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PI's Signature \_\_\_\_\_ Date \_\_\_\_\_  
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**Royal Thai Army, AFRIMS**  
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## **I. INTRODUCTION**

### **A. General**

Collaborative studies into infectious diseases of military importance have been conducted at the Armed Forces Research Institute of Medical Sciences (AFRIMS) by both the U.S. Army Medical Component (USAMC) and the Royal Thai Army Medical Component (RTAMC) for 4 decades. Studies leading to develop drugs and vaccines to combat tropical diseases of military relevant importance.

### **B. Statement of Work**

Administrative, logistical and scientific personnel required to support the ongoing U.S. Army AFRIMS research efforts, and utilities and maintenance required to support the U.S. Army AFRIMS research effort.

### **C. U.S. ARMY AFRIMS Research Efforts at Department of Entomology**

Department of Entomology research efforts are the following:

1. Use of GIS to Assess Relative Risk in Different Biotopes within Endemic Scrub Typhus Areas
2. Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines
3. Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

### **D. U.S. ARMY AFRIMS Research Efforts at Department of Immunology**

Department of Immunology research efforts are the following: See page 26-28

### **E. U.S. ARMY AFRIMS Research Efforts at Department of Enteric Diseases**

Department of Enteric Diseases research efforts are the following:

1. Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand
2. Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens

3. Characterization of Enteric Pathogens Isolated from Children in Phnom Penh
4. Characterization of *Campylobacter jejuni* Isolates

#### **F. U.S. ARMY AFRIMS Research Efforts at Department of Veterinary Medicine**

Department of Veterinary Medicine research efforts are the following:

1. Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (*Macaca mulatta*)/*Plasmodium cynomolgi* Malaria Models
2. Care and Maintenance of Rhesus (*Macaca mulatta*) and *Cynomolgus* (*Macaca fascicularis*) monkeys and Management of Breeding Colonies
3. Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance/Quality Surveillance Program
4. A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs
5. Characterization and Validation of *Anopheles dirus* Sporozoite-Induced Mouse Malaria Models (ICR mouse/*Plasmodium berghei* and *P. yoelli*) for Screening Exoerythrocytic Antimalarial Drugs

#### **G. U.S. ARMY AFRIMS Research Efforts at Department of Virology**

Department of Virology research efforts are following:

1. The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue
2. A Phase II, Prospective, Randomized, Double Blind, Placebo Controlled Field Efficacy Trial of a Candidate Hapatitis E Vaccine in Nepal, WRAIR #749, HSRRB Log #A-9117.1
3. Prospective Study of Dengue Virus Transmission and Disease in Primary School and Villages in Kamphaeng Phet, Thailand
4. A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naïve Children
5. Training and Workshops
6. Influenza Surveillance in Southeast Asia

7. Sentinel Surveillance for Emerging Diseases Causing Dengue-like or Acute Encephalitis Syndrome in the Philippines (SEDP)

#### **H. U.S. ARMY AFRIMS Research Efforts at Department of Retrovirology**

Department of Retrovirology research efforts are following:

1. Screening and Evaluation of Potential Volunteers for a Preventive HIV-1 Vaccine Trials in Thailand (RN 148, HSRRB)

2. A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp 120 B/E (AIDSVAX® B/E) Boosting in HIV-uninfected Thai Adults (RV144, HSRRB Log No. A-11048, BB-IND 8795)

#### **I. Space and Utilities Required**

Funding under the cooperative agreement is also directed by the Principal Investigator to the provision of site maintenance including space and utilities management for both the RTAMC and the USAMC in support of research activities.

## **II. BODY**

### **A. Department of Entomology, AFRIMS FY06 Research Accomplishments**

**1. Title of Research Project:** Assessment of malaria transmission in China and Korea in comparison with Thailand

**a. Investigator:**

Dr. Jetsumon Prachumsri, Ph.D

**b. Objectives:**

Comparing malaria transmission in China, Korea and Thailand with emphasis on biology of parasites and Anopheles vectors

**c. Methods:**

Experimental design:

1. Study of *P. vivax* parasites:

(a) *P. vivax* infected blood will be collected from patients (male and female, age 15 years or older) at selected clinics in Thailand (Maesod and Mae Kasa, Tak province), Korea (Munsan, Kyonggi province) and China (Jiangsu, Anhui and Hubei provinces). Blood will be used for mosquito feeding to produce sporozoites and cryopreserved in liquid nitrogen for short-term *in vitro* culture (established by Udomsangpetch R., Mahidol University) and for study on molecular genetic of the parasites.

(b) To compare development of short and long incubation types of *P. vivax* in human, the *in vitro* culture of liver parasite established at AFRIMS will be used. Briefly, human liver cells (HC04) will be plated 2 days prior to sporozoite invasion. Sporozoites will be harvested from *P. vivax* infected mosquitoes and added to culture of human hepatocyte cell line. Liver parasites will be examined at 4, 7, 14, 28 days post invasion. Monitoring of parasite development will be accomplished by IFA staining of parasite specific proteins (such as CSP, AMA1, MSP, Hsp70) and quantitative PCR of parasite genomic DNA.

(c) Patients plasma will be separated and kept for study of human immune responses to *P. vivax* in each region using IFA and ELISA for anti-malaria antibody against blood stages (asexual and sexual) and sporogonic stages (ookinete and sporozoite).

(d) We will investigate the population diversity of *P. vivax* in China and Korea compared to Thailand, using two polymorphic genetic markers, the merozoite surface protein-3a (MSP3a) and the circumsporozoite protein (CSP)(Cui, *et al* 2003).

### ***Plasmodium vivax* vectors:**

Mosquitoes will be collected from malaria endemic areas in Thailand (Tak and Kanchanaburi), Korea (Kyonggi Province) and China (Jiangsu, Anhui and Hubei provinces) during low and high malaria transmission period (April and June to August), using man-biting collection methods. Mosquitoes will be identified, by morphology and genotype, and tested for malaria parasites (by CSP ELISA and PCR).

### **Work schedule**

#### **Year 1:**

1. Human use protocol to work in China and Korea was established. Similar to protocol used for study in Thailand will be applied to work in Korea and China for collection of blood samples.

2. Scientists from AFRIMS visit Korea and China to obtain baseline information on malaria transmission, control program and case management at local clinics or public health centers. Clinics in Korea and China were selected for blood collection. Malaria endemic areas were selected for mosquito collection. These mosquitoes were



used for blood feeding and sporozoite production. Species verification was accomplished by the Walter Reed Biosystematics Unit (WRBU).

3. Scientists in Korea and China received training at AFRIMS in conducting artificial membrane feeding of mosquitoes, human blood collection and preparation for in vitro culture, and cryopreservation of infected blood to facilitate study.

4. Mosquito collection was accomplished with local staff in each region. Mosquito identification was accomplished at local laboratories and confirmed by molecular biological methods developed at AFRIMS, WRBU and Jiangsu Institute. Malaria parasites infections were examined at AFRIMS by using CSP ELISA and PCR after species identification.

### **Year 2 and Year 3:**

1. We collected isolates of *P. vivax* from each country. Additionally, we collected blood samples, membrane feeding to the mosquitoes and perform in vitro culture of liver stage parasites are done at each local laboratory.

2. Study and compare the respective genotypes and phenotypes of parasites from each area.

3. Mosquitoes were field collected for 7 to 14 days at each endemic area during low and high malaria transmission to ascertain primary vectors in each region.

4. Analyzed relationship between parasite and vector species and malaria transmission patterns comparing between tropical (Thailand) and temperate *P. vivax* (China and Korea).

### **d. Results:**

#### Summary of sample collection and experiments in China

1. Total of 122 blood samples was collected, 50 samples were used for drug sensitivity testing and 64 cases were used for mosquitoes feeding.

2. Total of 24/50 cases were successfully cultured for drug assay. The IC50 for each drug is shown in the attachment [drug results from China].

3. Study of parasite development in two vector species using a membrane feeding method showed that

(a) The oocyst positive rate of *An. sinensis* was 72.6% and the sporozoite rate was 19.4%.

(b) The oocyst positive rate of *An. anthropophagus* was 70.9% and sporozite rate was 25.8%.

(c) Mean oocyst number of *An. sinensis* was 22.27 oocysts/ mosquito.

(d) Mean oocyst number of *An. anthropophagus* was 33.82 oocysts/ mosquito.

4. Genotyping analysis of *P. vivax* isolates from China: Sixty nine blood samples infected with *P. vivax* were confirmed by PCR. From these, 60 samples were able to further analyzed by PCR-RFLP for MSP3-alpha gene diversity. The majority of these samples (54 out of 60 samples) were found to contain the MSP3 gene similar to that of the Sal I strain. The remaining six samples could be divided into two types: Four were found to have about 400 bp deletion compared to that of the Sal I strain whereas the other two had larger deletion of about 900 bp. Sequencing of these genes is now being conducted.

### **Summary of sample collections and experiments in Korea**

1. *P. vivax* infected blood from patients was fed to different F1 mosquito species. The results are summarized in the attached file [Korea mosquito]

2. Field mosquitoes collected in 2005 by using light trap and identified as *An sinensis* were tested by PCR to confirm mosquito species.

3. Data for drug sensitivity of the parasites in Korea is being analyzed.

4. Genotyping analysis of Plasmodium vivax isolates from Korea: Thirty PV samples were collected during malaria transmission season and confirmed by PCR to be PV. Only 15 samples were able to be further analyzed for genetic diversity. Of these 15 samples, 12 were found by PCR-RFLP to contain the MSP3 gene, similar to that of the Sal I strain. PCR analysis of the MSP1 gene showed that the products of nine samples had the same length as that of Sal I strain (720 bp); those of 5 samples had the same length as that of Balem strain (680 bp); whereas 1 had a mixed infection with 2 different size PCR products. Sequencing of these genes is now in progress.

### **e. Future plans:**

We have all the samples from China and Korea. Samples have been tested for different assays. There will be no more sample collection in 2007 under MIDRP due to the end of funding for this project in 2006. But China and Korea group will continue some parts of this study under different funding sources, hopefully from local governments. The data will be shared among scientists from China, Korea and AFRIMS on the progress as well.

### **2. Title of research project: Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines**

**a. Investigators:**

LTC James W. Jones, Ph.D  
Dr. Kriangkrai Lerdtusenee, Ph.D

**b. Objectives:**

1. Conduct genetic characterization of *O. tsutsugamushi* infecting 12 colonies of *Leptotrombidium* chiggers sps. maintained at AFRIMS.

2. Evaluate the ability of each of the 12 chigger colonies to transmit *O. tsutsugamushi* to laboratory mice. Down-select 4-5 key chigger colonies for further studies. These chigger colonies should be infected with different strains of *O. tsutsugamushi* and should produce consistent, high infection rates when fed on mice.

3. Focus efforts on building up down-selected chigger colonies to the high levels required for potential vaccine studies.

4. Develop methods for assessing the efficacy of candidate vaccines using the chigger/mouse model. Criteria used to assess efficacy must include quantification of rickettsemia in the mice; however, additional methods (clinical or immunological responses) may also be assessed.

**c. Methods:**

1. Characterization of Strains/Isolates of *Orientia tsutsugamushi*: The goal is to characterize the 12 strains of *O. tsutsugamushi* infecting our 12 chigger colonies in order to determine phenotypic and genotypic relationship between different strains. The sequencing of *O. tsutsugamushi* strains currently being conducted in collaboration with Walter Reed Army Institute of Research.

2. Continue to evaluate the efficacy of chigger colonies to transmit *O. tsutsugamushi* to mice

3. Build-up key chigger colonies to levels sufficient to support vaccine challenge studies: The rearing and maintenance of *Leptotrombidium* chiggers is a long, slow process. The total life cycle (from egg to egg-laying adult) requires approximately 3 months (this is in contrast to 2-3 weeks for most mosquitoes). Each female chigger will only produce about 1000 eggs over her lifetime. Once a chigger colony is selected for use in vaccine trials, it requires approximately 6 months to build it up to a level required to support the trial.

4. Assess the efficacy of candidate vaccines using the chigger/mouse

model. Initial efforts focus on determining the course of rickettsemia over time in the Lc-1 strain of *O. tsutsugamushi* and on the development and/or confirmation of diagnostic procedures (PCR, ELISA, etc.) to quantify rickettsemia in challenged mice. We will also continue to evaluate the effect of chigger infection with specific strains of *O. tsutsugamushi* on potential indicators of immunity, to include lymphocyte transformation, morbidity (as quantified by food consumption, weight gain/loss, activity, etc.), and mortality (time to death following chigger infection).

#### **d. Results:**

For the first (single-dose vaccination) group: forty-five (45) 8-week-old ICR mice were vaccinated by a single dose administration of the scrub typhus candidate vaccine (25 µg Kp-r56, 10 µg CpG and 60 µL Montanide per mice).

The second (double-dose vaccination) group: twenty five (25) of 4-week-old ICR mice (25 mice) were vaccinated by a double dose administration of the scrub typhus candidate vaccine (with a 4-week interval for boosting).

The third (triple-dose vaccination) group: twenty five (25) of 4-week-old ICR mice (25 mice) were vaccinated by a triple dose administration of the scrub typhus candidate vaccine (with a 4-week interval for boosting).

Each vaccinated mouse was subcutaneously injected with 120 µL Kp-r56 of the scrub typhus vaccine candidate, 60 µL at each side of dorsum neck areas.

The control groups of non-vaccinated mice consisted of 25, 8-week-old, ICR mice. They were subcutaneously injected with adjuvant (10 µg CpG and 60 µL Montanide per mice). Twenty non-vaccinated mice were challenged by scrub typhus-infected Lc-1 (Karp-like) strain chiggers, while the other 5 are being challenged by non-scrub typhus-infected chiggers.

The orbital bleeding of mice was performed 1-3 days before immunization/vaccination and 1 week before chigger-challenging. Serum samples were collected in order to determine any immune response developed by mice.

Regarding the age of vaccinated mice when they were challenged by scrub typhus-infected Lc-1 (Karp-like-strain) chiggers were as followed: 12-week old mice for the single- and double-boosting groups and 16-week old mice for the triple-boosting group. All vaccinated and control mice were clinically observed for 21 days (3-week-period) post challenging by chiggers.

For the single-dose vaccination of a total of 45 mice, 3 mice survived (7%) after 21-day-observations, in which 2 of them showed no clinical signs of sickness and one mouse become sick and recovered after day 16th. Three mice died at the typical

time of death from a scrub typhus infected mite feeding procedure, and 39 mice died (94%) within day 13 to Day 20.

For a double-dose vaccination of a total of 25 mice, 8 mice survives (32%) after 21-day-observations, in which 3 of them demonstrated no clinical signs of sickness and 5 mice became sick and recovered after day 21. No mouse died during the characteristic time of death resulting from scrub typhus infected mite feeding, and 17 mice died (68.00%) between day 13 to Day 20.

For the triple-dose vaccination of a total of 25 mice, 6 mice survived (24 %) after 21-day-observations, in which 3 of them showed no clinical signs of sickness and 3 mouse become sick and recovered after day 21st. No mouse died during the normal period of death by scrub typhus feeding procedure, and 19 mice died (76%) within day 13 to Day 20.

The most important observations were made on those mice which exhibited delayed death observed in all 3 groups. The majority of mice died on day 15-16 in all three groups 39 out of 78 mice (50%): 22 out of 39 for single-dose vaccinated mice, 7 out of 17 for double-dose vaccinated mice and 10 out of 19 for triple-dose vaccinated mice, respectively, when compared to only 3 mice of the single-dose vaccinated group which died normally on day 9-12.

All mice in the control groups of non-vaccinated mice which were challenged by scrub typhus-infected Lc-1 (Karp-like strain) chiggers died on day 9- 12. While another control group of non-vaccinated mice which were challenged by non-scrub typhus-infected chiggers all survived.

The second evaluation of scrub typhus vaccine candidates by assessing protection afforded vaccinated mice by was conducted as follows:

1. Single-dose (immunized) vaccination group: 4-week & 8-week-old ICR mice were immunized by a single dose of a scrub typhus vaccine candidate (25 µg Kp-r56, 10 µg CpG & 60 µL Montanide per mouse).
2. Double-dose vaccination group: 4-week ICR mice were immunized by two doses of scrub typhus vaccine candidate (25 µg Kp-r56, 10 µg CpG & 60 µL Montanide per mouse);

Each immunized mouse was subcutaneously injected at each side of the dorsum neck areas. They were challenged (fed on) by scrub typhus-infected Lc-1 (Karp-like-strain) chiggers.

