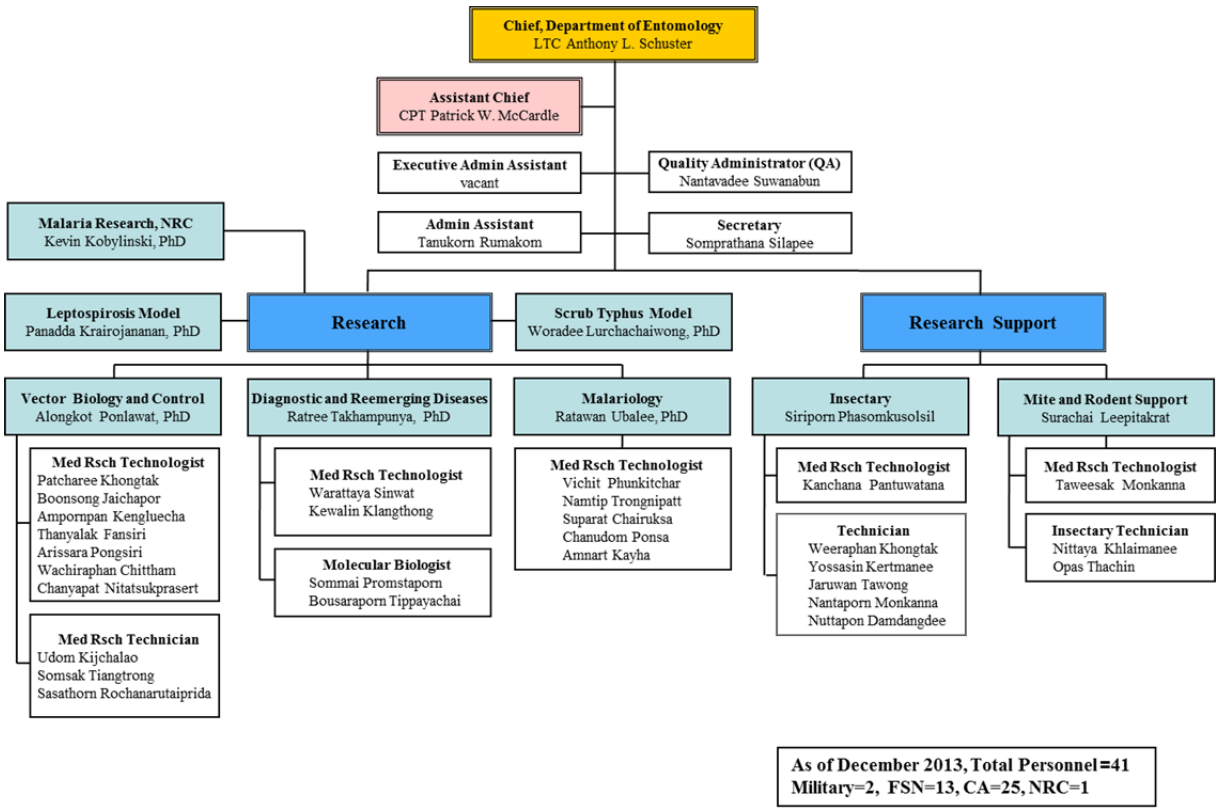
DEPARTMENT OF ENTOMOLOGY

MISSION

To identify vector- and rodent-borne disease threats of military and global health importance and to develop and evaluate interventions and products in order to mitigate those threats.

PERSONNEL



IN-HOUSE TRAINING PROGRAMS AND OUTSIDE TRAINING OF PERSONNEL

In-House Training

- Accident, Illness and Complaint Reporting, Records and Investigations
- Animal Bites and Other Injuries/Reports
- Animal BSL-3 Hazard Training
- B-Virus Post-Exposure Intervention and Prophylaxis
- Bio-Safety in Laboratory
- Blood-Borne Pathogens Exposure Control Plan
- BSL-3 Laboratory Training Program
- Computer Security Awareness
- Composite Risk Management
- Chemical Safety
- Counter Intelligence, Operations Security, and Anti-Terrorism
- Equal Employment Opportunity and Prevention of Sexual Harassment (EEO & POSH)
- Ethics Training
- Fire Prevention, Protection, Report and Investigation

- Fire Fighting Training
- Human Subject Protection Program
- The Humane Care and Use of Lab Animals
- Hazard Communication Program
- IACUC Training
- Laboratory Waste Management
- Occupational Health Program & Communication
- Post-Exposure Prophylaxis and Interventions
- Respiratory Protection Program
- Routine Prophylaxis and Screening and Post-Exposure Prophylaxis and Intervention
- Staff Training on Command SOPs, Protocols, Departmental SOPs
- Safety Equipment Usage
- Basic Supervisory Skills Workshop, Foreign Service Institute U.S. Department of State

