



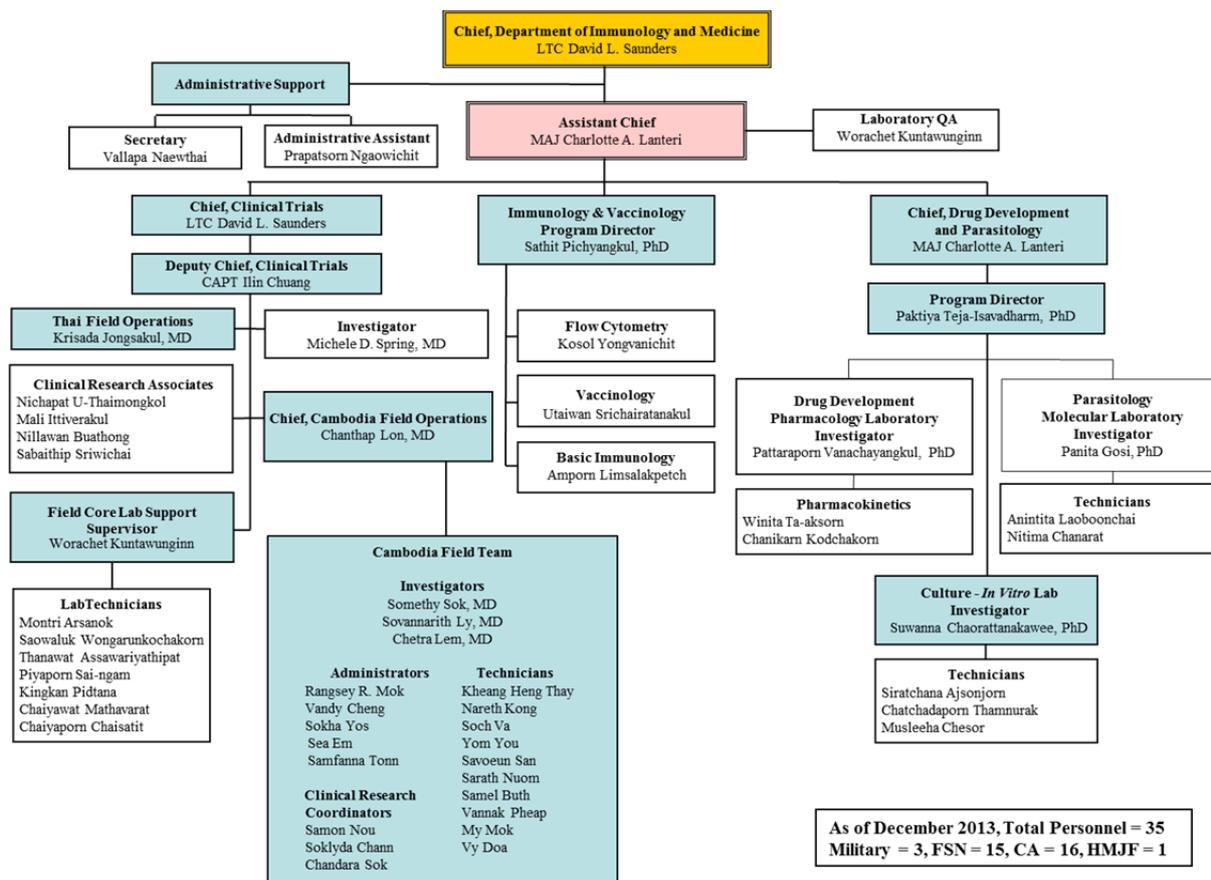
DEPARTMENT OF IMMUNOLOGY AND MEDICINE

MISSION

To support medical product development to protect the war fighter and host nation citizens, **and** conduct surveillance of diseases of military importance in Southeast 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