The control groups consisted of 4-week-old non-vaccinated ICR mice which were being subcutaneously injected with adjuvant (10 µg CpG and 60 µL Montanide per mice). One group of 10 non-vaccinated mice was challenged with scrub

typhus-infected Lc-1 strain chiggers, while another group of 10 non-vaccinated mice was challenged with uninfected chiggers.

Orbital bleeding of mice was performed 1-3 days before immunization/vaccination and 1 week before chigger-challenges began. Serum samples were collected in order to determine immune response. Experimental mice were weighed daily and their body weights recorded. Mice were observed daily for clinical manifestations for 21 days (3-week-period) post challenging by chiggers.

### **Result of 2nd (Scrub Typhus Vaccine Kp r56) Vaccine Trial - Challenge with *Orientia tsutsugamushi*-infected *Leptotrombidium chiangraiensis* (Lc-1) chiggers**

1. Immunized by a single-dose of Kp r56 vaccine: Results from our second (Scrub Typhus) Vaccine Trial on 4-week-old mice which were immunized with a single-dose of scrub typhus Kp r56 vaccine showed 3 of 10 mice survived for the period of our clinical observation (21 days period). The survival rate in our 2nd Vaccine Trial is higher than the results of the 4-week old mice challenged with a single-dose of Kp r56 vaccine in the 1st (Scrub Typhus) Vaccine Trial which only 3 of 45 mice survived and also higher than the results of 8-week-old mice inoculated with a single-dose of Kp r56 vaccine in which only 1 of 10 mice survived challenge.

Of the three of ten 4-week-old surviving mice, 2 mice became ill on day 11 and another mouse on day 12. Two mice recovered on day 18 and one mouse on day 20. The single surviving 8-week-old mouse became ill on day 9 and recovered on day 12.

Survival time of the mice that became sick and eventually died was increased over the challenged non-vaccinated group. The normal time for mice to die from challenge normally falls from day 10 to day 13.

**1st Vaccine Trial:** 13 out of 42 four-week-old mice with a single-dose of Kp r56 or 30.95% died between days 10 - 13.

**2nd Vaccine Trial:** 2 out of 7 four-week-old mice with a single-dose of Kp r56 or 28.57% died between days 10 - 13.

**2nd Vaccine Trial:** 3 out of 9 eight-week-old mice with a single-dose of Kp r56 or 33.34% died between days 10 - 13.

Mice appeared to survive longer in our 2nd vs. the 1st Vaccine Trial:

**1st Vaccine Trial:** 11 out of 42 (5 on day 15 + 3 on day 16 + 3 on day 17) four-week-old mice with a single-dose of Kp r56 or 26.18% died on day 15 - day 17,

**2nd Vaccine Trial:** 3 out of 7 (2/7 on day 15 + 0/7 on day 16 + 1/7 on day 17) four-week-old mice with a single-dose of Kp r56 or 42.85% died on day 15 - day 17,

**3rd Vaccine Trial:** 4 out of 9 (2/9 on day 15 + 0/9 on day 16 + 2/9 on day 17) eight-week-old mice with a single-dose of Kp r56 or 44.44% died on day 15 - day 17, also in this group one mouse died on day 19.

2. Immunized by a double-dose of Kp r56 vaccine:

Results from challenges during our 2nd (scrub typhus) Vaccine Trial on 4-week-old mice immunized with a double-dose of scrub typhus Kp r56 vaccine showed 3/10 mice (30%) survived through out the course of our clinical observations (21 days period). The survival rate of 30% in our 2nd Vaccine Trial is similar to the results of the 4-week old mice challenged with a single-dose of Kp r56 vaccine during the 1st (scrub typhus) Vaccine Trial (32%). Of those 3 surviving mice observed in our 2nd Vaccine Trial, all 3 mice exhibited a delayed onset of illness by becoming ill on day 12, one mouse recovered on day 14, while the other 2 mice recovered on day 20. While the other 7 mice in this 2nd Vaccine Trial group died, these mice survived longer than normal from challenge.

When compared the results obtained from the 1st & 2nd scrub typhus vaccine trials of the double dose immunization, it appears that more mice in the 2nd group exhibited delayed onset of illness than the 1st group as well as more mice survived longer.

Based upon our preliminary results on the scrub typhus vaccine trials on either the single-dose and/or double-dose immunized mice, which revealed similar protection in both groups. Results are still inconclusive as to which regime would provide a better protection.

### **Results of 3<sup>rd</sup> Scrub Typhus Vaccine Trial (Chigger-Mouse-Model):**

The 3<sup>rd</sup> scrub typhus vaccine trial-"Mouse Model", (delayed 2 months due to the installation of the decontamination system in the AFRIMS BSL-3 suite) started in August 2006 has been completed. Results are as follows:

Single-dose vaccination group of 4-week-old ICR mice:

- 20 vaccinated & 20 non-vaccinated (control) mice were challenged by scrub typhus-infected, Lc-1 chiggers.
- All control mice died.
- Of the vaccinated group, 4 mice survived {4/20 = 20% survival rate (3 mice never got sick, one mouse got sick and recovered within 2 weeks)}.
- Of 16 dead mice (16/20 = 80%), no mouse died during the normal mortality period (day 10-13), they exhibited a delayed onset to disease and a prolonged illness period prior to death): 3 mice died on day 14; 9 mice died on day 15-17 and 4 mice (4/16=25%) died on day 18-23.

Double-dose vaccination group of 4-week-old ICR mice:

- 20 vaccinated & 20 non-vaccinated (control) mice were challenged by scrub typhus-infected, Lc-1 chiggers.
- All control mice died.
- Of the vaccinated group, 2 mice survived {2/20 = 10% survival rate (both mice ever got sick)}.
- Of 18 dead mice (18/20 = 90%), 2 mice died during the normal mortality period (day 10-13). Of the remaining 16 mice, which exhibited a delayed onset of disease and a prolonged illness period prior to death): 5 mice died on day 14, 4 mice died on day 15-17 and 7 mice (7/18 = 39%) died on day 18-23.

### **Summary of All Scrub Typhus Vaccine Trials (Chigger-Mouse-Model):**

- Survival rate in single-dose group was 23.3% and the multiple doses were 24.0% for both double- and triple-Dose groups.
- About 18% of dead-mice died during the normal mortality period (17.4% in single-dose group & 18.4% in double-dose group). No mouse in the triple-dose group died during the normal mortality period.
- Of the vaccinated-mice, 82.6% and 81.6% of dead-mice in single- and double-dose groups exhibited a delayed onset of disease and a prolonged illness period prior to death. While in the vaccinated-mice of the triple-dose group, all mice (100%) showed a delayed onset of disease and a prolonged illness period prior to death.
- Among those vaccinated-mice with a delayed onset of disease and a prolonged illness period prior to death, more than half of them died during days 14-17 as demonstrated in single-, double- and triple-dose groups of 65.2%, 55.3% and 73.7% respectively.
- About a quarter total of vaccinated mice with a delayed onset of disease and a prolonged illness period prior to death died during days 18-23 as evidenced in the single-, double- and triple-dose groups with mortality percentages of 17.4%, 26.3% and 26.3%, respectively.

### **Statistical Analysis on Survival time:**

Statistical Analysis on Survival time of the Vaccinated-mice versus the Reference-mice:

- Using the Kaplan-Meier Test, the statistical analysis of our preliminary results, revealed that there were significant differences of “Median Survival Time (=By determining the 50% probability of survival; @ 95% Confidence Interval)” of the single- and double-dose vaccination groups and the reference group and



experiment-control groups (with the normal mortality period), indicated a significant delay in illness and death occurred in the vaccinated mice.

### **Overall Preliminary Conclusion**

- Our overall preliminary conclusion revealed that there was no difference in the survival rates between the vaccinated-mice of single-&multiple-dose groups. However, our data indicated that the vaccinated-mice of the multiple-dose groups had a longer delay in becoming ill. Therefore, they had the potential to be living longer when compared with mice of the reference group.

### **Future Experiment for FY07:**

- The needle challenge in utilizing the “Mouse-model” (by which IP injection of the *Orientia tsutsugamushi* Lc-1 isolate) will be conducted in order to compare the results with the chigger challenge model.
- Determining the immune response developed by the vaccinated-mice from the serum samples by the ELISA technique.
- Conducting the evaluation on other scrub typhus vaccine candidates; i.e., kp r47 KD.

#### **e. Future Plans:**

Continue to conduct mouse-challenge evaluation of candidate vaccines in FY06.

**3. Title of Research Project:** Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

#### **a. Investigator:**

Dr. Jetsumon Prachumsri, Ph.D

#### **b. Objectives:**

1. Provide mosquitoes with consistent, reproducible salivary gland infections to support *Plasmodium vivax* sporozoite challenge.

2. Provide live *P. vivax* sporozoite-infected mosquitoes and/or harvested, purified *P. vivax* sporozoites (on wet- or dry-ice) to WRAIR/NMRI investigators or collaborating institutions.

3. Provide live, frozen and antigen slides of blood and exo-erythrocytic stages of *P. vivax* parasites for vaccine studies.

### c. Methods:

1. Validation of basic system to provide sporozoite-infected mosquitoes in support of STEP/STO requirements. This system will be based on our current ability to feed mosquitoes directly on *P. vivax*-infected patients reporting to local malaria clinics. In brief, adult patients report to local malaria districts when they think they may have malaria. Ministry of Public Health (MOPH) personnel make thick blood smears and check the patients for malaria. As part of an approved Human Use Protocol, Department of Entomology personnel allow to draw 20 ml of patient blood to feed mosquitoes using membrane feeding technique. These studies are conducted weekly at Mae Kasa and Mae Sod malaria clinics. Aliquots of each blood sample will be spotted on filter paper and smeared on glass slide. Confirmation of parasite species will be accomplished by PCR of filtered blood and microscopic examination of blood smears. Infected mosquitoes are returned to AFRIMS and maintained in the AFRIMS insectary. Five to 10% of mosquitoes from each mosquito feed are checked for the presence or absence of oocysts approximately 7-10 days after infection. These mosquitoes are thereafter available for use in malaria sporozoite challenge studies.

2. Refined Sporozoite Challenge System. In 2007 we will continue refining the system in order to reduce the variability in the mosquito infections (critical for ensuring consistent challenges) and to eliminate the risk of concomitant mosquito infections. The goal will be to develop a system that will i) consistently provide mosquito infection rates with >60% of blood-fed mosquitoes having +3/4 (>100 sporozoites) salivary gland infections, and ii) provide *P. vivax*-infected mosquitoes that do not harbor concomitant pathogens. Consistency in the challenge is a critical component of any vaccine trial. *Plasmodium vivax*-infected patients reporting to local malaria clinics will serve as the starting point for development of the "refined system". Mosquitoes will be fed on venous blood provided to them in an artificial membrane feeding system. A series of carefully controlled experiments will be conducted using the membrane feeding system. We hypothesize that pooling blood from infected patients will reduce inherent variability in mosquito infections. In addition, pooling blood from several patients may offer the added benefit by increasing the genetic diversity of the sporozoite challenge, and thus may more truly evaluate the efficacy of any candidate vaccine. Replacement of Patient Sera with Commercial Sera: Blood will be collected from patients, and packed red blood cells separated from the sera and subsequently reconstituted with commercial sera. The reconstituted blood will be fed to mosquitoes in a membrane feeding system and mosquito infections quantified. This method has the advantage of removing anti-malaria antibody that may affect gametocyte infectivity (2) and replaces patient sera that is potentially infected with concomitant cellular pathogens with commercial sera that is known as pathogen-free.

3. Parasite Characterization: In the absence of an in vitro culture system, it will be necessary to feed mosquitoes on a *P. vivax*-infected volunteer or the blood from a volunteer. Since it will be impossible to ensure that mosquitoes are infected with a single *P. vivax* clone (as is currently done with *P. falciparum*), it is critical that we develop a method of characterizing the parasites (i.e., genetic diversity of the parasites,

resistance to antimalarial drugs, etc.). Once mosquitoes are infected, parasites from the infectious blood meal will be characterized by PCR using polymorphic gene targets, such as the nonapeptide repeat region of the circumsporozoite protein (PvCSP), and the region between interspecies conserved blocks 5 and 6 of the merozoite surface protein (PvMSP1).

4. We will continue our effort to establish a short term culture technique to produce gametocyte and study gametocyte infectivity. In this portion of the study we propose to evaluate the ability of produce infective gametocyte by in vitro culture of cryopreserved blood to infect mosquitoes. Effort will focus on establishment of cryopreservation techniques that will maximize parasite viability. Development of procedures to culture infective gametocytes would allow for production of sporozoites from *P. vivax* specimens obtained from throughout the world. These sporozoites could then be used in a variety of experimental models (i.e., we could use sporozoites obtained from East Timor in our hepatoma cell model to evaluate resistance of Exo-erythrocytic stage parasites to primaquine and tafenoquine). We have used different cryopreservatives to freeze *P. vivax* infected blood. The frozen samples will be thawed and cultured for short periods to evaluate the viability of the parasites and gametocyte infectivity to mosquitoes in 2007.

**d. Results** (accomplishments during the period of January 2006-December 2006):

1. New human use protocol to collect blood samples from patients has been completed and we have received an approval letter from the Thai Ministry of Public Health.

2. Updated standard operating procedure (SOP) has been prepared for the 2007 studies.

3. Sporozoites have been produced and used for preparation of IFA slide antigens and *in vitro* culture of liver stage parasites. *In vitro* culture of *P. vivax* parasites in human liver cells, HC04, have been started. Parasite infected liver cells have been collected and prepared for study of parasite genes and proteins.

4. Analysis of genetic variation of vaccine gene candidates for *P. vivax* among Thai isolates were done. The data from this study will help us to estimate the case number of blood the will be required for *P. vivax* vaccine human challenge model which is planned for 2007.

**e. Future Plans:**

Different stages of parasites will be produced as listed below.

1. Blood stage parasites from different field sites will be cryopreserved and kept in liquid nitrogen to be used as starting parasites for in vitro culture.

2. Parasite pellets and antigen preparation as IFA slides will be prepared from *P. vivax* infected blood and kept frozen at -70 before being transported to AFRIMS and/or CONUS laboratories. Short term culture for blood stage *P. vivax* will be performed to obtain synchronous stage of the parasites. Separation of different blood stage will be done by using percoll gradient method.

3. Produce *P. vivax* sporozoites or sporozoite infected mosquitoes, blood from malaria clinics in Thailand will be fed to *Anopheles dirus* using membrane feeding method. Parasite development will be examined 7 and 14 days post feeding before harvesting of salivary gland sporozoites or transportation of live infected mosquitoes to CONUS laboratories. Shipment of *P. vivax* infected blood, sporozoites and/or infected mosquitoes will be coordinated by the PI (Department of Entomology, AFRIMS) and CONUS investigators.

4. Exo-erythrocytic (EE) parasites will be produced by an in vitro culture technique using HC-04 hepatoma cells established at AFRIMS. Different developmental stages of liver parasites will be harvested and antigen slides will be prepared at different times for early EE parasites (day2), EE merozoites (day 7-14) and hypnozoite/no development forms (day 28) after sporozoite inoculation.

We plan to collect *P. vivax* parasites from 300 patients for this study in FY2007. Approximately 100 isolates of *P. vivax* will be used for mosquito feeding that will produce at least 30 batches of sporozoite infected mosquitoes. Sporozoites will be harvested from mosquitoes to prepare IFA slides, keep as sporozoite pellets and to be used for production of EE IFA slides. Blood stage parasites from 150 isolates will be cryopreserved, prepared for IFA slides and kept as frozen pellets for further genetic studies.