Outside Training Provided By Department

- One student from the Bhutan Ministry of Public Health was sponsored by the Asia Pacific Malaria Eradication Network. He received hands on training in all sections for 60 days.
- Two students from a local university trained for 90 days with the Vector Biology and Contol as well as Diagnostic and Reemerging Diseases sections.
- Two students from a local university received training for 30 days in general molecular diagnostic techniques with the Diagnostics and Reemerging Diseases section.
- One public health professional from Rodophe Merieux Laboratory, Faculty of Pharmacy, University of Health Sciences, Cambodia trained in the molecular detection and identification of *Leptospira* species in the Diagnostic and Reemerging Diseases Section. This training was part of the BiodivhealthSEA Project in collaboration with Dr. Serge Morand (Montpelier University-Paris/TropMed-Kasetsart University).
- Two public health professionals from Bhutan were sponsored by the WHO for two weeks and received hands on training in the diagnosis and identification of scrub typhus vectors and pathogens. They receive training in PCR/ELISA techniques from the Diagnostic and Reemerging Diseases and Ectoparasites Sections.
- One student from a local university received training in tick-borne disease detection and identification for 120 days in the Diagnostic and Reemerging Diseases Section.
- Twenty five Pubic health officials from 5 provinces in Thailand received one day of training in proper calibration and use of pesticide application equipment by the Vector Biology and Control Section.
- Two local university students received training for 4 weeks in polymorphism and genetic analysis of *P. vivax* genomes using a Microsatellite Assay method in the Malariology Section.

RESEARCH AREAS

Vector Biology and Control: Develops and evaluates mosquito surveillance traps; evaluates control or preventive measures including insecticide barrier treatments, insecticide-impregnated bed nets and tents, and personal-use and spatial repellents. Studies are aimed at understanding basic vector biology and behavior through vector competence and ovi position assays. Taxonomic keys are continually developed and updated for the mosquitoes of Thailand. Apply Geographic Information Systems (GIS)-based tools to evaluate the risk of vector-borne disease outbreaks. This capability incorporates environmental factors and field collection data of vector/pathogen distributions in order to delineate vector-borne disease such as dengue, Chikungunya and Japanese encephalitis viruses, and malaria risk areas.

Diagnostic and Reemerging Diseases: Develops and evaluates conventional and rapid field assays for the detection of emerging/re-emerging pathogens. Conduct cutting edge bio-informatics and molecular research on arthropod-borne disease vectors (molecular identification of mites) and pathogens (*Leptospira*, *Orientia*, *Bartonella, Plasmodium*, Chikungunya and dengue viruses), and the relationships among the pathogen, vector reservoir, and host.

Malariology: Conducts research on malaria biology and transmission. On-going research includes studies on malaria parasite development in mosquitoes, erythrocyte and liver cells. Develop *in vitro* systems for the screening of antimalarial compounds against exoerythro-cytic, gametocytic and sporogonic stages of malaria parasites. Studies are also focused on game-tocytogenesis in *Plasmodium falciparum* and the production of different stages of human and animal malaria parasites in order to support drug and vaccine development, and transmission blocking strategies.

Leptospirosis Model: Conducts research on leptospirosis pathogen biology and host-vector relationships. Evaluate environmental factors that affect transmission and maintenance of pathogen across diverse geographical differences. Correlate vector, reservoir, and host genotypes across divergent geographical areas with a focus on phylogenetic relationships.

Scrub Typhus Model: Conducts research on scrub typhus epidemiology within vectors, reservoirs and hosts. Establish and maintain colonies of *Leptotrombidium* mites to conduct vector competency and vaccine challenge studies. Develop repeatable standardized chigger-mouse model to further investigate transmission studies. Develop chigger-nonhuman primate model to more closely mimic human reactions to chigger feeding and pathogen transmission in order to support drug and vaccine development and transmission blocking strategies.

Insectary: Produce world class superior quality standard mosquitoes for malaria, dengue, and Japanese Encephalitis research. Rears and researches biology of 5 endemic Thai malaria vectors for transmission studies in support of internal Veterinary Medicine, Entomology and Immunology transmission blocking, vaccine, and prophylactic compound challenges.

Mite and Rodent: Maintain endemic scrub typhus chigger colonies for biological studies and ongoing transmission studies. Provide infected and non-infected chigger mites in support of NHP model and mouse model development for scrub typhus vaccine evaluations. Additionally, supports *Orientia tsutsugamushi* transmission and immunological investigations involving rodents and mites. Collects and identifies rodents/mites in support of rodent-borne disease surveillance and transmission studies.

ACCOMPLISHMENTS

1. **Title: Development of an Integrated Push-Pull System for the Control of Biting Flies**

Funding Source: MIDRP

Project Summary: The overall goal of this research was to develop an integrated push-pull system for the control of arthropod vectors of disease. Attractants were evaluated under semi-field conditions in the Large Mosquito Enclosures, Kampang Phet. The attractants were evaluated for their efficacy in pulling biting flies away from potential hosts and into traps where they are killed. Spatial repellents were evaluated for their efficacy in pushing biting flies away from potential hosts. The semi-field experiment was completed and results are being analyzed.

Future Plan: Data will be analyzed and published.