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- Dr. Chetra Lem, MD

IN-HOUSE TRAINING PROGRAMS AND OUTSIDE TRAINING OF PERSONNEL

In-House Training

- Malaria Slide Reading Training for visiting medical student from New York University. January 2013
- Annual Briefings for ABSL-3, B-virus Hazard. Occupational Health & Safety (OHS) Program. February 2013
- AFRIMS Biosafety & BSC Certification Workshop. April 2013
- AFRIMS Department of Immunology and Medicine, Molecular Biology Section Trained Cambodian Field Team Medical Research Technicians on PCR Techniques To Support Clinical Studies, Including Proper Pipette Use, PCR Master Mix Preparation, Extraction of DNA and RNA from Clinical Samples, Real-Time PCR Assay Methods and Machine Programming. Battambang Referral Hospital, Cambodia. 12-31 August 2013
- AFRIMS Clinical Lab and Entomology Training Course. Anlong Veng Referral Hospital, Cambodia. 6-30 September 2013
- Surveillance Investigation and Outbreak Response to Rapid Response Team (RRT) Exercise Workshop. Battambang Referral Hospital, Cambodia. 6-7 November 2013
- AFRIMS Fire Fighting Training. November 2013
- DoD Information Assurance Awareness Training. November 2013
- DoD Ethics Training. November 2013
- Prior to initiating WR#1917, AFRIMS Bangkok staff conducted training of local field team staff at Srisaket and Ubon Ratchathani, Thailand, on protocol performance, SOPs, SSPs, GCP
- Prior to initiating WR# 2017, AFRIMS Bangkok staff conducted training of clinical study team at KRCH, Sangkhlaburi, Kanchanaburi, Thailand on Protocol Performance, SOPs, SSPs, and CITI.
- AFRIMS English Proficiency Course, on-going

Outside Training

- Electrocardiogram (ECG) Training by medical experts from Calmet Hospital. Anlong Veng Referral Hospital, Cambodia. 4-6 January 2013
- Data Safety Monitoring Board (DSMB) Final Meeting for WRAIR#1849 with CNM Director and study team. CNM Headquarters, Phnom Penh, Cambodia. 5 March 2013
- Surveillance Investigation and Outbreak Response to Rapid Response Team (RRT) Workshop Led by Cambodian CDC. Apsara Angkor Hotel, Siem Reap, Cambodia. 1-7 June 2013
- Improving International Health Reporting Requirements (IHR) in Cambodia to support DTRA project on febrile illness detection. Siem Reap, Cambodia. 3-6 June 2013
- USAMMDA Human Subject Protection (HSP) and Good Clinical Practice (GCP) training of clinical research staff. Bangkok, Thailand. June 2013
- 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.



- Hosting of Ph.D candidate from UNC-Chapel Hill at AFRIMS-Bangkok and at field labs in Cambodia to assist with his ph.d dissertation project and to train up in molecular biology techniques applied to the study of malaria transmission.

AWARDS

Non-Applicable

ACCOMPLISHMENTS

Methods: The Department of Immunology and Medicine applies a variety of classical and state-of-the-art technologies to execute a multi-faceted clinical and preclinical research program. A mobile epidemiology team allows for conducting clinical work in adverse, remote field locations in malaria endemic areas, including field sample collection and processing screening, reference microscopy, assessment of rapid diagnostics for various tropical infectious diseases, and a staff well-versed in performing clinical trials to GCP and ICH standards. Drug susceptibility profiling of fresh clinical *Plasmodium falciparum* and *P. vivax* isolates from malaria patients is routinely conducted using antibody- and microscopy-based methods to help interpret efficacy outcomes, detect cases of drug resistant infections, and actively track drug resistance geographical and temporal trends in Thailand and Cambodia. Routine cultures of reference *P. falciparum* clones are maintained and new drug resistant isolates are cloned for molecular and phenotypic characterization. State-of-the art molecular methodologies are available for the study of the efficacy of vaccine and drug candidates, to include advanced molecular biology methods such as sequencing, SNP analysis, and real-time PCR. Technologies for detecting and monitoring safety signals in clinical trials include high resolution melting real-time PCR detection of glucose-6-phosphate dehydrogenase (G6PD) deficiency mutations and electrocardiogram monitoring of patients administered test drugs. Cellular immunology techniques are available, such as flow cytometry and sorting technologies, ELISPOT, and molecular methods. The preclinical research teams are all trained in laboratory animal research and regulations, current AAALAC requirements, and laboratory animal test and observation methods. Pharmacology assays include HPLC, LC-MS, and LC-MS/MS analyses of *in vivo* levels of drugs and drug candidates evaluated in nonhuman primates and in clinical trials, as well as a unique malaria bioassay to measure the *ex vivo* anti-malarial bioactivity of preclinical and clinical plasma samples to ascertain correlations between pharmacokinetics and pharmacodynamics of evaluation antimalarials.

