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FOREWORD

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S. Sangkharomaya Jan. 28, 2002

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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 US 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 US Army AFRIMS research efforts, and utilities and maintenance required to support the US Army AFRIMS research effort.

C. US ARMY AFRIMS research efforts at Department of Entomology

Department of Entomology research efforts are the following:

Development of an in vitro hepatoma cell invasion assay to evaluate the efficacy of Plasmodium falciparum and P. vivax pre-exoerythrocytic vaccine candidates.

Development of a chigger-challenge model for the evaluation of candidate scrub typhus vaccines.

In vitro and In vivo evaluation of arthropod repellents against chiggers.

Transmission-dynamics of anti-biotic resistant scrub typhus.

Development of a standardized system for assessing dengue risk based on surveillance for adult dengue vectors.

Genetic variation in Plasmodium falciparum and P. vivax: Role of the anopheline mosquito as a selective force in the transmission of certain strains of parasite.

Longitudinal and spatial study on the epidemiology of malaria: Development of a Geographic Information System to assess the threat of malaria transmission by Anopheles mosquitoes.

Simultaneous identification of vector species and militarily-relevant human pathogens by molecular diagnostics.

D. US ARMY AFRIMS research efforts at Department of Immunology and Entomology

Optimization of sporozoite production to support a human Plasmodium vivax sporozoite-challenge model.

Development of an in vitro assay to evaluate the exo-erythrocytic activity of antimalarial drugs against Plasmodium falciparum.

Tafenoquine (WR-238605) and artelinic acid: Evaluation of transmission-blocking activity against Plasmodium falciparum and P. vivax.

E. US ARMY AFRIMS research efforts at Department of Enteric Diseases

Department of Enteric Disearses research efforts are the following:

Surveillance for Antimicrobial Resistance patterns of Enteric pathogens in Thailand, Vietnam and Nepal.

F. US ARMY AFRIMS research efforts at Department of Veterinary Medicine

Department of Veterinary Medicine research efforts are the following:

Assessment of new anti-malarial drugs in a non-human primate relapsing malaria model (rhesus-*P. cynomolgi.*

A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs.

Surveillance of Emerging and Re-emerging Flavivirus Infections in Sangkhlaburi District By Using Sentinel Animals

G. US ARMY AFRIMS research efforts at Department of Virology

Department of Virology research efforts are the following:

Febrile disease surveillance, Kathmandu, Nepal

Hospital-based EID surveillance, Kamphaeng Phet, Thailand

Influenza surveillance

The Dengue Hemorrhagic Fever Project II: Continued Prospective Observational Studies of Children with Suspected Dengue

Prospective Study of Dengue Virus Transmission and Disease in Primary School Children.

H. 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 FY01 Research Accomplishments

1a. Development of an in vitro hepatoma cell invasion assay to evaluate the efficacy of Plasmodium falciparum and P. vivax pre-exoerythrocytic vaccine candidates.

1. Introduction:

Malaria remains one of the most significant military threats in many areas of the world. Control of the disease is complicated since i) malaria parasites have developed resistance to many drugs used for treatment and ii) effective vaccines are not available. One of the priorities of the Military Infectious Disease Research Program (MIDRP) is to develop falciparum and vivax malaria vaccines.

Exoerythrocytic stage *Plasmodium* parasites are of key interest to STEP/STO F (Malaria vaccine research). Since the primary goal of STEP F is to prevent all clinical manifestations of malaria, STEP F research is focused on "antigens expressed by irradiated sporozoites within hepatocytes and on the surface of sporozoites." However, in spite of the need for a model system with which to evaluate the efficacy of sporozoite or EE-stage vaccines, there is no single standard system.

Hepatocyte invasion by malaria sporozoites involves specificity between sporozoites and hepatocyte surface membranes (Hollingdale et al., 1983, Scheller et al., 1995), implying that not all hepatocytes or hepatoma cells are suitable hosts for parasite development. Cultivation of liver forms of falciparum malaria has been established in primary hepatocyte cultures of humans (Mazier, et al., 1985) and a new world monkey (Millet, et al., 1991). Complete development of *P. falciparum* liver stages in a human hepatoma cell line has been reported (Kanasuta, et al 1995) but the infection rate was very low (0.009%). Unfortunately, these models do not yield reproducible results or an easily quantifiable infection rate and are thus impractical for studies involving hepatic stage parasites.

In order to establish reproducible, highly efficient production of EE-stage *P. falciparum* parasites, the following 3 requirements must be met: 1) production of infective gametocyte cultures with which to infect mosquitoes, 2) production of large numbers of infective sporozoites from mosquito salivary glands, and 3) development of stable hepatoma cell lines that can be maintained in continuous culture. Once these requirements have been met it will be possible to standardize a system of producing EE-stage parasites for drug or vaccine studies.

The Departments of Entomology and Immunology, AFRIMS have standardized the production and maintenance of *P. falciparum* gametocyte cultures and methods for the infection of anopheline mosquitoes. The Department of Entomology routinely harvests *P. falciparum* sporozoites from these mosquitoes. In addition, *P. vivax* sporozoites are routinely obtained from mosquitoes fed on gametocytemic patients reporting to Thai malaria clinics. As part of an ILIR project (FY98-FY00: Development of human hepatoma cell lines for high density production of exoerythrocytic stage of human malaria, *Plasmodium falciparum*), Dr. Jetsumon Prachumsri has recently established a long-term culture of a human hepatoma cell line (Cell Line ID#: HC-04,

Passage #63, >17 months in continuous culture). Reproducible infection rates of 0.5-1.0% are achieved using an inoculum of $5x10^4$ sporozoites. These infection rates are significantly (approximately 100-fold) higher than those previously reported. In addition, trophozoite infection of red blood cells overlaid onto the hepatoma cell lines has been observed, indicating that complete *in vitro* cultivation of EE-stage *P. falciparum* parasites has been achieved.

In this study we will refine the culture system as established under Dr. Jetsumon's ILIR. Primary emphasis will be placed on i) modifying *P. falciparum* sporozoite inoculation and hepatoma culture procedures to increase infection rates to an even higher level and ii) developing a "user-friendly" method of quantifying EE-stage infection rates. Once this system is standardized to produce maximum yield of EE-stage parasites, subsequent efforts will focus on use of the model system in drug efficacy and vaccine development studies and to provide further understanding of the biology and genetics of exoerythrocytic stage parasites. Additional emphasis will be placed on the adaptation of the *P. falciparum* model to allow for studies on *P. vivax*.

2. Objectives:

Transition a successful ILIR project (FY98-FY00) into the core MIDRP program. Our objective is to establish a standard method of evaluating the ability of candidate vaccines to prevent sporozoite invasion of hepatocyte cells. The method will be based on a continuous culture system for maintaining human hepatoma cell lines capable of producing exo-erythrocytic stage *Plasmodium falciparum* parasites. The goal will be to standardize culture procedures and methods of quantifying EE-stage parasites. This system can then be used to precisely assess the ability of sera from vaccinated monkeys to prevent sporozoite invasion of hepatocytes. Emphasis will be placed on modifying techniques currently used to maintain *P. falciparum* cultures for *P. vivax*.

3. Methods:

Primary focus of this project will be to optimize established procedures for the production of EE-stage P. falciparum parasites. Success will be defined as a reproducible (>75% of inoculations) 10-fold increase in the number of EE-stage P. falciparum parasites observed in hepatoma cell culture (an increase from the currently achievable level of 0.5-1.0% infection rates following inoculation of 50,000 sporozoites to a 5-10% infection rate).

Optimization procedures will focus on i) preparation of sporozoites (diluents, time from harvesting to inoculation, etc.), ii) processing of sporozoites (addition of Kuppfer cells to hepatoma cultures), and iii) culture media/procedures.

Secondary focus of the project will be to develop simplified procedures for quantifying EE-stage parasites in the hepatoma cells. Current procedures (preparation of thick and thin films, staining, and subsequent counting infected cells) are laborious, time-consuming, and require an experienced microscopist. Emphasis will be placed on the development of a monoclonal-antibody based ELISA and/or quantitative (TAQ-Man) PCR.

Finally, emphasis will also be placed on modifying procedures used for the production of EE-stage P. falciparum parasites so that a standard model can be developed for EE-stage P. vivax parasites.

4. Results:

The Departments of Entomology and Immunology, AFRIMS have standardized the production and maintenance of P. falciparum gametocyte cultures and methods for the infection of anopheline mosquitoes. The Department of Entomology routinely harvests P. falciparum sporozoites from these mosquitoes. In addition, P. vivax sporozoites are routinely obtained from mosquitoes fed on gametocytemic patients reporting to Thai malaria clinics. As part of an ILIR project (FY98-FY00: Development of human hepatoma cell lines for high density production of exoerythrocytic stage of human malaria, Plasmodium falciparum), Dr. Jetsumon Prachumsri (Sattabongkot) has recently established a long-term culture of a human hepatoma cell line (Cell Line ID#: HC-04; a patent application for this cell line has been submitted). Reproducible infection rates of 0.5-1.0% in this cell line are achieved using an inoculum of 50,000 sporozoites. These infection rates are significantly (approximately 100-fold) higher than those previously reported. In addition, trophozoite infection of red blood cells overlaid onto the hepatoma cell lines has been observed, indicating that complete in vitro cultivation of EE-stage P. falciparum parasites has been achieved.

In FY00 we conducted studies on P. vivax exo-erythrocytic development and found that cell line HC-04 support development of EE-stage P. vivax. In initial experiments, HC-04 cells resulted in a higher level of sporozoite invasion 4-days post inoculation than did HepG2-A16 cells. The mean invasion rates were 0.23 % (range 0.13-0.39%) and 0.73% (range 0.48-0.99%) for HepG2 and 0.36% (range 0.18-0.64%) and 1.69% (range 0.39-3.0%) for HC-04 cells. Early-stage trophozoites were observed in red blood cells overlaid on the hepatoma cells 10 days after sporozoite inoculation. These results clearly demonstrated that exoerythrocytic merozoite development had occurred. Although invasion rates of the red blood cells were low, this may have been due to the fact that P. vivax normally invades young red cells or reticulocytes. Red blood cell invasion rates may be improved if reticulocytes are used for the cultures.

In FY01 efforts focused on refining the hepatoma culture system, with primary emphasis placed on 1) modifying sporozoite inoculation and hepatoma culture procedures to increase infection rates to an even higher level, and 2) developing an improved method of quantifying EE-stage infection rates. Primary focus of this project was to optimize established procedures for the production of EE-stage P. falciparum and P. vivax parasites. Success was defined as a reproducible (>75% of inoculations) 10-fold increase in the number of EE-stage P. falciparum and P. vivax parasites observed in hepatoma cell culture (an increase from the currently achievable level of 0.5-1.0% infection rates following inoculation of 50,000 sporozoites to a 5-10% infection rate). Optimization procedures focused on 1) screening additional hepatoma cell lines that may yield higher invasion rates, 2) preparation of sporozoites (diluents, time from harvesting to inoculation, etc.) for use in the assay, and 3) identification of culture media/procedures that will enhance EE-stage parasite production. A Summary of results from these experiments is provided in Table 1-3. Although no candidate vaccines have yet been tested in this model system, we have evaluated the activity of candidate antimalarial drugs. Results are promising, and it should be feasible to evaluate sera from vaccinated individuals (or animals) at any time.

Secondary focus of the project was to develop simplified procedures for quantifying EE-stage parasites in the hepatoma cells. Results from these experiments are summarized below:

1) During FY01, it was established by using slot blot analysis using stage specific probes, PfA and PfC (Li et al. 1993. Exp. Parasitol. 76:32-38), that two types of ribosomal RNA (rRNA) are expressed in different developmental stages of P. falciparum. Type A rRNA is preferentially expressed during the asexual stage (blood stage), whereas type C or type S rRNA is dominantly expressed during the sexual stage (sporozoite).

2) Stage specific primers and probes for TaqMan analysis were designed from P. falciparum small subunit (SSU) rRNA region. The specificity of the primers and probes were determined by using sporozoites and blood stage culture of P. falciparum. Type C primers and probe could detect fluorescence signal from sporozoites but not from the blood stage parasites. Whereas, type A primers and probe could detect the signal only from blood stage parasites but not from sporozoites.

3) While the TaqMan primers and probes were analyzed for their specificity, conventional RT-PCR using P. falciparum SSU rRNA primers was performed on total RNA samples from sporozoite-infected and uninfected HC04 cells with and without drug treatment to estimate if the drugs would affect parasite development. It appeared that of all the drugs tested (BN66378, KKPII-61, Primaquine, and Tefnoquine), Primaquine is the only one that has no effect on the parasite development. The 400 bp RT-CR product from sporozoite-infected cells with Primaquin treatment was observed at the similar level as that from infected cells without treatment. However, no RT-PCR product was detected from infected cells treated with other drugs.

4) To exclude the possibility that the drug (other than Primaquine) treatment has an effect on the host cells not the parasites, RT-PCR of a house keeping gene (GAPDH) was carried out from infected and uninfected cells with and without treatment. It was found that the 160 bp RT-PCR product was detected from all samples tested with no significant difference in their signals. Therefore, drug treatment as well as infection has no effect on the normal function of the cells. Taken together, it is likely that the drugs, BN66378, KKPII-61, and Tefnoquine but not Primaquine, could prevent P. falciparum development in liver cells. The molecular mechanism of this prevention has to be further investigated.

5. References:

Sattabongkot J, N Yimamnuaychoke, R Udomsaengpetch, S Leelaudomlipi, M Rasameesoraj, TG Brewer and RE Coleman. Complete in vitro maturation of exo-erythrocytic stage Plasmodium falciparum in a novel human hepatocyte cell line. Proceedings of the National Academy of Sciences, U.S. A. (In preparation).

Sattabongkot J, R Udomsaengpetch, R Jenwithisuk, N Ratchapaew, S Leelaudomlipi, DE Kyle and RE Coleman. Evaluation of the exo-erythrocytic activity of selected antimalarial drugs against Plasmodium falicparum and P. vivax in human hepatocyte cell line (HC-04). Transaction of the Royal Society of Tropical Medicine (In preparation).

1b. Development of a chigger-challenge model for the evaluation of candidate scrub typhus vaccines.

1. Introduction:

Trombiculid mites are the vectors of Orientia tsutsugamushi, the etiologic agent of scrub typhus. Currently, AFRIMS maintains the only DoD colony of these scrub typhus vectors. The revised (FY00 and FY01) strategic plan for STEP J focuses on the development of a vaccine capable of protecting military troops from scrub typhus. As part of the vaccine development process, it will be necessary to develop an animal model for challenge studies that mimics the natural transmission of scrub typhus. This proposal focuses on i) identifying relevant factors that affect the ability of chiggers to become infected with and transmit O. tsutsugamushi, ii) identifying and characterizing several chigger-borne strains of O. tsutsugamushi (to include both antibiotic susceptible and resistant strains) that will be used for vaccine testing, and iii) developing a reliable chigger/mouse model with which to conduct vaccine testing.

AFRIMS is the only DoD medical research laboratory that contains all of the resources required to bring this project to successful completion. The following is a summary of the critical resources available at AFRIMS that are required for completion of this project:

- Trained scientific and technical staff: Dr. Kriangkrai Lerdthusnee heads a 5-person ectoparasites team (1 Ph.D., 2 M.S., and 2 technicians) that is currently developing a chigger-challenge mouse model with which to evaluate candidate vaccines. CPT Tom Kollars heads an 8-person molecular biology team (2 Ph.D., 3 M.S., and 3 B.S. scientists) that is currently conducting studies on the genetic diversity of scrub typhus. In addition, this team is characterizing O. tsutsugamushi from infected-chigger colonies maintained at AFRIMS.

- Immediate access to field sites: Active scrub typhus study sites are maintained in Chiangrai and Nonthaburi provinces, Thailand. Seasonal vector abundance and population densities have been quantified at each of these sites.

- Animal Use Protocols: AFRIMS Protocol #97-02 allows for the maintenance of infected chigger colonies on laboratory mice. Additional protocols to i) quantify antibiotic-resistance and virulence of O. tsutsugamushi in mice, and ii) conduct epidemiological investigations on scrub typhus are currently under review.

- All chiggers required for the project: The AFRIMS insectary maintains the worlds only colonies of Leptotrombidium fletcheri, L. deliense, L. imphalum, and L. chiangraiensis. Both Orientia tsutsugamushi-infected and uninfected colonies are maintained.

- Proven capability in this area of research: The Department of Entomology, AFRIMS, has a long history of demonstrated (through publication in peer-reviewed journals) excellence on the repellent studies. Selected references are provided (1-8).

2. Objectives:

This is Year-2 of a STEP-J funded project. The objectives of this project are to:

1) Identify reference strains of O. tsutsugamushi that represent the full range of phenotypic diversity present in SE Asia.

2) Precisely characterize these reference strains of O. tsutsugamushi for antibiotic sensitivity and virulence using an in vivo mouse model. Ensure validated methods (reproducible, quantifiable, and feasible) are available with which to quantify O. tsutsugamushi development in infected mice.

3) Characterize O. tsutsugamushi in the 12 infected-chigger colonies of Leptotrombidium sps. maintained at AFRIMS. Ensure that the strains of O. tsutsugamushi present in the chigger colonies reflect the strains identified in objective #1. If not, ensure that additional strains are acquired (either by collecting chiggers from the field and establishing infected-colonies or by developing methods of infecting chiggers with well-characterized strains of O. tsutsugamushi.

4) Standardize a chigger-challenge model for the evaluation of candidate scrub typhus vaccines.

3. Methods:

1) Production of "Reference Strains" of Orientia tsutsugamushi: The goal is to culture representative strains of O. tsutsugamushi that are antibiotic-sensitive and "antibiotic-resistant." Success is defined as production of quantities (multiple aliquots) of each strain sufficient to meet the needs of AFRIMS and NMRI. Successful completion of this goal is a pre-requisite to most of the following goals. The selection of which strains should serve as "Reference Strains" will be based on review of all available (clinical, in vitro, and in vivo) data. We propose to produce 6 anti-biotic sensitive strains, 6 anti-biotic resistant strains (selected from isolates produced during Dr. George Watt's study in Chiangrai), and 6 virulent strains (selected from patients who were too ill to be included in Dr. Watt's study). A panel of 8 prototype strains (Karp, Kato, Gilliam, TH1817, TA 686, TA 678, TA 716, and TA 763) will be included.

2) Characterization of Strains/Isolates of Orientia tsutsugamushi: The goal is to characterize the "Reference Strains" of O. tsutsugamushi (produced under Goal 1) in order to determine phenotypic and genotypic relationship between different strains. Initially we will evaluate Or. tsutsugamushi response to antibiotic agents and growth rates in a mouse model. We will also use heteroduplex PCR and gene sequencing of the r56 and 16SrDNA genes to characterize each of the reference and prototype strains.

3) Identify factors affecting chigger transmission of O. tsutsugamushi: Infected and non-infected colonies of mites will be tested for variation in their ability to passage and acquire various strains of O. tsutsugamushi (including anti-biotic resistant strains). Colonies of mites will be characterized by PFGE and DNA amplified fingerprinting to identify colony lines with the highest rate of infectivity to laboratory mice. These colonies will be monitored over several generations to ensure a "hot" strain capable of infecting >90% of recipient animals is maintained. In addition, control lines of uninfected chiggers will be monitored. This will ensure that the mite colonies that are the best vectors of scrub typhus will used in the mouse protection model. Additional studies will be conducted to determine if intrathoracic inoculation of O. tsutsugamushi into uninfected chiggers can be used as a means of developing new colonies of infected chiggers. The ability to rapidly develop colonies of chiggers infected with newly identified strains (to include antibiotic resistant strains) of O. tsutsugamushi will allow us to evaluate vaccine candidates against a wide variety of pathogens. Finally, we will attempt to characterize mechanisms by which Leptotrombidium deliense and L. chiangraiensis become

infected with O. tsutsugamushi, to include analysis of vertical (mite to mite) and horizontal (vertebrate to mite to vertebrate) transmission. The ability to infect mites from infected hosts would allow us to evaluate the ability of potential vaccine candidates to prevent the transmission of O. tsutsugamushi from mites to vertebrates and subsequently back into mites. This ability of a vaccine candidate could have important epidemiological implications.