2. **Title: Field Evaluation of Surveillance Traps for Malaria Vectors**

Funding Source: MIDRIP

Project Summary: We evaluated the efficacy of the Centers for Disease Control and Prevention light traps (CDC Light Trap) and modified CDC Light Traps (a BG-sentinel fan was

attached below the CDC Light Trap collection net) using different attractive sugar baits (ASB) for the collection of malaria vectors in Thailand; and the efficacy of ASB compared with a control (cotton ball soaked with water). The field experimental study against malaria vectors was carried out in the village of Huay Pla Kong, Mae Lamad district, Tak Province, western Thailand. AFRIMS has worked in this area due to its high mosquito biting rates and high incidence of malaria. To limit effects of differences between test-days and trap-position, treatments were rotated so that each treatment was tested in each trapping station 3 times following a 6 x 6 Latin Square design. For each replicate, we placed traps over a period of 6 consecutive nights from 1800 to 0600 hours each night. Mosquitoes collected in the different traps were separated, counted, morphologically identified to species, and the parity rate determined. We collected 4,572 mosquito samples which consist of *Anopheline* (56.10%), *Aedes* (29.33%), *Culex* (12.25%), *Armegeres* (2.17%), and *Uranotaenia* (0.15%). A total of 22 species of Anopheline mosquitoes (2,565 adults) were collected in the current study. The most common Anopheles species collected were *An. (Cel.) sawadwongporni* (44.41%), *An. (Cel.) minimus* (20.74%), *An. (Cel.) maculatus* (17.35%), *An. (Cel.) kochi* (6.16%), *An. (Ano.) barbirostris* (2.88%), and *An. (Cel.) jamesii* (2.65%). The effectiveness of each trap configuration (as measured by the mean number of *Anopheles* mosquitoes collected) is being analyzed.

Future Plan: Data will be analyzed and published.

3. Title: Evaluation of a Sticky Trap Baited with Candidate Phytochemical Attractants for the Control of *Aedes aegypti* (L.)

Funding Source: MIDRP

Project Summary: The overall goal of this research was to evaluate the relative attractivenessof plant infusion-baited sticky traps for the control of yellow fever mosquitoes, *Aedes aegypti* under semi-field conditions. Three plant infusions were used in this experiment: lemongrass (*Cymbopogon citrates*), basil (*Ocimum tenuiflorum*) and dried hay. To prepare the infusions, the plants were immersed in filtered water in closed containers for one week at room temperature before use at a rate of 250 g/L, 5 g/L, and 10 g/L for lemongrass, basil, and hay, respectively. Laboratory strains of *Ae. aegypti* males, host-seeking females, blood-fed females and gravid females (4 days after blood feed) were used in the experiment. They were maintained under laboratory conditions, at 28°C ± 2 at 80% relative humidity under a photo regime of 12:12 h (L:D) by the Insectary, Department of Entomology, AFRIMS, Thailand. A 4 x 4 Latin Square experimental design was used in this study. Four mosquito mesh nets (3.0 x 3.0 x 2.5 m) containing infusion-baited sticky traps and one control treated with water only were placed at 4 corners of large mosquito enclosures. One hundred and twenty *Ae. aegypti* (30 males, 30 blood-fed females, 30 host-seeking females, and 30 gravid females) were released at the center of each large mosquito enclosure. The sticky sheets were collected 4 hours after releasing mosquitoes. Mosquitoes stuck on each sticky trap were sorted by gender and counted. Temperature and relative humidity inside the LMEs were measured. Significantly fewer numbers of blood-fed mosquitoes was collected by sticky ovitraps baited with basil leaf infusion. The lower number of gravid mosquitoes was attracted by lemongrass infusion than other infusion. No significant correlations were detected in the proportion of males and host seeking females among these plant infusions and water control.

Future Plan: N/A

4. Title: Testing and Validation of Commercial Mosquito Control Spray Equipment and Insecticides to Protect Personnel from Mosquito-Borne Diseases

Funding Source: MIDRP

Project Summary: The project evaluated the effectiveness of two insecticides (ULD® BP300 and NyGuard®) and to compare the efficacy of two sprayers (Thermal fog and ULV) with

the insecticide (9M Plus2) using spray application equipment that the Thai MoPH currently uses for *Aedes* species control in Thailand. In an attempt to reduce the incidence, transmission of dengue fever and control of mosquito vectors, Department of Entomology has a desire to study the efficiency of two insecticides (ULD® BP300 and NyGuard®) which used for indoor spraying in order to control mosquitoes inside houses as well as the efficacy evaluation of two commercial sprayers (Thermal Fog (Patriot) and ULV (Twister)). Moreover, the effectiveness of these two insecticides sprayed by two types of sprayers was compared with the insecticide (9M Plus2) that Thai MOPH is currently used for control *Aedes* population in Thailand. Villages located in Ban Rai and Khon Tee Subdistrict, Muang District, Kamphange Phet Province were selected as study areas. The research team selected nine groups of houses located in the study area. The permission from the owner of each house, forty houses for each group, is required prior to the experiment. Treatment houses were sprayed inside using different type of sprayers and insecticides. The insecticides applied in this study have been approved by Environmental Protection Agency (US-EPA) and these are rated as slightly toxic insecticide. The risks from the sprayed insecticide are minimal, safe for human and domestic animals and can be used to exterminate insects from house in accordance with the attached MSDS. To determine the effectiveness of sprayers and insecticides whether these chemicals can control or minimize mosquito vector population or not, adult mosquitoes were collected from fifteen treatment houses of each group after insecticide application using the BG sentinel traps operated for 24 hrs. Mosquitoes were collected for 7 time points as follow; 5 and 10 days before spraying and post spraying at 1, 5, 10, 15 and 20 days, respectively. The Twister yielded significantly lower counts than the Patriot, indicating that the Twister is more effective than the Patriot in killing *Aedes* females. In addition, Deltamethrin yielded significantly lower counts (and hence is a more effective pesticide) than BP. Furthermore, the presence of Nyguard larvicide yielded lower counts than the absence of Nyguard (though the difference is not significant: Presence < Absence), indicating that Nyguard contributed a positive effect on killing Aedes females. In summary, it appears that a combination of Twister, Deltamethrin, and Nyguard would potentially maximize efficacy and effectiveness.