## B. Department of Immunology AFRIMS FY06 Research Accomplishments

### 1. Title of Research Projects:

Number	Projects	Status
1	MRDD Phase III (687-2001)	In life completed 2002; report writing completed; FDA-IND in process
2	MRDD Phase IIIb – Venous vs. Fingertick	In life completed 2003; data analysis complete
3	Human Malaria Vivax Challenge	Protocol in development. Undergoing ethical review.
4	Rhesus Pf AMA-1 Combinations Vaccine	Study complete; publication in progress

5	Immunologic studies of SIV/p27 adenovirus as a model system for future malaria antigen studies	Study completed; publication in progress
6	PvDBP Polymorphisms	Study completed; publication in progress
7	Tafenoquine Cure/Radical Cure Dose Ranging NIH	In life completed Jan 2005; manuscript preparation in progress
8	Phase II Assessment of Azithro-Quinine Combos	In life completed; results reported at ASTMH; publication in progress
9	Azithro-Quinine and Azithro Artesunate in Pf Rx	Study completed; manuscript published
10	AS Bioequivalence study (comparing WRAIR IND formulation vs. Guilin non GPO product)	Protocol prepared; scientific approval obtained; study canceled
11	Bioassay/HPLC/LC-MS Validation - FDA	AS complete; LC-MS equipment validated; Method Validation protocol completed
12	Artesunate Phase II Protocol Development	In progress; in life study will begin March 2007
13	Fever Surveillance in Sangklaburi	Results reported to local health authorities; manuscript published
14	Cambodia Malaria Prevalence Study	In life and slide reading completed; initial data analysis completed; publication in progress
15	Leptospirosis in Sangklaburi	Manuscript published; PCR optimized
16	Molecular Assessment of Nepal Malaria Isolates	In life and data analysis completed; manuscript published
17	Bangladesh In Vitro Pf Resistant assessment	Protocol approved; Second of 5 year project completed
18	Cambodia In Vitro Pf Resistant assessment	Protocol development and in life portion of study completed, data analysis ongoing
19	Thailand In Vitro Pf Resistant assessment	completed; initial data analysis complete; publication in progress
20	PF MSP-1 Genotyping – assay development	In progress;
21	Vivax Genotyping - PV Mahidol	Data analysis

22	Gametocyte Production for Entomology	In Progress
23	Set up and Maintenance of Regulated LC-MS Laboratory	Continuing
24	Pharmacodynamic/kinetic of lead antimalarial drugs in rhesus malaria model	Ongoing
25	Safety and Immunogenicity of <i>Plasmodium vivax</i> circumsporozoite Vaccine in Rhesus Monkeys	Protocol submitted
26	Development of Real Time PCR Assay for Diagnosis of <i>Plasmodium falciparum and vivax</i>	Optimization completed
27	Development of PCR Assay for Diagnosis of Leptospirosis	Optimization completed
28	Surrogate Markers of Malaria	Data generated; report and publication in progress
29	Pre-clinical Safety and Immunogenicity Primate Studies to Support INDS	Data generated; publication in progress
30	Evaluation and Testing of <i>P. falciparum</i> Parasite Antigens for Use in Active Immunizations Against Malaria	Data generated; publication in progress
31	Evaluation of Avian Influenza hemagglutinin sequences in wild birds	Data generation in progress
32	Kwai River Christian Hospital surveillance of influenza like illness	Protocol development in progress

**a. Investigators:**

Dr. Mark Fukuda, MD  
Dr. Bryan L. Smith, MD  
Dr. Sathit Pichyangkul, Ph.D.  
Dr. Paktiya Teja-Isavadharm Ph.D.  
Dr. Krisada Jongsakul, MD  
Dr. Harald Noedl MD, PhD  
Dr. Delia Bethell, MD  
Dr. Youry Se, MD  
Dr. Kurt Schaecher, PhD

**b. Objectives:**

1. To protect, project and sustain the military soldier against disease threats produced by the two major species of malaria, *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv). To support this mission through the evaluation of new or improved vaccines, prophylactic and therapeutic drugs, rapid diagnostic kits, and the maintenance of a center for excellence focused on the basic biology and epidemiology of malaria.

2. To assess emerging febrile diseases along high-risk regions in Thailand and throughout SE Asia.

**c. Methods:**

The Department of Immunology and Medicine has applied as many kinds of classical and state-of-the-art technologies as possible to the above multi-faceted research. Clinical research included mobile epidemiology team able to work in adverse conditions where malaria is present, including field sample collection and processing screening, reference microscopy, assessment of rapid diagnostics for various tropical infectious diseases, and a staff well-versed in conduct of clinical trails to GCP and ICH standards. The animal research teams are all trained in laboratory animal research and regulations, current AALAAC requirements, and laboratory animal test and observation methods. State-of-the art methodologies are available for the study of vaccine and drugs to include advanced molecular biology methods such as sequencing, SNP analysis, and real-time PCR. Cellular immunology techniques are available which include flow cytometry and sorting technologies, ELISPOT, and molecular methods. Pharmacology assays include HPLC, LC-MS, a unique malaria bioassay to measure the *in vivo* antimalarial bioactivity of potential new antimalarial medications, sustained malaria cell culture and radioisotopic uptake, and antibody based methods for measuring *in vitro* drug sensitivity patterns of malaria strains against standard malaria drugs.

**d. Results (accomplishments during the period of January-December 2006):**

**1. Malaria Drugs STEP Q, STO-AQ, STO-A4, STO-A5**

Managed the implementation of departmental quality practices for the execution of studies in agreement with MRMC policies and US FDA standards in support of IV AS drug development program. Work involved the generation and/or revision of nearly 50 SOPs; upkeep of personnel training and qualification records; space utilization for LCMS lab, sample repository, and field clinical lab; establishment of a controlled sample tracking and inventory system; qualification of equipment used for regulated studies; and continued interaction with Medical Maintenance and service contractors. Helped integrate Departmental QA/QC efforts with those of the subsequently established QA units at the AFRIMS, WRAIR and MRMC. Participated in the IPT teleconferences, providing metabolism and pharmacokinetics insight. Chaired the PK SC undertaking tasks to address related concerns. Completed the implementation of a metabolism-based drug interaction study for AS using markers of enzymatic activity and rhesus monkeys; presented the data the ISSX and ASTMH meetings. Wrote protocol and built field site for a Phase II IV artesunate dose-ranging study that will begin in March 2007 (MIDRP funded AQ0073\_06\_AF).

The method of AS/DHA analysis in human plasma using LC/MS was validated according to a WRAIR approved protocol. Current efforts, are focused on measuring AS/DHA samples from ongoing Phase II Artesunate clinical trials. (AQ0047\_06\_AF: Maintenance of a cGLP Analytical Lab).

Published methods paper describing a simple, nonisotopic, semiautomated bioassay for the measurement of antimalarial drug levels in plasma or serum based on the quantization of histidine-rich protein II in malaria culture is presented. The assay requires only small sample volumes and was found to be highly sensitive and reproducible. The results closely paralleled those obtained with isotopic bioassays ( $R = 0.988$ ,  $P < 0.001$ ) and high-performance liquid chromatography-electrochemical detection ( $R = 0.978$ ,  $P < 0.001$ ).

Analyzed data from a clinical trial of tafenoquine monotherapy in adults for evaluation of radical curative ability and pharmacokinetics in *P. vivax* malaria. This study was fully successful in demonstrating that tafenoquine monotherapy can effectively eliminate both blood and liver stage parasites in a manner compatible with current therapies. Funded with NIH co-development grant with GSK, and partnered with Hospital of Tropical Diseases, Faculty of Tropical Medicine, Mahidol University.

Supported parasitology requirements for continuing efforts to develop a hepatocyte cell line to screen activity in the liver of antimalarial drugs and vaccine candidates. Collaboration with Department of Entomology. MIDRP funded.

### **3. Diagnostics/Rapid Diagnosis of Malaria STEP-L/STO-L**

Continued development of real-time PCR method to reliably diagnose Pv and Pf malaria from human blood. Developed new primer and probe sets to detect malaria generically (all *Plasmodium* species) and specifically for *P. falciparum* and *P. vivax*. This development used DNA alignment software to compare the sequence of the 18S rRNA gene of *Plasmodium falciparum*, *vivax*, *malariae*, and *ovale* and *Homo sapiens sapiens*. For generic primer and probe sets, common sequences for *Plasmodium* were chosen that had differences with human sequence. For specific *Plasmodium* primer and probe sets, sequences unique for only *P. vivax* or *P. falciparum* were chosen. Chosen sequences were then checked via BLAST search for homology with any other sequence. Finally, primer and probe sets were designed using PrimerExpress™ (Applied Biosystems, Foster City California). The most optimal primer/probe set for Generic, *P. falciparum*, and *P. vivax* were determined. All PCR assays (Generic, *P. falciparum*, *P. vivax*) were optimized for MgCl<sub>2</sub> concentration, probe concentration, and primer concentration. Also, limits of detection and quantitation were completed along with a small diagnostic specificity study.

Continued work with leptospirosis diagnostic tests at the Sangkhlaburi febrile diseases study site. Surveyed 214 paired samples for IgM titers to leptospirosis using ELISA diagnostic tests (PanBio Inc., Baltimore, M.D.). Data published in January 2006.

Developed and assessed primer sets as a diagnostic for leptospirosis. Developed 5 primer sets and tested annealing temperatures and cycle curves for each set on four different cultured serovars of leptospirosis. Using one of the primer sets, confirmed the presence of leptospirosis in four clinical samples that were positive by



culture. Also, determined that whole blood or packed cells were the best samples for use in leptospirosis diagnostics using PCR. We also ran 16 samples in two different runs from Nepal suspected of being positive for leptospirosis. Along with conventional PCR, two primer/probe sets were developed to detect LipL32, an outer membrane protein found only in pathogenic lepto, using real time PCR. Both sets were optimized for MgCl<sub>2</sub>, probe, and primer concentrations. Primer sets were tested against laboratory strains of leptospirosis and also tested for limits of detection and quantitation.

Using ELISA, conducted an assessment of two different proteins (*P. vivax* Circumsporozoite protein and Merozoite Surface Protein) to detect antibody responses that could be used as a surrogate marker of malaria exposure. We developed (antigen concentration and secondary antibody dilution) the ELISA with 40 different known negatives to clear background reactions. We then ran 587 different samples from a variety of anti-malarial drug clinical trials against both antigens to assess feasibility to detect increases in antibody response from malaria pre-exposure to post exposure. We found that PvCSP could detect antibody changes from baseline better in individuals who had several prior malaria exposures, but naive subjects had less of a response. PvMSP was a much better indicator. Also, antibody responses were most robust in subjects receiving no treatment or receiving mefloquine. The draft report and publication for this study is still pending.

We evaluated the level of malaria recrudescence and/or re-infection in 16 subjects from a clinical drug resistance study in Bangladesh. The alleles of MSP1, MSP2, and GLURP were tested at pre- and post-drug treatment in malaria parasite DNA collected from each individual. Publication still pending. We evaluated 57 samples collected from malaria subjects in Trat province, Thailand for MSP1, MSP2, GLURP for PCR correction (recrudescence/re-infection) and also ran restriction fragment length polymorphism assays for MSP1 and MSP2 on the same samples. We are currently running samples from our drug resistance surveillance study in Tesan, Cambodia with the same parameters.

We have established a quality system for microscopy that involves the creation of a certified set of standard testing slides, a quality approved SOP for testing slide readers, and a system for evaluating the performance and training of tested microscopists. This set was recently used in support of the Artesunate Phase IIb DrT study.

In FY2007, we plan to publish data on our optimization characteristics for the Plasmodium real time PCR, publish leptospirosis real time PCR assay data, support ongoing efforts to develop a multiplex diagnostic PCR for use with GEIS fever study objectives, and build upon our malaria drug resistance surveillance efforts by development of real time PCR assays capable of assessing molecular markers associated with resistance.

## **Emerging Infectious Diseases (GEIS)**

### **Epidemiology of Falciparum Malaria Drug Resistance Patterns in Asia:**

Continued surveillance activities throughout Southeast Asia (Bangladesh, Myanmar, Thailand and Vietnam) for threat assessment of multi-drug resistant malaria. Assessed potential new field sites in Cambodia, eastern Bangladesh, Nepal, and northern Thailand (Chiang Dao). Parasite isolates continue to be analyzed longitudinally to assess for trends in antimalarial drug sensitivity patterns potentially signaling a diminution in the utility of the present armamentarium of malaria medications. GEIS funded, and coordinated with Public Health departments in the various countries.

Validated under field conditions a new non-isotopic method for *in vitro* drug resistance assays, which is simpler, as robust, and avoids radioisotopes. The methods has been made available free of charge to the malaria research community as a public service (see <http://malaria.farch.net>). The test shows very reliable comparisons to the WHO microtest using a much simpler methodology. Funded by GEIS with support from Mahidol University and University of Vienna.

Continued a combined *in vitro-in vivo* antimalarial drug efficacy trial of standard artesunate/mefloquine therapy in Trat province in southeast Thailand. This study, intended to investigate previously anecdotal reports of high failures using this regimen is of paramount importance in informing malaria drug policy for the greater southeast asia area. The study will be conducted employing directly observed therapy of all dosed antimalarial drugs and will draw inferences between IC50s measured using the above mentioned non-isotopic *in vitro* drug resistance assay. This study has been expanded to examine the same parameters in Tesan, Cambodia.

### **Surveillance of Febrile Diseases along the Thai-Myanmar Border:**

Published results of a multi-year effort to establish infectious etiologies to undifferentiated fevers along the Thai-Myanmar border in Kanchanaburi province: *Am. J. Trop. Med. Hyg.*, 74(1), 2006, pp. 108–113

**Abstract:** A hospital-based study was conducted along the Thai-Myanmar border to provide greater knowledge of the causes of febrile illness and to determine what zoonotic and vector-borne emerging infectious diseases might be present. A total of 613 adults were enrolled from June 1999 to March 2002. Cases were classified based on clinical findings and laboratory results. An etiologic diagnosis was made for 48% of subjects. Malaria was the most common diagnosis, accounting for 25% of subjects, with two-thirds *Plasmodium falciparum*. Serologic evidence for leptospirosis was found in 17% of subjects. Other etiologic diagnoses included rickettsial infections, dengue fever, and typhoid. The most frequent clinical diagnoses were nonspecific febrile illness, respiratory infections, and gastroenteritis. Clinical associations were generally not predictive of etiologic diagnosis. Apparent dual diagnoses were common, particularly for

malaria and leptospirosis. Findings have been used to modify treatment of unspecified febrile illness in the area.

### **Malaria Prevalence Study in Cambodia:**

Final analysis and publication of a completed 2005 investigation from the Department of Immunology and Medicine country wide malaria prevalence survey in Cambodia. Results indicated that malaria prevalence is generally highest in clusters located in Rattanakiri, Stung Traeng, Preah Vihear and northern areas of Kampong Thom and Kratie. This is reflected in prevalence calculations by domain, which show that mean prevalence in domains 1, 2 and 3 were 6.9%, 2.8% and 0.2% respectively. Corresponding figures for prevalence by domain at mini-prevalence sites were 9.2%, 1.6% and 1.0%. The table below shows the prevalence of different species of malaria parasite by domain. Final molecular analysis of drug resistance markers is ongoing.

### **Parasite prevalence by domain from cross-sectional blood slide survey during household survey**

<b>Domain</b>	<b>P. falciparum</b>	<b>P. vivax</b>	<b>Pf + Pv</b>	<b>Other*</b>	<b>Total Positive</b>	<b>Negative</b>
1	5.4 (128)	1.2 (31)	0.2 (4)	0.1 (4)	6.9 (167)	93.1 (2718)
2	1.3 (45)	1.4 (39)	0.04 (2)	0.03 (1)	2.8 (887)	97.2 (2723)
3	0.1 (5)	0.1 (5)	0 (0)	0.02 (1)	0.2 (11)	99.8 (2729)
<b>Total</b>	<b>1.8 (178)</b>	<b>0.8 (75)</b>	<b>0.1 (6)</b>	<b>0.04 (7)</b>	<b>2.7 (266)</b>	<b>97.3 (8159)</b>

\*Other species = 7 (*P. malariae* = 6, mixed Pm+Pv = 1)

#### **e. Future plans:**

We plan to continue our multi-faceted emphasis on support for malaria product development in diagnostics, new drugs, and new vaccines. We anticipate heavy participation in a DoD wide effort on malaria microscopy QA procedures and have hired a dedicated expert teaching microscopist charged with the responsibility of developing a rigorous microscopy teaching and certification standard. Furthermore, we anticipate being the lead overseas lab for field-testing intravenous artesunate in phase I and II, as possibly phase III testing. We will continue efforts for tafenoquine development, especially towards an indication of radical cure for *Plasmodium vivax*. We will continue safety and immunogenicity testing of candidate malaria vaccines in rhesus, and progress towards vivax challenge studies for eventual human testing of vivax vaccines in Thailand. Emerging infection work in Sangkhlaburi will continue with emphasis on flaviviruses, leptospirosis and typhoidal illnesses, and this study will be expanded to another targeted site in Nepal. Lastly, we anticipate an expand role in regional malaria surveillance with a combination of *in vivo*, *in vitro* and genetic methods to define expanding malaria drug resistance.