4) Develop a chigger/mouse model: Finally, we will use infected chigger colonies to develop a chigger/mouse model with which to evaluate vaccine candidates. Initial efforts will focus on developing a reliable, reproducible system with which infected mites can be used to challenge immunized mice. Studies will focus on determining the course of rickettsemia over time for various strains of O. tsutsugamushi (to include antibiotic resistant strains) and on the development and/or confirmation of diagnostic procedures (PCR, ELISA/IIP, etc.) to quantify rickettsemia in challenged mice. In addition, we will quantify the effect of chigger infection with specific strains of O. tsutsugamushi on various 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).

4. Results:

1) Characterization of Orientia tsutsugamushi strains/isolates: Kollars et al. (Manuscript in preparation) evaluated a 300 base pair sequence of the 56-kDA antigen gene from 27 O. tsutsugamushi human isolates, to include 12 from northern Thailand (Attachment 1). In brief, genomic DNA was isolated from cultures using the Ready Amp kit (Promega) as specified by the manufacturer• s protocol. Amplification was accomplished by PCR using 2 primers: OtP 56.809 5¢-GATTTAGAGCAGAGCTAGGGTTTATGTACC-3¢ and OtM 56.1221 5¢-TGGATTTCCA-ACAGGATTAGGGTTACC-3¢, which amplify a 291 base pair region of TSAg of O. tsutsugamushi Karp strain. Automated DNA sequencing was accomplished using the ABI PRISM 377 System (Perkin Elmer) following the manufacturer's protocol. Sequences are currently being submitted to GENBANK. A 300 bp product corresponding to 818-1,118 bp region of the TSAg of Karp strain was aligned and compared using Cluster Analysis Percent Disagreement using Statistica (Statsoft). Two of these isolates (C3 and C27) that Kollars et al. examined were those that Watt et al. (1996) had reported as antibiotic resistant in his initial paper on antibiotic resistant scrub typhus in Thailand. A third isolate (SV-193) was highly resistant to doxycycline, chloramphenicol and azrithromycin in the mouse model (Attachment 2). Somewhat surprisingly, there was high disagreement in the 300 base pair sequence of these 3 isolates. The C27 isolate was most similar to the Kato strain, while the C3 isolates (6 each) were most similar to Gilliam. The SV-193 strain was quite distinct from each of these other strains. Although based on only a small segment of a single gene, this initial data suggests that putative antibiotic resistant isolates may not be closely related.

Kollars et al. (Manuscript in Preparation) also used the same methods to evaluate the r56 gene from a number of O. tsutsugamushi chigger isolates, to include an isolate from L. chiangraiensis and isolates from 7 L. imphalum (Li-1 thru Li-7) colonies (Attachment 1). Procedures for this evaluation follow those described for his evaluation of human isolates. Interestingly, the L. chiangraiensis isolate was most similar to human isolate C3, while L. imphalum isolates Li-5 and Li-6 were more similar to Karp and isolates Li-1 thru Li-4 and Li-7 were more similar to Kato and human isolate C27. Human isolates C3 and C27 are reportedly

antibiotic resistant (Watt et al. 1996). Although based on only a small segment of a single gene, this initial data suggests that putative antibiotic resistant isolates may not be closely related.

Of the 12 scrub typhus-infected chigger colonies of Leptotrombidium species (1 Leptotrombidium deliense, 7 L. imphalum and 4 L. chiangraiensis) maintained at Department of Entomology, AFRIMS, 5 strains of Orientia tsutsugamushi were successfully isolated and cultured in L929 cell lines. These are as followed: 2 isolates of L. chiangraiensis (Lc-1 and Lc-5 of scrub typhus-infected chiggers collected from drug-resistant scrub typhus area) and 3 isolates of L. imphalum (Li-3, Li-4 and Li-7 a typical scrub typhus vector). The r56 KD gene of all 5 O. tsutsugamushi isolates are currently being characterized/identified using an automated DNA-sequencer (ABI 310, Applied Biosystems) and DNA-amplified fingerprinting techniques in order to determine their phenotypic and genotypic types. About 650 bps of the r56 KD gene have already been identified. New primer sets will be designed in order to finish the rest of the sequence of the r56 KD gene. Results of known DNA sequences of these 5 O. tsutsugamushi isolates will compared to the reference and/or prototype strains of O. tsutsugamushi (i.e., Karp, Kato, Gilliam, TH1817, TA 686, TA 678, TA 716, and TA 763) as well as the anti-biotic sensitive strains, anti-biotic resistant strains, and virulent strains.

2) Development of a chigger-challenge animal model for scrub typhus: As part of the vaccine development process, it is necessary to develop an animal model for challenge studies that mimics the natural transmission of scrub typhus. Therefore, we designed animal (rodent) experiment to develop methods of quantifying chigger transmission rates and to develop methods of quantifying disease progression and rickettsemiae.

Each of our chigger colonies has been evaluated for their ability to transmit O. tsutsugamushi to mice (Lerdthusnee et al., paper submitted to AJTMH), with transmission rates ranging from 7-88%. A summary of the transmission rates for each colony is presented in Attachment 3. Transovarial transmission rates have been evaluated for several of these colonies, with rates ranging from 93-100% (Tanskul et al. 1998). Most recently, Kollars et al. (2001) demonstrated that there were temporal changes in O. tsutsuagmushi infection rates in the progeny from a single infected evaluated chigger, and that there were differences in the pathogenicity of O. tsutsugamushi from several colonies of L. chiangraiensis and L. imphalum (Kollars et al. 2000). Our laboratory is currently determining relationships between strains of O. tsutsugamushi isolated from chiggers and strains isolated from humans and rodents. Particular emphasis is being placed on isolates from areas with putative antibiotic resistance.

On-going studies on the clinical effects of O. tsutsugamushi in mice resulted in the following data (Attachment 4; Tables 1-6):

a. Daily-observations found differences in mortality rates in mice fed on by individual versus multiple chiggers. Mice fed on by higher numbers of O. tsutsugamushi-infected chiggers become sick sooner than did those fed on by fewer chiggers. The mean number of days that mice survived after individual versus multiple chiggers fed on them was 13.6 vs 12.8 days for L. chiangraiensis; 14.8 vs 14.1 days for L. imphalum and 19.3 vs 19.3 days for L. deliense. Based upon our current available data we conclude that virulence of the 3 species is as follows: L. chiangraiensis > L. imphalum > L deliense.

b. Data on the effect of O. tsutsugamushi infection on weight loss, food/water intake, core body temperature and urine/faeces volume in ICR mice is presented in

Attachment 4. Data from these experiments was obtained using metabolic activity cages (weight, food intake, water intake, urine volume, and faeces weight) and subcutaneous transponders (core body temperature). Weight loss, (day 14), food intake (days 11-14), and core body temperature (days 13-14) appeared to be the best predictors of infection, while significant increases in water intake and urine volume (days 12-13) were noted in infected mice. Mouse infection had no impact on amount of faeces.

c. A series of additional experiments was conducted and showed that weight loss was higher in the pooled-chigger feeding groups than in the individual-chigger feeding groups as followed: 2.7 vs 1.9 gms for L. chiangraiensis; 2.5 vs 0.7 gms for L. imphalum and about the same in L. deliense (of 0.2 gm). However, when considered the total weight lost within the groups, we found that in the individual-chigger feeding groups the L. chiangraiensis (1.9 gm) > L. imphalum (0.7 gm) > L deliense (0.2 gm), but in the pool-chigger feeing groups the L. chiangraiensis (2.7 gm)= L. imphalum (2.5 gm) > L deliense (0.2 gm). Quantitative measurement of food consumption by experimental mice in both the individual- and pool-chigger feeding groups demonstrated the continuing decrease in the amount of food consumed by sick/ill mice to the time of death, when they stopped eating. This decrease was significantly greater than that observed in the control mice.

d. In a final experiment, we used PCR to determine the point at which O. tsutsugamushi could be detected after mice were fed on by 2 different species of chiggers (Attachment 4, Table 1). Blood and various tissues were examined. Results revealed that the liver and spleen were the first tissues (days 7-8 post-feeding) in which O. tsutsugamushi could be detected.

5. References:

Coleman RE, V Sangkasuwan, J Sattabongkot, C Eamsila, N Suwanabun, AL Richards, D Rowland, P Devine and K Lerdthusnee. Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to Orientia tsutsugamushi in Thailand. Amer. J. Trop. Med. Hyg. In Press.

Kollars, TM, D Bodhidatta, D Phulsuksombati, B Tippayachai and RE Coleman. Variation in the 56-kDa type-specific antigen gene of Orientia tsutsugamushi isolated from patients in Thailand. Amer. J. Trop. Med. Hyg. (In Review).

Kollars TM, K Wongkalasin and RE Coleman. Detection and identification of murine and scrub typhus using one primer set by polymerase chain reaction and restriction enzyme digestion. J. Med Entomol. (In Review).

Lerdthusnee K, N Khlaimanee, S Mungviriya, KJ Linthicum, SP Frances, TM Kollars Jr. and RE Coleman. Efficiency of Leptotrombidium chiggers at transmitting Orientia tsutsugamushi to laboratory mice. J. Med. Entomol. (In Review).

Kollars, TM, Jr, B Tippayachai, P Tanskul, N Khlaimanee, T Monkanna, K Lerdthusnee and RE Coleman. Molecular evidence that Leptotrombidum chiangraiensis (Acari: Trombiculidae) may be a vector of drug resistant Orientia tsutsugamushi, the etiologic agent of scrub typhus. J. Clin. Micro. (In Preparation)

Kollars, TM, Jr., A. Kengluecha, N. Khlaimanee, and P. Tanskul. 2001. Temporal changes in prevalence of scrub typhus rickettsia (Orientia tsutsugamushi) infecting the eggs of Leptotrombidium imphalum (Acari: Trombiculidae). J. Med. Ent. 38: 108-110.

Ic. In vitro and In vivo evaluation of arthropod repellents against chiggers.

1. Introduction:

Larval Trombiculid mites (chiggers) are important vectors of scrub typhus in Southeast Asia, Korea, Japan, Australia, and the Pacific Islands. Humans are infected by coming into contact with the rodent-host and infected chiggers. Chemical repellents and toxicants applied to the skin and clothing have been used to minimize contact with chiggers (Frances & Khlaimanee, 1996; Frances, 1994; Frances et al.,1992; Ho & Ismail, 1991; Buescher et al.,1984; Kulkarni, 1977). We propose to conduct preliminary tests on the effectiveness of chemical repellents using *in vitro* and *in vivo* systems. The following is a summary of the critical resources available at AFRIMS that are required for completion of this project:

- Trained scientific and technical staff: Dr. Kriangkrai Lerdthusnee heads a 5-person ectoparasites team (1 Ph.D., 2 M.S., and 2 technicians) that is currently conducting studies on the efficacy of repellents against chiggers.

- Immediate access to field sites: Active scrub typhus study sites are maintained in Chiangrai and Nonthaburi provinces, Thailand. Seasonal vector abundance and population densities have been quantified at each of these sites.

- Approved Animal Use Protocols: AFRIMS Protocol 96-08 "Evaluation of New Repellents or Repellent Formulations of Military Importance" received initial review and approval in July 1996. This protocol allows for testing of candidate repellents on mice and rabbits.

- Approved Human Use Protocols: WRAIR Protocol #310 (Laboratory evaluation of insect repellents using human volunteers) has been modified to allow for testing of piperidine repellents and diastereomers on humans at AFRIMS. In addition, AFRIMS Human Use Protocol #99-04 (Field evaluation of arthropod repellents and repellent formulations in Thailand) is currently undergoing scientific review and should be approved for implementation NLT March, 2000.

- All chiggers required for the project: The AFRIMS insectary maintains the worlds only colonies of *Leptotrombidium fletcheri*, *L. deliense*, *L. imphalum*, and *L. chiangraiensis*. Both *Orientia tsutsugamushi* infected and uninfected colonies are maintained.

- Proven capability in this area of research: The Department of Entomology, AFRIMS, has a long history of demonstrated (through publication in peer-reviewed journals) excellence on the repellent studies. Selected references are provided.[1-3, 6--11]

2. Objectives:

The objective of this project is to develop an *in vivo* laboratory system for testing and evaluating chemical repellents against the chigger vectors of scrub typhus (*Orientia tsutsugamushi*).

3. Methods:

1) Evaluate new repellent compounds against mosquito vectors of malaria and dengue using the human-skin bioassay: An amendment to WRAIR Human Use/Ethical Protocol #302 (Log #A-5216; "Laboratory evaluation of Insect Repellents using Human Volunteers") allowing for testing of candidate repellents on human volunteers in Thailand was recently approved. This protocol allows AFRIMS to evaluate candidate repellents approved by the Ethical Committee on military volunteers. Testing of these compounds was based on the methods described by Debboun and Khlun (1999). In brief, a 6-compartment feeding chamber was used for all testing. Ten 7-10 day old female mosquitoes of a given species were placed in each of the six compartments. Mosquitoes were provided water only for a period of 6-24 hours (exact period varied depending on the species of mosquito used) prior to testing. Repellent was applied to 6 separate 3.5 x 4.5cm areas on each leg and allowed to air-dry for 5 mins. A feeding chamber was strapped to the leg and a plastic slide removed thereby allowing mosquitoes access to the leg. The number of mosquitoes probing on each of the 6 sites was recorded every minute for 5 mins (for Aedes mosquitoes) or every other minute for 10 mins (for Culex and Anopheles mosquitoes). The total number of probes recorded on each site was recorded. A Probit Anaylsis procedure was used to calculate effective doses that repelled 50% (ED50) and 95% (ED95) of mosquitoes.

a. Repellent Preparation: For each test, five serial dilutions of each repellent were prepared in 100% ethanol, with controls consisting of ethanol only. We initially used 1:4 dilutions of each repellent to establish an approximate range of effective doses. Subsequent experiments used 1:1 dilutions of repellent to precisely establish ED50 and ED95 doses. A random number generator was used to select the site for application of each of the 5 repellent dilutions and the control on the leg.

b. Replication of Tests: On a given day of testing, up to 18 tests were conducted on each individual volunteer. There is room for 3 test sites (left side, middle, and right side) on the quadriceps of a large adult male (myself). Both legs are used, so there are 6 test sites available. On each site, 3 tests are conducted (i.e., for Aedes mosquitoes, repellent is applied, allowed to air dry for 5 mins, followed by the first cage for 5 mins, the second cage for the next 5 mins, and the last cage for 5 additional mins).

2) Evaluate new repellent compounds against chigger vectors of scrub typhus using an in vitro assay: We will determine ED50 and ED95 concentrations of up to 10 experimental compounds against Leptotrombidium deliense and L. imphalum. Deet will serve as the appropriate reference compound. Narrow bands of filter paper will each be treated with a given concentration of a given repellent, with control filter paper treated with ethanol only. A total of 4 concentrations of repellent will be required in order to calculate the ED50/95 using a PROBIT Analysis Procedure. A band of repellent-treated filter paper will be placed in a small plastic tube along with an individual chigger. The number of times that each chigger crossed the band will be determined every minute for 10 mins, and ED50 and ED95 doses (dose that repelled 50% and 95% of chiggers, respectively) calculated. The test will be replicated a minimum 10 times for each tested dilution of each repellent.

3) Select field sites: Potential study sites will be evaluated to determine whether arthropod numbers are high enough to conduct repellent testing.

4. Results:

1) In Vivo (Human) Testing against Mosquitoes: We evaluated the efficacy of racemate mixtures of two candidate arthropod repellent compounds (the piperidine compounds AI3-37220 and AI3-35765) using Deet as our "gold-standard." Experimental compounds (100mg/ul in ethanol) were provided by Dr. Jerry Klun, USDA, Beltsville, MD. Each piperidine was provided as a mixture of racemates (exact ratio blinded to this laboratory). Testing of these compounds was based on the methods described by Debboun and Khlun (1999). Laboratory colonies of Anopheles dirus A, An. sawadwongporni, An. minimus, Aedes aegypti, A. albopictus, and Culex quinquefasciatus are available for repellent testing. Effort in this FY focused on evaluating the racemate mixtures of the two piperidine compounds AI3-35765 and AI3-37220 against An. dirus and Ae. albopictus. The ED50 for the Deet/An. dirus combination was 6.3 mg Deet/cm2 (CI=), for AI3-37220 it was 13.1 mg/cm2 (CI=) and for AI3-35765 it was 8.5 mg/cm2. The ED50 for the Deet/Ae. albopictus combination was 1.1 mg/cm2 (CI=), while for AI3-35765/Ae. albopictus it was 2.4 (CI=) and for AI3-37220/Ae. albopictus it was 1.5 (CI=). These data clearly indicate that the racemate mixtures of AI3-35765 and AI3-37220 provide protection comparable to deet using this model system. Data is presented in Attachment #1.

2) In Vitro Testing against Chiggers:

a. Permethrin-treated Rain-Gear: We continued previous studies to evaluate the efficacy of a proprietary method of treating military rain-gear with permethrin. Studies were initiated in FY00 and completed in FY01. Studies in FY00 found that the method of treating the rain-gear was not effective. Three lots of Matricap-treated rain-gear were evaluated in FY00. The rain-gear was treated with 1.25 grams Matricap encapsulated Permethrin per square meter of material. The repellent was applied in either a 1% or 2% formulation, with material applied once, twice or three times to the rain-gear (variables therefore are treated vs control, 1% vs 2% formulation vs control, and 1x, 2x, or 3x treatment vs control). No significant differences were found in between any of the treatments and the controls, or between treatments. In FY01, the CRADA partner provided us with additional samples of treated rain-gear. This 3rd lot of treated rain-gear was significantly more effective than previous lots. Data is presented in Attachment #2

b. Evaluation of repellent activity against chiggers: We evaluated the efficacy of DEET, DEPA, DM-9, DM-34-1, DM-36, DM-153, DM-155, DM-159, DM-163, DM-165 and an unknown repellent against the chigger Leptotrombidium imphalum. A number of the compounds appeared to be as effective or better than deet at repelling this species of chigger. Data is provided in Attachment 3.

5. References:

Lerdthusnee K, N Khlaimanee, T Monkanna, S Mungviriya, W Leepitakrat, M Debboun and RE Coleman. Techniques for evaluating repellents against the chigger, Leptotrombidium imphalum (Acari: Trombiculidae), a vector of scrub typhus. J. Med. Entomol. (In Preparation).

1d. Transmission-dynamics of anti-biotic resistant scrub typhus.

1. Introduction:

Over 1 billion people are at risk for scrub typhus (Rosenberg, 1997), and during World War II scrub typhus caused more mortality to U.S. troops than did malaria. However, shortly after WWII the discovery that chloramphenical and tertracyclines cured scrub typhus greatly reduced the military threat of scrub typhus. In 1996, Watt et al. reported that scrub typhus patients in the Chiangrai region of Thailand were responding poorly to appropriate antibiotic therapy, and, on the basis of clinical patient data and *in vitro* and *in vivo* (mouse model) laboratory data, concluded that chloramphenicol- and doxycycline-resistant strains of *O. tsutsugamushi* were present in the region. The Department of Entomology at AFRIMS recently discovered a new species of *Leptotrombidium* chigger that was transmitting scrub typhus in active rice agriculture areas, a habitat not previously known to support scrub typhus transmission (Tanskul et al., 1998). Transmission of scrub typhus in this "new" habitat was closely associated with the homes of patients hospitalized with anti-biotic resistant infections, and the authors suggested that the significant health implications of this finding warranted further study. This study is an effort to conclusively determine whether *Leptotrombidium chiangraiensis* is the sole vector of anti-biotic resistant scrub typhus, and whether this newly emerging disease entity is associated solely with active rice fields.

2. Objectives:

Conduct an epidemiological investigation to determine the vectors of anti-biotic resistant scrub typhus. Scrub typhus is one of the most militarily relevant acarine-borne infectious diseases, with a long history of outbreaks in military populations in the CINPAC Area of Operations.