Future Plan: N/A

5. Title: Rodent-borne Surveillance in Thailand Focusing on Leptospirosis, Scrub Typhus and Rickettsial Disease

Funding Source: GEIS

Project Summary: To better understand the sylvatic epidemiology of leptospirosis, scrub typhus, and rickettsiosis through systematic sampling of rodents from the North, East, and South regions of Thailand. Six provinces were included in this study: Chiang Rai/Phayao (North), Rayong/Trat (East), Chumphon/Surat Thani (South). Rodent tissues and their associated ectoparasites were screened for the presence of leptospirosis, scrub typhus, and rickettsial pathogens. We collected 988 rodents and 573 of their associated ectoparasites (chigger, tick, and flea). *B. indica* (389) is the most predominant species followed by *R. rattus* (303), and then *R. exulans* (103). Leptospirosis was found in 17 out of 988 rodents (1.7% prevalence rate) mostly in Rayong and Chiangrai Provinces, 10 and 4 respectively. *R. rattus*, *B. indica*, and *R. norvegicus* are the three species consistently found to carry pathogenic *Leptospira sp*. From 16S rRNA sequence and phyliogenetic analyses, most of *Leptospira sp*.-positive samples group within *L. interrogans* species except one sample (from Chiangrai Province) groups into *L. borgpetersenii*. There was no rodent positive for scrub typhus and rickettsiosis during this surveillance. Chiggers collected from these rodents were pooled by host and 17 pools were positive for scrub typhus (17 out of 466 pools, 3.6% prevalence rate). The infection of *O. tsutsugamushi* in chiggers was found in just two locations; Chiangrai and Phayao Provinces, with 14.2% and 2.7% prevalence rate, respectively. Almost all genotypes (Karp-like, Gilliam, TA716-like, TA678-like, and Kato) were found in this vector. High prevalence of *Rickettsia sp*. infection was found in ticks collected

from rodent hosts (44 out of 56 pools, 78.6%), while no *Rickettsia sp*. infection was found in rodent host itself. High prevalence rate of *Rickettsia sp*. was found in ticks from Phayao Province (38 out of 42 pools, 90.5%), followed by ticks from Rayong (4/12, 33.3%), and one positive was found in Chiangrai and Trat Provinces. Ticks collected from *Bandicota* rodent have high prevalence of *Rickettsia sp*. infection. Only one species of *Rickettsia sp*. (Mie 180) was found in tick samples from this study.

Future Plan: Extend surveillance into other regions of Thailand.

6. Title: Development of a Molecular Taxonomic Key for the Identification of Medically-Relevant Trombiculid Species from Korea, Japan, and Thailand

Funding Source: GEIS

Project Summary: Develop a molecular taxonomic key from field collections of mites originating from Korea, Japan, and Thailand in order to distinguish among species belonging to the family Trombiculidae, the most medically-significant family of mites. Each morphologically identified species can be associated with DNA barcode (COI gene, 12-16S rDNA and ITS2). Chiggers were collected from wild-caught rodents trapped from two provinces, Chiang Rai and Rayong, located in different parts of Thailand (Figure1, Table1). Chiang Rai is the northernmost province of Thailand whereas Rayong is located on eastern region of Thailand. The distance between the two provinces is approximately 1,000 kilometers. One chigger group was subjected to molecular identification by DNA sequencing and another was slide-mounted to identify genus and species by morphology. Three chiggers found on each rodent were selected for processing in molecular identification. Chiggers from colonies maintained at AFRIMS were used as references for *Leptotrambidium chiangraiensis*, *L. imphalum*, and *L. deliense* in molecular and morphological identification.

A total of seventy-eight chiggers from 26 field-caught rodents, 39 chiggers from each province (Chiang Rai and Rayong), were subjected to PCR amplification and DNA sequencing. The three fragments covering the complete COI gene of 1,509 bp and two fragments covering 16S-12S rRNA region of 1,515 bp were amplified from each chigger and sent for DNA sequencing. Sixty-two sequences of COI gene, forty-one sequences of 12S rRNA, and thirty-six sequences of 16S rRNA of field chiggers successfully generated by this method. The phylogenetic trees were constructed from the partial (5' conserved region) and full-length COI gene, 12S rRNA, and 16S rRNA sequences. The maximum likelihood (ML) method using and Seaview programs were used to analyze the evolutionary relationship among field chiggers, reference colonies at AFRIMS, and the reference sequences retrieved from GenBank database. Our results showed that the genetic information of COI gene, especially amino acid sequence, is the most useful data in terms of species identification compare to 12S or 16S ribosomal DNA sequences. However, the nucleotide sequence of COI was too diverse and should not be used to differentiate chigger species. Trees of both full-length and partial (5' conserved region) regions can be used to differentiate chigger species successfully (*L. deliense*, *L. chiangraiensis*, *L. imphalum*); however, trees constructed from full-length COI is preferable since higher bootstrap values (>95% confident) were observed on the nodes that separate each chigger species.