Results: Accomplishments during the period of January-December 2013:

1. Malaria Drug Development

1.1 Preclinical malaria drug development in nonhuman primates

The AFRIMS Department of Immunology and Medicine, Pharmacology Section, supports preclinical development of new drug candidates for the U.S. Military Malaria Research Program (MMRP). We investigate in rhesus monkeys the pharmacokinetic/pharmacodynamic (PK/PD) properties of promising lead candidate molecules. These compounds, ideally representing diverse chemical classes with novel mechanisms of action, are selected based on available preclinical data. Detailed PK models in healthy Rhesus are developed and compared with *ex vivo* activity of plasma from monkeys administered test compounds against human *P. falciparum* blood stages to prioritize compounds for advanced preclinical development. We analyze plasma samples via LC/MS or LC/MS-MS (when greater analytical sensitivity is needed) to determine if the parent molecule and/or a metabolite(s) is responsible for antimalarial activity. Besides



analyzing plasma, we are developing dried blood spot methods that require only 15 μ l of blood per timepoint applied to cellulose-based filter papers to analyze blood PK. These blood levels can serve as markers of the intraerythrocytic parasite's exposure to the compound. Furthermore, potential safety concerns identified previously in small animal models will be fully explored using appropriate clinical and laboratory endpoints. The most promising drug candidates are further evaluated in a rhesus *P. cynomolgi* efficacy model, using the dosing regimen identified in the healthy rhesus model likely to be safe and efficacious.

The healthy rhesus PK/PD model is efficient, as results important to guiding further drug candidate development decisions can be acquired relatively quickly and using few animals. Each compound is dosed and evaluated in Rhesus in as few as 7-14 days, depending on the experimental conditions. PK (in plasma and blood) and PD (*ex vivo P. falciparum* bioassay) analyses can usually be completed within two months following testing in Rhesus. Animal resources are conserved because the same monkey is used to test different doses or routes of dosing (as needed) of a particular compound by applying a wash-out period in which no test compound is applied prior to testing the next dose of interest. This approach also avoids the potential confounder of inter-individual differences in metabolism or other pharmacological factors that can occur when analyzing the PK of a compound in different monkeys.

Using this model, the PK/PD properties and safety of test candidates are evaluated in monkeys. We determine key PK parameters in plasma and blood, such as maximum concentration (C_{max}), elimination half life ($t_{1/2}$) and the area under the plasma concentration-time curve (AUC). The AUC is an especially important PK parameter because it provides an indication of the relative degree of *in vivo* exposure for a test compound and its metabolites. The AUC can be compared to the *P. falciparum ex vivo* bioassay results to derive an estimated *in vivo* concentration required to kill 50% of blood stage parasites (IC_{50} value).

Compounds are also prioritized based on safety assessments. Our protocol includes daily observations of monkey behavior, including grooming, mood and eating habits. In addition, tolerability of test compounds during and after administration is carefully evaluated. Routine blood chemistries are performed, such as CBCs, HCT, Hgb, reticulocytes counts, etc., Liver function tests (ALT, AST) are also conducted to detect any potential liver toxicities. Furthermore, we have the capacity to measure other safety endpoints, as needed based on the particular compounds or chemical class being evaluated. For example, AFRIMS Veterinary Medicine staff has conducted daily measurements of met-hemoglobin formation as a marker for primaquine-induced oxidative stress. Preclinical safety results in other species and information about the compound class can therefore guide the design of safety endpoints to be monitored for each study.

The most promising candidate(s) are tested for efficacy in *P. cynomolgi* -infected rhesus using the optimal dose identified in studies conducted in the healthy rhesus PK/PD model. The combined results attained in the PK/PD rhesus model and the subsequent efficacy studies are critical information necessary to support an FDA investigative new drug (IND) application. Such lead candidates will be identified as demonstrating adequate oral absorption, having acceptable safety and tolerability, and exerting antimalarial activity in the *ex vivo* bioassay against chloroquine-resistant *P. falciparum* parasites. Such promising compounds will be evaluated in a Rhesus malaria model at the dose identified most likely to be efficacious (based on bioassay results related to PK data) and safe.

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 four 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.

In 2013, the AFRIMS Department of Immunology and Medicine evaluated the PK/PD properties of a dozen preclinical drug candidates for the MMRP. The results of these investigations lead to selection of a new drug class for further development as a new prophylaxis agent for malaria. Furthermore, in 2013 the Department collaborated with the Medicines for Malaria Venture on a project investigating the pharmacology and malaria prophylactic and radical curative properties of a new drug candidate. Further studies with MMV are currently being planned to explore the PK/PD properties of another preclinical drug candidate.