## **C. Department of Enteric Diseases, AFRIMS FY06 Research Efforts**

### **1. Title of Research Project: Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand**

#### **a. Investigators:**

Dr. Ladaporn Bodhidatta  
Kaewkanya Nakjarung  
Tasawan Singhsilarak  
Rungnapha Phasuk  
Sasikorn Silapong  
Chittima Pitarangsi  
Boonchai Wongstitwilairoong  
Apichai Srijan  
Paksathorn Puripanyakom

#### **b. Objectives:**

Monitor diarrhea etiology and antimicrobial resistance of enteric pathogens at multiple sites within Thailand.

#### **c. Methods:**

Hospitals and regional laboratories in several sites in Thailand to include Trang, Mae Hong Son, Ubon Ratchathani, Samutsakhon, Bangkok, and other locations agreed to participate. The study protocol was approved by both the US and Thai authorities. Several visits were made to each site by the Principal Investigator, as well as nursing and laboratory staff prior to study initiation. Additional equipment and supplies were provided to the microbiology laboratory at the each site. Onsite trainings were conducted. Stool samples were received at each participating site for initial assessment followed by confirmatory tests and additional laboratory studies to include molecular studies and antimicrobial susceptibility testing at AFRIMS in Bangkok Thailand.

#### **d. Results:**

Over 2,500 stool samples have been received and over 1,000 of bacterial isolates have been collected with Campylobacter and non typhoidal Salmonella as leading pathogens. Real time PCR assays to detect Norovirus were applied to investigate 1,819 samples and 113 positives were sequenced to identify genotypes.

#### **e. Future plans:**

Continue study.

## **2. Title of Research Project: Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens**

### **a. Investigators:**

Orntipa Sethabutr  
Rungnapha Phasuk  
Sasikorn Silapong  
Kaewkanya Nakjarung  
Tasawan Singhsilarak

### **b. Objectives:**

Develop and standardize realtime PCR assays for the detection and characterization of enteric pathogens to include Shigella, Salmonella, Campylobacter, Cryptosporidia, Cyclospora, and Noroviruses.

### **c. Methods:**

Based on literature review and best available sequence data, multiples sets of primers and probes were designed for each pathogen of interest. The sets were initially evaluated against cultured material. Selected sets of primers and probes were then tested against frozen stool samples collected and archived from multiple Department of Enteric Diseases studies. Lower limits of detection for several sets of primers and probes were determined.

### **d. Results:**

Probes and primer sets have been developed and evaluated for Campylobacter, Shigella, Enterotoxigenic *E. coli*, Enterohemorrhagic *E. coli*, Salmonella, Cryptosporidia, and Noroviruses. Over 1,000 stool samples have been tested.

### **e. Future plans:**

Transfer validated and evaluated assays to JBAIDS platform.

## **3. Title of Research Project: Characterization of Enteric Pathogens Isolated from Children in Phnom Penh**

### **a. Investigators:**

Dr. Bryan Smith  
Dr. Ladaporn Bodhidatta  
Boonchai Wongsatitwilairoong  
Apichai Srijan  
Chittima Pitarangsi

**b. Objectives:**

Determine diarrhea etiology and antimicrobial resistance of enteric pathogens from young children with diarrhea presenting to the National Pediatric Hospital in Phnom Penh, Cambodia.

**c. Methods:**

The study protocol was approved by both the US and Cambodian authorities. Additional equipment and supplies were provided to the microbiology laboratory at the National Pediatric Hospital in Phnom Penh. After obtaining informed consent, stool specimens were collected from diarrhea cases on presentation. An effort was made to obtain a matched asymptomatic control for each case. Initial stool examination and culture was performed at the National Pediatric Hospital. Confirmatory tests and additional studies to include molecular studies and antimicrobial susceptibility testing were conducted at AFRIMS in Bangkok.

**d. Results:**

Over 1200 stool samples have been received and common diarrheal pathogens identified thus far including Rotavirus and Shigella. Real time PCR assays to detect Shigella, Campylobacter, Enterotoxigenic *E. coli* and Norovirus were applied to investigate over 900 stool samples.

**e. Future plans:**

This study was completed during late CY 2006.

**4. Title of Research Project: Characterization of *Campylobacter jejuni* isolates**

**a. Investigators:**

Oralak Serichantalergs  
Piyarat Poothong  
Panida Nopthai

**b. Objectives:**

Identify and characterize a safe human challenge strain of *Campylobacter jejuni* for future vaccine evaluation.

**c. Methods:**

*C. jejuni* isolated from adult travelers during Cobra Gold exercises in 1998-2003 and Bumrungrad hospital in 2001-2002 including *C. jejuni* isolates from

Multicenter studies, 2005 were selected and serotyped for Penner heat stable and Lior heat labile serotyping systems. Standardized PFGE was performed on each *C. jejuni* isolate with 2 restriction enzymes. Characterization of representatives of each major cluster was done by PCR for virulence and Guillain-Barre syndrome (GBS) related genes. Isolates not containing genes associated with GBS related illness will be identified for further study.

**d. Results:**

Three Hundred and Forty Six *C. jejuni* isolates were screened by PFGE analysis. One hundred and six *C. jejuni* from each 105 PFGE major cluster were characterized by PCR for virulence genes and GBS related genes. Partial sequence of these genes has been completed, analyzed and compared to *C. jejuni* sequences in the GenBank. Potential candidate strains that lack of GBS related genes were identified and sent for further studies.

Finalized results of sequences analysis from 106 *C. jejuni* isolates (virulence genes and GBS genes) in FY 06 were completed for 4 GBS genes [*cgtA*, *cgtB*, *cstII* (HS2), *cstII*(HS19)]and 8 virulence genes (*cdtA*, *cdtB*, *cdtC*, *flaA*, *cmeC*, *peb1A*, *cadF*, and pVirB11) . All sequences were aligned and blasted to partial amino acid sequence of the relevant genes from *C. jejuni* NCTC 11168 or *C. jejuni* 81-176. The results of alignments using Sequencher and Mega software were completed. The *flaA* gene (1596 bp) is the most variable gene amongst 106 isolates since there were both changes in deletion and addition in nucleotides compositions as shown in 4 groups of *flaA* amino acid sequence. Sequences of 106 *flaA* gene were submitted for *fla* typing in Campylobacter database web site.

Five isolates from 106 *C. jejuni* were identified to lack GBS related genes and these five Thai isolates were sent for further testing at NMRC for human challenge study.

Characterization of more 45 Thai *C. jejuni* isolates (Multicenter studies, 2005) was carried out in FY 06 for detection of virulence, protein antigens and GBS related genes. Developing of Multilocus sequence typing (MLST) was initiated at Dept. of Enteric Disease to use as a new typing technique by sequencing of 7 house-keeping genes for 45 +106 *C. jejuni* isolates.

**e. Future Plan:**

Continue study.

**5. Title of Research Project: The Use of Rhesus Monkeys for the Establishment of the Intra-gastric Challenge Model for Wild Type Shigella Dysenteriae-1, 1617 Strain**

**a. Investigators:**

Dilara Islam, Ph.D  
Nattaya Ruamsap  
Ajchara Aksomboon  
Patchariya Khantapura  
Chittima Pitarangsi  
Boonchai Wongstitwilairoong  
Wilawan Oransathit  
Paksathorn Puripanyakom  
Sawat Boonnak  
Songmuang Piyaphong

**b. Objectives:**

The goal of this project is to establish a dependable and reproducible *S. dysenteriae* 1 challenge regimen in the rhesus monkey model using the wild-type 1617 strain to document the reactogenicity and the immune response elicited by this wild-type 1617 strain and to demonstrate that previous infection with wild-type 1617 strain will protect monkeys against a subsequent challenge with the same organism. Three groups of 5 rhesus monkeys were intragastrically challenged sequentially with the three escalating doses of *S. dysenteriae* 1,1617 strain to establish the optimal challenge dose required to produce dysentery in 3-4 monkeys of 5 ( $\geq 80\%$  attack rate). The doses are: Group-1:  $2 \times 10^8$  cfu (challenge) &  $2 \times 10^9$  cfu (re-challenge); : Group-2:  $2 \times 10^9$  cfu (challenge) &  $2 \times 10^{10}$  cfu (re-challenge); Group-3:  $2 \times 10^{10}$  cfu (challenge) &  $2 \times 10^{10}$  cfu (re-challenge).

**c. Methods:**

The study protocol was approved by the AFRIMS' IACUC. In order to establish the animal model of *S. dysenteriae* 1 infection, various specimens were taken for analysis, including, blood, stool, colonic lavage and colonic biopsies. Various clinical, immunologic, bacteriological, and histological tests were conducted on animal specimens from all monkeys. Serum IgA, IgG and IgM antibody titers, antibody secreting cells, fecal secretory-IgA and fecal cytokines were measured at different time points of the study.

All monkeys were monitored by direct observation for 30 minutes (after challenge) for occurrence of immediate adverse reactions. Also, beginning on day (-3) and continuing until day 7 or until all animals are clinically normal the monkeys were closely observed and scored twice daily using a clinical observation report (VM Form B20). Animals showing any signs of clinical disease were removed from the cage daily, physically examined by a veterinarian, and provided appropriate veterinary medical care.



**d. Results:**

The optimal dose that is established by this study is:  $2 \times 10^9$  cfu of *S. dysenteriae*-1 1617 strain. With the dose  $2 \times 10^{10}$  cfu all 5 monkeys had dysentery and one of the monkey died within 24 h.

Colonization of *S. dysenteriae* 1 was monitored by shedding of *S. dysenteriae* 1 in fecal samples as detected by culture method. Shedding of *S. dysenteriae* 1 can continue up to 12 days, but in the monkeys which received antibiotic, shedding stopped within 48 hours of antibiotic treatment.

Group	Inoculum <i>S. dysenteriae</i> 1 1617 (dose)	Clinical observation	Histologic changes in colonic biopsies	Hematologic changes
1	Challenge: $2 \times 10^8$	Dysentery: 2 of 5	Minimal AI: 1 of 5	4 of 5
	Re-challenge: $2 \times 10^9$	Dysentery: 1 of 5	No change	1 of 5
2	Challenge: $2 \times 10^9$	Dysentery: 5 of 5	Minimal changes: 4 of 5	LS&TC: 5 of 5 L&N: 2 of 5
	Re-challenge: $2 \times 10^{10}$	Soft stool with mucous: 2 of 5	Minimal changes: 1 of 5	LS&TC: 3 of 5
3	Challenge: $2 \times 10^{10}$	Dysentery: 5 of 5  (1 monkey died within 24 h)	ANC: 3 of 4	Significant LS&TC: 4 of 4
	Re-challenge: $2 \times 10^{10}$	No significant clinical signs	CEI: 3 of 4	Eosinophilia: 4 of 4

M&B: mucus and blood; LS&TC: left shift and toxic change

L&N: leukocytosis & neutrophilia; AI: acute inflammation

ANC: acute necrotizing colitis; CEI: colonic eosinophilic inflammation.

Dysentery is at least one watery or loose stool containing blood and mucus.

Antibody titers against protein antigen (Invaplex) and *S. dysenteriae* 1 LPS were measured in multiple plasma samples by ELISA. 4-fold increase (convalescent sample compared to baseline plasma sample) of antibody titers was noted after challenge although the increase was not dose dependent. However in each group of monkeys antibody titers increased several fold after re-challenge. Levels of cytokines in fecal extract samples were dose dependent; levels were highest in group 3 monkeys after challenge, but levels were very low after re-challenge compared to the levels after challenge.

**e. Future plans:**

Rhesus monkey model is suitable for the pre-clinical evaluation of the Shigella vaccine candidates'. The reactogenicity and the elicited immune response in monkeys closely mimic the disease and immune response seen in humans. Future plan is to evaluate different *S. dysenteriae*-1 vaccine candidates in this monkey model and compare to the existing *S. dysenteriae*-1 vaccine WRSd1. An effective vaccine could reduce: environmental risk from contaminated food and water, prevent loss of combat effectiveness due to dysentery and preclude the need for expensive new antimicrobials to treat multi-drug resistant species of *S. dysenteriae*-1.

**D. Department of Veterinary Medicine AFRIMS FY06 Research Accomplishments**

**1. Title of Research Project: Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (*Macaca mulatta*)/*Plasmodium cynomolgi* Relapsing Malaria Model**

**a. Investigator:**

Dr. Montip Gettayacamin  
Ms. Pranee Hansukjariya  
Dr. Jetsumon Prachumsri  
Mr. Srawuth Komchareon  
LTC Robert S. Miller  
Dr. Dennis E. Kyle

**b. Objectives:**

1) Use the rhesus monkey/*P. cynomolgi* model to determine the effectiveness of new causal prophylactic and radical curative compounds which are being synthesized and developed by the US Army antimalarial drug development program.

2) Use the rhesus monkey/*P. cynomolgi* blood-stage malaria model to evaluate new antimalarial compounds for their blood schizonticidal activity.

**c Methods:**

Malaria is one of the most important parasitic diseases worldwide. Traditional treatment for malaria includes drugs used to prevent disease (prophylaxis) and to cure the infection (therapeutic). Antimalarial drug screening in the rhesus monkey model is very effective for making comparisons between drugs. It is fairly rapid, relatively inexpensive, and makes reliable predictions of how drugs will in act in man. Antimalarial drug screening in the rhesus monkey has played a key role in the development of every antimalarial drug licensed in the the US for the past 30 years.

This model provides a mechanism to identify effective new drugs for the enhanced prevention and treatment of malaria infections.

**d. Results:**

Three experiments were conducted in this fiscal year using 36 monkeys. The NPCB and other 4 FDA approved compounds (tinidazole, triamterene, mebendazole and chlorazaniil) were screened for radical curative activity. Four compounds (WR294716, WR299849, WR299872 and WR299548) were tested for causal prophylactic and radical curative activities in a combined test. NPCB had radical curative activity and the Minimal Effective Doses (MED) of 3-day treatment was 6 mg/kg base/day. Triamterene at and Tinidazole showed radical curative effect in one of the two treated monkeys but mebendazole and chlorazaniil showed no activity. WR299548 had a slight prophylactic activity to delay parasite patency and relapse. The other compounds had no activity.

**e. Future plans:**

We anticipate conducting at least two experiments over the next calender year.

**2. Title of Research Project: Care and Maintenance of Rhesus (*Macaca mulatta*) and *Cynomolgus (Macaca fascicularis)* Monkeys and Management of Breeding Colonies**

**a. Investigators:**

Dr. Montip Gettayacamin  
Mr. Sawuth Komcharoen

**b. Objectives:**

Maximize the production of specific pathogen-free rhesus monkeys in the USAMC-AFRIMS production colony, using the best and most humane husbandry care, maintenance procedures, veterinary care, and disease surveillance and environmental enrichment procedures available.

**c. Methods:**

USAMC-AFRIMS maintains a breeding colony of rhesus macaques using a closed colony system. Approximately 150 rhesus monkeys are used in the breeding program. Two types of breeding are managed: compatible male and female pairs are housed in special paired-type caging, and multiple harem groups are established and maintained in large gang cages. Harems consist of one breeding sire and 5-15 adult females. Newborn monkeys are weaned at approximately 6 months of age, and then are reared to adulthood in gang cages with other weanlings. All colony primates are

tested routinely for the presence of infectious diseases that pose a threat to either the health of the colony or to personnel working with the primates. Humane use of the animals is assured by the intense oversight of the Institutional Animal Care and Use Committee. Veterinary and technical care is extensive and continuous.

Whenever possible, animals are re-utilized in multiple protocols in order to optimize the use of this limited and essential resource.

**d. Results:**

Forty-two (42) baby rhesus macaques were born in the colony in the last year.

**e. Future plans:**

These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free nonhuman primates to support USAMC-AFRIMS research needs. Maintain and expand the colony by obtaining 16 new breeding males, increasing the number of paired housing cages, and placing breeding pairs in these new cages into additional animal rooms in the vivarium. Importing monkeys is pending on the funding.

**3. Title of Research Project: Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance/Quality Surveillance Program**

**a. Investigators:**

Dr. Montip Gettayacamin  
Ms. Anchalee Tungtaeng

**b. Objectives:**

Maintain a breeding colony of specific pathogen-free laboratory rodents to meet the scientific research needs of the USAMC-AFRIMS, using state-of-the-art knowledge, equipment, and facilities.

**c. Methods:**

USAMC-AFRIMS maintains breeding colonies of laboratory rodents to meet the needs of AFRIMS research. Using state-of-the-art equipment, knowledge, and facilities, production is matched to the anticipated needs of individual research projects. Extensive and thorough recordkeeping ensures that outbred strains remain outbred, and that inbred strains remain truly inbred. An extensive quality assurance/quality surveillance program, which includes serologic assessments as well as necropsy/histopathologic analysis, ensures that the colony produces only

high-quality disease-free animals. When necessary, new breeder stock is procured from a reliable vendor in the United States or Japan. Veterinary and technical care is extensive and continuous.