A multiplex assay, nested PCR method, was developed for chigger species identification. However, only medically important chigger species found in Thailand, *L. chiangraiensis*, *L. imphalum*, and *L. deliense*, were included in the assay. The assay worked very well with chigger colonies maintained at AFRIMS laboratory; however, nonspecific signals were observed when performing this assay with field caught chiggers. Further research into the natural biome of wild caught chiggers should be investigated to account for the nonspecific signals.

Future Plan: N/A

7. Title. Rodent-Borne Disease Surveillance in Southeast Asia (Thailand, Laos, Cambodia, Philippines)

Funding Source: GEIS

Project Summary: To estimate the prevalence of rodent-borne diseases (leptospirosis, scrub typhus, rickettsial diseases) in reservoir hosts and vectors throughout Southeast Asia (Thailand, Laos, Cambodia, Philippines). To identify and characterize the risk factors and diseases distribution associated with rodents and vectors throughout SEA. Rodent specimens from Cambodia (22 kidneys), Laos (84 kidneys), Vietnam (285 kidneys) were received from Dr. Serge Morand through collaboration between AFRIMS and University of Montpellier, France. Rodent and ectoparasite collections were completed in 3 regions and 6 provinces in Thailand; 988 rodents and 109 of ectoparasite (flea, lice, and tick) pools from cats and dogs were collected. Rodent and ectoparasite collections in Philippines were completed through collaboration between Dr. Serge Morand, University of Montpellier, France and the Director of Research Institute for Tropical Medicine, Philippines. Seventy-six rodents and 149 pools of ectoparasites (lice, flea, and tick) were collected. All specimens were screened for leptospirosis, rickettsiosis, and scrub typhus. *Bandicota indica*, *R. argentiventer* and *R. tanezumi* represented the most predominant rodent species in Southeast Asia (Thailand, Cambodia, Laos, Vietnam, and Philippines). Pathogenic *Leptospira sp*. was detected from several *Rattus* (*R. rattus*, *R. norvegicus*, *R. tanezumi*, *R. argentiventer*) and *Bandicota indica* rodents. Prevalence of leptospirosis in rodents was 2.9% (40/1379). Two main species of leptospirosis, *Leptospira interrogans and L. borgpetersenii,* were found in *Rattus* and *Bandicota* rats according to DNA sequence and phylogenetic analyses of 16S rDNA gene. Scrub typhus agent was detected mostly in rodent-associated chiggers (3.6%, 18/500 pools), and almost all genotypes (Karp-like, Gilliam, Kato, and rodent source such as TA716-like, TA678-like) are equally distributed in nature. High prevalence rates of *Rickettsia sp*. were found in ticks and fleas collected from dogs and rodents (26.8%, 98/365 pools). Species identification of these *Rickettsia*-positive samples using *glt*A sequence analysis reveals two *Rickettsia* species were detected: *R. felis* and *Rickettsia sp*. Mie180.

Future Plan: N/A

8. Title: Screening of Antimalarial Compounds in Non-Human Primate for Blood and Liver Stage Activities

Funding Source: In-house collaboration with Department of Veterinary Medicine

Project Summary: In collaboration with Department of Veterinary Medicine, we provide *Plasmodium cynomolgi* (monkey malaria) infected mosquitoes and live sporozoites harvested from salivary glands for the screening of antimalarial compounds. *An. dirus* mosquitoes fed on *P. cynomolgi* infected monkeys are maintained at the Entomology Department. Approximately 6-9 days or 9-11 days after feeding on malaria infected blood, 5-10% of the mosquitoes infected with *P. cynomolgi* are checked for the presence or absence of oocysts respectively. At approximately 12-14 days, the sporozoites are harvested from infected salivary glands and provided to the Department of Veterinary Medicine for monkey injection. The malaria infected monkeys are used to evaluate blood schizontocidal activity and causal prophylactic activity screening of new compounds.

Future Plan: Continue to provide *P. cynomolgi* sporozoites and other species of the non-human primate parasites to support several drug and vaccine development studies.

9. Title: Evaluation of *Plasmodium vivax* Susceptibility to Chloroquine in Western Thailand

Funding Source: GEIS (Sustaining)

Project Summary: This study is the evaluation of the susceptibility of the parasite to chloroquine *in vitro*. Individuals with *P. vivax* positive blood film examinations and that met

appropriate criteria were enrolled. Blood was collected from *P. vivax* infected prior to treatment for *in vitro* drug susceptibility assay. Polymorphism of parasite genome will be analyzed among clinical *P. vivax* isolates.

In FY13, an *in vitro* chloroquine sensitivity test was conducted following the method by Chotivanich *et. al.* with some modification. Five fresh isolated *P. vivax* infected blood samples were collected from malaria infected patients. The samples were tested against different concentrations of chloroquine; i.e., 0, 3.9, 7.8, 15.6, 31.2, 62.5, 125 ng/ml, and 250 ng/ml and then incubated at 37°C with 5% CO_2 for 24 and 40 hrs. Thick and thin films were made and complete schizogony of parasites was evaluated per 200 infected red cells.