1.2 Severe malaria clinical investigations

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 reporting these findings was published in 2013.

1.3 *P. vivax* human challenge model

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.

1.4 Clinical trials to support malaria prophylaxis drug development for the U.S. military

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 two 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. Results from this DHA-piperaquine efficacy trial were published in PLoS One in 2014.

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 was 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 was halted once a pre-determined cardiac safety signal was reached. The results from this important clinical safety and efficacy study were presented at the annual meeting of the American Society of Tropical Medicine and Hygiene and is undergoing review in the journal Antimicrobial Agents and Chemotherapy.



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 emerging and spreading of resistance to artemisinins, as signaled by a trend of increasing ACT treatment failures on the Thai-Cambodian border, which has historically been an epicenter of drug resistant malaria. 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 (*ex vivo*). AFRIMS' proven ability to monitor artemisinin resistance with a consistent regionally applied method and standards for its *ex vivo* 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 investigate why the artemisin-based treatment failures described by the Thai National Malaria Program 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 (6 mg/kg daily for 2 days), MQ (25 mg/kg split into 2 doses) and PQ (0.5 mg/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 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 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 worked on these trials as collaborative efforts to study and better understand artemisinin resistance resulting in malaria treatment failures, an important public health policy concern.

The *in vivo* component of these efforts aimed 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. Due to the changed local epidemiology of malaria in Trat and the malaria containment efforts in border districts in Trat, this site did 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 and submission of a final manuscript reporting results from these trials.

Concerns with regard to artemisinin resistance in *P. falciparum* parasites have extended to the Thai-Myanmar border, where cases of poor response to standard antimalarial regimens are rapidly emerging. Furthermore, *P. vivax* parasite resistance to chloroquine (CQ), the recommended first-line drug for treatment of vivax malaria, appears to be spreading towards Thailand, evidenced by elevated IC₅₀ levels to CQ 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 (RTA) Medical Department, in 2013 we expanded our anti-malarial drug resistance work to include malaria patients presenting to RTA facilities along Thailand's other international borders. AFRIMS Department of Immunology and Medicine staff recently initiated a new malaria drug resistance surveillance clinical study with the RTA. This on-going trial, WRAIR 1917, entitled "Evaluation of Molecular Markers of Antimalarial Drug Resistance and *In Vitro* Antimalarial Drug Sensitivity in *P. falciparum* Malaria Parasites from Patients Presenting to Thai Military Health Facilities in Thailand" involves active surveillance of malaria drug resistance in *P. falciparum* cases occurring at sentinel sites of multidrug resistance emergence along the Thai-Cambodian border. Emergence of artemisinin resistance, especially along the Thai-Cambodian border, is currently the largest threat to malaria control.

The findings from WRAIR 1917 are of significant global public health relevance. The team is responsible for providing malaria disease and drug resistance surveillance data for military populations not covered by other Thai national surveillance mechanisms. The team is developing geospatial and longitudinal associations of *in vitro* drug sensitivity trends, newly identified molecular resistance markers and clinical data to dramatically improve the ability to monitor and curb emerging resistance to the artemisinins and partner drugs. Through this new AFRIMS-RTA initiative, *in vitro* drug sensitivity and molecular data are being obtained to 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 initiated in June 2013, with continuing ongoing patient enrollment. We expected to enroll 40-50 cases per year during the 5 year study period.

The solidarity of the joint AFRIMS Immunology and Medicine and RTA staff was critical in establishing successful study sites capable of supporting clinical trials of rigorous international regulatory standards. Starting in 2012, the AFRIMS team skillfully and efficiently conducted field site development at two sites: a military healthcare facility at Pusaron in Kantharaluk District, Srisaket Province and at a second site in Kabchoeng District, Surin Province. The team professionally demonstrated diplomacy to surmount logistical and administrative challenges, such as acquiring RTA command level approval and training in laboratory research and study design procedures for local healthcare staff who never before conducted clinical studies. The team's training efforts successfully resulted in developing a local healthcare staff team capable of independently conducting key clinical trial procedures, such as applying subject selection criteria, obtaining informed consent from prospective volunteers, data collection, blood collection per specific testing, adverse events recording and reporting, and timely specimen shipment to a central laboratory in Ubol Ratchathani. A further testament to the team's dedication to the AFRIMS research mission is building Thai healthcare capacity to achieve compliance with the International Conference on Harmonization of Good Clinical Practices guidelines.