**d. Results**

One thousand three hundred sixty (1,360) ICR mice (*Mus Musculus*) were produced for 7 active protocols. Quality assurance procedure monitors the health status of the animals produced in the colony and purchased from a local vendor.

**e. Future plans:**

These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free rodents to support USAMC-AFRIMS research needs.

**4. Title of Research Project: A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs**

**a. Investigators:**

Dr. Montip Gettayacamin  
Ms. Pranee Hansukjariya  
Ms. Anchalee Tungtaeng

**b Objectives:**

To evaluate potential antimalarial chemotherapeutic agents in the *P. berghei* ICR mouse - the modified Thompson Test model.

**c. Methods:**

The test system used for the determination of antimalarial activity of the compounds is a modification of the suppressive test known as the Thompson Test. Typically in this test, up to 22 groups of 8 mice are inoculated intraperitoneally (IP) with *P. berghei*-infected erythrocytes then treated with candidate drugs to determine the antimalarial activity. Infected erythrocytes are provided from donor mice. On experiment day 0, the donor mice are anesthetized then exsanguinated via cardiac puncture, the blood pooled and the level of parasitemia determined. The pooled blood is then diluted with normal mouse serum to a concentration of  $1 \times 10^6$  *P. berghei*-infected erythrocytes per inoculum (0.1 ml). The groups of experimental and control mice are inoculated with this parasitized blood on day 0. On day 3, 4, and 5 mice are treated with either the candidate antimalarial drug or with vehicle alone, to serve as the negative control. The drug is administered orally (PO), subcutaneously (SC), intramuscularly (IM), and/or intraperitoneally (IP) up to three times a day, based on the individual and unique pharmacodynamics of the test compound. Each experimental group receives a different

dose level, with up to 7 different dose groups per compound. A standard antimalarial drug may be tested along with the candidate drug for structure-activity determination and for quality assurance of the model. Blood films and body weights are taken on the third and sixth days post-infection, then at weekly intervals through day 30. Blood films are stained, examined by light microscopy, and the percent parasitemia determined. All mice are observed twice a day to assess their clinical signs. All mice with negative smears at 30 days are considered cured.

**d. Results:**

A total of 5 compounds were tested in one experiment.

**e. Future plans:**

This mouse model for screening new candidate antimalarial compounds has been used for over 30 years and is very effective for making comparisons between drugs. It is rapid, relatively inexpensive, and makes reliable predictions of how drugs will act in higher mammalian hosts, including humans. This is a core capability of the USAMC-AFRIMS Department of Veterinary Medicine and will be maintained so that many more compounds can be tested. The protocol expired in FY 06. The renewed protocol was submitted and approved in Jun 06 (Protocol No 06-5). New compounds screening will be continued.

**5. Title of Research Project: Characterization and Validation of *Anopheles dirus* Sporozoite-Induced Mouse Malaria Models (ICR mouse/*Plasmodium berghei* and *P. yoelii*) for Screening Exoerythrocytic Antimalarial Drugs**

**a. Investigators:**

Dr. Montip Gettayacamin  
Dr. Jetsumon Prachumsri  
Ms. Anchalee Tungtaeng  
Dr. Robert S. Miller  
Dr. Dennis Kyle

**b. Objectives:**

To evaluate potential causal prophylactic antimalarial agents in the *P. yoelii* mouse exoerythrocytic (EE) model at AFRIMS.

**c. Methods:**

A model involves infecting mice with sporozoites harvested from infected *Anopheles dirus* mosquitoes on day 0. The infected mice are dosed with test compound on day -1, prior to inoculation of sporozoites and then on day 1. There are 6-16 groups of 5 mice or up to 80 mice in each experiment. The routine test consists of 1 to 3 dosage

levels of up to 5 compounds administered by one or two routes. One group will receive vehicle alone to serve as the control. Blood films, weight and clinical signs are followed to 30 days post-infection. Mice with negative smears at 30 days are considered to be protected.

**d. Results:**

This protocol successfully established sporozoite-challenge mice malaria model using mice malaria (*P. berghei* ANKA strain) in ICR mice in Phase I. The optimized model, *P. berghei* ANKA strain was chosen. The model was characterized and validated in Phase II studies.

**e. Future plans:**

Phase III study is currently conducted and screens new antimalarial compounds against the exo-erythrocytic (liver stage parasites) *P. berghei* in mice.

**6. Title of Research project: Development of an *Ex Vivo* G6PD Hemolytic Toxicity Assay Using Rhesus Monkeys**

**a. Investigators:**

Mr. Srawuth Komcharoen BSc, MSc  
Dr Geoffrey S. Dow, PhD  
Dr. Montip Gettayacamin  
CPT Kirsten Smith  
CPT Mara Kreishman-Deitrick  
CPT Tiffany Heady  
LTC Lisa Cardo  
Carson Friedman  
Dr. Rawiwan Im-erbsin  
LTC Colin Ohrt

**b. Objectives:** To validate a potential *ex vivo* G6PD hemolytic toxicity assay developed at WRAIR using Rhesus monkeys by showing that plasma from tafenoquine and primaquine, but not chloroquine-treated monkeys induces dose-related hemolysis of human G6PD deficient red cells *in vitro* using appropriate endpoints.

**c. Methods:** Pre-treatment blood samples were obtained from 56 rhesus monkeys on day -21. Monkeys were dosed orally with the drug vehicle, primaquine, tafenoquine and chloroquine on days 0-6. Post-post-treatment draw were obtained from each monkey at 2, 12 and 4 hours after the final dose of primaquine, tafenoquine and chloroquine respectively.

**d. Results:** Samples were delivered to WRAIR, Division of Experimental Therapeutics. Result is pending.

e. **Future plans:** The project was finished.

## **7. Active and Passive Protection of Mice against Japanese Encephalitis Virus**

### **a. Investigators:**

Dr. Yvonne Van Gessel  
Dr. Rober Putnak (WRAIR)  
Dr. Shailesh Dewasthaly (InterCell Austria)  
Dr. Montip Gettayacamin  
Dr. Robert Gibbons

### **b. Objectives:**

To establish a reliable mouse model of Japanese Encephalitis Virus (JEV) infection at AFRIMS. To evaluate a new second generation Japanese Encephalitis purified-inactivated vaccine (JE PIV) for active and passive cross-protection in this model when challenged with homologous and heterologous JEV strains.

### **c. Methods:**

The study was divided into 4 distinct experiments.

In experiment 1, "Determination of optimum challenge dose", lethal dose 50 (LD50 = the virus challenge dose at which 50% of the animals challenged develop clinical signs of JE) and optimal challenge dose (the dose at which 80-100% of animals challenged consistently develop clinical signs of JE) was determined for multiple JE strains. To determine LD50 groups of 10, 6-week-old, female, ICR mice were challenged with escalating doses of JEV after disruption of the blood brain barrier by intracranial injection of sterile saline. LD50 was calculated using probit analysis. Using this data, 10 LD50, 50 LD50 and 100 LD50 were then tested for each strain to determine the lowest dose at which 80-100% morbidity was achieved (optimal challenge dose).

In experiment 2 "Passive protection model establishment" high titer and low titer sera from JE PIV vaccinated humans volunteers was tested for passive protection in the mouse model using JEV SA14 strain. Groups of 6-7-week-old female ICR mice (N=10) received either 0.5 ml of high-titered serum (PRNT50 = 256), low-titered serum (PRNT50 = 26) or JE non-immune human serum via intraperitoneal (IP) injection. The mice were challenged by the IP route approximately 18 hours later with a lethal dose (50 LD50) of JEV SA14 strain after disruption of the blood brain barrier by intracranial injection of sterile saline. The animals were observed for clinical signs of JE for 21 days.



In experiment 3 ‘Determination of vaccine-induced (active) cross-protective potency against JE virus challenge’, the candidate JEV vaccine (JE PIV IC-51) and current JEV vaccine (JE-VAX) were tested at various doses for active protection in the ICR mouse against challenge with SA-14 and Beijing JEV strains. Groups of 6-7-week-old female ICR mice (N=10) were vaccinated twice 2 weeks apart with 170  $\mu$ l of either JE PIV, JE-VAX or adjuvant via intraperitoneal injection (IP). The mice were challenged by the IP route 1 week after the second vaccination with either a lethal dose (50 LD50) of JEV SA14 strain or 500 PFU of JEV Beijing strain, after disruption of the blood brain barrier by intracranial injection of sterile saline. Mice were observed for 21 days.

Experiment 4, ‘Determination of serum antibody induced (passive) cross protection against JE’ was not conducted in this fiscal year. In addition to the experiments listed above, an in-vivo potency assay (Exp 5) was run concurrently with all viral challenges in experiments 1 thru 4 to prove correct challenge dose.

#### **d. Results:**

Experiment 1: The approximate dose resulting in 50% mortality/morbidity (LD50) for SA14, KE093 and KE92-001 was determined to be 19.0, 78.7 and 231.1 PFUs respectively. Optimal challenge dose was shown to be approximately 50 LD50 for all strains at 1,000, 4,000 and 10,000 PFU for SA14, KE093 and KE92-001 respectively. The JEV Beijing strain tested did not produce reproducible 80-100% morbidity/mortality at any dose tested; however, a dose dependent effect was observed. LD50 could not be calculated for this strain. Several dose optimization experiments were performed in an attempt to find a challenge dose that consistently resulted in at least 60% morbidity/mortality; 500 PFU was chosen as the optimal challenge dose for the Beijing strain. JEV Nakayama strain produced only rare sporadic morbidity/mortality; this strain was dropped.

Experiment 2: At the end of the 21 day observation period, 9 of 10 mice receiving non-immune control serum developed clinical disease or died, whereas no animals in the high titer group and only 1 of 10 mice in the low titer group developed any signs of disease.

Experiment 3: The dose of at which 50% of the animals challenged with 50LD50 (lethal dose) of SA-14 (effective dose 50 or ED50) was determined for both JE-PIV and JE-VAX. A definitive ED50 for challenge with Beijing strain could not be determined due to inconsistent mortality in control groups, however the results suggest that high doses of both vaccines confer protection against JEV-Beijing challenge.

#### **e. Future plans:**

Complete all animal manipulations for Experiment 4. Submit written reports to Intercell Austria of all animal studies for inclusion in the new product licensing packet submitted to the U.S. Food and Drug Administration. The AFRIMS ICR JE

mouse model may also be used to test other promising prophylactic and therapeutic treatments for JEV.

## **E. Department of Virology, AFRIMS FY06 Research Accomplishments**

### **1. Title of Research Project: Prospective Study of Dengue Virus Transmission and Disease in Primary Schools and Villages in Kamphaeng Phet, Thailand**

#### **a. Investigators:**

##### 1. Principal Investigators:

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3. Ananda Nisalak, M.D., Consultant in Arbovirology
4. Charity Ann M. Ypil-Butac, M.D., Consultant in data analysis
5. Butsaya Thaisomboonsuk, Ph.D., Head, Arbovirology Section
6. Chonticha Klunghong, Ph.D., Head, Molecular Research Section

##### Department of Entomology

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#### Thai Ministry of Public Health (MOPH):

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3. Daniel H. Libraty, M.D., Assistant Professor
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6. Francis A. Ennis, M.D., Director

Department of Entomology, University of California, Davis:

1. Thomas W. Scott, Ph.D., Professor of Entomology and Director, Davis Arbovirus Research Unit
2. Amy C. Morrison, Ph.D., Assistant Research Entomologist

Department of Geography, San Diego State University:

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**b. Objectives:**

The goal of the proposed study is to identify those factors that have the strongest influence on determining the early events in acute DV infections, and the eventual clinical manifestations of disease. An equally important goal is to characterize protective immune responses (e.g. CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses, neutralizing antibody responses) as we have found that low levels of pre-existing neutralizing antibodies to a subject's own infecting virus isolate do not necessarily protect from symptomatic DV infection. We plan to prospectively identify host-specific factors (e.g. pre-existing memory T and B cell responses to DV, HLA genetic polymorphisms, viral burden and replication in the host), virus-specific factors (e.g. DV serotype, serotype infection sequence), and environmental factors (e.g. mosquito population patterns, mosquito viral burden) for asymptomatic and symptomatic secondary DV infections, particularly severe infections (DHF/DSS). Multi-year investigations are crucial to this study due to the year-to-year variations in the incidence and prevalence of circulating serotypes. An improved understanding of the correlations between the host, viral, and environmental factors and dengue disease severity will contribute to DV vaccine development and testing.

**c. Study Specific Hypotheses:**

1. Subjects with pre-existing neutralizing dengue antibodies above a definable threshold will be protected from DV infection or severe disease on subsequent exposure to virus.

2. The frequency of pre-existing CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and their specific cytokine responses to stimulation with DV antigens will correlate with disease severity (protection or enhancement) and the plasma viral RNA levels measured in secondary DV infections.

3. Specific serotype sequence combinations of DV infections will elicit qualitatively and quantitatively distinct immune responses associated with illness of varying severity.

4. Higher viremia levels will be seen in secondary DEN-2 and DEN-4 virus infections in subjects with higher levels of *in vitro* antibody-dependent enhancing capability of pre-illness blood samples.

5. DV infection rates will cluster in households around a DV-infected index case and a correlation will exist between the number of susceptible contacts, and associated mosquito density, and mosquito infectivity (viral RNA levels).

6. DV disease severity will correlate with peak plasma viremia levels and associated mosquito density and mosquito infectivity (viral RNA levels).

7. Genes encoded within the human MHC, the NK killer inhibitory receptor (KIR) gene complex on chromosome 19, and the Fc gamma receptor gene complex on chromosome 1 influence the susceptibility, severity and resistance to primary and secondary DV infections.

#### **d. Methods:**

In this study, we

i) Continue the successful prospective, school-based, study platform to study dengue epidemiology in primary school children in KPP province, and

ii) Conduct a village-based, cluster surveillance study.

(a) This is a prospective school-based study of 2,000 children, which began in 2003 and will end in January 2008. Students in K2 to grade 6 are recruited and enrolled into the study. Baseline demographics are recorded and study numbers assigned. Each subsequent year, new K1-Grade 5 students are newly enrolled. Students are followed until they are either disenrolled, withdrawn by their parent/guardian, graduate from Grade 6 or when the study ends. Every year, plasma

(PBMCs for Dengue Season 1 only) is collected from the entire cohort at the beginning of the surveillance period (June). Plasma and PBMCs are collected from the entire cohort at the end of the surveillance period (January). The hemagglutination inhibition (HAI) assay is performed on paired sera from the beginning and end of the surveillance period to assess for flavivirus seroconversion. Plasma and PBMCs obtained at the end of the surveillance period in January serve as pre-illness samples in subjects who have a DV infection that same calendar year.

During the active surveillance period extending between June and November, those children who are absent from school (or who report ill to the teacher), will be evaluated either by a village health worker or AFRIMS nurse using a questionnaire and oral temperature measurement. Any child who has a documented fever (temperature  $\geq 38$  C) or reports illness with subjective fevers during the prior 7 days, is transported to the Public Health Office (PHO) where a public health nurse will do an evaluation. An acute blood specimen will be drawn. The child will be referred to the hospital at the discretion of the public health nurse. About 14 days later, an AFRIMS nurse visits the child to administer another questionnaire and to draw a convalescent blood specimen. The acute and convalescent specimens are evaluated by the AFRIMS dengue/JE IgM/IgG ELISA and HAI. The acute specimen will be evaluated further by dengue RT-PCR (and virus isolation techniques).

(b) Cases 'triggering' a cluster investigation are identified between Monday and Thursday of each week during the School-Based Component active surveillance period. Most specimens from acutely ill children arrive at the field station laboratory by 3pm each day. Upon arrival of the specimen, the database is reviewed to assess whether the child meets all index case inclusion and exclusion criteria. The field teams are notified of a possible case. The DV RT-PCR result (positive or negative) will normally be available by 11AM the following morning. No more than 30 positive and 30 negative clusters (as defined by the RT-PCR result of the index case) will be initiated in any given year. Once triggered, an Advance Team composed of a nurse and an entomological team supervisor visits the village and begins the consent form process. The exact location of all houses in each participating village has previously determined using a Global Positioning System (GPS) unit. Data points will be used to construct a digital map which will enable the team to precisely identify houses located within 100 meter radius of the index case and rapidly assess the likelihood of enrolling a minimum of 10 contacts. Once at least 10 contacts have been consented, the field teams will be dispatched to the village where the consent form process will continue. A clinical nurse will review the consent form, answer questions, address parental concerns, and obtain informed consent from the parents of susceptible contact children (ages 6 mo-15 yrs) residing within a 100 meter radius of the index household. Following the acquisition of parental consent, blood samples will be collected from 10-25 contacts. Those parents (and children) who are unavailable to be consented (and bled) are visited that same evening or the following morning. The clinical team will return to these homes approximately 5, 10 and 15 days after the initial visit to perform clinical assessments. The children bled on day 0 (initial specimen) are re-bled on approximately day 15 (follow-up specimen). DV RT-PCR will be performed on all acute specimens. If the day

15 blood is positive RT-PCR we will go out to draw blood on day 30 for doing ELISA. Dengue IgM/IgG ELISAs are performed on paired initial and follow-up specimens.