Future Plan: Continue to evaluate the susceptibility of the parasites to chloroquine in vitro and conduct parasite genetic analysis to determine and analyze genetic polymorphism among clinical *P. vivax* isolates.

10. Title: Active Surveillance for *P. falciparum* Drug Resistance with Assessment of Transmission Blocking Activity of Single Dose Primaquine in Cambodia

Funding Source: Collaboration with Department of Immunology and Medicine (GEIS)

Project Summary: This study is a two-arm open label treatment study comparing the efficacy, safety, tolerability and pharmacokinetics of three-day course of Dihydroartemisinin-Piperaquine (DP) with or without single-dose primaquine (PQ) in patients with uncomplicated *P. falciparum* malaria at selected sites of malaria drug-resistance in Cambodia. On the last day of DP therapy, volunteers will be randomized to receive either a single dose of PQ or DP only (no PQ). Resistance to DP and DP-PQ will be assessed by a combination of clinical, pharmacologic, and parasitological parameters including genomic signatures of selection during careful weekly follow-up visits for 42 days. Investigators will also evaluate the effect of PQ on the asexual stages of malaria parasite and potential transmissibility of infection to *Anopheles dirus* mosquitoes as compared to those not treated with PQ using membrane feeding assay.

This is an opportunity for technology transfer and capacity building in the development and implementation of the membrane feeding assay. Malariology section provided the training to the identified personnel from the community, RCAF and/or CNM Cambodia. We also provided training in the maintenance of colony reared *An. dirus* mosquitoes, membrane feeding for mosquito infection, midgut dissection, oocyst enumeration and preservation, and sporozoite collection techniques for molecular analysis to staff of the Department of Immunology and Medicine prior to commencement of this. We provided on site assistance in the establishment of a field site laboratory for this study in Cambodia. Ten *P. falciparum* infected patients were enrolled and membrane feeding had been performed. The results are being analyzed.

Future Plan: Completed subject enrollment and the data is being analyzed.

11. Title: Vivax Malaria Vector Competence of *Anopheles sinensis* and *An. kleini* from Republic of Korea

Funding Source: No funding source/In-house

Project Summary: Vivax malaria has been endemic on the Korean Peninsula for centuries. Until recently (2005), *Anopheles sinensis* sensu lato was believed to be the primary vector in Korea. However, conflicting reports on the vector status among mosquitoes in the hyrcanus group have been reported. To understand the epidemiology of malaria in ROK, we examined the potential vector competence of *An. sinensis* sensu stricto and *An. kleini* to *P. vivax* infections. *Anopheles sinensis* s.s. and *An. kleini* were colonized from wild caught specimens collected from a beef farm in Tongilchon, near the demilitarized zone and a malaria high-risk area, and reared at the Entomology Department, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. *Anopheles dirus* (control) and F13-16 generation *An. sinensis*

s.s., and *An. kleini* were fed concurrently on *P. vivax* infected blood from Thai patients under an approved protocol using artificial membrane feeding method. On day 7 post-feed, midguts were dissected to assess oocyst development. Salivary glands were dissected on days 14, 21, and 28 post-feed and examined for sporozoites. While similar numbers of oocysts were observed on the midguts of *An. sinensis* s.s. and *An. kleini*, the mean size (diameter) of oocysts in *An. kleini* were significantly larger ($P < 0.05$, t-test or other stat value) than those of *An. sinensis* s.s. Sporozoite rates were significantly higher in *An. kleini* compared to *An. sinensis* s.s ($P < 0.05$, stat value). In addition, the mean sporozoite index (MSI) for *An. sinensis* s.s. was lower (+2 MSI) than for *An. kleini* (+2.6 MSI). These data support the hypothesis that *An. kleini* is one of the primary vectors of *P. vivax* in Korea, while *An. sinensis* s.s. is a secondary vector.

Future Plan: Completed and the data is being analyzed.

12. Title: Production of *Plasmodium cynomolgi*-infected blood products utilizing a Rhesus Monkey (*Macaca mulatta*) Malaria Model

Funding Source: Novartis

Project Summary: The Novartis Institute for Tropical Diseases has a mission to discover novel treatments and prevention methods for malaria. In developing countries where these diseases are endemic, Novartis will make treatments readily available, without profit, to poor patients. The Novartis Institute for Tropical Diseases has an active anti-malarial drug discovery program. The program has an interest and a history of developing new *in vitro* assays which target the parasite liver stages for the purposes of drug screening.

AFRIMS has the capabilities in the Department of Entomology and Veterinary Medicine to infect non human primates with *Plasmodium cynomolgi* malaria parasites and feed mosquitoes on infected animals. This will enable AFRIMS to supply and ship freshly isolated *P. cynomolgi* sporozoites to Novartis for development of an *in vitro* anti-hypnozoite assay. Development of this assay at Novartis will allow for screening of chemical libraries to identify new compounds with potential radical curative activity.