AFRIMS Department of Immunology and Medicine also initiated a second malaria drug study in Thailand in 2013. The clinical trial WRAIR 2017 is a DoD GEIS effort involving close collaboration of 3 different DoD research labs in Kenya, Peru, and Thailand at our site at the Kwai River Christian Hospital (KRCH) in Sangkhlaburi, close to the Thai-Myanmar border. The study required extensive coordination and frequent teleconferences and site visits to plan a methods-harmonized trial and to achieve IRB approval. The main focus of the study is to investigate artemisinin resistance trends in the 3 countries by examining the efficacy of artesunate-mefloquine (AS-MQ) in uncomplicated *P. falciparum* malaria patients and studying key parasitological, pharmacological, molecular drug resistance marker, and immunological endpoints. AS-MQ is one of the five ACT combinations recommended for the treatment of uncomplicated *P. falciparum* malaria, is commonly used in many malaria



endemic regions, and is a standard treatment regimen in all three study venues. This integrated *in vivo-in vitro* therapeutic efficacy surveillance study was initiated at the AFRIMS' malaria research center at KRCH in October 2013, and to date 8 subjects were enrolled and have completed 42 days follow up without recurrence. A total of 59 evaluable volunteers are required, and active enrollment is on-going.

2.2 Cambodia

Data from AFRIMS' earlier Artemisinin Resistance in Cambodia trial 1 (ARC1) study conducted in Western Cambodia in 2006 suggest that along parts of the Cambodian-Thai border there are *P. falciparum* isolates that 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 (4 mg/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 Artemisinin Resistance in Cambodia 2 (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 Tassan Health Center in Western Cambodia, due south of Pailin and close to the border with Thailand. Tassan 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 (6 mg/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 6 mg/kg dose level.

In vitro drug sensitivity assays have been used as a tool to characterize the drug susceptibility phenotype of clinical *P. 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 ARC2 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 ongoing genome-wide association studies.

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 (Dec 31 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.

Late 2012, the department integrated 2 new components in our anti-malarial surveillance studies that will address 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 45 mg given on day 3.

Isolates of Thai and Cambodian malaria patients with treatment failures were screened for molecular markers of artemisinin resistance, in collaboration with the University of Maryland. The findings from these efforts were presented in several research symposia at recent annual meeting of the American Society of Tropical Medicine and Hygiene and also were published in peer-reviewed scientific literature. This molecular biology research has



proven to be useful in measuring the present extent of resistance and guiding 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 Medicines for Malaria Venture (MMV), a non-profit foundation created to discover, develop and deliver new, affordable anti-malarial drugs through effective public-private partnerships. The MMV project findings are soon to be published in the journal *Antimicrobial Agents and Chemotherapy*.

In 2012-2013, AFRIMS Department of Immunology and Medicine initiated a malaria drug treatment study in Cambodia that produced findings of great significance to the U.S. Military Malaria Research Program (MMRP) and the global health community. The focus of the trial, WRAIR 1877, entitled “Active surveillance for *P. falciparum* drug resistance with assessment of transmission blocking activity of single dose primaquine in Cambodia” was to evaluate the efficacy and safety of dihydroartemisinin-piperaquine (DP), recently adopted as first-line artemisinin combination therapy in Cambodia. WRAIR 1877 was a two arm, open-label treatment study of adults with acute, uncomplicated *P. falciparum* malaria comparing the efficacy (42 day PCR-corrected malaria recurrence rate), safety, tolerability and pharmacokinetics of a three day course of DP with or without a single dose of primaquine. The trial was carried out from 10 December 2012-19 February 2013, with the goal of enrolling 150 patients, but was ended earlier after evaluating 96 patients because the team found DP treatment failure rates that were elevated compared to a previous AFRIMS trial conducted only 3 years earlier in the same region. The team’s findings were timely for helping guide the Cambodian national malaria control program’s revision of malaria treatment guidelines.

The team professionally and efficiently worked together to successfully complete this innovative trial conducted in a challenging, austere field setting. A major finding from WRAIR 1877 was unacceptable levels of DP treatment failures (approximately only 64% per-protocol efficacy). The team’s interdisciplinary research approach provided data suggesting that failures appear in part a result of emerging piperaquine resistance, as evidenced by failed patients having *in vivo* plasma levels of piperaquine below *ex vivo* piperaquine 50% growth inhibitory values against their *falciparum* infections. These findings suggestive of piperaquine efficacy being lost to resistance were critical to the MMRP’s decision to de-prioritize DP as a potential malaria prophylaxis drug for use in protecting deployed Soldiers. Furthermore, the outcome of this trial is also being applied by Cambodian and World Health Organization public health officials in updating national malaria treatment guidelines to find an alternative to DP as first-line treatment of drug resistant malaria in Southeast Asia.

