A/H1N1(2009), B, A/H3N2. Despite occasional occurrence of human cases in Western Cambodia, no influenza A/H5N1 has been detected at AFRIMS sentinel sites through laboratory surveillance. Also in 2012, 15 influenza genomes from 2011 and 10 from 2012 (A/H1N1(2009) and A/H3N2) have been sequenced at Institut Pasteur in Phnom Penh and analyzed by the Bioinformatics Section at the WRAIR Virology Branch.

Future Plan: The surveillance project will be maintained with further specimen collections. No further sentinel sites are planned for the time being. A manuscript about spatial analysis of genetic diversity of influenza viruses is under preparation.

Capacity-Building: The department established a joint team with the CNM which includes 12 highly trained Cambodian research staff of physicians, nurses and laboratory technicians. The team is capable of conducting high quality, regulated clinical studies to international standards. In 2012, the team continued to build capacity in expert malaria microscopy, biosafety and occupational health, the ethical conduct of research, protection of human subjects, and good clinical practices.

OVERVIEW OF RESEARCH PROJECTS:

1. Active Clinical Surveillance for *P. falciparum* Drug Resistance and Therapeutic Efficacy Monitoring with Assessment of Evidence for Transmission Blocking Activity of Primaquine in Cambodia

Status: Study initiated in December 2012 and paused due to dry season.

2. Study at the Thai-Myanmar Border as Part of a Harmonized Global Malaria Drug Resistance Surveillance Program at Overseas DoD Laboratories

Status: Protocol approved. Study initiation planned for end of 2013.



3. Evaluation of Molecular Markers of Antimalarial Drug Resistance and *In Vitro* Antimalarial Drug Sensitivity in *P. falciparum* Malaria Parasite from Patients Presenting to Royal Thai Army Health Facilities in Thailand
Status: Protocol approved. Site initiation planned in March 2013.
4. Efficacy of Artesunate-Mefloquine Combination Therapy for the Treatment of Uncomplicated *Falciparum* Malaria in Trat Province, Thailand
Status: Manuscript in preparation.
5. Evaluation of *In Vitro* Cross-Reactivity with Avian Influenza H5N1 Virus in Healthy Volunteers Vaccinated with a Prime Boost Regimen of Seasonal Influenza Vaccine
Status: Manuscript in press
6. Survey for *In Vitro* and Molecular Markers of Antimalarial Drug Resistance in Cambodia
Status: On hold; data entry and analysis conducted in 2012.
7. Human Influenza Sentinel Surveillance in Cambodia
Status: On-going at 3 sentinel sites-over 500 ILI-specimens collected since 2010-25 whole influenza genomes obtained for spatial molecular analysis. Manuscript under preparation
8. A Randomized, Double Blind, Placebo-Controlled Clinical Trial of Monthly DHA-Piperazine for Malaria Prevention in Cambodia
Status: Study initiated in May and halted in June 2012 pending DSMB review.
9. An Active Malaria Epidemiology Cohort Study in Personnel and Dependents of The Royal Cambodian Armed Forces with Evaluation of a 2 Day Versus 3 Day Treatment 7 Regimen of DHA-Piperazine for Patients with Uncomplicated Malaria
Status: Data analysis
10. Artemisinin Resistance in Cambodia II (ARC2 study)
Status: Approved by WHO and Cambodia; parasite data included by WWARN/-MORU for meta-analysis.
11. Sentinel Human Surveillance for Influenza in Thailand
Status: New protocol approved by Thai MOPH IRB. Site re-initiated in March 2012.
12. Comparison of Malaria SYBR Green I Fluorescence (MSF) and Histidine-Rich Protein 2 Enzyme-Linked Immunosorbent (HRP2 ELISA) Assays for measuring *In Vitro* Drug Susceptibility of *Plasmodium falciparum* Reference Clones and Fresh *Ex Vivo* Field Isolates from Cambodia
Status: Manuscript under preparation
13. *Ex Vivo* Activity of Anti-Malarial Candidate Compounds from MMV against Multi-Drug Resistance *P. falciparum* Isolates from Western Cambodia
Status: Manuscript under preparation
14. Anti-Malarial Drug Susceptibilities in Fresh *Ex Vivo* Isolates of *Plasmodium falciparum* from Cambodia in 2008 to 2012: in Association with *pfmdr1* and *pfprt* Gene
Status: Data analysis
15. A Comparison between *Ex Vivo* and *In Vitro* Antimalarial Susceptibilities for *P. falciparum* from Cambodia
Status: Laboratory experiment in process
16. Analysis of Plasma Samples from Malaria Patients for *Ex Vivo* Antimalarial Activity in a *P. falciparum* Bioassay to Help with Interpretation of Clinical Findings in Malaria Trials
Status: Continuing project