Future Plan: Ongoing

13. Title: *Plasmodium vivax* Liver Stage Cultivation Using Cryopreserved Human Hepatocytes

Funding Source: FY14 MIDRP

Project Summary: In the present study, we proposed to develop *in vitro* system for *P. vivax* liver stage cultivation using cryopreserved human hepatocytes (CPHH). Five batches of CPHH and HepG2 cell will be tested for their ability to support sporozoite infection and development. To achieve superior sporozoite infection rates, different numbers of sporozoites will be loaded into different cell types and cell densities. Ability among batches of CPHH and HepG2 cell to support parasite infection and development will be compared. Optimum ratio of parasite number and cell density generates the maximum infection rate will be selected for further studies.

Detection and quantification of the parasite infection load, and development of the liver stage will be accomplished by Quantitative Real-Time PCR (qRT-PCR) of 18s rRNA gene. The qRT-PCR condition will be developed and optimized. A measure of parasite burden can be calculated as the ratio of parasite 18S rRNA to human β-actin control plasmid equivalents. The development of the liver stage parasites will be also confirmed using HSP70 Immunofluorescent staining (IFA). For further studies, the developed *in vitro* culture system will be used to evaluate the blocking efficacy of immune sera for sporozoite invasion using ISI assay.

Future Plan: Ongoing

14. Title: Maintenance of the Scrub Typhus Chigger Challenge Model Utilizing *Orientia tsutsugamushi* Infected *Leptotrombidium* Chigger-Mite Colonies

Funding Source: MIDRP

Project Summary: This is the only DoD laboratory which maintains *O. tsutsugamushi* infected mite colonies. We currently maintain three species of *Leptotrombidium* mites with 11 infected lines: *L. chiangraiensis* (Lc) (3 lines); *L. deliense* (Ld) (1 line) and *L. imphalum* (Li) (7 lines).

O. tsutsugamushi infected mite colonies are maintained in an Animal Biosafety Level-3 (ABSL-3) containment facility. The procedures are conducted in the BSL-3 facility for the infected colonies as well as in the BSL-2 laboratory for the non-infected colonies. These mites have a complex 3-4 month life cycle during which *O. tsutsugamushi* is maintained transovarially and transstadially. Only the larvae (chigger) are parasitic, feeding on extra-cellular fluid of their mammalian hosts in order to develop to the next stage. We are rearing +80,000 mites/year. Standard protocols are used to raise both *O. tsutsugamushi* infected and non-infected *Leptotrombidium* mites to support:

1. Chigger challenge model
2. Scrub typhus immunopathophysiology model in nonhuman primates (STIMP)
3. Evaluation of r56 vaccine candidates in a homologous chigger challenge mouse model (NMRC collaboration)

Future Plan: Continue with research in support of MIDRP, and GEIS goals. Continue support of collaborative project with MORU to establish a non-human primate model for scrub typhus investigations. Expand support and collaborative projects with other researchers working on scrub typhus prevention and elimination.

15. Title: Scrub Typhus Immunopathophysiology Model in Nonhuman Primates (STIMP)

Funding Source: National Institute of Health-IAA

Project Summary: Overall objective is to establish a non-human primate model to more closely mimic the immunopathophysiological parameters of natural infection with, and vaccine-induced protection against scrub typhus. The immediate objective is to establish effective mechanical infections of mites with the human pathogenic Karp strain of *Orientia tsutsugamushi*. This is a collaborative effort with Mahidol Oxford Research Unit (MORU). We established the intial conditions for microinjection (micropump pressure, injection time and volume) using methylene blue.

Microinjection of Leptotrombidium mites. The microneedle is filled with methylene blue and it is approaching the ventral inoculation site.

When initial conditions were standardized, uninfected adult female *Leptotrombidium chiangraiensis* (Lc) mites were selectively injected with L929 cell culture alone (control) or a culture prepared with infected mouse liver-spleen homogenate (treatment). All microinjection steps were performed in the Animal Biosafety Level 3 (ABSL3) laboratory. Injected adult mites were maintained until they produced offspring (F1 generation). Uninfected adult female *Leptotrombidium imphalum* and *Leptotrombudium deliense* were also injected with semi-purified *O. tsutsugamushi* in L929 cell. The new (F1) progenies were identified to species using multiplex PCR. The trans-stadial and trans-ovarial infection rates were analyzed using qPCR.

Times	Mites species	Inoculum	No. of mites	Survival rate	Transovarian rate
First mechanical infection	Lc	non purified Ot from L929 cell culture	3 controls	3/3 (100%)	2/3 (66.66%)
			9 experiments	6/9 (66.66%)	4/9 (44.44%)
Second mechanical infection	Lc	non purified Ot from L929 cell culture	5 controls	3/5 (60%)	3/5 (60%)
			15 experiments	8/15 (53.55%)	3/15 (20%)
Third mechanical infection	Lc	non purified Ot from L929 cell culture	6 controls	5/6 (83.33%)	5/6 (83.33%)
			18 experiments	17/18 (94.44%)	12/18 (66.67%)
Fourth mechanical infection	Li	semi-purified Ot from L929 cell &semi-purified Ot from tick cell	24 experiments	23/24 (95.83%)	21/24 (87.5%)
Fifth mechanical infection	Ld	semi-purified Ot from L929 cell	24 experiments	20/24 (83.33%)	20/24 (83.33%)

Results of Five Mechanical Infection Runs

We established microinjection conditions as: injection pressure 85 KPa; injection time 0.1-0.4 s; injection volume 0.07-0.1 µL; and inoculum concentration is 1.40 x 10^6 organism/µL. The inoculum was prepared using AFRIMS human-pathogenic *O. tsutsugamushi* strains cultured in L929 cells provided by MORU. In addition, we developed a "Chigger Retention Device" (CRD) to place chiggers on non-human primates. This will be used to retain chiggers at a specific location on the non-human primate body.