3. Methods:

This study is being conducted in the vicinity of Chiangrai, in northern Thailand. Two field sites have been established: one closely associated with a foci of anti-biotic resistant scrub typhus and the other without reported anti-biotic resistance. In order to determine whether transmission of the anti-biotic resistant scrub typhus occurs in only specific habitats, each field site has been divided into 4 distinct habitats (village outskirts, active rice fields, ecotone, and wood/forest). In each habitat we have establish a grid system consisting of 80 traps (40 small live-traps and 40 medium live-traps). Collections are made on 6 consecutive days each month. The rigid experimental design of this study has allowed us to collect a variety of chigger species from a variety of animal hosts from distinct habitats associated with anti-biotic resistant scrub typhus and from habitats with no known history of anti-biotic resistant scrub typhus.

The following procedures are used to determine which chiggers species are infected with anti-biotic resistant and/or anti-biotic sensitive O. tsutsugamushi. In brief, blood and tissue samples and all ectoparasites (i.e., chiggers) are collected from all trapped animals. A sub-sample of each blood and tissue sample is assessed for the presence of O. tsutsugamushi by PCR, as are 25% of the chiggers collected from each animal. If a given sub-sample (blood, tissue or chigger) is positive, we subsequently attempt to isolate O. tsutsugamushi from the parent sample. In order to increase our success at isolating O. tsutsugamushi, we inoculate both laboratory mice and cell culture (L-929 cells) with triturated blood, tissue, or chiggers. Once O.

tsutsugamushi has been isolated from each PCR-positive sample, we will then assess resistance to chloramphenicol, doxycycline, and azithromycin in laboratory mice (this portion of the study has not yet been initiated). Groups of 10 mice each will be inoculated with 100-LD50's (calculated for each strain) or 5x10\6/ infected cells of each O. tsutsugamushi isolate. By conducting experiments that inoculate both a known number of rickettsia (i.e. 5 million infected cells) as well as a known number of LD50's we can control for variation in virulence/pathogenicity in the different isolates, while still quantifying anti-biotic resistance. Each group of mice will be treated with serial dilutions of chloramphenicol, doxycycline, or azithromycin on days 5, 6, and 7, with control animals receiving diluent only. The maximum dose given will be approximately 6 times the human dose (300mg/kg/day, 18mg/kg/day, and 50mg/kg/day for chloramphenicol, doxycycline, and azithromycin, respectively) according to Watt et al. (1996).

In addition to quantifying the degree of anti-biotic resistance for each isolate, we will also characterize strain variation in each isolate using the p56 and 16S rDNA portions of the genome (Due to the late start date of the project, this portion of the study has not yet been initiated). We will identify and characterize the field isolates using molecular diagnostic techniques (heteroduplex PCR and DNA sequencing). Results from the field isolates will be compared with 8 "reference" strains (Karp, Kato, Gilliam, TH1817, TA 686, TA 678, TA 716, and TA 763). At the conclusion of this study we will have determined whether there is any clearly defined association between particular species of chiggers, ecological habitat, and the transmission of anti-biotic resistant scrub typhus. In addition, we will have characterized genetic strain variation in chigger and rodent isolates and can then compare these results with data from human isolates in the same area. If it appears that there is a clear association between anti-biotic resistant scrub typhus and particular chiggers and/or habitats, it will be possible to develop a precise "threat assessment" (i.e., Geographic Information System) for military use.

4. Results:

Rodents were collected during the course of 7 monthly trips to the Chiangrai study site. Data presented is from the February to August 2001 trip; however, monthly trips through November 2001 have been completed and the study is continuing. During the 7 months of the study, a total of 24,960 trap-nights (12,480 sherman traps and 12,480 medium-size live traps) were set in the 4 distinct habitats/areas in the 2 different study sites (Pagook village apperas to be a foci of antibiotic resistant scrub typhus and Maesad village appears to be a foci of antibiotic sensitive scrub typhus). In each habitat, we set up 40 rodent-traps and 40 Sherman-traps by alternating between the grids and transect systems. A total of 525 rodents were captured. Blood and tissue samples and all ectoparasites (i.e., chiggers) were collected from all trapped animals. A sub-sample of each blood and tissue sample was assessed for the presence of O. tsutsugamushi by PCR, as were 25% of the chiggers collected from each animal. The 525 rodents represented 10 species. A total of 11,997 chiggers were removed from these 525 rodents. The predominant species of rodents captured were Bandicota indica (58%; 309/525), Rattus rattus (22%; 116/525), Rattus losea (10%; 53/525), and Mus caroli (6%; 33/525). See Table 1 for details on the rodent collection.

The majority of chiggers were removed from Bandicota indica (70%; 8366/11997), Rattus rattus (17%; 2056/11997), Rattus losea (7%; 802/11997), and Mus caroli (2%; 293/11997). See Tables 2-3 for details on the chigger collections.

Chiggers were sorted and identified to species. A total of 4 species were found: Leptotrombidium chiangraiensis, L. imphalum, a species of Blankaartia and a species of Schoengastia (Table 4). The numbers of each species are as follows: Schoengastia spp. (42%; 5015/11997), Leptotrombidium imphalum (42%; 4991/11997), Leptotrombidium chiangraiensis (7%; 808/11997), Blankaartia spp. (8%; 934/11997) and unidentified specimens (2%; 249/11997).

Rodent tissue samples (liver, spleen, kidney, and whole blood samples) and chiggers were assessed for the presence of O. tsutsugamushi using PCR. Four primers, selected from the DNA sequence of the gene encoding the 56 kD protein of the Karp strain, were used. DNA was extracted from the tissue samples and chiggers using the Wizard Genomic DNA Purification kit (Promega, Wisconsin,USA). Nested PCR was performed as previously described (Horinuouchi et al. 1996).

Although all samples have not yet been assesed by PCR, O. tsutsugamushi was detected from liver and spleen samples from 31 rodents (5.9%; 31/525). We subsequently assesed blood and kidney samples and chiggers collected from a number of these O. tsutsugamushi-positive rodents. Only 1/10 (10%) of the O. tsutsugamushi-positive rodents had infected blood, while 0/10 (0%) kidney samples {0.0% (0/10)} was positive for O. tsutsugamushi. Chiggers from 6 O. tsutsugamushi-positive rodents were tested: only 33% (2/6) of the infected rodents tested has O. tsutsugamushi-infected chiggers on them (Table 5).

We also conducted animal-passage experiments in order to determine the chronology of onset of O. tsutsugamushi infection in ICR mice. ICR mice were fed on by two species of infected chiggers (Leptotrombidium chiangriensis and L. Imphalum). The animals were euthanized daily for 14 days after being fed on, and blood and tissue samples collected for the assessment of O. tsutsugamushi by PCR.

Results from the ICR mice demonstrated that O. tsutsugamushi was first detected in the liver and spleen samples on day 7 post-infection, while the positive kidney and blood samples were not detected until days 8-9 or later.

The entire r56 gene of all O. tsutsugamushi isolates is currently being sequenced. Samples from each isolate are inoculated into animals and subsequently plaque-purified in L929 cells. Once plaque-purified isolates are obtained, samples from each isolate are being sent to the University of Maryland (Drs. Abdu Azad and Suzanna Radulovic), where they will be assessed for antibiotic resistance markers. Subsequent studies (FY02/03) will examine the in vivo and in vitro responses of selected isolates to antibiotics.

5. References:

Coleman RE, V Sangkasuwan, J Sattabongkot, C Eamsila, N Suwanabun, AL Richards, D Rowland, P Devine and K Lerdthusnee. 2002. Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to Orientia tsutsugamushi in Thailand. Amer. J. Trop. Med. Hyg. In Press.

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Kollars TM, K Wongkalasin and RE Coleman. Detection and identification of murine and scrub typhus using one primer set by polymerase chain reaction and restriction enzyme digestion. J. Med Entomol. (In Review).

Lerdthusnee K, N Khlaimanee, S Mungviriya, KJ Linthicum, SP Frances, TM Kollars Jr. and RE Coleman. Efficiency of Leptotrombidium chiggers at transmitting Orientia tsutsugamushi to laboratory mice. J. Med. Entomol. (In Review)

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1e. Development of a standardized system for assessing dengue risk based on surveillance for adult dengue vectors.

1. Introduction:

Dengue is the most significant vector-borne disease threat to military operations in SE Asia. Dengue is widespread throughout Thailand, and in 1998 Thailand experienced the 2nd worst dengue epidemic ever recorded in the country. There are no prophylactic drugs or vaccines capable of preventing dengue, and the best means of preventing the disease is through rigorous use of personnel protection measures (PPM) and targetted vector-control aimed at controlling the vectors in high-risk areas.

Unfortunately, in the absence of symptomatic human disease there are currently no effective means of estimating the threat of dengue nor of focusing control efforts on specific areas. It currently is not possible to use mosquito data alone to predict the threat of dengue. An effective method of conducting surveillance for dengue vectors is therefore required in order to 1) assess the threat of dengue, and 2) to determine when and/or where control methods should be used to minimize the military threat.

This collaborative project will be conducted with scientists at WRAIR (Drs. Jeff Ryan and Michael Perich), USDA at Gainesville (Drs. Dana Focks), the Institute of Vector Biology, University of California at Davis (Drs. Tom Scott and John Edman), and the Department of Virology, AFRIMS (Dr. Tim Endy).

The study site will be in Kamphaeng Phet Province, Thailand. The Department of Virology, AFRIMS, is currently conducting a prospective epidemiological study ("Prospective Study of Dengue Virus Transmission and Disease In Primary School Children") on primary and secondary dengue infections in this region. 2,214 children from 12 primary schools were enrolled

during the first year of the project. From June to November, the peak dengue transmission months, 10,592 student-absent-days were evaluated of which 1,183 students had a history of fever or documented fever. Of these acute illnesses, there were 3 children with primary dengue fever and 71 with secondary dengue (57 not hospitalized and 14 hospitalized with severe dengue disease). In addition, there were 78 children who had evidence of dengue infection by antibody titer of which 28 had no school absence or reported illness. The overall incidence of dengue virus infection was 6.7% over 6 months of observation with an overall subclinical to mild to severe dengue disease ratio of 2:8:1. Some schools had transmission rates as high as 25-30%, while other schools had transmission rates less than 1%. Active daily mosquito trapping at the school with the highest incidence of dengue demonstrated a correlation between disease incidence and vector abundance. In addition, forensic analysis of the blood meals within the mosquito gut was matched to the human host in the majority of cases, allowing for a determination of vector-host-dengue disease interactions.

The Department of Entomology at AFRIMS has a long, productive history on dengue. AFRIMS is currently the only laboratory in the USAMRMC that has all of the required components necessary to fully develop the proposed dengue surveillance system. The following is a summary of the facilities and support available at AFRIMS:

- An insectary that produces approximately 30,000-40,000 *Aedes aegypti, Ae. albopictus*, and *Toxorhynchites splenectens* each month. The AFRIMS insectary is maintained by a staff of 9 individuals whose only mission is to raise mosquitoes (in contrast to other institutions where 1 or 2 part-time individuals work in the insectary). These 9 individuals have an average of over 15 years experience rearing mosquitoes.

- Comprehensive dengue diagnostic capabilities, to include: ELISA, *in vitro* culture, IFA, HIA, and PCR.

- A trained 10-person team (1 Ph.D., 2 M.S., 3 B.S. scientists, and 4 technicians) with an average of 14 years experience working on dengue virus.

- Active dengue study sites in Mae Sod (Collaborative project with Drs. Tom Scott, John Edman, and Dana Focks), Kamphaeng Phet (Collaborative project with Dr. Tim Endy), and Ratchaburi (Collaborative project with Dr. Mike Perich).

- An approved human use protocol (Research on *Aedes aegypti*, the Mosquito Vector of Dengue Virus: Multiple blood feeding by mosquitoes: HSP Log No. A-9147; Approved for implementation 16 August, 1999). The purpose of this protocol is to evaluate factors that affect the transmission of dengue virus, and to develop a predictive computer model with which to evaluate dengue threat. In addition, the Department of Virology has an approved protocol (Prospective study of dengue virus transmission and disease in primary school children: WRAIR Protocol #654; Approved for implementation 22 October, 1997) for the study of dengue in Kamphaeng Phet.

- A long history of demonstrated (through publication in peer-reviewed journals) excellence in research on the epidemiology of dengue and on the development of surveillance and control strategies and tools. A list of selected references is provided.[1-9]

2. Objectives:

Develop a simple-to-use, field deployable method of assessing the risk of dengue based on vector surveillance. Specific objectives are to

1) FY00-01: Develop a standardized, field-deployable apparatus for dengue vector surveillance.

2) FY01-02: Evaluate this standardized surveillance apparatus as a tool for predicting dengue threat. This evaluation will be in the form of a carefully controlled prospective study, and shall evaluate vector sureillance in relation to actual dengue transmission (both symptomatic and asymptomatic) rates.

3) FY01: Evaluate field-deployable dipstick assays (currently under development: SBIR with NAVIX, Inc, COR: MAJ Jeff Ryan) as a means of determining dengue threat.

4) FY01-02: Develop a computer model to estimate dengue risk to military personnel.

3. Methods:

This is a 3-phase project that will be conducted at AFRIMS (laboratory component) and in the vicinity of Kamphaeng Phet Province, Thailand (field component). The Department of Virology, AFRIMS, is currently conducting a prospective epidemiological study on primary and secondary dengue infections in Kamphaeng Phet. Our study on dengue surveillance will be conducted in close association with this prospective epidemiological study. The advantage for this project is that precise transmission (both symptomatic and asymptomatic primary and secondary dengue) rates will be determined in a cohort of over 2,000 school children at 12 area schools. The field component of this study will be conducted at 4 of these schools (the 2 schools with the highest dengue transmission rates and the 2 schools with the lowest transmission rates).

Phase-1 of the project will take place in FY00 (STEP-U funded project) and FY01. In this phase we will determine the most effective method of monitoring adult dengue vector (primarily Aedes aegypti and Ae. albopictus) populations. In FY00 we will compare standard vector monitoring methods (i.e., light traps, gravid traps, back-pack aspirators, and biting collections) with various novel methods (shannon traps, American Biophysics "Mosquito Magnet" trap, collapsible resting-box trap, sticky panel trap, and a Nokkosia "pickle-jar attractant trap"). We will evaluate trap efficacy based on 1) ability to collect dengue-infected female Ae. aegypti (see next parasgraph) and 2) simplicity and ease of use. In Similar studies are also being conducted at NAMRID, Peru and at NAMRU-2, Indonesia. Initial trap evaluation conducted at these 3 laboratories in FY00 will result in the selection of 2-3 of the most promising traps. The primary investigators at each of these overseas laboratory will coordinate with the manager of the Dengeu Vector Control System program at WRAIR to make this decision. The 2-3 most promising traps will be further evaluated in FY01, to include the efficacy of promising new mosquito atractants currently under development at the USDA laboratory in Gainesville, Florida. At the conclusion of Phase-1 (end of FY01) we will have selected the most effective method of monitoring dengue vector populations.

In Phase-1 of the project we will also evaluate the efficacy of a field-usable dipstick assay (currently under development by NAVIX, Inc) for the detection of dengue virus in mosquitoes. The sensitivity/specificity of this assay will be compared with that obtained using the dengue-typing ELISA, cell culture, and nested PCR. Successful laboratory evaluation of the product will be followed by field validation using mosquitoes collected over the course of the project. The goal will be to ensure that the field-usable dengue dipstick assay can be used to determine mosquito infective rates. This dipstick assay is a critical component of the dengue surveillance system, as it can be used by field PVNTMED units to precisely identify the threat (by combining surveillance for dengue vectors with a determination of infectivity we identify high risk areas. Emphasis (in the form of area avoidance, targetted mosquito control, or use of personal protective measures), can then be focused on these high risk areas.

In Phase-2 (FY01-02) of the project we will use the selected surveillance apparatus/methodology and dengue diagnostic assays (both the field-usable dipstick assay and the dengue typing ELISA) to evaluate mosquito transmission of dengue in 4 schools in Kamphaeng Phet. Mosquitoes will be collected daily in 1/3 of the classrooms in each school. By associating mosquito surveillance data and mosquito infection rates with actual dengue transmission rates (obtained in the prospective epidemiological study) throughout each school, we will be able to identify factors that can be used to predict dengue transmission. Our data will also allow us to track the introduction and spread of dengue throughout the schools. This portion of the project will serve to validate the efficacy of our selected surveillance and diagnostic tools.

During Phase-3 of the project (FY01-02), we will work with Drs. Tom Scott, John Edman (both at the University of California at Davis), and Dana Focks (University of Florida) to incorporate mosquito and dengue transmission data obtained in this study into the mosquito and dengue transmission simulation models (CIMSiM & DENSim)(12). The ultimate goal will be to determine whether use of random sampling for adult mosquitoes can serve as a means of estimating dengue risk, and of determining population levels of Ae. aegypti that are required to maintain transmission of dengue. During Phase-3 of the project we will validate the dengue surveillance system that we have developed by testing it at 3-4 selected sites throughout Thailand, to include urban and rural areas.

The eventual success of this project depends on the ability of AFRIMS to effectively collaborate with other ongoing studies on dengue. The Department of Entomology, AFRIMS, is currently involved in 2 long-term, collaborative studies on dengue transmission (NIH-funded projects entitled "Multiple blood feeding by mosquitoes" to Dr. Tom Scott, University of California, and "Prospective study of dengue virus transmission and disease in primary school children" to Dr. Francis Ennis, University of Massachusetts). Although we receive no funding from these projects, the links and collaborative efforts that we have established are critical to our success in this proposed MIDRP-sponsored project.

The following is a description of the specific methods to evalute mosquito traps in FY01:

1) Man-biting collections for adult female Ae. aegypti are the "gold standard" against which other collection methods are measured. However, surveillance through man-biting collections is dangerous because of exposure to disease as well as the difficulty in standardization. Standardized techniques are required to monitor changes in mosquito

abundance, evaluate the effectiveness of mosquito control efforts, and for surveillance of arboviruses in mosquito populations. Adult mosquito traps will likely be the principal DVCS surveillance component for monitoring changes in adult dengue vector populations. The traps will additionally be used to provide specimens for determination of the dengue minimum infection rate in adult mosquito populations through the use of dengue panel assay dipsticks. Three adult traps were evaluated for efficacy in monitoring adult dengue vector populations (primarily Aedes aegypti and Ae. albopictus populations) in FY01. Fay-Prince omni-directional traps, Wilton traps, and sticky panel traps were compared against man-biting collections. This was the second year in which adult mosquito traps were evaluated by AFRIMS to select the most efficient adult trapping device for the Dengue Vector Control System (DVCS).

Tambon Song Tham, Amphur Muang, Kamphaeng Phet Province, Thailand was selected to evaluate adult surveillance devices. Village number 12 in this subdistrict historically has high Ae. aegypti populations and high incidences of dengue. Preliminary sampling was conducted to determine which houses had the highest populations of larval and adult mosquitoes. Houses were divided in to groups of four based on mosquito populations. For example: the four houses with the highest mosquito populations were placed in group1, the next four in group 2, etc. A total of 32 houses were used for the evaluation with 16 houses or 4 groups used one night and 16 different houses or 4 different groups used the following night. Each of the three traps plus a man-biting collection was represented in each of the groups. A 4x4 Latin square design was used to evaluate the traps. Traps ran from 0800 until 1700 during the sampling periods. Each trap type was put at each location randomly. Traps were rotated among houses in each grouping to ensure that each trapping device and man-biting collection was rotated once through each house in the grouping. Traps were located in the interior of houses and the suction portion of the traps or sticky board placed at 1 meter above the floor. An insulated container holding 1 kg of dry ice was located directly above the Fay-Prince and the Wilton traps.