An entomological team collects mosquitoes, administers questionnaires, and performs insecticide spraying within the pre-determined meter radius of the index household. Another entomological team will collect mosquitoes but not perform insecticide spraying around the classroom and school bathroom areas of the index case.

## **e. Results:**

### **1. School cohort study**

At the beginning of the active surveillance period (1 June 2006), there were a total of 2,086 children enrolled in the school-based cohort. By the end of the surveillance period (1 December 2006), there were a total of 2,045.

To make up for the expected efflux of school cohort subjects due to graduation from Grade 6 in March 2007, 387 new children were enrolled so that a total of 2432 children underwent scheduled blood draw in January 2007 from 11 participating schools. Of the new enrollees, 204 (53 %) were in Grade K1.

In the school-based active surveillance period in 2006, 1837 school absences were evaluated. Of these, 871 were related to acute or recent (reportedly within prior 7 days of evaluation) febrile illness and 764 (88 %) of these had acute blood draws for RT-PCR testing and EIA testing. Eighty-four cases were positive for dengue by EIA. Seventy-two children were positive for dengue by RT-PCR: 43 were DEN-1 and 21 were DEN-4. Six JEV infection were diagnosed serologically but had no signs and symptoms of encephalitis. Twenty-two children with positive dengue RT-PCR were admitted to Kamphaeng Phet Provincial Public Hospital. All were discharged in good condition from the hospital.

Based on RT-PCR results from acute specimens from ill children in the school-based surveillance, 39 cluster investigations were initiated from June until November 2006. Twenty-two of these were based on dengue positive index cases and 17 were based on dengue negative index cases. The dengue positive index cases were identified as thirteen DEN-1, one DEN-2, eight DEN-4.

Overall during the 2006 active surveillance period, there were significantly more dengue cases than during the prior two surveillance years (2004 and 2005). Analysis of this season's obtained data is ongoing.

### 1.1 Number of cases related to fever and bled in 2006

School No.	T $\geq$ 38° C on evaluation during a given illness	T<38° C but child reports subjective fevers during prior 7 days	Children with either reported or recorded fever	Bled (acutely and 14 $\pm$ 4 days later)	Percent of recent fevers who were bled
01	33	14	47	41	87.23
02	32	10	42	35	83.33
03	75	50	125	118	94.40
04	20	9	29	26	89.66
05	26	1	27	26	96.30
06	32	14	46	40	86.96
07	81	53	134	115	85.82
08	58	55	113	93	82.30
09	46	39	85	84	98.82
10	69	48	117	101	86.32
11	46	60	106	85	80.19
Total	518	353	871	764	87.72

### 1.2 Serology with Dengue RT-PCR results

Serology	Serotype					Total
	DEN1	DEN2	DEN4	Indet.	NEG	
Pending	3	1	0	1	29	34
Acute Primary Dengue Infection	1	0	0	0	2	3
Acute Secondary Dengue Infection	41	0	21	0	12	74
Acute Secondary Flavivirus Infection	0	0	0	0	1	1
JEV Infection	0	0	0	0	4	4
Need Follow Up Specimen	0	0	0	0	1	1
No Evidence of Recent Flavivirus Infection	3	0	0	0	631	634
Recent JEV Infection	0	0	0	0	2	2
Recent Primary Dengue Infection	1	0	0	0	2	3
Recent Secondary Dengue Infection	0	0	0	0	4	4
Recent Secondary Flavivirus Infection	0	0	0	0	1	1
Single specimen	0	0	0	0	3	3
Total	49	1	21	1	692	764

### 1.3 School-based Acute Illness RT-PCR Dengue Serotype with confirmed EIA

School No.	Serotype					Total
	DEN1	DEN2	DEN4	Indet.	NEG	
01	0	0	1	0	40	41
02	2	0	0	0	33	35
03	2	0	0	0	116	118
04	2	0	0	0	24	26
05	0	0	7	0	19	26
06	0	0	3	0	37	40
07	3	0	6	0	106	115
08	6	0	2	0	85	93
09	0	0	0	0	84	84
10	25	0	0	1	75	101
11	9	1	2	0	73	85
Total	49	1	21	1	692	764

## 2. Village-based study

During the active surveillance period (1 June to 30 November 2006), a total of 39 cluster investigations were performed: 22 positive clusters (involving 339 child contacts) based on dengue PCR positive index cases and 17 negative clusters (involving 233 child contacts) based on dengue PCR negative index cases.

Dengue RT-PCR was performed on day 0 and day 15 specimens of the child contacts (ages 6 months to 15 years) of index cases. Of these specimens, 45 were positive for dengue by PCR in the 22 positive clusters; 6 were positive for dengue by PCR in the 17 negative clusters.

Four contacts were hospitalized for acute secondary dengue infection. All of them were discharge having recovered from their illness.

Out of 1442 *Aedes aegypti* mosquitoes collected from cluster investigations, 8 were positive for dengue by RT-PCR (4 DEN-1, 1 DEN-2, and 3 DEN-4). All of these positive mosquitoes were collected from positive clusters and the dengue serotype of these mosquitoes were the same as their respective positive index cases.

Cluster investigations:

Cluster No.	Subject No. of Index case	School No.	Type of cluster	Serotype of Index Case	Serology	Number of Enrollees		Number of Houses
						F	M	
4-01	112121KDS4 1	11	Positive Cluster	DEN1	Acute Secondary Dengue Infection	5	7	30
4-02	113431KDS4 1	11	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	5	8	17
4-03	071347KDS4 1	07	Positive Cluster	DEN4	Acute Secondary Dengue Infection	7	7	27



4-04	072750KDS4 1	07	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	3	8	38
4-05	061088KDS4 1	06	Positive Cluster	DEN4	Acute Secondary Dengue Infection	6	11	16
4-06	062666KDS4 1	06	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	6	9	31
4-07	071442KDS4 1	07	Positive Cluster	DEN4	Acute Secondary Dengue Infection	11	12	17
4-08	081433KDS4 1	07	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	10	11	17
4-09	101848KDS4 1	10	Positive Cluster	DEN1	No Evidence of Recent Flavivirus Infection	7	4	14
4-10	112079KDS4 1	11	Positive Cluster	DEN1	Acute Secondary Dengue Infection	11	14	48
4-11	112938KDS4 1	11	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	6	5	19
4-12	061116KDS4 1	06	Positive Cluster	DEN4	Acute Secondary Dengue Infection	6	5	27
4-13	050966KDS 41	05	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	8	10	29
4-14	102539KDS4 2	10	Positive Cluster	DEN1	Recent Primary Dengue Infection	8	12	21
4-15	101890KDS4 1	10	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	4	6	26
4-16	072727KDS4 1	07	Positive Cluster	DEN1	Acute Secondary Dengue Infection	12	4	23
4-17	083237KDS4 1	08	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	7	6	31
4-18	040780KDS4 1	04	Positive Cluster	DEN1	Acute Secondary Dengue Infection	9	12	25
4-19	040770KDS4 1	04	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	9	11	17
4-20	023081KDS4 1	02	Positive Cluster	DEN1	Acute Secondary Dengue Infection	7	8	22
4-21	030609KDS4 1	03	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	7	3	12
4-22	101904KDS4 1	10	Positive Cluster	DEN1	Acute Secondary Dengue Infection	14	9	21
4-23	030453KDS4 2	03	Positive Cluster	DEN1	Acute Secondary Dengue Infection	12	4	24
4-24	103301KDS4 1	10	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	3	7	15
4-25	030613KDS4 2	03	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	5	5	27
4-26	071307KDS4 1	07	Positive Cluster	DEN4	Acute Secondary Dengue Infection	7	10	19

4-27	071289KDS4 1	07	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	7	4	5
4-28	081482KDS4 2	08	Positive Cluster	DEN1	Acute Secondary Dengue Infection	3	11	22
4-29	081509KDS4 2	08	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	2	9	35
4-30	050955KDS4 1	05	Positive Cluster	DEN4	Acute Secondary Dengue Infection	6	5	11
4-31	052715KDS4 1	05	Positive Cluster	DEN4	Acute Secondary Dengue Infection	6	8	34
4-32	113445KDS4 1	11	Positive Cluster	DEN4	Acute Secondary Dengue Infection	6	4	35
4-33	073199KDS4 1	07	Positive Cluster	DEN1	Acute Primary Dengue Infection	4	6	22
4-34	072735KDS4 2	07	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	11	8	28
4-35	103302KDS4 1	10	Positive Cluster	DEN1	Acute Secondary Dengue Infection	9	1	39
4-36	113432KDS4 2	11	Positive Cluster	DEN2	Unknown	3	7	20
4-37	101778KDS4 1	10	Positive Cluster	DEN1	Acute Secondary Dengue Infection	8	6	8
4-38	093277KDS4 1	09	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	12	7	23
4-39	050966KDS4 2	05	Positive Cluster	DEN4	Acute Secondary Dengue Infection	4	12	30

**Positive Clusters:**

Cluster No.	Index PCR	No. Contacts	Serologically			DAY 0		DAY 15			Mosquito PCR (Female <i>Ae.aegypti</i> )		
			1°	2°	*Other	DEN1	DEN4	DEN1	DEN2	DEN4	DEN1	DEN2	DEN4
4-01	DEN-1	12		2/1				1			1		
4-03	DEN-4	14		1/1	1		1			1			1
4-05	DEN-4	17		3			1						
4-07	DEN-4	23	7	8/1		1	9	1		1			1
4-10	DEN-1	25	1	2		1							
4-12	DEN-4	11											
4-14	DEN-1	20											
4-16	DEN-1	16		3	2	1					1		
4-18	DEN-1	21			1			1					
4-20	DEN-1	15		1							1		
4-22	DEN-1	23	1	4		1			1				
4-23	DEN-1	16											
4-26	DEN-4	17			1								1
4-28	DEN-1	14		1									
4-30	DEN-4	11	1										
4-31	DEN-4	14		3	1		2			1			
4-32	DEN-4	10					1						
4-33	DEN-1	10											
4-35	DEN-1	10		1		1		1			1		
4-36	DEN-1	10										1	
4-37	DEN-1	14		3		2		1					
4-39	DEN-4	16											
<b>Total</b>	<b>339</b>	<b>10</b>	<b>10</b>	<b>32/3</b>	<b>6</b>	<b>7</b>	<b>14</b>	<b>5</b>	<b>1</b>	<b>3</b>	<b>4</b>	<b>1</b>	<b>3</b>

Negative Clusters:

Cluster No.	Index PCR	No. Contacts	Serologically			DAY 0		DAY 15			Mosquito PCR (Female Ae.aegypti)		
			1°	2°	*Other	DEN1	DEN4	DEN1	DEN2	DEN4	DEN1	DEN2	DEN4
4-02	NEG	13											
4-04	NEG	11											
4-06	NEG	15			0/1					1			
4-08	NEG	21											
4-09	NEG	11		1/1		1		1					
4-11	NEG	11											
4-13	NEG	18											
4-15	NEG	10		3		2							
4-17	NEG	13											
4-19	NEG	20											
4-21	NEG	10											
4-24	NEG	10											
4-25	NEG	10											
4-27	NEG	11						1					
4-29	NEG	11											
4-34	NEG	19											
4-38	NEG	19	1										
Total		233	1	4/1	0/1	3		2		1			

**f. Future plans:**

The post surveillance period blood draw of the school cohort is currently being undertaken. Data analysis of all obtained data will be performed in the next several months. Several manuscripts detailing interim study analyses are being prepared for publication. The final active surveillance period will occur from June 2007 to Nov 2007. Interaction with human subjects will end with the final post surveillance blood draw in January 2007. Data analysis and laboratory testing will continue beyond that point.

**2. Title of Research Project: The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue**

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(University College London)

**b. Objectives:**

To identify the immunopathological mechanisms of dengue hemorrhagic fever (DHF), to analyze differences between DHF resulting from primary versus secondary infections, to identify a sensitive method for detection of plasma leakage, and to characterize the dengue specific T cell response. The project encompasses studies from 2003 to 2007.

**c. Study Specific Objectives:**

1. Characterize genetically and functionally the dengue virus-specific T lymphocyte response during, and after dengue virus infections (intracellular cytokine staining, HLA tetramers, T cell receptor gene usage).
2. Analyze interactions between dengue virus, virus-specific antibodies, and target cells in PBMC during acute dengue virus infections (quantify and characterize immune complexes, define the major cellular compartments in PBMC supporting dengue viral replication).
3. Determine if ultrasound or interstitial fluid albumin levels can predict early plasma leakage and shock. The ability to detect these shifts early in disease progression may help in prediction algorithms for DHF and permit early intervention with new therapies in the at-risk population.
4. Assess the utility of plasma sNS1 levels in predicting disease severity for subjects with primary or secondary infection due to any of the four dengue serotypes.

5. Analysis of the activation of innate immune responses in vivo during acute dengue virus infections (chemokine gene expression, inhibitory and activating NK receptor expression).

6. Identification of polymorphisms in immune response genes associated with disease manifestations and cellular immune responses during dengue virus infections (MHC class I and II, Fc $\gamma$  receptor gene, KIR genes, NK receptors) and MHC class I chain-related (MIC) genes (ligands for lectin-like receptors),

7. Quantitation of viral burden in plasma and cell subsets of peripheral blood mononuclear cells (PBMC) for all four serotypes in primary and secondary dengue virus infections and determine if there is a correlation between viral load in these compartments and disease severity.

8. Measurement of neutralizing antibody elicited by primary infections, over an extended period of time. Few long-term studies of antibody titer following dengue infection have been performed previously. Neutralizing antibody will be measured on study day 1, 6 months, 1 year, and annually thereafter. Understanding wild type responses will help to set realistic standards for vaccines. Mature secondary responses determined by neutralization six months or more after infection will be correlated with class II HLA type.

9. Determination of memory T-cell responses following primary and secondary dengue infections, over an extended period of time. Understanding wild type responses and the durability of these responses over time will be crucial in setting standards for testing of candidate dengue vaccines.

10. Continue sequencing portions of the dengue genome from patients with mild dengue fever and those with severe DHF/DSS to test a hypothesis that severity of disease is strain related. In addition, compare the kinetics of plasma viral load and immune responses in primary and secondary infections with different DV serotypes.

11. Evaluate the accuracy of sequentially measured semi-quantitative d-dimer assay, as compared to standard clinical parameters, at predicting the clinical progression to severe clinical dengue.

#### **d. Methods:**

Children were enrolled if they were suspected of having an early DV infection (without evidence of DHF) or a fever without an identifiable source. Inclusion criteria included an oral temperature  $\geq 38.5^{\circ}\text{C}$ , fever onset not longer than 72 hours prior to the initial evaluation, weight  $> 6\text{kg}$ , flushed face, signed consent by parent or guardian. After informed consent is obtained, subjects are admitted to the hospital and a blood specimen obtained. The result of the plasma test for DV RNA by RT-PCR is available the morning of study day 2. Children who are DV RT-PCR-negative are given

the opportunity to leave the study, or to continue in the study for clinical observation. Those children remaining in the hospital undergo inpatient observation until one day following defervescence (fever day +1). Clinical information is collected and recorded daily. Radiographic studies are performed as outlined in the protocol. Serial blood samples are collected and analyzed for routine and dengue-specific blood and plasma tests were conducted to include, but not limited to:

1. CBC, WBC differential, AST, Albumin
2. Hemagglutination inhibition (HAI) assay for dengue
3. Antibody-capture DV IgM/IgG enzyme immunoassay (EIA)
4. RT-PCR for dengue, Plasma viremia titers
5. Dengue virus isolation in *Toxorhynchites splendens* and typing
6. IL-15, IL-18, MIP-1a, MIP-1b, and MCP-1, CD69, CD38, and Ki-67
7. Labeled antibodies to identify T cell subsets, NK cells and B cells
8. NS1 (soluble NS1 and anti-NS1 antibodies)
9. Complement assays

**e. Results:**

There were 43 positive PCR cases (DEN 1 = 17; DEN 2 = 1; DEN 3 = 15; DEN 4 = 10; Negative PCR = 50. All subjects had ultrasound evaluation for plasma leakage. A subset (n=33) of dengue positive cases had interstitial fluid sampling done. One case was lost to follow-up (non-dengue diagnoses). No serious adverse events occurred.