Future Plan: Determine *O. tsutsugamushi* transmission rates to scrub typhus naieve non human primates from mechanically injected adult F1 generation chiggers. Determine the most appropriate number of chiggers and the location of attachment on non-human primates to achieve efficient and consistent pathogen transmission and disease.

16. Title: Evaluation of r56 Vaccine Candidates in Homologous Chigger Challenge Mouse Model

Funding Source: MIDRP

Project Summary: Evaluate the protective efficacy of recombinant protein r56 in a homologous chigger challenge mouse model. This is a follow-on study to an earlier MIDRP project, "Establishment of a Chigger Challenge Model. Determination of Optimum Inoculation Doses of Two *Orientia tsutsugamushi* Strains in ICR Mice", and "Evaluation of Scrub Typhus Vaccine Candidates by Natural and Artificial Challenge Models in ICR Mice (*Mus musculus*)".

In order to eliminate contributing factors due to homologous versus heterologous immunogens to elucidate protective immune responses and simplify data analysis, we amplified the *Orientia* 56kDa gene from Lc-1 infected chiggers and produced the recombinant protein r56Lc-1. The protective efficacy of this r56Lc-1 was evaluated by challenge with chiggers infected with *Orientia* strain Lc-1. For this study, we divided mice into 3 experimental sets and each experimental set was divided into 2 groups. Sixty mice of group 1 from all experimental sets are control animals which were challenged by adjuvant only whereas sixty mice of group 2 from all experimental sets were vaccinated with homologous scrub typhus vaccine candidates. After 4 weeks of vaccination, the vaccinated mice were challenged by naturally infected and uninfected chigger feeding.

	Experiment 1		Experiment 2		Experiment 3	
	Group1	Group2	Group1	Group2	Group1	Group2
immunization	Vaccine	Adjuvant	Vaccine	Adjuvant	Vaccine	Adjuvant
Number of mice	10	10	10	10	10	10
Un-infected Lc-1 chigger	√	√	√	√	√	√
Infected Lc-1 chigger		√	√	√	√	√
Total mice	120					

Immunization with r56Lc-1 vaccine and chigger challenge models

No mice died after challenge by uninfected chiggers, with or without the vaccine candidate r56Lc-1. No survived in the groups of mice immunized with adjuvant only and challenged by an infected chigger. Only 3/10 mice immunized with r56Lc-1+adjuvant survived the challenge of an infected chigger in Experiment 1 and 2. In Experiment 3, the survival rate was even worse (2/10). Our data demonstrated that r56Lc-1 provided 20-30% of protection consistently upon challenge by an infected chigger which carries the same *Orientia* strain based on the sequence of 56 kDa antigen.

Future Plan: N/A.

17. Title: Mosquito Rearing for Experiment Support

Funding Source: MIDRP

Project Summary: Rear and maintain vigorous and human pathogen free colonies of seven *Anopheles* mosquito species: *An. dirus*, *An. cracens*, *An. minimus*, *An. sawadwongporni*, *An. campestris*, *An. kleini* and *An. sinensis*; 2 spp. of *Aedes* mosquito, *Ae. aegypti*, *Ae. albopictus*; and *Toxorhyncites splendens*. These mosquitoes support basic mosquito biology, pesticide resistance, and pathogen transmission efficacy studies within the Entomology Department as well as other departments within AFRIMS, WRAIR and other collaborators. Research focus is disease elimination and prevention in Thailand, South East Asia and worldwide.

Laboratory-reared mosquitoes are provided on request are disease-free, of the same age, and in impeccable condition. The first step of rearing starts when eggs are immersed in water in larval rearing trays. After 1-2 days, eggs will hatch. On day 2 after hatching, larvae will be fed in the rearing tray with ground fish food. When pupation starts, pupae will be collected and kept in a tub of fresh water until emergence. Adults will be fed with vitamin syrup until 5-7 days old when females are needed for experiments or for natural/artificial mating to maintain the colony. Females will be fed a blood meal via an artificial membrane feeding apparatus to provide protein for egg production.

Numbers of mosquitoes produced to support 2013 experiments are recorded in the table below.

Mosquito sp.	Number of females produced
An. dirus	206,000
An. sawadwongporni	11,000
An. campestris	6,800
An. minimus	18,800
An. cracens	9,100
An. kleini	10,300
An. sinensis	10,200
Ae. aegypti	9,880,000
Toxorhynchites splendens	10,580 (adult M&F)

Future Plan: Establish new colonies of *An. stephensi*, one normal strain and one GMO strain resistant to plasmodium infections. These colonies will be used to evaluate effectiveness of the GMO strain to resist *Pv* and *Pf* infections in Thailand.