2) The following is a description of the specific methods to evalute the dengue dipstick assays in FY01:

VecTest dengue diagnostic dipstick panel assays are based on the dual monoclonal antibody sandwich principle. The dipsticks are able to detect the presence or absence of specific peptide epitopes of DEN 1-4 by the formation of Ag-Ab-gold complexes when a dipstick is placed in a homogenized sample. These complexes migrate through test zones containing immobilized DEN-1, DEN-2, DEN-3, or DEN-4 specific monoclonal antibodies. Unbound dye complexes migrate through the test zone to the control zone and are captured. A reddish-purple control line develops if antigen is present. A control line will always develop if the test is performed correctly. The dipsticks were first evaluated for range of sensitivity to the dengue virus serotypes 1, 2, 3, and 4 seed stocks that were used to inoculate Ae. aegypti mosquitoes for subsequent testing. To accomplish this, dipsticks were placed directly into undiluted seed stock. The evaluator waited for 15 minutes, and checked for results. (Positive results are indicated by the presence of a line, other than the control line, corresponding to the particular serotype being tested.). If a positive band developed dipsticks were further tested in serial dilutions (using grinding solution) of seed stock at 1:10, 1:100, and 1:1000. During the initial series of tests, dipsticks were then evaluated against inoculated mosquitoes regardless of the of test results with seed stocks. However, during subsequent tests if dipstick evaluations of

any dengue serotype seed stock were repeatedly negative, then we halted the study for that particular dipstick lot. A weak positive panel assay result in undiluted seed stock, with corresponding ELISA result, was required before proceeding with dengue serotype sensitivity tests using inoculated mosquitoes. If the dipstick or the ELISA evaluation failed to detect a particular seed stock serotype, then both the dipstick and ELISA were re-evaluated. If a negative result occurred again then both dipstick and ELISA were re-evaluated using a new batch of seed stock. To evaluate inoculated mosquitoes, 500 ml of grinding solution was used to homogenize individual female Ae. aegypti inoculated with dengue virus. Homogenization was accomplished using a manual grinder and performed on an ice tray. Two hundred- fifty ml of the homogenate was sent to the Department of Virology to conduct a standard plaque assay. One hundred-twenty ml of the homogenate was used to test each dipstick. The test dipstick was placed into the mosquito suspension in the grinding tube with the arrows pointing down. After 15 minutes had elapsed, the strip was removed and checked for results. Test results must be read within 30 minutes of test initiation. One hundred ml was used for dengue antigen capture ELISA The remaining homogenate was used to prepare further dilutions for testing that would follow if a strong positive reaction is observed from the dipstick. Uninfected female Ae. aegypti were used as negative controls

4. Results:

1) Results for Trap Evaluations: During 32 trap days, man-biting collections obtained 1085 female Ae. aegypti (Table 1). The Fay-Prince trap collected 119 and the Wilton trap collected 93 female Ae. aegypti. The sticky panel traps collected 1 female and 1 male Ae. aegypti. The sticky panel trap also collected 5 Culex spp. mosquitoes but only Ae. aegypti was collected via the other trapping methods. The collection percentage of total was 84% for man-biting collections, 9% for the Fay-Prince traps, and 7% for the Wilton traps. Parity rates were 79% for females from man-biting collections, 79% for Fay-Prince collections, and 74% for Wilton trap collections.

2) Results for dipstick evaluations: To summarize the results from 6 evaluations conducted this FY; strong detection bands were evident from dipstick lots 11160 and 120800 tested against seed stock solutions of dengue serotype-4 (1.0 x 104 Pfu / ml). A strong detection band was obtained from lot 120800 against stock solutions of dengue serotype-2 (7.5 x 104 Pfu / ml) and a strong but weaker band was also obtained for lot 11160. However, both lots had weak reactions to stock solutions of dengue serotype-1 (1.75 x 105 Pfu / ml) and very weak to no reaction to dengue serotype-3 (2.25 x 105 Pfu / ml). Similar reactions were evident when dipsticks were evaluated against inoculated mosquito homogenates. Unfortunately, virus titers corresponding to homogenates of mosquitoes inoculated with one of the four dengue serotypes are unavailable. The grinding solution was toxic to plaque assay cells and so it was impossible to get virus. Initially it was suspected that detergent was killing cells. Collaborators sent a non-detergent grinding solution that was used to homogenize samples. Cells likewise died following use of the non-detergent grinding solution and it was discovered that the grinding solution contained azide, which is lethal to the cells. A new grinding solution has been received from our collaborators that does not contain detergent or azide and will be used to evaluate new lots of dipsticks.

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1f. Genetic variation in Plasmodium falciparum and P. vivax: Role of the anopheline mosquito as a selective force in the transmission of certain strains of parasite.

1. Introduction:

Human malaria is only transmitted by anopheline mosquitoes; however, not all species of *Anopheles* mosquitoes can transmit malaria. Factors that determine whether a particular species of *Anopheles* mosquito is capable of transmitting malaria are not fully understood; however, various physiological factors such as lifespan and the presence or absence of specific gut or salivary gland receptors are important.

Published studies of the repeat region of the circumsporozoite protein (Pv-CSP) have identified two distinct immunophenotypic strains of *P. vivax* in Thailand: the Vk-210 and Vk-247 variants.[1-3] Genetic variation occurs within each of these phenotypic strains. Preliminary studies conducted by the Department of Entomology, AFRIMS, have indicated that genetic variation also occurs within Thai strains of *P. falciparum*. Published studies evaluating variation in the circumsporozoite protein (Pf-CSP) of *P. falciparum* isolates from throughout the world have identified clearly defined variants (CSP25-CSP32).[4] Studies at AFRIMS have indicated that CSP strains 26-29 occur in Thailand (Tom Kollars, unpublished data). The distribution of the different strains of *P. falciparum* and *P. vivax* throughout Thailand and SE Asia is unknown. However, published studies have clearly demonstrated that patients in different regions of the Thailand can be infected with more than one strain of vivax malaria, and unpublished data from this laboratory have shown that patients can be infected with up to 4 CSP variants of *P. falciparum*.

A recent study by Gonzalez-Ceron et. al.[5] demonstrated that different species of *Anopheles* mosquitoes transmit the Pv-247 and Pv-210 strains of *P. vivax* in South America. These results have significant implications, as the distribution of each variant may be limited by the distribution of the specific vector. In terms of vector control, efforts targeted against a particular species of vector may reduce the transmission of one *Plasmodium* variant but not the other. This may be particularly true where one vector in endophilic and the other exophilic, or where significant behavioral differences differentiate the vectors of different *Plasmodium* variants.

In this study we propose to precisely determine whether different strains of *P. falciparum* and *P. vivax* are preferentially transmitted by certain species of *Anopheles* mosquitoes. This effort will focus on evaluating genetic diversity of the *Plasmodium* population within the human host and within mosquitoes fed on that host. The genetic diversity of sporozoites (output) produced within the mosquito will be compared with that found in the infective blood-meal (input). If differences are observed, we will then evaluate the genetic diversity of zygotes/ookinetes, oocysts, and sporozoites to determine where the selective bottleneck is occurring. In addition, we will evaluate genetic diversity in *Plasmodium* isolated from 2 discrete human populations. One population occurs in an isolated valley where the majority of inhabitants work in the immediate area, rarely travel, and have little contact with outside populations. Mosquito transmission appears to primarily occur in the village. The second population is found less than 30km from the first; however, the population is relatively mobile with frequent travel into surrounding areas in Thailand and Myanmar. There is little local mosquito transmission. We hypothesize that the genetic diversity of the *Plasmodium* population found at the first (mobile population) site will be far greater than that occurring at the second (isolated) site.

AFRIMS is the only DoD medical research laboratory that contains all of the resources required to bring this project to successful completion. The following is a summary of the critical resources available at AFRIMS that are required for completion of this project:

- All mosquitoes required for the project: The AFRIMS insectary produces 40,000 *A. dirus*, *A. minimus*, *and A. sawadwongporni* mosquitoes each month. The insectary is maintained by a staff of 9 individuals whose only mission is to raise mosquitoes (in contrast to other institutions where 1 or 2 part-time individuals work in the insectary). These 9 individuals have an average of over 15 years experience rearing mosquitoes. This insectary has been established for over 30 years. Approximately 25% of the mosquito output of the insectary can be dedicated to support this project.

- Trained scientific and technical staff: Dr. Jetsumon Sattabongkot heads a 9-person team (1 Ph.D., 1 M.S., 3 B.S. scientists, and 4 technicians) with an average of 14 years experience infecting mosquitoes on malaria patients and quantifying mosquito infections. The primary mission of this team is to study the transmission of malaria. This team was responsible for first demonstrating the existence of the VK-247 variant of *P. vivax*. Dr. Thomas Kollars heads an 8-person molecular biology team (2 Ph.D., 3 M.S., and 3 B.S. scientists) that is currently conducting studies on the genetic diversity of *P. falciparum* and *P. vivax* in Thailand.

- Routine production of *P. vivax* and *P. falciparum* infected mosquitoes by feeding on infected patients: The Department of Entomology currently feeds 6,000 *A. dirus*, *A.*

minimus, and A. sawadwongporni mosquitoes on 30-50 *P. vivax*-and *P. falciparum*-infected patients each week. Since 1982, over 50% of all mosquito feeds on *P. vivax*-infected patients have resulted in infected mosquitoes. This long, continuous history of excellence in this field exceeds that available anywhere else in the world.

- Immediate access to field sites: Active malaria study sites are maintained in Kanchanaburi, Sanghlaburi, and Mae Sod. Laboratory-reared mosquitoes are routinely (weekly) infected on malaria patients at each of these sites.

- Approved Human Use Protocols: An approved, ongoing human use protocol (Comparative susceptibility of known and suspected species/strains of Thai *Anopheles* to *Plasmodium* parasites: WRAIR #044, Log #A-6768) that allows for feeding of laboratory-reared anopheline mosquitoes on malaria patients. An additional protocol (Mosquito Acquisition of Malaria (MaM): Role of host and vector factors as determinants of mosquito infection) that specifically supports this project has completed scientific review and is currently undergoing ethical review. This protocol should be implemented NLT February, 2000.

- Proven capability in this area of research: The Department of Entomology, AFRIMS, has a long history of demonstrated (through publication in peer-reviewed journals) excellence on the mosquito transmission of *Plasmodium vivax*. Selected references are provided.[1-3, 6--11]

2. Objectives:

Our goals are to determine: 1) the degree of genetic variation that occurs *in P. falciparum* and *P. vivax* in SE Asia, and 2) whether the vector mosquito serves as a selective force that only permits the transmission of certain strains of *P. falciparum* and *P. vivax*. This collaborative project is being conducted with Dr. Dina Fonseca at the Walter Reed Biosystematics Unit (WRBU). Dr. Fonseca is submitting a separate STEP U proposal complements this AFRIMS proposal.

3. Methods:

The original proposal indicated that the methods outlined in Section 1 (below) would be used. Unfortunately, we were unable to obtain a large number of cases where were were able to feed all 3 species of mosquito on a single malaria patient. Because of this problem, we decided to expand the study to include an evaluation of P. vivax and P. falciparum genetic diversity in 2 locations in Thailand. Details on the methods used for this portion of the study are provided in Section 2.

1) Role of the anopheline mosquito as a selective force in the transmission of certain strains of parasite:

Anopheles dirus, A. minimus, and A. sawadwongporni mosquitoes were simultaneously fed on blood obtained from Plasmodium vivax or P. falciparum infected volunteers reporting to malaria clinics throughout Thailand. Approximately 30-40 individual mosquitoes of each species (i.e., 90-120 total mosquitoes) were placed in a 1-pint ice cream carton. Unengorged mosquitoes were removed from the carton within 1-hour of feeding. Blood samples were collected from the malaria patients at the time of the mosquito feed for genetic analysis of the Plasmodium circulating in the human donor. In order to evaluate the efficiency of

each mosquito species as a malaria vector, oocyst and sporozoite infection rates and absolute numbers were calculated 10 and 21 days, respectively, after the mosquitoes were infected.

The following procedures were used to determine whether preferential selection of certain Plasmodium strains is occurring in the different mosquito species. Sporozoites were harvested from the mosquitoes 21 days after receiving the infectious blood-meal. DNA was isolated from the original host-blood and from these sporozoites using the Ready-AMP gene kit (Promega). PCR primers and DNA probes from published sources and from those developed at AFRIMS will be used to investigate malaria strain variation in the sporozoites and the infective blood. Emphasis will be place on the CSP, Merozoite Surface Protein -1 (MSP), and/or other heterologous targets. The P. vivax CSP ELISA will be used to differentiate P. vivax-246 from P. vivax-210 sporozoites. Currently chemi-luminescent probes are being used to identify CSP26, CSP27, CSP28 and CSP29 genotypes of P. falciparum from Thailand. It is possible that other genotypes occur in Thailand. Specific probes will be developed based upon sequence data from each of the known genotypes. With the ability to distinguish genotypes, multiple infections can be detected in patients and mosquitoes, and we can determine if differential selection is occurring in the mosquito (comparison of source blood meal genotype with sporozoite genotype). Complementary studies will be conducted by Dr. Fonseca at the Walter Reed Biosystematics Unit; however, the focus of her effort will be based on the use of microsatellite markers as a means of differentiating parasite strains. If it appears that certain mosquitoes are only transmitting certain parasite strains (i.e. the strains that appear in the salivary glands do not reflect all of the strains present in the host blood), subsequent efforts will focus on identifying the site where preferential selection occurs. This will be done by harvesting zygotes/okinetes, oocysts, and sporozoites and characterizing strain variation in each parasite stage. Stock samples of each prototype strain will be preserved at AFRIMS and WRBU and made available for other researchers.

Finally, as part of a longitudinal study on malaria, we will compare genotypic variation in Plasmodium populations from 2 discrete populations: In the immediate vicinity of Sanghlaburi (mobile population with little local transmission) and in the village of of Ban Kong Mong Tha (an isolated, non-mobile population with primarily local transmission). We hypothesize that genetic variation will be minimal in the isolated population and that it will be great in the mobile population.

2. Molecular analysis of Plasmodium diversity in an isolated population in western Thailand:

a. Study site: Samples were collected between May 2000 and August 2001 in the village of Kong Mong Tha, Thailand. The study was approved by the Ethics Committee of the Ministry of Public Health, Thailand, and by the US Army Human Subjects Research Review Board.

b. Patients and sample collection: 646 adults and children (>1 year) were enrolled in the study. Informed consent was obtained from all individuals participating in the study, along with demographic information. During a 4-day period each month, 3 teams of investigators went house to house collecting fingerprick blood-samples from all consenting individuals. Thick/thin blood films were prepared and 5 drops of blood spotted onto filter paper for subsequent molecular diagnosis. Thick and thin films were stained with 10% Giemsa solution

and examined at 1,000x by a microscopist with over 30 years experience. The parasite density was counted per 500 leukocytes and was then expressed as the number of trophozoites per microliter by assuming a leukocyte count of 7,000/• Ý1.

c. Primary DNA extraction: Plasmodium DNA was extracted from patient¡¦s blood dried on filter paper using the saponin-chelex method of Plowe et al. (1995). Briefly, the red blood cells were lysed by 0.5% saponin-PBS solution and the DNA was purified by 20% chelex solution.

d. PCR differentiation of Plasmodium species: Plasmodium parasites were identified by means of nested PCR, with primers selected from the DNA sequence of the small subunit ribosomal DNA gene (Kimura & Kawamoto 2000). The first set of primers (P1F and P2R) are specific for genus Plasmodium; the nested primers (FR, MR, OR, and VR) are species-specific. The first amplification contained P1F and P2R primers and the second one contained P1F as the forward primer and each of the species-specific primers (FR, MR, OR, and VR) as reverse primers. The PCR product was resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide, and observed under ultraviolet transillumination. The expected PCR products are 140-160 bp and 110 bp for the first and second amplification, respectively.

e. PV VK210 vs VK247 differentiation: The primers PV5 and PV6 were selected from the DNA sequence of the circumsporozoite protein (CSP) gene of Plasmodium vivax (Rosenberg et al. 1990). The expected PCR product is 650-700 bp. The differentiation of P. vivax subtype was performed by slot-blot hybridization using specific probes (VK210, 5-S-CCA GCA GGT GAT AGA GCA G-3-S and VK247, 5-S-GGC AAT CAA CCA GGA GCA AAT GG-3-S) (Rosenberg et al. 1990). The chemiluminescence assay (Amersham) was carried out as recommended by the manufacturer. The signal was detected by exposure to X-ray film.

f. Merozoite surface protein-3a (MSP-3a) genotyping: Genotyping of P. vivax using the Msp-3a locus was based on procedures described by Bruce et al. (1999, Am J Trop Med Hyg. 61:518-25). In brief, the protocol uses a combination of nested PCR and restriction fragment length polymorphism (RFLP). Nested PCR amplification of PvMsp-3a genes was carried out in reaction volumes of 20 ul using 1-2 ul of DNA in the primary round and 1 - 0.01 ul of the primary reaction in the nested round. Primary and nested oligonucleotide PCR primers were designed from conserved regions of the PvMsp-3• Ñ gene. Products were visualized under UV illumination after electrophoresis on 0.8% agarose gels containing 0.25ug/ml ethidium bromide, with sizing of products based on a standard curve using DNA markers. For RFLP, ca. 4ul of each PCR product was digested individually with restriction enzymes Hha I and Alu I in 20 ul reaction volumes. DNA fragments were visualized and sized as above.

4. Results:

1) Role of the anopheline mosquito as a selective force in the transmission of certain strains of parasite:

A total of 25 feeds were conducted on P. vivax patients using A. dirus, A. minimus, and A. sawadwongporni. Blood from these patients was frozen at -70 degrees C. Mosquitoes were maintained for 21 days post-feeding and were then stored frozen at -70 degrees C. All of these samples were sent to Dr. Dina Fonseca (Walter Reed Biosystematics Unit,

Washington, DC) for molecular anlaysis of microsatelite markers. Results from her analysis of these samples are reported under MIDRP Proposal #U0025-01-WR (Vectors as bottlenecks: what does knowing the species of mosquitoes present tell us about malaria population genetics).

2) Molecular analysis of Plasmodium diversity in an isolated population in western Thailand:

a. Plasmodium prevalence: 6,886 blood films were collected from 646 individuals (average of 10.6/person) over a 16 month period (May 00 thru Aug 01). By microscopy, 371 (5.4%), 275 (4.0%) and 9 (0.1%) films were positive for P. vivax, P. falciparum, and P. malariae, respectively (Fig 1). P. vivax parasite rates were low (average of 296 trophozoites and 98 gametocytes/ul; mode of 56/ul and 14/ul, respectively), with only 8% of individuals reporting fever or other symptoms at the time the positive blood smear was taken. Mixed infections were relatively rare, with microscopy revealing only 5 of 370 (1.4%) P. vivax-infected individuals concomitantly infected with P. falciparum and PCR revealing 18 (6.7%), 2 (0.7%) and 1 (0.4%) P. vivax-positive individuals co-infected with P. falciparum, P. malariae, and P. ovale, respectively. Molecular analysis of 3,781 filter paper samples demonstrated P. vivax in 276 (7.3%) samples. The type of P. vivax variant (Pv-210 or Pv-247) could be determined in 67% (185) of positive samples, with Pv-210 found in 92% (171) of samples and Pv-247 in 18% (34) of samples. 11% (20) of samples contained both Pv-210 and Pv-247.

b. Msp-3a Analysis (P. vivax): A total of 201 P. vivax samples collected in Ban Kong Mong Tha were used in the study (Attachment 1). Plasmodium DNA was successfully extracted from 131 (65%) of these samples, with allelic diversity at the PvMsp-3a locus successfully evaluated in 93 (71%) of the 131 samples (Attachment 2). There were 88 unique samples and 5 paired samples (taken from the same person on the same day). In order to confirm the reliability of the method, the 5 paired (whole blood and filter paper) samples from the same individual were evaluated. All 5 paired samples produced identical PCR products and RFLP patterns when using restriction enzymes Hha I and Ilu I. We subsequently evaluated samples taken from 13 individuals at intervals ranging from 1-5 months. Identical RFLP patterns were obtained in samples from 5 of these 13 individuals, while in 8 individuals the patterns were different.

Initial analysis revealed a major size polymorphism, with products predominantly of 3 sizes (approximately 1200, 1600 and 1900-2100 bp). Digestion with either Hha I or Alu I yielded fragment sizes that were highly polymorphic between samples, with the sum of RFLP fragment sizes significantly greater than the size of the uncut product in some samples (indicating the presence of more than 1 allele).