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

Future Plan:

The AFRIMS Department of Immunology and Medicine is continuing active surveillance of antimalarial resistance in Thailand and Cambodia, and aims to establish new capabilities to strengthen our research program. Ongoing efforts involve augmenting current capabilities, to include adding new real-time PCR assays to screen clinical samples for the latest molecular markers of drug resistance and establishing a new microscopy-based phenotypic assay for detecting artemisinin resistance, referred to as the ring stage survival assay. In addition, in 2013 we developed and are currently actively implementing a new *P. vivax* drug susceptibility assay involving a *P. vivax* lactate dehydrogenase (LDH) ELISA to monitor *vivax* drug resistance in fresh isolates. Moreover, we will clone *P. falciparum* isolates from patients who failed DP therapy from Cambodia as a means to study molecular mechanisms associated with DP resistance and also to establish clones valuable as positive controls for our phenotypic and molecular assays.



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 as 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-piperazine 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.

MIDRP funding was obtained in FY12 and FY13 to develop a multi-plexed bead array assay (Luminex), which allows for measurement of titers to multiple malaria antigens in a single well. To date, serum from volunteers enrolled in the completed WRAIR IRB-approved study WR 1737 “An active malaria epidemiology cohort study with evaluation of a 2 day versus 3 day treatment regimen of DHA-Piperazine for patients with uncomplicated malaria” has been tested in this new assay. In February 2013, Dr. Alexander Kayatani of WRAIR, and co-principal investigator on the FY12 MIDRP proposal, came to AFRIMS for two weeks to transfer the Luminex technology to the Department of Immunology and Medicine. Antigens selected for testing included: the repeat and N-terminal regions of *P. falciparum* CSP, the C-terminal 42-kDa region of *P. falciparum* MSP1 alleles 3D7 and FVO, *P. vivax* MSP1 (allele Sal I), *P. falciparum* AMA1 allele 3D7, and *P. falciparum* LSA1. All recombinant protein antigens are current/past vaccines candidates or peptides of candidates (RTS,S). Results indicate that levels of antibodies (IgM, IGG, and all 4 IgG subclasses) to various vaccine antigens overall were low, although a wide range of values was seen amongst subjects. There was no indication that level of antibody conferred protection against infection or recrudescence, thus antibodies in this low to moderate transmission area are more indicative of a marker of exposure. A vaccine candidate introduced in this area would be able to boost antibody responses and target immune responses toward a more protective role. For FY13, additional vaccine candidate antigens will be added to the assay (*Pf* and *Pv* CELToS and others) and serum from 2 clinical studies (WR 1849 and WR 1877) tested. A consultation visit by Dr. Evelina Angov from WRAIR in 2014 will aid in optimization and interpretation of all results. Genotyping/sequencing of the 42-kDa region of MSP1 for all *Pf* parasite isolates is also planned for FY13 to give additional information on MSP1 antibody cross-reactivity.

The Vaccinology Section has pre-clinically evaluated multiple recombinant vaccine candidates against *Plasmodium vivax*. Two versions of vaccines were made: a soluble recombinant protein and a particulate antigen fused with HBsAg. Antibody and T cell responses were equally generated. However, antibodies to AGDR region, a potentially protective B cell epitope were only detected in the rhesus monkeys receiving a particulate antigen. Manuscript of this work was published in Vaccine. 2013; 31: 6216. In 2012, the Vaccinology Section also completed pre-clinical testing of a *P. knowlesi* CelTOS formulated with a Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE). In this study, we also tested if *P. falciparum* CelTOS would induce a cross-species protective response. Our results showed that both PkCelTOS/GLA-SE and PfCelTOS/GLA-SE elicited non-protective immune response



against *P. knowlesi* challenge. Manuscript of this experiment is being prepared. The established *P. knowlesi* sporozoite challenge will be a very useful model to investigate the mechanism of protective immunity against malaria infection in the liver (MIDRP proposal FY14).