**f. Future plans:**

Long-term clinical follow-up is ongoing for prior years of enrollment and we are preparing for enrollment next year. Analysis for markers that predict disease severity (d-dimer, NS1 protein/antibody levels, immune activation markers), that indicate plasma leakage is or will occur, and that indicate immunity will be done. Statistical analysis of DHF resulting from primary versus secondary DV infections with regard to the role viral serotype, viral burden and virus-antibody complexes plays on resulting disease severity is planned. Characterization of the dengue specific T cell response with regard to the magnitude of T cell expansion during infection and the functional characteristics of these cells is also planned.

**3. Title of Research Project: A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Infants**

**a. Background:**

The US Army seeks to acquire a licensed vaccine capable of protecting soldiers and their families from disease caused by infection with the dengue viruses. The Kingdom of Thailand shares this goal. For over 50 years the US Army has been active in developing and testing various vaccine candidates. This study represents the

first use of the most promising Army dengue vaccine candidate in an overseas (Thailand), infant population.

**b. Principal Investigators:**

Robert V. Gibbons, LTC, MC (USAMC-AFRIMS)  
Veerachai Watanaveeradej, MD, Phramongkutklao Hospital  
(PMK), Thailand

**Sub Investigators:**

Rudiwilai Samakoses, MD (PMK Hospital)  
Angkool Kerdpanich, MD (PMK Hospital)  
Veerachai Watanaveeradej, MD (PMK Hospital)  
Ananda Nisalak, MD (AFRIMS)  
Bruce L. Innis, MD (GSK)  
Wellington Sun, MD (WRAIR)  
Robert V. Gibbons, MD (USAMC-AFRIMS)  
Stephen J. Thomas, MAJ, MC (WRAIR)  
Kenneth H. Eckels, PhD (WRAIR)  
J. Robert Putnak, PhD (WRAIR)  
Celia Barberousse (GSK)

**c. Objectives:**

The primary objective of this study is to compare the reactogenicity in terms of solicited symptoms within the 21-day follow-up period after dose 1 and to assess the immunogenicity in terms of seroconversion 30 days post-dose 2 of the WRAIR tetravalent dengue live-attenuated virus (LAV) vaccine in flavivirus naïve infants, extend the follow-up period to 4 years after dose 2 to assess the kinetics of dengue neutralizing antibodies to each dengue virus serotype one and four years following dose 2 of dengue/control vaccination and to conduct passive surveillance for hospitalized dengue during the follow-up period.

**d. Methods:**

1. Screen and enroll 51 healthy, flavivirus naïve, Thai infants between the ages of 12 and 15 months.
2. Provide 2 doses of the WRAIR tetravalent dengue vaccine as outlined in the study protocol.
3. Closely monitor the infants following each dose of vaccine for safety and tolerability.
4. Assess the immunogenicity of the dengue vaccine as outlined in the study protocol.

5. Follow-up for four year following dose 2 of dengue/control vaccination to assess for dengue-related hospitalizations and dengue antibody kinetics.

**e. Results:**

Four years period following dose 2 of dengue/control vaccination to assess for dengue-related hospitalizations and dengue antibody kinetics (Protocol Amendment 5) was approved. Forty-nine infants (except subject number 36: migration from study area) have been enrolled. One year follow-up and telephone contact have been completed. The clinical follow-up visit year 1 and six-month telephone contact have been completed. No subjects were hospitalized with dengue during the follow-up period. There were no SAE cases reported.

**f. Future plans:**

The investigators desire to administer a third (booster) dose of F17 dengue vaccine to subjects in 2 doses of dengue vaccine group enrolled in the DEN-001 amendment 5, between 2 and 3 years following the primary (dose 1 and 2) vaccination series (Protocol Amendment 6). This amendment would allow for the collection of peripheral blood mononuclear cells (PBMCs) at the time of, and 1 month following booster vaccination from all subjects.

**4. Title of Research Project: A Phase I/II, Open, Five-Year, Clinical Follow-Up Study of Thai Children Who Participated in Dengue-003 ("A Phase I/II Trial of A Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Children") With Evaluation of A Booster Dose Given One Year After Primary Dengue Vaccination Series**

**a. Investigators:**

Robert V. Gibbons, LTC, MC, USAMC-AFRIMS  
Sriluck Simasathien, MD, Phramongkutklao Hospital (PMK), Bangkok,  
Thailand

**b. Objectives:**

The primary objective of this study is to assess the immunogenicity of a booster dose of dengue vaccine administered approximately one year following the second dose.

**c. Methods:**

1. Enroll seven flavivirus antibody-naïve Thai children who participated in Study Dengue-003.



2. Provides one dose of dengue vaccine given one year after the last dose of dengue vaccine in Dengue-003

3. Assess the persistence of antibody one year after completion of the two-dose primary dengue vaccination series

4. Assess the safety and immunogenicity of a booster dose of dengue vaccine administered one year after the second dose and to assess antibody persistence one year after the booster dose.

5. Four annual visits follow-up for passive surveillance for hospitalized dengue.

**d. Results:**

The study was approved to enroll 7 flavivirus antibody-naïve Thai children who participated in study Dengue-003 who received two doses of dengue vaccine. Seven subjects were enrolled. There were no subject withdrawals from the study. All subjects were administering a booster dose in February 2005 (Year-1) and completed followed up in March 2006 (Year-2). Next follow up visit (Year-3) will be scheduled in March 2007.

A preliminary analysis of N antibody data collected at the time of, and following, a booster vaccination indicated a marked decline in N antibodies to all serotypes among all study subjects. Neutralizing antibodies measured one month following a booster dose (i.e. after the third dose) of vaccine failed to demonstrate expected robust rises consistent with a secondary response. One possible explanation for the failure to boost N antibody responses following a third dose of vaccine is that the primary vaccination series (dose 1 and 2) induced significant T and B cell memory which subsequently inhibited vaccine viral replication following a third dose.

**e. Future plans:**

The protocol will be amended (Protocol Amendment 1) to allow for acquire peripheral blood mononuclear cells (PBMCs) and sera to characterize cell-mediated immunity responses to vaccination and correlate these with N antibody titers at year 3 follow-up.

**5. Title of Research Project: A Phase II, Prospective, Randomized, Double Blind, Placebo Controlled Field Efficacy Trial of a Candidate Hepatitis E Vaccine in Nepal WRAIR# 749, HSRRB Log# A-9117.1**

**a. Investigators:**

**Principal Investigators:**  
M. P. Shrestha (WARUN)  
R. M. Scott (WARUN)

**Associate Investigators:**

S. B. Bajracharya (SBH)  
M. P. Mammen (USAMC-AFRIMS)  
R. A. Kuschner (WRAIR)  
K. S. A. Myint (USAMC-AFRIMS)  
P. R. Pandey (SBH)  
K. J. B. Rana (SBH)  
K. N. Rayamajhi (SBH)  
J. Seriwatana (WRAIR)  
G. R. Shakya (SBH)  
G. B. Thapa (SBH)  
S.K. Shrestha (WARUN)  
N. Thapa (SBH)  
C. Jhang

**b. Objectives:**

To evaluate the protective efficacy for the prevention of definitive and probable hepatitis E disease provided by the candidate hepatitis E vaccine administered according to a 0, 1 and 6 month schedule.

**c. Methods:**

A candidate recombinant baculovirus expressed hepatitis E virus (HEV) vaccine was found to be safe and immunogenic in 88 American and 44 Nepalese volunteers. A 20µg formulation was selected for further evaluation in a randomized double blind placebo controlled efficacy trial in susceptible, active duty Nepal Army volunteers. The clinical phase started 30 April 2001 at the Nepalese Army Shree Birendra Hospital. Of 5,571 consenting volunteers screened, 3,692 were susceptible to HEV. Two thousand volunteers (8 females, 1,992 males) were enrolled, receiving either placebo or 20µg of active candidate vaccine. Volunteers were vaccinated at 0, 1, and 6 months with sera collected at months 0, 1, 3, 6, 7, 13, and 24. One tenth of the volunteers were followed on days 1, 3, 5, and 7 after each vaccination for local and general solicited adverse events (SoAE). Non-serious adverse events (NSAE) were recorded for 30 days after each vaccination and serious adverse events (SAE) were also collected throughout the study period. Sera and stool from cases meeting clinical and biochemical criteria compatible with viral hepatitis, were examined for HEV RNA by a reverse transcriptase-polymerase chain reaction, and serologically for HEV IgM and IgG, HAV IgM, HBsAg, HBcIgM and HCV IgG. Pregnancy was also recorded as SAE. Two subjects became pregnant during the study, about one year after third dose. The outcome was favorable in both cases. Seven deaths were documented and verified by Data Safety Monitoring Board (DSMB). They were 4 killed in action, 1 accident, 1 cholangiocarcinoma and 1 undetermined.

Of the 2000 enrolled subjects, 2000 received dose 1, 1890 received dose 2 and 1794 dose 3 (and 31 received dose 1 and 3). A total of 1566 subjects returned for concluding visit (Encounter# 8). Clinical study encounter with the subjects was concluded in January 2004.

**d. Results:**

The clinical phase of the trial has been successfully completed according to the protocol amendment 9. The DSMB unblinded 111 cases of suspected hepatitis following SOP provided by GSK on 23 June 2004. The results of the unblinding of the cases were announced in Washington D.C. on 12 December 2005 in the HEV Symposium of the American Society of Tropical Medicine and Hygiene (ASTMH) annual meeting.

In determining the vaccine efficacy 14 days after the third dose of vaccine (primary end point), there were 66 cases of definite hepatitis E among the volunteers receiving the placebo and 3 cases among those who received 20µg of the r-HEV vaccine. The vaccine was found to be 96 % (86-99) effective (95% CI) among those who received 3 vaccine doses.

In determining the vaccine efficiency between 14 days after the second dose and the third dose (secondary end point), there were 8 cases in the placebo group and 1 case in the 20 ug of the r-HEV vaccine group. There was statistically significant vaccine protection with efficacy in the total cohort receiving 2 vaccine doses of 87%. The 95% confidence interval for this estimate is wide, due to the small number of cases.

The vaccine was well tolerated, and no safety signals were identified.

**e. Future plans:**

The WRAIR protocol # 749 reached five year term in October 2006. Memorandum requesting extension of this protocol for 18 months has been submitted to Office of Research Management (ORM) on 12 January 2007. This is required for allowing the complete unblinding of all the volunteers, to notify the volunteers as to the inocula that they received and to receive the final study report from the sponsors.

The manuscript "Safety & Efficacy of a Recombinant Hepatitis E Vaccine" is in review by an international peer-review journal.

The source document in all subject folders have been scanned and secured. The electronic files have also been secured off-site. Management of hard copy source documents and volunteer registry data sheet needs further discussion.

## 6. Title of Research Project: Japanese Encephalitis Surveillance in Nepal

### a. Investigators:

Robert V. Gibbons LTC, MC (USAMC-AFRIMS)  
Sanjaya Kr. Shrestha, MBBS, MD (WARUN, Kathmandu)

### b. Objectives:

To determine JE diagnosis validation in Nepal, to determine the percentage of Japanese encephalitis and other causes of encephalitis among blinded samples provided to AFRIMS from Nepal.

### c. Methods:

1. Blinded samples provided by National Public Health Laboratory (NPHL) to WARUN without personal identifier.
2. The specimens will then be processed, packaged, and shipped to the Department of Virology, AFRIMS, Bangkok.
3. Definitive quality control testing for JE diagnostics will be done at USAMC-AFRIMS.

### d. Results:

Nine hundred and twenty-nine samples including 338 cerebral spinal fluid (CSF) and 591 serum samples were sent from National Public Health Laboratory (NPHL) in Nepal to AFRIMS for JE IgM-capture ELISA assay. The result was shown in the table below.

Result of blinded samples tested by JE IgM-capture ELISA assay

Specimen	Total	JE (IgM > 40 units)	Negative (IgM < 40 units)
CSF	338	63 (18.63 %)	275
Serum	591	139 (23.51 %)	452
Total	929	202 (21.74 %)	727

Sixty three of 338 CSF samples (18.63 %) showed positive result. One hundred and thirty nine of 591 serum samples (23.51 %) showed positive result. For overall, we can detect JE IgM 21.74 % (202 samples) of all 929 samples from NPHL.

**e. Future plans:**

Plans for CY2007 include, Analysis of the data collected to date; Discussion of the data with investigators at WARUN and NPHL, and Composition of a manuscript reporting the data.

**7. Title of Research Project: Influenza Surveillance in Southeast Asia**

**a. Background:**

Influenza is an important cause of morbidity and mortality among populations at the extremes of age. Continuous viral surveillance and isolation of influenza viruses provides important information for the creation of annual vaccine formulations based on the identification of new and emerging strains of influenza. AFRIMS has been actively involved in influenza surveillance in Thailand and Nepal for several years. Expansion of AFRIMS influenza surveillance activities in the region will enhance DoD's ability to detect and respond to an outbreak of pandemic influenza early in the course of the pandemic.

**b. Investigators:**

Khin Saw Myint, M.D. (USAMC-AFRIMS)  
Robert V. Gibbons, M.D. MPH (USAMC-AFRIMS)  
Richard G. Jarman, Ph.D (USAMC-AFRIMS)  
Kamnuan Ungchusak, M.D., MPH (MOPH, Thailand)  
Chusak Pimgate, M.D. (USAMC-AFRIMS)  
John Mark Velasco, M.D. (USAMC-AFRIMS)  
Charity Ann Ypil-Butac, M.D. (USAMC-AFRIMS)  
Sanjaya K. Shrestha, M.D. (WARUN, Nepal)  
Rodney Coldren, M.D. (GEIS, Thailand)  
Linda C. Canas, B.Sc. (AFIOH, U.S.A.)

**c. Objectives:**

- (a) To collect and characterize influenza viruses circulating within the human population in Asia including Thailand, Nepal, the Philippines and from the US Embassy/Consulate in the region.
- (b) To provide influenza surveillance data to the US CDC and WHO surveillance network towards the annual re-formulation of the influenza vaccine.
- (c) To report the circulating influenza strains and other respiratory pathogens to the Ministry of Health of host countries.

#### **d. Methods:**

Samples were collected from patients with clinically suspected influenza infection (case definition includes fever or history of fever  $\geq 38^{\circ}\text{C}$  within 72 hours with cough or sore throat). Participating physicians and staff identified patients who met the case definition during routine clinic visits. Emphasis was placed on quality samples that may provide genetic data for future influenza vaccines rather than a large number of samples to be tested for incidence and prevalence data. Clinical history forms, including basic demographic and clinical information, were completed by the OPD nurse or AFRIMS research nurses. Nasal specimens were collected and tested at field sites for rapid feed-back to the physicians and patients. Nasal/throat swabs were collected and placed in viral media and stored at  $-70^{\circ}\text{C}$ . All specimens were shipped on dry ice to AFRIMS for subtyping using molecular techniques. An aliquot is shipped to Armstrong Laboratory, San Antonio, Texas, for virus isolation and other definitive diagnosis.

#### **e. Results:**

AFRIMS continues to work in close collaboration with the US and Thai CDCs, the Thai Ministry of Public Health, and with NAMRU-2. The influenza surveillance is divided into individual country projects each for Thailand, Nepal, US Embassies in the region and the Philippines. Provision of staff, equipment, infrastructure development, and training is well underway. AFRIMS has recently set up training on molecular diagnostics in Kathmandu, Nepal and has plans to do similar trainings in Kamphaeng Phet and in the Philippines. A dedicated respiratory pathogens laboratory is near completion. This will allow immediate processing of influenza samples, and ensure on-time reporting. Construction of a BSL-3 laboratory is also underway. The respiratory laboratory will be equipped with a real-time pcr machine, serology set up, viral isolation, computers for data entry, and capabilities for specimen storage and archiving.