The total number of different alleles was not determined in this initial analysis; however, it appears that there are at least 9 different alleles present in this single village. A single allele (Attachment 1: lanes 2, 7, 10, 12, and 14) accounted for over 15% (17/88) of samples. Although this allele occurred throughout the course of the study, 94% (16/17) of isolates were restricted to the eastern (grids A, C, and D), with only 1 isolate found in grid F. Although there is extensive parasite polymorphism in the village of Kong Mong Tha, our preliminary data clearly demonstrates that certain parasite clones are widely circulated. Further analysis will be required to determine whether observed patterns have a temporal or geographic basis.

c. MSP-1 Analysis (P. falciparum): A total of 46 P. falciparum-positive samples collected between Oct 2000 and May 2001 in the village of Ban Kong Mong Tha were sent to Dr. Kazu Tanabe (Osaka Institute of Technology) for analysis of the MSP-1 haplotypes. 94% (43/46) of the samples were positve for MSP-1. Of 24 possible haplotypes, only 8 were found in the study. In contrast, a previous study in Mae Sod found 18 haplotypes. In addition, each individual had a mean of only 1.4 haplotypes, which is lower than that found in Mae Sod (mean of 1.8/person). All 8 haplotypes persisted for the entire period, which suggests that there is little or no gene flow from other areas. One exception was that one haplotype first appeared in March 2001 and persisted therafter, suggesting that this may have been a novel genotype. In fact, this haplotype was first found in a visitor to the area, and was subsequently in 3 village residents. These studies on the genetic diversity of Plasmodium are ongoing.

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Ig. Longitudinal and spatial study on the epidemiology of malaria: Development of a Geographic Information System to assess the threat of malaria transmission by Anopheles mosquitoes.

1. Introduction:

Most malaria vectors in Thailand are members of complexes that consist of morphologically identical species. Species within a complex vary in their vectorial capacity, ecology and behaviour. The primary vectors in Thailand are mosquitoes in the *Anopheles dirus* complex. We are conducting detailed studies on the role of these mosquitoes towards malaria transmission. High priority is placed on the development of molecular tools to identify sibling

species, and on ecological investigations into the distribution and role of *An. dirus* in disease transmission. Studies on the population dynamics of *An. dirus* sibling species clearly indicate that the predominant species in an area is dependent on the ecological habitat and that the prevalence of each species varies with the season.

Remote sensing is a powerful tool that can assess the location and extent of vegetation formations that are difficult to examine on the ground. Instead of using a traditional ground-based approach, this study will use Remote Sensing (RS) and Geographic Information System (GIS) technologies to develop predictors of *An. dirus* sibling species abundance. Abundance will be considered an indicator of potential human vector contact and malaria transmission risk.

A key element in malaria control is the development of new methods for controlling larval mosquitoes. Successful larval control is dependent on the ability to identify larval habitats and distinguish between high and low-producing sites in a timely manner. Larval habitat quality may be inferred by using vegetation as an indicator of environmental condition. Remote sensing data can be used at a regional scale to identify and monitor temporal and spatial vegetation characteristics which can then be used to identify larval habitats.

AFRIMS is the only DoD medical research laboratory that contains all of the resources required to bring this project to successful completion. The following is a summary of the critical resources available at AFRIMS that are required for completion of this project:

- Trained scientific and technical staff: Dr. Jetsumon Sattabongkot heads a 9-person team (1 Ph.D., 1 M.S., 3 B.S. scientists, and 4 technicians) with an average of 14 years experience studying the mosquito transmission of malaria. Mrs. Ratana Sithiprasasna heads a trained 10-person field team (3 M.S., 3 B.S. scientists, and 4 technicians) that has extensive experience with GIS. Dr. Benjawan Khunirat heads an 8-person molecular biology team (2 Ph.D., 3 M.S., and 3 B.S. scientists) that is currently conducting studies on the genetic diversity of *Anopheles dirus* mosquitoes throughout Thailand.

- Proven capability in this area of research: The Department of Entomology, AFRIMS, has a long history of demonstrated (through publication in peer-reviewed journals) excellence on the GIS as a means of studying vector-borne diseases in Thailand and on the epidemiological study of malaria. Selected references are provided.[1-11]

- Immediate access to field sites: Active malaria study sites are maintained in Kanchanaburi, Sanghlaburi, and Mae Sod.

- Approved Human Use Protocols: An approved, ongoing human use protocol (Comparative susceptibility of known and suspected species/strains of Thai *Anopheles* to *Plasmodium* parasites: WRAIR #044, Log #A-6768) that allows for feeding of laboratory-reared anopheline mosquitoes on malaria patients. An additional protocol (Mosquito Acquisition of Malaria (MaM): Role of host and vector factors as determinants of mosquito infection) that specifically supports this project has completed scientific review and is currently undergoing ethical review. This protocol should be implemented NLT February, 2000.

2. Objectives:

The goal of this project is to assess factors affecting the transmission of falciparum and vivax malaria in western Thailand and to develop a Geographic Information System (GIS) to predict malaria threat and as a tool for developing targetted vector control strategies. This study was initiated in August, 1999 in the vicinity of Sanghlaburi, Kanchanaburi Province, Thailand. We are conducting a longitudinal study on the dynamics of malaria transmission and the role of factors that affect mosquito infection with malaria. We are currently developing a GIS that incorporates malaria infectivity, mosquito larval and adult habitats, and environmental data. The ultimate goal is to develop the ability to 1) Use remote sensing (satellite imaging) to identify ecological habitats in Thailand where malaria vectors are found, and 2) Determine if remote sensing and ground data can be integrated into a Geographic Information System (GIS) that can be used to monitor populations of malaria vectors so that malaria threat can be predicted.

3. Methods:

1) This project is a longitudinal study of malaria transmission and control in the village of Ban Kong Mong Tha, Tambon Laivou, Kanchanaburi Province, Thailand. The study was initiated in June, 1999 and is scheduled to continue thru September 2004. The goal of this study is to develop a Geographic Information System that will allow us to identify key areas that are susceptible to targeted malaria control efforts and to subsequently implement selected control measures (as part of the proposed U.S. Army Malaria Vector Control System). The specific aims of this study are to use GIS to:

A. identify larval habitats that produce key vector species (such as Anopheles minimus, A. maculatus, and A. dirus),

B. examine the temporal and geographic distribution of man-biting adult Anopheles mosquitoes to determine whether there is a link between adult mosquito distribution and location of larval habitats,

C. examine the temporal and geographic distribution of human malaria cases to determine whether certain individuals are key to the maintenance of malaria transmission, and whether distribution of certain species of mosquitoes is directly related to malaria distribution,

D. incorporate our findings into a decision matrix that will allow us to identify key areas that are critical to maintaining malaria transmission and that would be susceptible to targeted control efforts,

E. implement selected control measures, and

F. evaluate impact of control measures on mosquito abundance and distribution and on actual malaria prevalence.

2. The following is a summary of methods used in the different components of this study:

A. Site Mapping: LANDSAT images (30 meter resolution) of the village and surrounding area have been acquired and used to establish the GIS using ERDAS software. IKONOS images (1 meter resolution) of the study site are on order.

B. Adult Mosquito Collections: Each month, adult Anopheles mosquitoes are collected while attempting to bite collectors at selected sites throughout the village. The village has been divided into 11 grids (A-I, School, and Wat) -- monthly collections are made at 8 separate sites (Grids A-H). Mosquitoes are identified to species, parity determined, and the abdomen and thorax of each individual mosquito tested by ELISA for P. falciparum, P. vivax-210, and P. vivax-247 circumsporozoite protein. The geographic and temporal distribution of each species is mapped out.

C. Larval Mosquito Collection: Each month, larval Anopheles mosquitoes are collected within the village and surrounding areas to determine if there is an association between larval mosquito habitat and adult mosquito distribution in the town. GPS is used to mark the site of each larval mosquito collection. Larvae are returned to AFRIMS and emerging adults are identified to species.

D. Human Malaria Surveillance: A human use protocol for this project received final approval from the US Army HSRRB and the Thai Ministry of Public Health in April, 2000. Villagers were entered into the study beginning in May, 2000, when baseline demographics and whole blood (200ul/person) from the entire volunteer population was obtained by fingerprick. During each subsequent month (currently May 2000-Nov 2001), 50-75% of the cohort was evaluated for malaria by fingerprick (thick- and thin-blood smears) and dipstick assay to determine Plasmodium prevalence. Additional blood samples were stored on filter paper and in microcapillary tubes for subsequent genetic analysis of parasites and determination of transmission-blocking antibody levels. Individuals positive for malaria in any given month have a 2.5-ml (individuals <10 years old) or 5-ml (individuals > 10 years old) blood sample drawn by venipuncture within 24 hrs of the finger-prick. Approximately 1-2ml of this blood sample is fed to mosquitoes in a membrane feeding system (this will allow us to determine which particular individuals are capable of infecting mosquitoes at that point in time). Duplicate thick/thin blood smears are made at the time of the mosquito feed. An additional sample (1-2 drops) is stored on filter paper for PCR analysis for malaria. The remainder of each blood sample is separated into 0.1ml aliquots for evaluation of factors that might affect transmission. Current focus is on conducting a genetic analysis of the parasite populations using the Merozoite surface protein (MSP) I for P. falciparum and MSP III for P. vivax. The goal is to determine the focality of transmission by tracking the spread of specific Plasmodium clones through the human population.

E. Establishment of the GIS and malaria modeling: Mapping out of the village (houses, road, building, rivers) and larval mosquito habitats has been completed. Current effort focuses on incorporating data (adult and larval mosquito collections, # of people per house, blood films collected, positive blood films, vegetation, etc.) into the GIS. LTC Leon Robert, Dr. Don Roberts and Mrs. Penny Masuoke in the Department of Preventive Medicine, USUHS, are collaborators on this project and are currently establishing the GIS. In addition, Dr. Richard Kiang at NASA is developing a plan for modeling of the interaction of vector mosquitoes and actual malaria. Dr. Simon Hay (Oxford University) and I have discussed his involvement in the project; however, his role has not yet been finalized.

F. Implementation of Malaria Control: Current effort has focused on evaluating the impact of the Thai Ministry of Public Health effort to spray all houses in the village with 5% Deltamethrin. Houses were sprayed in March and October 2000 and March and

July 2001. In addition, permethrin-treated bednets were provided to 123 families in the village in May 2001. The effect of residual spraying and treated bednets on malaria incidence and anopheles mosquitoe abundance is currently being determined.

4. Results:

1) Site Mapping and GIS. A GIS map of the village has been prepared. A total of 117 houses, divided into 9 grids, are included in the study. Demographic information (age, sex, house location, birth-date) on all 713 individuals living in the village has been entered into the GIS data-base. A total of 659 individuals have volunteered to enroll in the study (remaining individuals either chose not to enroll or we have been unable to contact them to ask them to enroll). The GIS data-base currently incorporates LANDSAT images of the site, house, and demographic information. LTC Leon Robert, Dr. Don Roberts, and Mrs. Penny Masuoke in the Department of Preventive Medicine, Uniformed Services University of the Health Sciences (USUHS) are currently preparing the GIS, to include incorporation of mosquito, human, and parasitological data. Dr. Richard Kiang at NASA will be producing land usage criteria and vegetation indices, and will conduct modeling of malaria transmission.

2) Adult Mosquito Collections: Monthly adult mosquito collections have been conducted since June 1999. As of September 2001, a total 9,618 adult anopheline mosquitoes comprising 29 species have been collected; however, 7 species predominate (Attachment 1). Of notable interest, all 3 groups of primary malaria vectors found in Thailand (A. dirus, A. minimus, and A. maculatus groups) are present in significant numbers. ELISAs have been conducted on 8,457 mosquitoes collected between June 1999 and June 2001. 0.2% (17/8,457) of mosquitoes were infected, to include 9/4632 (0.19%) A. minimus, 3/1201 (0.25%) A. maculatus, 2/1248 (0.16%) A. sawadwongporni, 2/478 (0.42%) A. campestris, and 1/2 (50%) A. hodgkini. Mosquito infections included 4 infected with P. falciparum, 8 with P. vivax 210, 3 with P. vivax 247, and 2 with mixed P. vivax 210 and 247. Preliminary statistical analyses have been conducted to determine temporal and geographic distribution of the vectors.

3) Larval Mosquito Collections: Monthly larval collections have been conducted since June 1999. As of September 2001, a total of 7,203 larval mosquitoes comprising 32 species were reared to adults and identified to species (Attachment 2). A total of 37 species (adults and larval collections) of Anopheles mosquitoes have been collected at the study site. Interestingly, approximately >50% (37/72) of the Anopheles species known to occur in Thailand are found in this small (10km2) area. Preliminary statistical analyses have been conducted to determine temporal and geographic distribution of the vectors, and to examine associations between larval mosquito habitats and distribution of adult mosquitoes throughout the village.

4) Human Malaria Surveillance: Between May 2000 and November 2001, a total of 8,268 blood smears were collected from the 659 individuals enrolled in the study. 9.1% (750/8,268) of these slides were Plasmodium positive (324 P. falciparum, 417 P. vivax, and 9 P. malariae) by microscopy (Attachment 3). Gametocytes were present in 15% (49/324), 25% (103/417), and 100% (9/9) of P. falciparum, P. vivax and P. malariae cases, respectively. The distribution (by house and by grid) of these cases has been mapped out (Attachment 4), as has the distribution of gametocyte carriers.

Molecular identification of the Plasmodium species present in 3,781 whole blood filter paper samples (collected between June 2000 and May 2001) has been determined.

Prevalence rates were 3.6% (135/3,781), 7.3% (276/3,781), 0.3% (10/3,781), and 0.05% (2/3,781) for P. falciparum, P. vivax, P. ovale, and P. malariae, respectively. Interestingly, both known strains of P. vivax (Pv-210 and Pv-247) are present at the study site. Molecular analysis revealed that 55% (151/276) of cases were pure Pv-210, 5% (14/276) were pure Pv-247, 7% (20/276) were mixed Pv-210 and Pv-247, and in 33% of cases (91/276) the strain of P. vivax could not be determined.

Current emphasis is being placed on examining the population structure of Plasmodium vivax and P. falciparum in Ban Kong Mong Tha. DNA has been extracted from a total of 201 P. vivax samples and from 46 P. falciparum samples. DNA from the P. vivax samples is is assessed for MSP-3 (in collaboration with Dr. Scott Miller, AFRIMS) while that from the P. falciparum samples is assessed for MSP-1 (in collaboration with Dr. Kazu Tanabe, Osaka Institute of Technology). Although these studies are continuing, our goal is to determine how many alleles of each species are circulating in this village, and whether there is any focality in the distribution of specific alleles. We hypothesize that certain alleles are maintained in extremely focal settings (several proximal houses).

5) Studies on Patient Infectivity: Blood from a total of 104 P. falciparum, 117 P. vivax, 6 P. malariae, and 75 negative (by microscopy) patients was fed to colonized A. dirus mosquitoes in a membrane feeding system (Attachment 4). Only 8.7% (9/104), 6.0% (7/117) and 0% (0/6) of membrane feeds conducted on P. falciparum-, P. vivax-, and P. malariae-infected blood yielded infected mosquitoes. Gametocytes were observed in only 33.3% (3/9) of the infectious P. falciparum samples and in only 28.6% (2/7) of the infectious P. vivax samples. All infections resulted in low oocyst loads (average of 1.2 oocysts per positive mosquito). Only 4.1% (12/296) of mosquitoes fed on the nine infectious P. falciparum samples developed oocysts, while 2.9% (9/311) of mosquitoes fed on the seven infectious P. vivax samples developed oocysts. The probability of a mosquito becoming infected with P. falciparum or P. vivax following a blood meal on a member of the human population of Ban Kong Mong Tha was estimated to be 0.014% (3.9% prevalence x 8.7% infectious x 4.1% mosquito infection rate) and 0.0087% (5% x 6% x 2.9%), respectively. Interestingly, only 0.18% (21/11,837) of mosquitoes fed on these 302 patients became infected. This number is almost identical to the rate found in wild mosquitoes in the village (0.20%; 17/8,457).

6) Initiation of Treatment Program: Although the goals of this project were to evaluate the efficacy of targetted mosquito control, actions taken by the Thai Ministry of Public Health (MOPH) have prevented us from initiating this portion of the project. The Thai MOPH conducted residual spraying with 5% Deltamethrin in all houses in the village on 4 separate occasions in 2000 (18-20 March and 21-22 October) and 2001 (21-23 March and 9-11 July). In addition, the MOPH distributed a total of 123 Permethrin-treated bednets throughout the village during May 2001. We decided to evaluate the impact of these actions on mosquito abundance and malaria rates prior to initiating any of our own control studies. We are currently completing analysis of this data; however, initial results suggest that neither treatment with residual insecticides nor distribution of treated bednets has had a marked effect on either anopheline mosquitoes or malaria.

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1h. Simultaneous identification of vector species and militarily-relevant human pathogens by molecular diagnostics.

1. Introduction:

A number of militarily-important infectious pathogens are vector-borne (transmitted by an arthropod such as a tick, mosquito, or chigger). For example, of the 18 STEPs/STOs that deal with specific infectious disease agents, 12 deal with diseases that are vector-borne. These vector-borne diseases include malaria; dengue and other flaviviruses such as Japanese encephalitis (JE) and West Nile virus (WNV); leishmaniasis; and rickettsial diseases such as scrub typhus and epidemic typhus.

Although the primary focus of the Military Infectious Disease Research Program (MIDRP) is the development of vaccines and prophylactic drugs, for many vector borne diseases neither are available. For example, dengue poses a major threat to military operations throughout much of the developing world. In the absence of a dengue vaccine and prophylactic drugs, control of the arthropod vector is the only practical means of intervention to protect military personnel.

The identification of military threat agents PRIOR to the onset of human disease is a key component of the DoD Preventive Medicine strategy. This is particularly important for diseases for which there are no vaccines or effective prophylactic drugs. In the absence of human disease, the identification of threat agents in arthropods can be used to assess the potential threat and to establish appropriate control measures.

In addition to identifying the vector-borne pathogen, it is important to identify the arthropod that is transmitting it. For example, only certain *Anopheles* mosquitoes can transmit malaria. Each vector species normally is found in a specific type habitat. By identifying the specific arthropod it is possible to focus control efforts (targetted control) on that arthropod and not waste resources controlling non-vectors.

Currently, there is no standardized system capable of simultaneously identifying a vector and determining what pathogens it is infected with. The only means of identifying an arthropod is by having a trained entomologist identify it using a microscope and morphological key. This procedure is laborious and time consuming. Also, the specimen is not available for testing for pathogens until it has been identified. This may significantly delay obtaining required information on the disease threat. In addition to the difficulty in identifying the vector, standardized diagnostic assays are not routinely available for many vector-borne diseases. For example, West Nile virus recently caused an outbreak in New York City but was initially misdiagnosed as Saint Louis encephalitis virus. Other vector-borne diseases are equally difficult to diagnose.

The purpose of this proposal is to develop and standardize molecular diagnostic procedures for the simultaneous identification of the vector and determination whether the vector is infected. Since Step L is developing a "Common Standard Diagnostic Platform" (ABI 7700, Cepheid Smartcycler, Roche Molecular Systems LC32, or ruggedized Idaho Technology RAPIDS Instrument), our goal is to develop a system that can be incorporated into the standard system as rapidly as possible.

Our laboratory has successfully used 16srDNA to differentiate 13 species of *Haemaphysalis,* 4 species of *Ixodes*, 2 species of *Dermacentor* and 2 species of *Amblyomma* ticks (Tom Kollars, unpublished data). We have also used 16srDNA in a RAPD PCR system to successfully differentiate a variety of *Anopholes*, *Culex*, and *Aedes* mosquito species in Thailand. Appropriate primers have been developed, as have methods for preparing arthropod specimens. We have also developed primers for the identification of many of the militarily important vector- and zoonotic-pathogens that occur in SE Asia, to include *Plasmodium falciparum*, *P. vivax*, and *Orientia tsutsugamushi*. Therefore, much of the baseline work required to complete this project has already been initiated.