Update: Two manuscripts published: *Vaccine*. 2013 Dec 16; 31(52): 6216-24 and *PLoS One*. 2013 Aug 12; 8(8): e70819.

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 male and 17 female, 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 published in *PLoS One*. 2013; 8(3): e59674.

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 published in *Am J Trop Med Hyg*. 2014; 90: 149.

3.3 Influenza Surveillance (DoD-GEIS)

This DoD-GEIS-funded project allows for ongoing surveillance of Influenza-Like-Illnesses (ILIs) and detection of influenza and highly pathogenic influenza among vulnerable military and civilian populations in Cambodia.

GEIS 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 Cambodia, 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. These surveillance data contribute 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

(ILI)

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 (KRCH) 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, with the study opening 17 May 2012 and the first enrolled case on 22 May 2012. During 2013, ILI disease surveillance was conducted at KRCH starting at 1 January through the time the study was closed out in September 2013. During this timeframe, there was a total of 11 ILI cases enrolled, with 2 of these patients being diagnosed by RDT as Influenza type A. The KRCH site was officially closed on 18 September 2013 as a result of low ILI case occurrence.

3.3.2 Sentinel Human Surveillance for Influenza in Western Cambodia

This surveillance project aims to characterize influenza types and subtypes and determine genetic heterogeneity and antiviral susceptibility of influenza A viruses circulating in Western Cambodia. Extension of this 8-year study from 2010-2018 is in process. 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, and training. Laboratory support for influenza-negative specimens is provided by the Department of Virology and reference laboratory support is provided by the Pasteur Institute in Phnom Penh. Surveillance data is 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 are collected and shared with the national ILI surveillance network on a weekly basis. After rapid diagnostic testing on-site, samples are 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 is contracted to the Pasteur Institute in Phnom Penh, which is also the National Influenza Center (NIC) in Cambodia. HA, NA, MP complete genes are routinely amplified and sequenced of selected isolates. Out of a subset of influenza isolates, full genome sequencing and uploading in GenBank is conducted. In addition, oseltamivir sensitivity assays are conducted by measurement of IC50 values using NASTar kit. Respiratory samples negative samples are sent to AFRIMS for further characterization by MasTag PCR. Data are 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 Northwestern Cambodia. The influenza season in Cambodia is well-defined and usually occurs in June through December. The AFRIMS clinical and laboratory teams continue to train and support Cambodian sentinel site and laboratory staff with study specific procedures and quality control and collaboration with Cambodian CDC to support outbreak investigator activities and laboratory tests.



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) was the most common virus identified in 2010 and 2013, whereas influenza B was 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 these 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 study from May 2010-Mar 2014, 891 subjects were enrolled from 4 sentinel sites in 4 provinces. Rapid antigen test and RT-PCR results were shared with study volunteers, sentinel site staff, provincial health department, Department of Communicable Disease, and DoD-GEIS. An overall influenza positivity rate of 29.4% (262 of the 891 specimens) collected and predominant influenza subtypes detected for 2010, 2011 and 2012 respectively were influenza B (15.2%), A/H1N1(2009) (6.2%), A/H3N2 (5.7%). Despite occasional occurrence of human cases in Western Cambodia, no influenza A/H5N1 has been detected at AFRIMS sentinel sites through laboratory surveillance. All the influenza positive samples were sent for sequencing at Institut Pasteur in Phnom Penh.

Future Plan: The surveillance project will be maintained with further specimen collections and expand to enroll ILI IPD patient (mild/moderate illness) in 2014. A manuscript describing spatial analysis of genetic diversity of influenza viruses is being prepared.

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 2013, 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 enrolment ceased on 19 Mar 2014, as a result of detecting a relatively high proportion of treatment failures with the artemisinin combination therapy (ACT) being evaluated, dihydroartemisin-piperaquine (DHA-PPQ).

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

Status: Study initiated in October 2014; active enrollment ongoing.



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: Study initiated in June 2014; active enrollment ongoing.

4. Efficacy of Artesunate-Mefloquine Combination Therapy for the Treatment of Uncomplicated Falciparum Malaria in Trat Province, Thailand

Status: Completed 2008. 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: Completed 2012. Manuscript published in Am J Trop Med Hyg. 2014; 90: 149.

6. Survey for *In Vitro* and Molecular Markers of Antimalarial Drug Resistance in Cambodia

Status: Active; Manuscript in preparation.