##### **1. Progress on influenza surveillance in U.S. citizens in U.S.**

Embassy/Consulate in Asia: the subject enrollment started since the protocol was approved by HURC on Feb 2006. There are now a total of 14 medical units/clinics from 13 countries in Asia participating in this study - Thailand (Bangkok), Burma (Rangoon), Bangladesh (Dhaka), India (New Dehli), Pakistan (Islamabad), Mongolia, Laos (Vientiane), Malaysia (Kuala Lumpur), Sri Lanka (Colombo), Vietnam (Hanoi and Ho Chi Minh City), Nepal (Kathmandu), China (Beijing), and Philippines (Manila). Total number of subjects enrolled since the study started is 40; majority was from the US Embassy Medical Unit in Bangkok. The gender distribution was about equal.

### 1.1 AFRIMS Real-time PCR Result

Influenza result determine by Real-time PCR	Positive for Influenza A	Positive for Influenza B	Negative for both Influenza A and B	Total samples
Number of Subjects from US Embassy Medical Unit-Bangkok, Thailand:	4	2	30	36
Number of Subjects from US Embassy Medical Unit-Rangoon, Burma:	0	0	1	1
Number of Subjects from US Embassy Medical Unit-New Dehli, India:	1	0	1	2
Number of Subjects from US Embassy Medical Unit-Dhaka, Bangladesh:	0	0	1	1
<b>Total</b> Number of Subjects from US Embassy Medical Units in Asia during 2006	5	2	33	<b>40</b>

### 1.2 Virus Isolation Results from Air Force Institute for Operational Health (AFIOH)

Influenza Result Determine by Real-time PCR	Pending Result	Positive for Influenza A and Sub type			Positive for Influenza B	Other Resp. Virus	Total Samples
		H1	H3	H5			
Number of Subjects from US Embassy Medical Unit-Bangkok, Thailand:	4	2	1	0	1	26 no resp. virus isolated 1 (Parainfluenza3) 1 (Herpes simplex virus)	36
Number of Subjects from US Embassy Medical Unit-Rangoon, Burma:	0	0	0	0	0	1 (Parainfluenza 2)	1
Number of Subjects from US Embassy Medical Unit-New Dehli, India:	2	N/A	N/A	N/A	N/A	N/A	2

Number of Subjects from US Embassy Medical Unit-Dhaka, Bangladesh:	0	0	0	0	0	no resp. virus isolated	1
<b>Total</b> Number of Subjects from US Embassy Medical Unit in Asia during 2006	6	2	1	0	1	27 no resp. virus isolated 1 (Parainfluenza2) 1 (Parainfluenza3) 1 (Herpes simplex virus)	<b>40</b>

2. Progress on influenza surveillance in Nepal: of the forty eight specimens collected since the approval of the protocol in 24 Aug 2006, fifteen tested positive for influenza B by Real-Time RT-PCR whereas only two have tested positive for flu B by Quick Vue rapid antigen test. The specimens have been transferred to AFIOH for further characterization of the isolates.

**f. Future plans:**

1. To expand surveillance sites to include Manila (National Children's Hospital and East Avenue Hospital) in the Philippines.
2. To expand surveillance to other countries in the region (Bhutan, Maldives and Bangladesh).
3. Set up BSL-3 facility at AFRIMS.
4. Set up Real-Time RT-PCR at KAVRU (Kamphaeng Phet) and PAVRU (Philippines).

**8. Title of Research Project: Sentinel Surveillance for Emerging Diseases Causing Dengue-like or Acute Encephalitis Syndrome in the Philippines (SEDP)**

**a. Principal Investigators:**

Armed Forces Research Institute of Medical Science (AFRIMS):  
Department of Virology

Mammen P. Mammen, Jr. LTC, MC (USAMC-AFRIMS)  
Maria Theresa Alera, M.D (San Lazaro Hospital, Manila, Philippines)

Other Study Personnel:

Armed Forces Research Institute of Medical Science (AFRIMS):  
Department of Virology



Charity Ann Ypil-Butac, M.D.  
John Mark Velasco, M.D.  
Robert V. Gibbons, M.D, MPH  
Richard G. Jarman, PhD  
Ananda Nisalak, M.D  
Butsaya Thaisomboonsuk, PhD  
Piyawan Chinnawirotpisan, PhD  
Thidararat Intararit, R.N.

Department of Entomology

James Jones, Ph.D., LTC, MSC

San Lazaro Hospital

Efren Dimaano, M.D

Philippine-Department of Health (DOH):

Lyndon Leesuy, M.D.  
Vito G. Roque, Jr., M.D.  
Mario S. Baquilod, M.D., MPH

World Health Organization (WHO)

Raman Velayudhan, M.D.

University of the Philippines – Entomology Department

Lillian A. de las Llagas, PhD

**b. Objectives:**

**Primary objectives: Epidemiology**

**General:**

To determine the spectrum of emerging diseases causing dengue-like syndrome (DLS) or acute encephalitis syndrome (AES) in the Philippines

**Specific:**

- (1) To determine the proportion of dengue-like syndrome that is caused by:
  - a. Dengue
  - b. Leptospirosis

- c. Chikungunya
  - d. Scrub typhus
  - e. Murine typhus
- (2) To determine the proportion of acute encephalitis syndrome that is caused by:
- a. Japanese encephalitis
  - b. Rabies
  - c. West Nile

### **Secondary objectives: Laboratory and Public Health Infrastructure**

- (1) To enhance the diagnostic capabilities of the Philippines.
- (2) To determine the mosquito species associated with the transmission of Japanese encephalitis (JE) and West Nile viruses.

### **c. Methods:**

#### **Study Design:**

This was a cross-sectional, hospital-based passive surveillance study conducted among admitted patients in San Lazaro Hospital, a tertiary government hospital located in Manila, Philippines

#### **Study Population:**

The study population consisted of male and female patients 2 years old and above who present with dengue-like syndrome or acute encephalitis syndrome and who signed an informed consent to participate in the study. Patients with DLS were enrolled in the study if they met the following inclusion criteria: any patient with history of fever (temperature of 38°C and above) within the past 2-7 days with either one of the following criteria: a positive tourniquet test, an eschar, migratory polyarthrits or calf pain **OR** two of the following, namely: headache, generalized rash, myalgias, arthralgias, retro-orbital pain or icteric sclerae. Patients with AES who present with acute onset of fever and a change in mental status (including symptoms such as confusion, disorientation, coma, or inability to talk) and/or new onset of seizures (excluding simple febrile seizures) were also enrolled. Criteria for exclusion were explainable causes of DLS or AES, AES preceded or associated with exanthem and known pregnancy.

#### **Data Collection:**

Patients who met the enrollment criteria were interviewed and examined. The patient's personal, demographic, clinical and laboratory data were recorded. Acute blood specimens were drawn. In addition, CSF samples were obtained from AES patients. Initial laboratory testing focused on probable etiologies given the recognized

endemic pathogens. Cases that remain undiagnosed after the initial stage of testing were further characterized using specialized testing to identify emerging pathogens. Patients were advised to come back 14 days after onset of illness where a convalescent serum was drawn.

Laboratory testing was conducted in Stages:

**Stage 1:** Laboratory tests routinely ordered by the San Lazaro Hospital clinical team as part of the management of the cases based on the prevailing standards of care of the various conditions in the Philippine setting

**Stage 2:** AFRIMS in-house DEN/JE Enzyme immunoassay (EIA) was performed to help rule in/rule out either dengue or JE etiology; specimen with negative/inconclusive results were subjected to the next level of testing

**Stage 3:** Laboratory tests performed for presumptive diagnosis

**Stage 4:** Confirmatory laboratory tests and other more advanced tests

Final diagnosis was based on study-specific case definitions. Cases which yielded negative results after Stage 4 testing were classified as those belonging to other etiologies.

The collaborating institutions were provided with laboratory test capabilities to perform AFRIMS in-house DEN/JE EIA. The investigators ensured that the materials required for the test performance were provided. On-site training of laboratory personnel and quality monitoring was conducted by AFRIMS staff. Proficiency evaluation was done.

#### **d. Results:**

##### **DLS Component**

As of 31 December 2006 a total of 260 patients were enrolled for the DLS component of the study. One hundred four subjects were enrolled from 1 September 2005 to 23 January 2006 while 156 subjects were enrolled from 21 July 2006 to 14 December 2006. Majority of the patients came from the National Capital Region (NCR).

As of October 2006, 143 patients have undergone laboratory testing for dengue infection of which 129 (90%) have been confirmed positive. The following diagnoses were noted among the 18 patients who were considered as non-dengue cases: 2 cases of possible leptospirosis, 1 typhoid, 1 *Plasmodium falciparum*, 1 chikungunya, 1 pneumonia, 1 disseminated tuberculosis, and 1 possible rickettsia. Specialized assays to diagnose the remaining non-dengue cases are still pending.

For subjects enrolled before April 2006 inclusive, all dengue serotypes were documented to be present while for subjects enrolled from April - October 2006, dengue

serotypes 2, 3, and 4 were documented. DEN-3 was noted to be the predominant serotype on both enrollment periods.

Preliminary sequencing on DENV-3 specimens collected before April 2006 inclusive, showed Asian genotype 1 strain. For specimens collected after April 2006, analysis of the clinical data and phenotypic identification and sequencing are still pending.

### **AES Component**

As of 31 December 2006, a total of 15 patients have been enrolled for the AES component. 13 have undergone laboratory testing for Japanese encephalitis (JE) with 4 subjects confirmed positive for JE. Of the confirmed JE cases, one was from Pampanga, another came from Bulacan, the third came from the province of Quezon and the last came from the city of Calococan. Specialized assays to diagnose the remaining non-JE cases are still pending.

For enrollment before April 2006 inclusive, 3 Serious Adverse Events (SAEs) were reported to HURC with 2 mortalities among the DLS component and 1 from the AES component. The 3 SAEs (3 mortalities) that were recorded were not attributed as a direct consequence of participation in the study.

#### **e. Future plans:**

We are requesting that Mammen P. Mammen Jr., M.D. be removed as co-principal investigator and In-Kyu Yoon, MD be added as a co-principal investigator. We are also requesting that the study duration be extended for 2 years from the current completion date of June 2007 to a proposed completion date of June 2009 in order to allow for ongoing laboratory testing and data analysis. No further subject contact will occur after the current completion date of June 2007.

## **9. Title of Research Project: Training and Workshops**

### **a. Background:**

The Department of Virology, Armed Forces Research Institute of the Medical Sciences (AFRIMS), Bangkok, Thailand, seeks to expand its diagnostic capabilities in South and Southeast Asia by improving regional laboratory capabilities through the dissemination of diagnostic kits and the training of technical personnel.

### **b. Objectives:**

1. To create and improve the laboratory infrastructure of South and Southeast Asian regional laboratories specializing in infectious disease surveillance.

2. To provide the training of laboratory personnel (technicians and supervisors) working in South and Southeast Asia and beyond in infectious disease diagnostic techniques.

**c. Activities:**

The department conducted numerous on-site and in-house diagnostic training activities.

1. Over 100 student scientists from Kasetsart University, Srinakharinvirot University, Chulalongkorn University, Phramongkutklao Medical College, Faculty of Tropical Medicine-Mahidol University, University of Texas Medical Branch and Air Force Institute for Operational Health (AFIOH) received 1 to 4 weeks of training at AFRIMS in diagnostic laboratory modalities. In addition, we provided our laboratory visit to the staff from WHO, US embassy (China and Burma), and the Federal Armed Forces Central Institute, Germany.

2. On-site training (Nepal and Philippines) in the proper performance of the AFRIMS DEN/JE IgM IgG EIA and basic instruction in QA and QC principles was provided to representatives of numerous Nepali health institutions including Nepal Public Health Laboratory (NPHL). The on-site training in serological assay was also provided to San Lazaro Hospital and Research Institute of Tropical Medicine in Philippines.

3. Training in the proper performance of the AFRIMS JE EIA and basic QA and QC principles was provided to visiting scientists from the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, and National Institute of Pediatrics, Hanoi, Vietnam.

4. During the past year diagnostic kits or training was provided to the following laboratories:

B.P. Koirala Institute of Health Sciences, Dharan, Nepal  
Nepal Public Health Laboratory, Kathmandu, Nepal  
Teku Hospital, Kathmandu, Nepal  
Bheri Zonal Hospital, Nepalgunj, Nepal  
Institute of Medicine, Kathmandu, Nepal  
ICDDR, Dhaka, Bangladesh  
Pasteur Institute, Ho Chi Minh City, Vietnam  
Research Institute of Tropical Medicine, Alabang, Philippines  
San Lazaro Hospital, Manila, Philippines

**F. Department of Retrovirology, AFRIMS FY06 Research Accomplishments**

**1. Title of Research Project: A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp120 B/E (AIDSVAX®)**

**B/E) Boosting in HIV-uninfected Thai Adults (RV144, HSRRB Log No. A-11048, BB-IND 8795)**

**a. Investigators:**

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**b. Objectives:**

Primary: To determine whether immunizations with an integrated combination of ALVAC-HIV (vCP1521) boosted by AIDSVAX<sup>®</sup> gp120 B/E prevent HIV infection in healthy Thai volunteers. Secondary: To determine whether immunization with this vaccine combination results in reduced HIV viral load “set point” among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To determine whether immunization with this vaccine combination results in an increased CD4 count measured at viral load “set point” among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To confirm the safety of this vaccine combination in Thai volunteers. To evaluate whether participation in this HIV vaccine trial is associated with behavior change that may increase the risk of HIV infection.

**c. Methods:**

This will be a community-based, randomized, multicenter, double-blind, placebo-controlled clinical trial (vaccine:placebo = 1:1). Screening of potential volunteers will be carried out under a separate protocol entitled “Screening and evaluation of potential volunteers for a trial in Thailand of a candidate preventive HIV vaccine” (RV148). Eligible volunteers will be enrolled over approximately one year. The statistical assumptions of the study will require that 16,000 persons enroll into the study. Vaccinations for each individual will occur over a 24-week period (0, 4, 12, 24 weeks). Women will be tested for pregnancy and pregnant volunteers will not be vaccinated. The volunteers will be followed with HIV testing every 6 months for 3 years after immunization. Blood will be collected for plasma (for diagnostics and HIV-specific antibodies) at 0, 24 and 26 weeks, and every 6 months during the follow-up phase. The blood collection at 0 and 52 weeks will also be used for cryopreservation and archiving of PBMCs (for HIV-specific cellular immune responses). At week 24 and at each six-month follow-up visit, volunteers will have HIV testing, preceded by pretest counseling and followed (approximately 2-3 weeks later) by post-test counseling. Assessment of HIV risk behavior will be performed at baseline and at each 6-month

follow-up visit. Education on risk behavior reduction will be given at each vaccination visit and at each post-test counseling visit.

**d. Results:**

The first volunteer injected on 20 October 2003. As of December 2005, 16,402 volunteers were enrolled and enrollment has ceased.

**e. Future Plans:**

All vaccinations will be performed by 31 July 2006. Volunteers will be followed for 3 years after vaccination. A Data and Safety Monitoring Board meeting was held 4 – 5 October 2005 and no safety concerns were raised. A Data and Safety Monitoring Board meeting is being held 25- 26 June 2006.

**2. Title of Research Project: Extended evaluation of the virologic, immunologic, and clinical course of volunteers who become HIV-1 infected during participation in a phase III vaccine trial of ALVAC-HIV and AIDSVAX<sup>®</sup> B/E (RV152, WRAIR #1184**

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**b. Objectives:**

This protocol seeks to establish whether a vaccine effect on HIV-1 viral load results in a reduction in the number of composite HIV-related clinical endpoints, which also includes a biomarker (CD4 count) component. This study also includes assays of both cellular (e.g., intracellular cytokine staining, CTL) and humoral (neutralizing antibody) responses to identify putative correlates of vaccine-associated immunity, as well as virologic characterization of infecting viruses by genotyping and selective sequencing to assess for selective vaccine efficacy.

**c. Methods:**

Volunteers attend study visits every 3 months to receive a clinical assessment, CD4 count, and viral load measurement, as well as collection of PBMC's and plasma for research assays.

**d. Results:**

The protocol began enrollment in May 2006. No analysis of data or abstracts has resulted from this study at this time.

**e. Future Plans:**

The protocol will continue to enroll HIV-infected participants from RV144 until the scheduled end of that protocol in August 2009.



### **III. APPENDICES:**

#### **PERSONNEL ASSIGNED UNDER AGREEMENT**

##### **Department of Administration**

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2. Mr. Weerasak Yeephu
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218. Ms. Kusum Bajracharya
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231. Mr. Rameshwor Ale Magar
232. Mr. Bharat Thapa
233. Mr. Harkha Bahadur Lama
234. Mr. Bishnu Rayamajhi
235. Mr. Shiva Ali Magar
236. Ms. Wimonsri Tinrum



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