AFRIMS is the only DoD medical research laboratory that contains all of the resources required to bring this project to successful completion. The following is a summary of the critical resources available at AFRIMS that are required for completion of this project:

- Trained scientific and technical staff: Dr. Thomas Kollars heads an 8-person molecular biology team (2 Ph.D., 3 M.S., and 3 B.S. molecular biologists) that is currently developing molecular diagnostic capability for the most militarily relevant arthropod-borne diseases in SE Asia. Mrs. Ratana Sithiprasasna heads a 10-person team (3 M.S., 3 B.S. scientists, and 4 technicians) with an average of 14 years experience in the taxonomy of vector mosquitoes.

- Immediate access to field sites: Active malaria, dengue, and scrub typhus study sites are maintained in Kanchanaburi, Sanghlaburi, Chaingrai and MaeSod. Field collections are made on a monthly basis at each of these sites. In addition, AFRIMS maintains the largest arthropod collection in SE Asia.

- Proven capability in this area of research: The Department of Entomology, AFRIMS, has a long history of demonstrated (through publication in peer-reviewed journals) excellence the taxonomy of arthropod vectors. Selected references from the last 5 years are provided.[1-8]

2. Objectives:

Develop a molecular method (multiplex PCR) of simultaneously identifying a vector and determining what pathogens it is infected with. A key goal will be to establish sample preparation procedures that can be effectively implemented by Army Field Preventive Medicine Units. Once the basic methodology has been established, a secondary objective will be to optimize the system to permit identification of the primary vectors and pathogens present in SE Asia.

3. Methods:

In order to validate the merit of our proposed strategy for simultaneous identification of vector species and pathogen, initial efforts will focus on a single vector/disease models: Anopheles mosquito transmission of malaria. For each vector/pathogen model, efforts will focus on 5 key objectives: 1) Sample preparation, 2) Vector identification, 3) Pathogen identification, 4) Simultaneous vector and pathogen identification using heteroduplex PCR, and 5) Assay optimization for inclusion in the Standard Diagnostic Platform.

Effort in FY01 will focus on objectives 1-3 (Sample preparation, vector identification and pathogen identification). If successful, eforts in FY02 will focus on objective 4 (simultaneous identification). Objective #5 will be addressed throughout the project by maintaining close contact with the STEP L (Dommon Diagnostic Platform) coordinator.

1) Sample Preparation (FY01): Effort will focus on developing field usable procedures that can be easily implemented by an Army Preventive Medicine Unit. Emphasis will be on standardizing the simplest possible procedures that will allow the Theater Army Medical Laboratory to successfully conduct a molecular analysis of the samples.

2) Vector Identification (FY01): The goal is to develop methods for the identification and differentiation of man-biting Anopholes mosquitoes that are common in Thailand (to include A. dirus, A. maculatus, A. minimus, and others). Initial effort will focus on use of laboratory colonies of arthropods to validate the system; however, field collected specimens of additional arthropod species will also be used. Our ultimate goal is to develop a molecular key to all of the medically important chiggers and the man-biting Anopheles mosquitoes that occur in Thailand (to include A. minimus, A. maculatus, A. dirus, A. sawadwongporni, A. varuna, A. kochi, A. barbirostris, A. nivipes, A. phillippinensis, A. aconitus, A. tessellatus, A. umbrosus, and A. campestris). Representative field-collected specimens of each of these species are available.

Methods for the preparation of DNA samples from both chiggers and mosquitoes have been standardized in our laboratory. Current efforts focus on developing primers based on the 16SrDNA that can differentiate the different Anopheles mosquitoes and Leptotrombidium chiggers. Preliminary results indicate that primers have been identified that can separate Anopheline species using a RAPD procedure. Development of primers for chiggers is ongoing. Heteroduplex mobility assays (HMA) allow for the rapid and cost-effective processing

of multiple specimens. Our laboratory has standardized the use of HMA assays as a means of differentiating vector species based on 16SrDNA.

3) Pathogen Identification (FY01): PCR primers have been developed that will detect and differentiate P. falciparum from P. vivax. Current efforts focus on the development of a well-characterized panel of blinded specimens with which to validate the assay. Procedures for sample preparation have been standardized.

4) Simultaneous Vector/Pathogen Identification (FY02): Our goal is to develop primers that can be used to test for multiple diseases and vectors simultaneously using a multiplex PCR system. Once the separate diagnostic components have been standardized it will be necessary to develop procedures and optimal conditions for the simultaneous identification of vectors and pathogens. We are currently preparing well-characterized panels of infected and uninfected Anopheles mosquitoes to use to validate this system.

5) Incorporation in the Standard Diagnostic Platform: Throughout all phases of this project we will work with the Coordinator of STEP L to ensure that procedures and methods that we have established can be incorporated into the Standard Diagnostic Platform.

4. Results:

1) A set of species specific primers from the ribosomal DNA internal transcribed spacer 2 (ITR2) gene was used to differentiate An. dirus complex species based on the various length of the amplified products (Walton et al. 1999. Med. Vet. Entomol. 13:24-32). Available laboratory rare mosquitoes (An. dirus A and An. dirus B) were employed to verify the specificity of the primers. When the recommended PCR condition was used, a lot of non-specific products were detected. The PCR condition was optimized and the annealing temperature had to be increased to 60 C instead of 55 C to reduce the background. The expect product of 560 bp was detected when DNA from An. dirus A was used and that of 510 bp was observed when DNA from An. dirus B was used. When DNA from Toxorhynchites splendans mosquito was used, no PCR product was obtained. Taken together, the results indicated that the primers are specific for An. dirus complex. However, there are seven known species of the An. dirus complex, five of which have been recognized in Thailand, An. dirus species A, B, C, D, and F. Additional studies with the rest of An. dirus complex as well as other Anopheles species, such as An. minimus, An. sawadwongporni, are required to confirm the specificity of the primers.

2) A set of primers from the 18s ribosomal DNA (rDNA) gene was used to detect Plasmodium DNA in patient• s blood (Kimura et al. 2000. The Toyota Foundation Mini-Symposium on Malaria, Bangkok, Thailand). All four human malaria species showed the expect PCR product of 160 bp. We routinely use these primers for identification of Plasmodium species from patient• s blood and laboratory rare An. dirus A infected with malaria parasite by artificial membrane feeding. Thus, this technique can be used to investigate if the field collected mosquitoes are infected with human malaria parasites.

3) In order to determine if a "one-tube PCR" with two sets of primers (one for mosquito and the other for malaria parasite) could be used to simultaneously identify the species of Anopheles dirus complex and its malaria infection state, the multiplex PCR condition had to be optimized such that there is no interference between these two sets of primers in amplifying each desired target. Since the mosquito ITR2 region contains the secondary structure that is

difficult to denature, addition of certain chemicals to the PCR reaction mixture is needed. Therefore, the condition of the mosquito primers was chosen for the multiplex reaction. Under this condition, the 160 bp product of malaria 18s rDNA was still observed from the An. dirus B DNA sample spiked with malaria DNA. Moreover, the 514 bp product of An. dirus B ITR2 was detected from the same reaction tube. Thus, it appeared that the "one-tube PCR" could be employed in the identification of An. dirus complex species as well as in the detection of malaria parasite present in that mosquito. Further investigation with field collected specimens is pursued to ensure that this multiplex system is practical.

5. References:

None

B. Department of Immunology and Entomology Diseases, AFRIMS FY01 Research Accomplishments

1a. Optimization of sporozoite production to support a human Plasmodium vivax sporozoite-challenge model.

1. Introduction:

Collaborate with the Department of Immunology, AFRIMS, in the development a sporozoite-challenge model for use in the evaluation of candidate *P. vivax* vaccines. The specific objective is to develop methods of producing mosquitoes with consistent, reproducible salivary gland infections that will minimize variation in the course of human infection following sporozoite challenge. A secondary objective is to develop methods to ensure that laboratory-reared *P. vivax* infected mosquitoes (infected by feeding on gametocytemic persons) are not capable of transmitting concomitant infections to challenge volunteers. This latter objective may be a critical requirement for ethical clearance of a vaccine-challenge study.

2. Objectives:

There are currently no reproducible methods of producing *P. vivax* sporozoite-infected mosquitoes that can be used in a GCP-quality Phase II vaccine trial. Use of sporozoite-infected mosquitoes to challenge vaccinated volunteers is preferred over inoculation of sporozoites harvested from mosquitoes. The process by which inoculated sporozoites reach the liver is different from that of mosquito-inoculated sporozoites, and use of mosquitoes to provide the sporozoite challenge provides a more realistic scenario than using harvested sporozoites.

It is possible to infect mosquitoes with *P. vivax* using a variety of techniques; however, none of the currently available methods are acceptable for use in a sporozoite challenge model. Although the *in vitro* culture of *P. vivax* has been reported,[1] recent efforts to re-establish this model at WRAIR have not produced sustained culture or gametocyte production. The use of *P. vivax*-infected monkeys to infect mosquitoes[2] which can then be used for sporozoite challenge of humans is unacceptable, due to the risk of transmission of concomitant infections (i.e. Cercopithecine herpes virus 1 or tuberculosis). Therefore, the feeding of laboratory-reared mosquitoes on individuals naturally infected with *P. vivax* will be the necessary route of

mosquito infection. This approach also has its shortcomings. Specifically, phenotypic and genotypic variation in the vivax strains and donor transmission blocking antibodies produce variability in mosquito infectivity rates. Also feeding on human subjects allows the risks of transmission of other malaria species and real and theoretical risks of transmission of concomitant infections (HBV, HCV, HIV, spirochetes, etc.). Although the risk of a mosquito transmitting a concomitant infection (ingested by the mosquito along with the *P. vivax* gametoctyes) to a volunteer in a vaccine trial is presumably minimal, we believe that it is prudent to investigate methods of minimizing this risk.

Therefore, alternate methods of infecting mosquitoes with *Plasmodium vivax* are desperately required in support of future Phase II vaccine trials. In this study we will focus our efforts on ensuring AFRIMS has a validated model system in place that is capable of routinely producing the required numbers of sporozoite-infected mosquitoes for human challenge and ensuring the safety of those volunteers.

AFRIMS is currently the only laboratory in the world that has all of the required components necessary to fully develop this system. The following is a summary of the facilities and support available at AFRIMS:

- An insectary that produces 40,000 *An. dirus*, *An. minimus*, *and An. sawadwongporni* mosquitoes each month. The AFRIMS insectary is maintained by a staff of 9 individuals whose only mission is to raise mosquitoes (in contrast to other institutions where 1 or 2 part-time individuals work in the insectary). These 9 individuals have an average of over 15 years experience rearing mosquitoes. This insectary has been established for over 30 years. Approximately 25% of the mosquito output of the insectary can be dedicated to support this project.

- A trained 9-person team (1 Ph.D., 1 M.S., 3 B.S. scientists, and 4 technicians) with an average of 14 years experience infecting mosquitoes on malaria patients and quantifying mosquito infections. The primary mission of this team is to study the transmission of malaria. 25% of the effort of this team can be dedicated to support this project.

- The Department of Entomology currently feeds an average of 6,000 mosquitoes on 30-50 *P. vivax*-infected patients each week. Studies requiring mosquitoes infected by feeding on *P. vivax* patients have been conducted since 1982. Since 1982, over 50% of all mosquito feeds on *P. vivax*-infected patients have resulted in infected mosquitoes. This long, continuous history of excellence in this field exceeds that available anywhere else in the world.

- Active malaria study sites in Kanchanaburi, Sanghlaburi, and Mae Sod. Laboratory-reared mosquitoes are routinely (weekly) infected on *P. vivax*-infected patients at each of these sites. Mosquitoes are routinely infected by either "direct feeding" (allowing mosquitoes to feed on a *P. vivax*-infected patients) or by "indirect feeding" (drawing blood from infected patients and allowing mosquitoes to feed on the blood using a membrane feeding system). Field laboratories are available at each of these sites.

- An approved, ongoing human use protocol (Comparative susceptibility of known and suspected species/strains of Thai *Anopheles* to *Plasmodium* parasites: WRAIR #044, Log #A-6768) that allows for feeding of laboratory-reared anopheline mosquitoes on *P. vivax*-

infected patients. This human use protocol allows for the production of sporozoite-infected mosquitoes for use in this study.

- A long history of demonstrated (through publication in peer-reviewed journals) excellence on the mosquito transmission of *Plasmodium vivax.* A list of selected references is provided.[3-16]

3. Methods:

The objectives of this project require 24-months for completion. This project is based on 3 sequential series of objectives. Objective #1 was completed in FY01, while Objectives #2-3 will be completed in FY02. Currently, P. vivax sporozoites and/or P. vivax-infected mosquitoes are available to DoD investigators for malaria studies at any time. Coordination for shipment of sporozoites and/or infected mosquitoes should be through LTC Gray Heppner (WRAIR) and LTC Russ Coleman (AFRIMS).

1) Basic Sporozoite Challenge System (Development completed in FY01): We have established a "basic system" to provide sporozoite-infected mosquitoes in support of STEP/STO requirements. This system is 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 feed 100-200 mosquitoes on each patient with detectable gametocytes. These studies are conducted weekly at Kanchaburi, Sanghlaburi, or Mae Sod MOPH malaria clinics. Infected mosquitoes are returned to AFRIMS and maintained in the AFRIMS insectary. 5-10% 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 (Development completed in FY02): Once we have established our "basic system," subsequent efforts will focus on 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." Instead of allowing mosquitoes to feed directly on gametocytemic patients, 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 patient blood and the membrane feeding system. Each of these experiments will provide "stand-alone" data to support the development of the sporozoite-challenge model; however, each experiment will build upon the information derived from the previous experiment. A description of each experiment follows:

a. Dilution of Blood to a standard Gametocyte Concentration: The number of gametocytes per 500 white blood cells (WBCs) will be calculated from the thick blood

smear. Blood group will be checked, and autologous uninfected blood will be used to dilute the infected blood to several defined gametocyte concentrations (i.e. 5, 10, and 20 gametocytes per 500 WBCs). Mosquitoes will be fed on each diluted blood sample, and mosquito infection rates compared to that of the original patient blood sample. This experiment will be repeated with 10 different patients.

b. Pooling of Blood Samples from 5 Infected Patients: Blood will be collected from 5 separate patients, pooled, and used to infect mosquitoes using a membrane feeding system. Controls will consist of blood from each separate patient. Percent of mosquitoes with oocysts, mean number of oocysts per mosquito, percent of mosquitoes with sporozoites in the salivary glands, and mean number of sporozoites in the glands will be the criteria used to evaluate variability. We hypothesize that pooling blood from infected patients will reduce inherent variability in the 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.

c. 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 (10) and replaces patient sera that is potentially infected with concomitant acellular pathogens with commercial sera that is known pathogen-free.

d. Use of Frozen Gametocyte Preparations to infect Mosquitoes: One of the key factors that limits production of P. vivax-infected mosquitoes is the requirement for fresh blood that contains infective gametocytes. Since there is no standardized in-vitro system capable of routinely producing infective gametocytes, this means that we must either bring the mosquitoes to the gametocytemic patient or collect fresh blood from the gametocytemic patient and allow mosquitoes to feed on it within 24 hours. A thorough review of literature back to 1966 found no published studies that have evaluated the ability of cryopreserved gametocytemic blood to infect mosquitoes. However, erythrocytic stages Plasmodium are routinely cryopreserved, while Collins et al. (18) were able to use frozen sporozoite preparations to infect non-human primates with the Salvador I strain of P. vivax. In this portion of the study we propose to evaluate the ability of cryopreserved gametocytemic blood to infect mosquitoes. Effort will focus on establishment of cryopreservation techniques that will maximize gametocyte viability and on procedures to maximize the percentage of mosquitoes that will feed on the frozen blood. Development of procedures to infect mosquitoes using cryopreserved blood 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).

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)(17).

4. Results:

We have established routine procedures for infecting mosquitoes with naturally-circulating strains of Plasmodium vivax and P. falciparum. In FY01 (1 Oct 2000 - 30 Sep 2001) a total of 227 mosquito feeds were conducted using blood obtained from P. vivax patients, while 30 feeds were conducted on P. falciparum patients. A summary of P. vivax and P. falciparum feeding success is presented in Attachment 1. A total of 252,374 anopheline mosquitoes were fed on blood obtained from these patients, with 112,045 (44%) of mosquitoes taking a blood meal (Attachment 2). From these feeds, a total of 8 million P. falciparum and 79 million P. vivax sporozoites were harvested in FY01 (Attachment 1).

In addition to establishing routine procedures for production of infected mosquitoes and sporozoites, we have conducted a variety of experiments/studies to 1) refine our protocol, and 2) use the established procedures to evaluate the efficacy of candidate vaccines and drugs. Results from these experiments are briefly summarized below.

1) Ensure relationship between direct feeding and membrane feeding: A total of 285 paired-feeds (comparison of direct vs membrane feeds using blood from 286 patients) were conducted. There was 83% (237/286) concordance between the 2 methods. In 17% (49/286) of the feeds there was some degree of discordance between results (typically due to all mosquitoes being negative by one method while a small percentage were positive by the other method). A manuscript from this study has been prepared and is under review (see reference list below).

2) Ensure reproducibility of membrane feeding system: A series of studies was conducted to determine the effect of various facors on mosquito feeding success (Attachment 3). The following is a summary of our results:

a. Concordance in infection rates when blood from a single patient was placed in multiple mosquito feeding chambers (Attachment 3, Table 1): Blood was collected from 99 individual patients in FY01, and the blood from each individual patient placed in multiple mosquito feeding chambers (range 3-22 aliquots). Groups of 100 mosquitoes were fed on each of these separate chambers, and infection rates determined. In 82% (81/99) of these patients there was a high degree of concordance in the number of mosquito feeding chambers that actually infected mosquitoes (in 19 cases all cups yielded all positive or all negative results, while in 62 cases >75% of cups yielded positive mosquitoes). However, in 18% (18/99) of the cases there was some disagreement (i.e., some of the aliquots resulted in positive mosquito feeds while others resulted in negative feeds). Undertanding the natural variation inherent in the membrane-feeding system is critical if we are to evaluate candidate vaccines.

b. Effect of mosquito age (Table 2), duration of feeding (Table 3), number of mosquitoes per carton (Table 4), length of time blood was held prior to feeding mosquitoes (Table 5), and effect of replacing patient sera with sera from donors with different blood groups (Table 6) were evaluated (these Tables are all found in attachment #3). None of these factors had any effect on the efficacy of the membrane feeding system.

3) Since FY00 we have been evaluating the efficacy of transmission-blocking drugs: Ongoing studies have evaluated the efficacy of selected transmission-blocking drugs (primaquine, tafenoquine, WR-250547, and others) using blood collected from P. vivax-infected individuals. Results clearly demonstrated that the procedures described above could be used to compare the efficacy of these drugs (Coleman et al., Submitted to the ASTMH).

4) Evaluation of transmission-blocking vaccines: Since FY00 we have also been evaluating the efficacy of selected transmission-blocking vaccine candidates using blood collected from P. vivax-infected patients. These studies have been conducted in association with scientists at the NIH Malaria Vaccine Development Unit (COL Alan MaGill) and at Ehime University in Japan. Blood was collected from P. vivax-infected patients, sera separated from the packed red blood cells, and the packed red blood cells reconstituted with sera from mice or rabbits vaccinated with an anti-ookinete vaccine. In this study we evaluated the effect of various concentrations of sera from the vaccinated animals on mosquito infectivity, as well as the effect of various adjuvants that were used when immunizing the host animals. Results clearly demonstrated that the assay system we developed provided quantifiable, reproducible results -- i.e., mosquitoes fed on blood reconstituted with control sera became infected, while mosquitoes fed on blood reconstituted with sera from the vaccinated animals did not become infected or developed very low infections. This study is continuing, and we are currently analyzing the data.