7. Human Influenza Sentinel Surveillance in Cambodia

Status: On-going at 3 sentinel sites—over 800 ILI-specimens collected since 2010—more than 30% of total specimens were influenza positive and genomes obtained for spatial molecular analysis. Manuscript in preparation

8. A Randomized, Double Blind, Placebo-Controlled Clinical Trial of Monthly DHA-Piperaquine for Malaria Prevention in Cambodia

Status: Study initiated in May 2012; halted in June 2012 after reaching prespecified cardiac safety endpoint; DSMB reviewed 15 Mar 2013. Manuscript submitted to Antimicrobial Agents and Chemotherapy.

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-Piperaquine for Patients with Uncomplicated Malaria

Status: Completed. Manuscript published in PLoS ONE. 2014; 9(3): e93138.

10. Artemisinin Resistance in Cambodia II (ARC2 study)

Status: Approved by WHO and Cambodia; parasite data included by WWARN/MORU for meta-analysis. Last manuscript in preparation.

11. Sentinel Human Surveillance for Influenza in Thailand

Status: Completed 2013. Data analysis ongoing.

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 published in Malaria Journal. 2013; 12: 239.

13. *Ex Vivo* Activity of Anti-Malarial Candidate Compounds from MMV against Multi-Drug Resistance *P. falciparum* Isolates from Western Cambodia

Status: Manuscript being revised and resubmitted for publication in Antimicrobial Agents and Chemotherapy.



14. *Ex Vivo* Drug Susceptibility and *Plasmodium Falciparum* Multidrug Resistance Gene 1 (pfmdr1) Profiling of Clinical Isolates from Cambodia in 2008-2013 Suggesting Emerging Piperaquine Resistance

Status: Manuscript in final stages of preparation.

15. A Comparison between *Ex Vivo* and *In Vitro* Antimalarial Susceptibilities for *P. falciparum* from Cambodia

Status: AFRIMS *In vitro* analyses of samples complete; undergoing molecular characterization at University of North Carolina.

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; sample testing and data analysis ongoing.

17. Building Capacity for Disease Surveillance in Cambodia, Defense Threat Reduction Agency (DTRA) Funded Project

Status: AFRIMS team is supporting and augmenting the febrile diseases surveillance mechanisms and reporting used by the Ministry of Health (MoH) of the Kingdom of Cambodia. Training on Surveillance Investigation and Outbreak Response, was conducted by AFRIMS on 3-5 June, 2013 at the Apsara Angkor Hotel, Siem Reap, Cambodia with 15 Trainers and 67 participants. Since 2013, our surveillance team in Cambodia detected 6 new human cases of avian influenza H5N1. To further build capacity of the Cambodian field team to detect a panel of common febrile illnesses, the AFRIMS team is conducting training and transfer of laboratory technology, to establish the capabilities of a multiplex real-time PCR assay with Fast Track Diagnostic kits, for CNM medical research technicians.

18. Development of a Safe and Reproducible Human Sporozoite Challenge Model for *Plasmodium vivax* for Use in Healthy Adults in Thailand

Status: Resubmission to WRAIR IRB DHSP as a new protocol for scientific review and remains under WRAIR IRB DHSP pre-review. New plan underway to collaborate with Faculty of Tropical Medicine, Mahidol University.

19. *Leptospira* (LPS assay) RAPID PCR Validation Using JBAIDS Molecular Assay Transition Package

Status: This MIDRP Sto L project completed in 2013. Poster of results presented at the ASTMH annual meeting in Washington, DC, Nov 2013. Manuscript in preparation.

20. A Multi-Centre, Double-Blind, Randomised, Parallel Group, Active-Controlled Study to Evaluate the Efficacy, Safety and Tolerability of Tafenoquine (SB-252263, WR238605) in Subjects with *Plasmodium vivax* Malaria, Phase 3 Trial in Cambodia (in Collaboration with GSK)

Status: NECHR approved and contract in negotiations with GSK

21. Comparison of Atovaquone-Proguanil and Artesunate Atovaquone-Proguanil for the Treatment of Uncomplicated *P. falciparum* Malaria in Areas of Multidrug Resistance in Cambodia (in Collaboration with NAMRU2)

Status: AFRIMS SRC approved and submitted to WRAIR IRB

22. Defining Effective, Appropriate, Implementable Strategies for Malaria Elimination in Military Forces in Cambodia as a Model for Mobile Migrant Populations

Status: Bill and Melinda Gates Foundation new research award 2013; Protocol preparation underway.