5) Studies on freezing of gametocytes: Gametocytemic blood from approximately 25 P. vivax patients was collected by venipuncture and frozen. Upon return to Bangkok, the blood was thawed out and placed in a membrane feeding system. None of the mosquitoes fed on these blood samples became infected. We subsequently placed thawed out samples into short-term in vitro culture and let the parasites develop for a period of 1-3 days. None of the mosquitoes fed on these samples became infected. Research on this critical area continues -- if successful, having the ability to infect mosquitoes from frozen blood will allow us to have samples sent to us from anywhere in the world. Sporozoites obtained from mosquitoes fed on these samples can then be used in various studies to evaluate drug or vaccine efficacy.

6) Molecular characterization of Plasmodium vivax: Current emphasis is being placed on examining the population structure of Plasmodium vivax and P. falciparum in Ban Kong Mong Tha (a small village in western Thailand). DNA has been extracted from a total of 201 P. vivax samples and from 46 P. falciparum samples. DNA from the P. vivax samples is being assessed for MSP-3 (in collaboration with Dr. Scott Miller, AFRIMS) while that from the P. falciparum samples is assessed for MSP-1 (in collaboration with Dr. Kazu Tanabe, Osaka Institute of Technology). Although these studies are continuing, our goal is to determine how many alleles of each species are circulating in this small village, and whether there is any focality in the distribution of specific alleles. In addition to the above study, we are also assessing the population structure of P. vivax in the Mae Sod region. A total of 50 P. vivax samples have been assessed for MSP-3 -- results are pending.

5. References:

Suwanabun N, J Sattabongkot, T Tsuboi, M Torii, N Maneechai, N Rachapaew, N Yim-amnuaychok, V Punkitchar and RE Coleman. 2001. Plasmodium vivax: In vitro culture of ookinetes from field isolates. J Parasitol. 87: 928-930.

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Sattabongkot J, N Maneechai, V Phunkitchar, N Eikarat, B Khuntirat, J. Sirichaisinthop, and RE Coleman. Comparison of artificial membrane feeding with direct skin feeding to estimate infectiousness of Plasmodium vivax gametocyte carriers to mosquitoes. Amer. J. Trop. Med. Hyg. (In Review).

1b. Development of an in vitro assay to evaluate the exo-erythrocytic activity of antimalarial drugs against Plasmodium falciparum.

1. Introduction:

Malaria remains one of the most significant military threats in many areas of the world. Control of the disease is complicated since i) malaria parasites have developed resistance to many drugs used for treatment and ii) effective vaccines are not available. One of the priorities of the Military Infectious Disease Research Program (MIDRP) is to develop effective methods for monitoring the efficacy of new antimalarial drugs and vaccines.

Exoerythrocytic stage *Plasmodium* parasites are of key interest to STEP/STO Q (Malaria drug discovery) and F (Malaria vaccine research). Since the primary goal of STEP F is to prevent all clinical manifestations of malaria, STEP F research is focused on "antigens expressed by irradiated sporozoites within hepatocytes and on the surface of sporozoites." Likewise, EE stage parasites are a key target of the STO Q research effort. However, in spite of the need for a model system with which to evaluate the effect of either antimalarial drugs or a malaria vaccine, no standard system has been established.

Hepatocyte invasion by malaria sporozoites involves specificity between sporozoites and hepatocyte surface membranes (Hollingdale et al., 1983, Scheller et al., 1995), implying that not all hepatocytes or hepatoma cells are suitable hosts for parasite development. Cultivation of liver forms of falciparum malaria has been established in primary hepatocyte cultures of humans (Mazier, et al., 1985) and a new world monkey (Millet, et al., 1991). Complete development of *P. falciparum* liver stages in a human hepatoma cell line has been reported (Kanasuta, et al 1995) but the infection rate was very low (0.009%). Unfortunately, these models do not yield reproducible results or an easily quantifiable infection rate and are thus impractical for drug efficacy tests or other studies involving hepatic stage parasites.

In order to establish reproducible, highly efficient production of EE-stage *P. falciparum* parasites, the following 3 requirements must be met: 1) production of infective gametocyte cultures with which to infect mosquitoes, 2) production of large numbers of infective sporozoites from mosquito salivary glands, and 3) development of stable hepatoma cell lines that can be maintained in continuous culture. Once these requirements have been met it will be possible to standardize a system of producing EE-stage parasites for drug or vaccine studies.

The Departments of Entomology and Immunology, AFRIMS have standardized the production and maintenance of *P. falciparum* gametocyte cultures and methods for the infection of anopheline mosquitoes. The Department of Entomology routinely harvests *P. falciparum*

sporozoites from these mosquitoes. In addition, *P. vivax* sporozoites are routinely obtained from mosquitoes fed on gametocytemic patients reporting to Thai malaria clinics. As part of an ILIR project (FY98-FY00: Development of human hepatoma cell lines for high density production of exoerythrocytic stage of human malaria, *Plasmodium falciparum*), Dr. Jetsumon Prachumsri has recently established a long-term culture of a human hepatoma cell line (Cell Line ID#: HC-04, Passage 63, >17 months in continuous culture). Reproducible infection rates of 0.5-1.0% are achieved using an inoculum of $5x10^4$ sporozoites. These infection rates are significantly (approximately 100-fold) higher than those previously reported. In addition, trophozoite infection of red blood cells overlaid onto the hepatoma cell lines has been observed, indicating that complete *in vitro* cultivation of EE-stage *P. falciparum* parasites has been achieved.

In this study we will refine the culture system as established under Dr. Jetsumon's ILIR. Primary emphasis will be placed on i) modifying sporozoite inoculation and hepatoma culture procedures to increase infection rates to an even higher level and ii) developing a "user-friendly" method of quantifying EE-stage infection rates. Once this system is standardized to produce maximum yield of EE-stage parasites, subsequent efforts will focus on use of the model system in drug efficacy and vaccine development studies and to provide further understanding of the biology and genetics of exoerythrocytic stage parasites.

2. Objectives:

Transition a successful ILIR project (FY98-FY00) into the core MIDRP program. Our objective is to establish a standard method of evaluating the exo-erythrocytic (EE) activity of antimalarial drugs. The method will be based on a continuous culture system for maintaining human hepatoma cell lines capable of producing exo-erythrocytic stage *Plasmodium falciparum* parasites.

3. Methods:

Primary focus of this project will be to optimize established procedures for the production of EE-stage P. falciparum parasites. Success will be defined as a reproducible (>75% of inoculations) 10-fold increase in the number of EE-stage P. falciparum parasites observed in hepatoma cell culture (an increase from the currently achievable level of 0.5-1.0% infection rates following inoculation of 50,000 sporozoites to a 5-10% infection rate).

Optimization procedures will focus on i) preparation of sporozoites (diluents, time from harvesting to inoculation, etc.), ii) processing of sporozoites (addition of Kuppfer cells to hepatoma cultures), and iii) culture media/procedures.

Secondary focus of the project will be to develop simplified procedures for quantifying EE-stage parasites in the hepatoma cells. Current procedures (preparation of thick and thin films, staining, and subsequent counting infected cells) are laborious, time-consuming, and require an experienced microscopist. Emphasis will be placed on the development of a monoclonal-antibody based ELISA and/or quantitative (TAQ-Man) PCR.

Finally, emphasis will also be placed on modifying procedures used for the production of EE-stage P. falciparum parasites so that a standard model can be developed for EE-stage P. vivax parasites.

4. Results:

The Departments of Entomology and Immunology, AFRIMS have standardized the production and maintenance of P. falciparum gametocyte cultures and methods for the infection of anopheline mosquitoes. The Department of Entomology routinely harvests P. falciparum sporozoites from these mosquitoes. In addition, P. vivax sporozoites are routinely obtained from mosquitoes fed on gametocytemic patients reporting to Thai malaria clinics. As part of an ILIR project (FY98-FY00: Development of human hepatoma cell lines for high density production of exoerythrocytic stage of human malaria, Plasmodium falciparum), Dr. Jetsumon Prachumsri (Sattabongkot) has recently established a long-term culture of a human hepatoma cell line (Cell Line ID#: HC-04; a patent application for this cell line has been submitted). Reproducible infection rates of 0.5-1.0% in this cell line are achieved using an inoculum of 50,000 sporozoites. These infection rates are significantly (approximately 100-fold) higher than those previously reported. In addition, trophozoite infection of red blood cells overlaid onto the hepatoma cell lines has been observed, indicating that complete in vitro cultivation of EE-stage P. falciparum parasites has been achieved.

In FY00 we conducted studies on P. vivax exo-erythrocytic development and found that cell line HC-04 support development of EE-stage P. vivax. In initial experiments, HC-04 cells resulted in a higher level of sporozoite invasion 4-days post inoculation than did HepG2-A16 cells. The mean invasion rates were 0.23 % (range 0.13-0.39%) and 0.73% (range 0.48-0.99%) for HepG2 and 0.36% (range 0.18-0.64%) and 1.69% (range 0.39-3.0%) for HC-04 cells. Early-stage trophozoites were observed in red blood cells overlaid on the hepatoma cells 10 days after sporozoite inoculation. These results clearly demonstrated that exoerythrocytic merozoite development had occurred. Although invasion rates of the red blood cells were low, this may have been due to the fact that P. vivax normally invades young red cells or reticulocytes. Red blood cell invasion rates may be improved if reticulocytes are used for the cultures.

In FY01 efforts focused on refining the hepatoma culture system, with primary emphasis placed on 1) modifying sporozoite inoculation and hepatoma culture procedures to increase infection rates to an even higher level, and 2) developing an improved method of quantifying EE-stage infection rates. Primary focus of this project was to optimize established procedures for the production of EE-stage P. falciparum and P. vivax parasites. Success was defined as a reproducible (>75% of inoculations) 10-fold increase in the number of EE-stage P. falciparum and P. vivax parasites observed in hepatoma cell culture (an increase from the currently achievable level of 0.5-1.0% infection rates following inoculation of 50,000 sporozoites to a 5-10% infection rate). Optimization procedures focused on 1) screening additional hepatoma cell lines that may yield higher invasion rates, 2) preparation of sporozoites (diluents, time from harvesting to inoculation, etc.) for use in the assay, and 3) identification of culture media/procedures that will enhance EE-stage parasite production. A Summary of results from these experiments is provided in Table 1-3. Although no candidate vaccines have yet been tested in this model system, we have evaluated the activity of candidate antimalarial drugs. Results are promising, and it should be feasible to evaluate sera from vaccinated individuals (or animals) at any time.

Secondary focus of the project was to develop simplified procedures for quantifying EE-stage parasites in the hepatoma cells. Results from these experiments are summarized below:

1) During FY01, it was established by using slot blot analysis using stage specific probes, PfA and PfC (Li et al. 1993. Exp. Parasitol. 76:32-38), that two types of ribosomal RNA (rRNA) are expressed in different developmental stages of P. falciparum. Type A rRNA is preferentially expressed during the asexual stage (blood stage), whereas type C or type S rRNA is dominantly expressed during the sexual stage (sporozoite).

2) Stage specific primers and probes for TaqMan analysis were designed from P. falciparum small subunit (SSU) rRNA region. The specificity of the primers and probes were determined by using sporozoites and blood stage culture of P. falciparum. Type C primers and probe could detect fluorescence signal from sporozoites but not from the blood stage parasites. Whereas, type A primers and probe could detect the signal only from blood stage parasites but not from sporozoites.

3) While the TaqMan primers and probes were analyzed for their specificity, conventional RT-PCR using P. falciparum SSU rRNA primers was performed on total RNA samples from sporozoite-infected and uninfected HC04 cells with and without drug treatment to estimate if the drugs would affect parasite development. It appeared that of all the drugs tested (BN66378, KKPII-61, Primaquine, and Tefnoquine), Primaquine is the only one that has no effect on the parasite development. The 400 bp RT-CR product from sporozoite-infected cells with Primaquin treatment was observed at the similar level as that from infected cells without treatment. However, no RT-PCR product was detected from infected cells treated with other drugs.

4) To exclude the possibility that the drug (other than Primaquine) treatment has an effect on the host cells not the parasites, RT-PCR of a house keeping gene (GAPDH) was carried out from infected and uninfected cells with and without treatment. It was found that the 160 bp RT-PCR product was detected from all samples tested with no significant difference in their signals. Therefore, drug treatment as well as infection has no effect on the normal function of the cells. Taken together, it is likely that the drugs, BN66378, KKPII-61, and Tefnoquine but not Primaquine, could prevent P. falciparum development in liver cells. The molecular mechanism of this prevention has to be further investigated.

5. References:

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1c Tafenoquine (WR-238605) and artelinic acid: Evaluation of transmission-blocking activity against Plasmodium falciparum and P. vivax.

1. Introduction:

Previous laboratory studies have clearly demonstrated that tafenoquine (WR-238605) has the capacity to prevent the transmission of *P. berghei* and chloroquine- sensitive and multi-drug resistant *P. falciparum* (Coleman, 1990; Coleman et al., 1992, 1994). In addition, artemisinin and its derivatives have a significant effect on gametocytogenesis, both in laboratory (Kumar & Zheng, 1990; Mehra & Bhasin, 1993) and field (Chen et al., 1994) studies.

Tafenoquine and artelinic acid are two of the most promising antimalarial compounds currently under development by the U.S. Army. Tafenoquine is currently entering Phase III clinical trials, and artelinic acid is scheduled to enter in Phase I trials this year. Although tafenoquine appears effective as both a radical curative and a prophylactic compound and artelinic acid is viewed pimarily as a radical curative compound, both agents have the potential to impact on malaria transmission. As such, they may have tremendous public health implications.

In addition to their primary role in reducing malaria morbidity and mortality, antimalarial drugs that prevent mosquito transmission of malaria can in theory prevent the spread of drug-resistant parasites. The mechanism by which a gametocytocidal and/or sporontocidal agent acts is distinct from the mechanism by which a schizontocidal compounds acts (Coleman et al., 1994). In drugs that exhibit both schizontocidal and gametocytocidal/sporontocidal properties, resistance to the schizontocidal properties is not necessarily correlated with resistance to the gametocyticidal/sporonticidal properties of the compound. In other words, even if schizontocidal resistance emerges against tafenoquine or artelinic acid, the gametocytocidal/sporontocidal activity of the drug may prevent transmission of the resistant parasites. Hypothetically, this could significantly delay the emergence and spread of resistance to these compounds.

Traditionally, the transmission-blocking activity of antimalarial drugs is evaluated by either assessing malaria infection rates in field collected mosquitoes or by feeding mosquitoes directly on malarious patients treated with a given compound. Using data on malaria infection rates in field collected mosquitoes to demonstrate gametocytocidal/sporontocidal activity is difficult at best – infection rates are normally so low it is impossible to statistically demonstrate that a drug has reduced the mosquito infection rate. Treating of malarious patients with a drug and then evaluating the gametocytocidal/sporontocidal activity of the compound requires that the drug either be in phase 3 clinical trials or already FDA approved for use. In addition, it is then necessary to repeatedly feed mosquitoes directly on the infected patients (something many patients are not willing to permit) and variability in the drug levels of individual persons must be taken into account afterwards.

2. Objectives:

Precisely quantify the transmission-blocking (gametocytocidal and sporontocidal) activity of tafenoquine (WR-238605) and/or artelinic acid using clinical (drug-treated) human sera samples and naturally occurring strains of Plasmodium falciparum and P. vivax.

3. Methods:

This project will focus on the development of a model with which to assess the tranmsission-blocking activity of experimental compounds. In order to validate the model, we will precisely define gametocytocidal and sporontocidal activity of tafenoquine and/or artelinic acid using clinical sera samples obtained from malaria-naive patients treated with a particular drug (i.e. Phase I trials). In order to evaluate gametocytocidal activity, blood will be drawn from malaria patients, packed red blood cells (RBC) will be separated from the sera, and the packed RBCs will then be reconstituted with the drug-containing sera samples. The validity of this technique will be ascertained in an initial series of experiments. In order to evaluate sporontocidal activity, mosquitoes will be infected on malaria patients and then allowed to re-feed on RBCs reconstituted with sera from the drug-treated patients.

Clinical sera samples will be obtained from patients treated with tafenoquine (a single 500mg dose) and/or artelinic acid (dose to be determined, but representative of normal curative dose). If sufficient sera samples are not currently available from pharmacokinetic studies, a human use protocol will be submitted that will allow us to collect these samples. Samples will be collected at 5 time-points: at peak level and at each of 4 drug halve-lives afterward. Samples will be required from 5 volunteers treated with each drug, with a total serum volume of 10ml required from each patient at each time point. For each of the 5 volunteers, multiple (approximately 20) 250ul aliquots of serum from each of these 5 time points and a control (pre-treatment) bleed will be stored at -70°C.

Assessment of Gametocytocidal Drug Activity: 10ml blood samples will be collected from patients reporting to local malaria clinics in Mae Sod or Kanchanburi, Thailand. Serum will be separated from the packed RBCs, and the packed RBCs (approximately 5ml) separated into twenty (20) 250ul samples. These samples will be reconstituted with drug-treated or control sera (as outlined in the previous paragraph) from 3 volunteers (5 drug-treated samples from different time points + 1 control/volunteer x 3 volunteers = 18 samples total). The 18 reconstituted blood/sera samples will be placed individually into a glass membrane feeder, and 50-100 mosquitoes fed on each sample. After feeding, unengorged mosquitoes will be removed, and oocyst and sporozoite infection rates determined 10 and 21 days later.

Sporontocidal Drug Activity: Mosquitoes will be infected by feeding directly on patients with malaria (Human Use Protocol WRAIR #044, Log #A-6768; Comparative susceptibility of known and suspected strains of Thai Anopheles to Plasmodium parasites). 4 days later, these mosquitoes will be fed on uninfected blood reconstituted with drug-treated sera (as in the previous paragraph). Infection rates will be determined by determining oocyst and sporozoite rates on day 10 and 21 of the infection, respectively.

These experimental procedures will allow us to determine the precise serum drug-levels that will be capable of preventing malaria transmission. Although serum drug concentrations are lower than that contained in the whole blood, gametocytes are normally exposed to drug in the sera, while sporogonic stage parasites are only exposed to drug that can penetrate the mosquito midgut and interact with the oocysts and or sporozoites.

4. Results:

The sporontocidal activity of tafenoquine, primaquine, artelinic acid, and WR-250547 was determined against naturally circulating isolates of Plasmodium vivax. Laboratory-reared Anopheles dirus mosquitoes were infected with P. vivax by feeding them on gametocytemic volunteers reporting to local malaria clinics in Tak province, Thailand. Four days after the infectious feed, mosquitoes were re-fed on uninfected mice treated 90 minutes previously with a given drug at a dose of 100 mg base drug/kg mouse body weight. Sporontocidal activity was determined by assessing both oocyst and sporozoite development. Primaquine was included as a negative control (previous studies reveal no sporontocidal activity) and WR-250547 was included as a positive control (previous studies reveal significant sporontocidal activity). Neither primaquine nor artelinic exhibited sporontocidal activity against P. vivax at a dose of 100mg/kg mouse body weight, whereas both tafenoquine and WR-250547 affected sporogonic development at this dose (Table 1).

A subsequent set of experiments determined the Minimum Inhibitory Dose (mg/kg mouse body weight) of tafenoquine and WR-250547 that affected sporogonic development. Tafenoquine had activity at doses as low as 12.5mg/kg (Table 2), while WR-250547 had activity at doses as low as 0.39 mg/kg (Table 3).

A final set of exeperiments evaluated the effect of these drugs when given 4, 8, 11, or 16 days after mosquitoes had been infected. Tafenoquine was only effect when given on day 4 (Table 4), while WR-250547 was effective on days 4 and 8 (Table 5).

5. References:

Coleman RE, N Polsa, N Eikerat, TM Kollar, Jr. and J Sattabongkot. 2001. Prevention of sporogony of Plasmodium vivax in Anopheles dirus mosquitoes by transmission-blocking antimalarials. Amer. J. Trop. Med. Hyg. 65: 214-218.

Polsa N, J Sattabongkot, P Kittayapong, N Eikerat and RE Coleman. Sporontocidal activity of selected antimalarials against Plasmodium vivax. Amer. J. Trop. Med. Hyg. (In preparation)

C. Department of Enteric Diseases, AFRIMS FY01 Research Efforts.

1a. Surveillance for Antimicrobial Resistance patterns of Enteric pathogens in Thailand, Vietnam and Nepal.

1. Introduction:

Much of the emerging antibiotic resistance observed today in the world originates in Southeast Asia. Southeast Asia is a patchwork of countries with differing economic development and differing approaches to health care & agriculture leading to widely varying exposures to antimicrobial agents. The region's extraordinarily high population density, continued close proximity to domestic animals by much of the population, and marginal public works provides ample opportunities for the transfer of genetic information between microorganisms. Unfortunately, surveillance for the development of antimicrobial resistance is

sporadic and characterization of the underlying mechanisms for the observed resistance is virtually nonexistent.

Diarrhea and dysentery remain a major cause of morbidity and mortality in young children in Southeast Asia. In population, clinic, or hospital based studies of the etiology of acute diarrhea and dysentery, generally from one-third to over one-half of the etiologic agents remain unidentified. Improper application and inadequacy of available diagnostic methods and culture techniques is a contributing factor. How much may be due to unrecognized pathogens or pathogenic variants of known species is unknown. The importance of Campylobacter species, for example, was not understand until the early 1980s and similarly Cyclospora until the early 1990s but both continue to be under diagnosed.

The Department of Enteric Diseases at the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand was founded to better understand diarrheal diseases in Southeast Asia. The department has conducted field and clinical studies and surveillance in Thailand, Vietnam, Bangladesh, Nepal, and elsewhere in Asia. The laboratory maintains active microbial and tissue culture capabilities, immunodiagnostics, and molecular techniques ranging from PCR through AFLP to sequencing. The department has collaborations with regional governmental and non-governmental organizations, as well as academic organizations in Asia and the US. Currently, there are five doctoral candidates working in the department at AFRIMS.

2. Objective:

Surveillance for the development of antimicrobial resistance

3. Methods:

Six different human populations were selected for study: US soldiers reporting to medical clinics with diarrhea (153 cases) during the Cobra Gold 2001 exercise held in northern Thailand (Phitsanulok); adult travelers with acute diarrhea and controls (100 cases and 55 controls) presenting to the CIWEC clinic in Kathmandu, Nepal; adult travelers with acute diarrhea and controls (207 cases and 207 controls) presenting to Bumrungrad Hospital in Bangkok, Thailand; Thai adults with acute diarrhea and controls (178 cases and 178 controls) presenting to Bumrungrad hospital in Bangkok, Thailand; children under 5 years of age with acute diarrhea and controls (106 cases and 99 controls) presenting to St.Paul's Hospital in Hanoi,Vietnam and children under 5 years of age with acute diarrhea and controls presenting to Kwai River Christian Hospital, Sangkhlaburi, Thailand (Enrollment started in October 2001)

The adult, non-immune population of US soldiers deploying to Thailand for Cobra Gold closely resembles travelers. The study for Cobra Gold in 2001 was conducted in Phitsanulok province in the northern part of Thailand. A treatment study for traveler's diarrhea comparing the efficacy of azithromycin and ciprofloxacin was continued from last year as well as the study of acute diarrhea etiology and immune response. Previous data had suggested that many Campylobacter isolates in Thailand were resistant to ciprofloxacin. The cases for the Cobra Gold study were recruited from personnel presenting with acute diarrhea to one of the clinic study sites.

CIWEC is a private clinic in Kathmandu, Nepal serving expatriates and travellers. This clinic continues to be the busiest travel clinic in Kathmandu with about 6000 patient visits per year. Approximately 30% of patients have diarrhea.

Bumrungrad Hospital was selected as it serves as the main referral hospital for foreigners residing in and visiting Bangkok, receiving about 400 foreign outpatient visits per day with about half from North America, Europe, Australia, and Japan. Approximately 20% of this population suffers from diarrheal disease. This hospital also serves the middle to upper class Thai population of Bangkok. Cases were adult travelers or expatriates from developed nations and Thai adults with acute diarrhea. Controls were recruited from non-diarrhea patients.

Saint Paul's Hospital is one of the major city hospitals in Hanoi. This 500 bed hospital receives approximately 100-200 child visits per day. Cases were children 3 months to 5 years old with acute diarrhea. Controls were enrolled from non-diarrhea children.

Kwai River Christian Hospital is about 360 kms northwest of Bangkok along the Thai-Myanmar border. AFRIMS has established AFRIMS-KRCH Clinical Center in this hospital as an active field laboratory performing studies on drug resistant malaria and fever. This setting is well-equipped with facilities for supporting field and clinical research. Cases were children 3 months to 5 years old with acute diarrhea and controls were non-diarrhea children. Patient enrollment was started in October 2001.

4. Culture techniques:

After enrolling subjects, rectal swabs or stool specimens were collected. The stool was cultured on MacConkey's, Hektoen, thiosulfate citrate bile salt sucrose (TCBS) agar before and after enrichment and incubated for 24 hours at 37C. Stool was also inoculated on Brucella agar with sheep blood by millipore filtration technique for Campylobacter isolation. Up to 5 lactose fermenting (Lac+) and 5 non-lactose fermenting (Lac-) of *E.coli* colonies as identified on MC agar were saved on Dorset Egg yolk media slant. *E.coli* isolates were tested by hybridization with specific Digoxigenin-labeled polynucleotide probes: LT and ST probes for Enterotoxigenic *E.coli*; EIEC probe for Enteroinvasive *E.coli*; SLT I and SLTII probes for Shiga-like toxin producing *E.coli*; EAE, EAF, and BfpA probes for Enteropathogenic *E.coli. Shigella, Salmonella, Vibrio spp.* and *Campylobacter* spp. were also identified. Isolates of *Shigella*, ETEC, *Campylobacter, Vibrio* and *Salmonella* were selectively tested for antimicrobial susceptibility to ampicillin, gentamycin,kanamycin,tetracycline,trimethoprim/sulfamethoxazole, nalidixic acid, ciprofloxacin and/or azithromycin by disk diffusion method using commercially prepared antibiotic disks or minimal inhibitory concentration (MIC) agar plate dilution methods. Initial plating and identification was performed in the field laboratories. Final identification, confirmation of the isolates, DNA hybridization and antibiotic susceptibility testing were performed in AFRIMS, Bangkok. For the field sites outside of Thailand stool culture was repeated at AFRIMS from swabs or samples saved in Cary Blair transport media by the field sites.

5. Results:

Stool samples from a total of 744 human cases and 539 human controls were cultured as shown in Table 1. A summary of the results for enterotoxigenic *E. coli* (ETEC), *Shigella* spp., *Salmonella* spp., and *Campylobacter* spp. by site is shown in Tables 2a & 2b. *Campylobacter* spp. were the most common pathogens isolated from the military personnel attended Cobra Gold exercise (36%), travelers in Thailand (14%) and travelers in Nepal(19%). Isolation rate of *Campylobacter* among the non-immune travelers with diarrhea was significantly higher than asymptomatic controls. *Salmonella* was frequently isolated from cases with diarrhea

in Thailand as well as in asymptomatic controls. Isolation rate of *Shigella* was reasonably low from both sites in Thailand. ETEC was identified in 7-8% of travelers and adult Thais with diarrhea at Bumrungrad hospital. Heat stable toxin (ST)was detected from 76%(13/17) of these ETEC isolates. Colonization factor antigens were characterized in 11 of these ETEC isolates as follows: CS6(55%); CS1,3(10%); PCFO159(10%); and CF-(27%). In Nepal, the other common pathogens identified in cases included *Shigella* (17%), cyclospora (17%)and ETEC (12%). Half of ETEC isolates from Nepal produced heat stable toxin(ST), 15% produced heat labile toxin (LT) and 35% produced both. Colonization factor antigens were characterized in 7 ETEC isolates and CS6 was the most common (43%) with the others being CFAI; CS1,3; CS2,3; and CS4,6. In Vietnam, *Salmonella* and *Shigella* were identified in 7% and 8% of children with diarrhea but were not commonly found in controls (2%). Interestingly, *Arcobacter*,the aerotolerant *Campylobacter spp*, was isolated from 1 cases and 5 controls in Vietnam.

Serotyping of Campylobacter and Shigella isolates is shown in in Tables 3a and 3b.

Antimicrobial resistance patterns for *Campylobacter* spp. and *Shigella* spp. isolates for selected antibiotics are shown in Tables 4a & 4b. The *Shigella* spp. isolates from all sites were generally resistant to trimethoprim/sulfamethoxazole as expected, but *Shigella* spp. resistance to ampicillin has surprisingly decreased to only about 10% in Nepal, Vietnam and Thailand. Ciprofloxacin is still very effective for *Shigella* at all sites. Among *Campylobacter* spp. isolates, the resistance to nalidixic acid ranged from 96% for Cobra Gold to 20% in Vietnam and resistance to ciprofloxacin ranged from 96% in Cobra Gold to 0% in Hanoi,Vietnam. High resistance to nalidixic acid and quinolones was detected among isolates from both sites in Thailand. Meanwhile, resistance to macrolide antibiotics, erythromycin and azithromycin, was detected in 7% of isolates from Thailand but none of the isolates from Nepal and Vietnam. MIC was performed by E-test and agar dilution technique for *Campylobacter* isolates from the Cobra Gold exercise. The MIC90 of azithromycin, ciprofloxacin, and levofloxacin were 0.125, >32 and >32 ug/ml, respectively

6. Publications:

Portions of this data have been summarized and accepted for poster presentation at the meeting of the Infectious Disease Society of America held in San Francisco in October 2001. Two abstracts have been prepared and submitted for presentation at the American Society of Microbiology meeting to be held in Salt Lake City, Utah in May 2002. Two manuscripts have been accepted for publication: "Comparative antibiotic resistance in diarrheal pathogens from Vietnam and Thailand, 1996-1999" accepted by the Journal of Emerging Infectious Disease and "Prospective study of Diarrheal Disease in a Cohort of Vietnamese Children along the Red River" published in Epidemiol Infect. 2001 Oct;127(2):229-36.

Table 1: Stool Samples Received and Cultured by the Department of Enteric Diseases at AFRIMS

Site	Number of Cases	Number of Controls
Cobra Gold, Thailand	153	0
CIWEC Clinic, Nepal	100	55
Bumrungrad Hospital, Bangkok - Foreigners	207	207
Bumrungrad Hospital, Bangkok - Thais	178	178
St.Paul Hospital, Vietnam	106	99
TOTAL	744	539

Table 2a: Shigella, Salmonella, Campylobacter, and ETEC Isolation Rates by Site for Cases

Site	Shigella	Salmonella	Campylobacter	ETEC
Cobra Gold	0%	13% (20/153)	36% (55/153)	0.6% (1/153)
Nepal	17% (17/100)	3% (3/100)	19% (19/100)	12% (12/100)
Bumrungrad-Foreigners	2% (5/207)	13% (28/207)	14% (29/207)	8% (17/207)
Bumrungrad-Thais	2% (3/178)	15% (26/178)	1% (2/178)	7% (13/178)
Vietnam	8% (8/106)	7% (7/106)	5% (5/106)	2% (2/106)

Table 2b: Shigella, Salmonella, Campylobacter, and ETEC Isolation Rates by Site for Controls

Site	Shigella	Salmonella	Campylobacter	ETEC
Nepal	0%	0%	5% (3/55)	2% (1/55)
Bumrungrad-Foreigners	0%	18% (37/207)	1% (2/207)	2% (4/207)
Bumrungrad-Thais	0%	17% (30/178)	3% (5/178)	1% (2/178)
Vietnam	2% (2/99)	2% (2/99)	0%	1% (1/99)

Table 3a: Lior Serotyping of *Campylobacter* isolates by site

Site	CJ2	CJ4	CJ5	CJ7	CJ11	CJ17	CJ19	CJ28	CJ36	CJ86	CJ94	CJ102	CJ114	Untypable CJ	CC	Camp Spp.
Cobra Gold (N=55)	2%	4%	20%	4%	11%		2%	4%	14%			5%	6%	30%		
Nepal (N=22)		5%						5%	18%	5%	5%		5%	36%	23%	
Bangkok (N=38)					3%	3%						3%		70%	8%	13%
Vietnam (N=5)		20%							20%					60%		

Table 3b: Serotyping of *Shigella* isolates by site

	S.dys9	S.flex	S.flex1b	S.flex2a	S.flex3a	S.flex6	S.boy11	S.sonnei
Nepal (N=17)	6%		6%	6%	6%	29%	6%	41%
Bangkok (N=8)				12%				88%
Vietnam (N=10)		10%			10%			80%

Table 4a: Antimicrobial Resistance Patterns for *Shigella* Isolates by Site

			Antibiotic	
Site	Trimethoprim/ sulfamethoxizole	Ciprofloxacin	Ampicillin	Nalidixic acid
Nepal	67% (12/18)	0%	11% (2/18)	33% (6/18)
Bangkok	100%(8/8)	0%	13% (1/8)	38% (3/8)
Vietnam	70% (7/10)	0%	10% (1/10)	0%

Table 4b: Antimicrobial Resistance Patterns for *Campylobacter* Isolates by Site

		Antibiotic		
Site	Nalidixic Acid	Ciprofloxacin	Erythromycin	Azithromycin
Cobra Gold	96% (53/55)	96% (53/55)	0%	0%
Nepal	59% (13/22)	50% (11/22)	0%	0%
Bangkok	87% (33/38)	82% (32/39)	6% (2/35)	5% (2/39)
Vietnam	20% (1/5)	0%	0%	0%

D. Department of Veterinary Medicine AFRIMS FY01 Research Accomplishments

1a. Assessment of new anti-malarial drugs in a non-human primate relapsing malaria model (rhesus-P. cynomolgi)

1. Introduction:

Although animal models of malaria may be imperfect predictors of the response in human malaria, they generally provide reasonably good screening systems for potential candidate drugs. The availability of a relapsing malaria model in non-human primates is required to accurately assess whether promising new compounds, as observed in vitro, have the capability to suppress or causally prevent relapsing malaria, or effect a radical cure of hypnozoite stages. Indeed, the development of WR 238605 was greatly furthered by testing the compound in vivax and vivax-like animal models (Puri, 1996). To that end, a model that is readily available at reasonable costs would be expected to greatly advance the development of promising new compounds.

The rhesus monkey/*Plasmodium cynomolgi bastianelli* model, developed by Schmidt et al (1982a,b), has been used to evaluate promising antimalarial compounds and it has been an important tool in the development of many of the antimalarials used today (Schmidt 1982, Dutta 1989; Shanks 1994). *P. cynomolgi* causes tertian relapsing malaria, biologically similar to *P. vivax* in several macaques in Asia, including *Macaca mulatta*. Pre-patent period is approximately 8-12 days in macaques, and the infection is characterized by low mortality, and relapses. Natural vectors include *Anopheles elegans*, *A. introlatus A. hackeri*, and *A. dirus*, the latter of which is reared at AFRIMS and can be used for infectivity studies. This model has been used at AFRIMS since 1975 to test drug candidates. This project was revived for the FY01 funding year and tested 2 analogs of the WR182393 (AFRIMS IACUC Protocol Number 01-01).

2. Objective:

Use the rhesus monkey/*P.cynomolgi bastianelli* 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.

3. Methods:

Candidate drugs are tested in monkeys using standard procedures established in the rhesus monkey/Plasmodium cynomolgi relapsing malaria model (AFRIMS protocol 01-01, which was developed and approved for up to 95 monkeys per year).

Testing Compounds: The compounds used in this protocol are new candidate antimalarial drugs, chemotherapeutic agents, or standard antimalarial drugs. All of the compounds are provided to the AFRIMS-DVM by the WRAIR-ET. Drugs are dissolved in an appropriate vehicle, as determined by ET-WRAIR, such as distilled water, 0.5% HEC:0.1% Tween 80 (HECT), peanut oil, or DMSO (<10% v/v) prior to administration. Solubility problems are noted. The selection of drugs to be tested, vehicles, the dosing regimens to be used, and the prioritization of the experiments is the responsibility of the WRAIR-ET.

Monkeys: One splenectomized rhesus monkey(*Macaca mulatta*) is infected to generate and donate gametocytes. Malaria-naive monkeys, weighing 2.5-5 kgs are pre-screened and randomized into groups. Two control animals are required per experiment. Approximately two to three rhesus monkeys are required per drug dose (up to 5 doses/compound per experiment).

Malaria: *Plasmodium cynomolgi bastianellii* infected erythrocytes frozen aliquots are used to expand the strain and produce gametocytes in the donor animal(s).

Vector: Laboratory reared *Anopheles dirus* mosquitoes are used for sporozoite development. Six to seven day old female mosquitoes are fed on the anesthetized donor animal during peak gametocytemia. On day 7, 5 mosquitoes of each batch are dissected to determine oocyst number (generally between 20-50). On day 12-14, the sporozoite number is calculated to estimate the number of mosquitoes necessary to provide 1 million sporozoites/monkey. On day 14-15, these sporozoites are harvested from the mosquitoes' salivary glands and diluted in PBS to prepare 1-ml inoculum containing 0.5 to 1 million sporozoites.

P. cynomolgi challenge model for causal prophylaxis test: The prophylactic drug is administered over three days (day -1, 0, 1). Intravenous sporozoite inoculation occurs on day 0. Blood smears are examined daily for 21 days, three times per week for 4 weeks, then twice/week until day 100. Control animals and all prophylactic failures are treated with chloroquine and primaquine.

P. cynomolgi challenge model for radical cure test: Following sporozoite challenge (day 0), blood smears are examined daily for patency beginning on day 6. When parasitemia exceeds 5,000/mm3, the animal is treated. Control monkeys receive a 7-day regimen of chloroquine and vehicle. Test animals receive chloroquine and the test compound. Blood smears are evaluated daily (days 6-21), three times per week for 4 weeks, then twice per week until day 100-post treatment. Control animals and all radical cure failures are treated with chloroquine and primaquine.

4. Results:

There were two experiments conducted in this fiscal year. Experiment 1 was a validation of the relapsing model and a radical curative test of one compound (WR182393). Experiment 2 is a causal prophylactic test of 3 compounds (WR182393, WR283193 and WR283205) at 1 or 2 different dosages.

The total number of animals used in two experiments including infected donors, controls and experimental monkeys was:

Experiment 1: 6 monkeys (1 donor, 2 controls and 1 group of 3 experimental monkeys); 1 compound (WR182393), IM route, at one dosage (30 mg/kg).

Experiment 2: 13 monkeys (1 donor, 2 controls and 5 groups of 2 experimental monkeys per group); 3 compounds (WR182393, WR283193 and WR283205), IM route, given once daily for 3 days on days -1, 0 and 1 .

(WR182393 at 30 mg/kg; WR283193 at 30 or 10 mg/kg; WR283205 at 30 or 10 mg/kg).

Grand total of monkeys used in this project = 19 monkeys.

Donor monkeys and Sporozoite Inoculum preparation: Two donor monkeys were splenectomized on 24 Apr 01. Each donor was infected 4 weeks prior to the planned day 0 of each experiment.

Experiment 1: The donor was infected on 29 Jun 01.

Experiment 2: The donor was infected on 8 Aug 01.

Sporozoite inoculations were performed on day 0.

Experiment 1: 27 Jul 01

Experiment 2: 5 Sep 01

Drug Preparation:

No solubility problems were indicated. In both experiments, all testing compounds were dissolved in DMSO prior to administration.

WR182393 at 30 mg/kg used in Experiment 1 and 2 was moderately soluble and became yellow turbid suspension.

WR283193 at 10 mg/kg was a clear solution and it completely dissolved. At 30 mg/kg, white sediment was noted.

WR283205 at 10 and 30 mg/kg were clear solution. It completely dissolved in DMSO.

Drug Administration:

Acute toxicity and other adverse effects: none

Study days:

Experiment 1 (radical curative test) started on 27 Jul 01 and continued to 24 Nov 01

Experiment 2 (causal prophylactic test) started on 5 Sep 01 and continued to 15 Dec 01

Results/conclusions:

Experiment 1(radical curative test): The relapsing model was validated. All 5 monkeys became positive, 9 days after sporozoite inoculation. In 2 control monkeys, a 7-day chloroquine treatment completely cured the parasite within 4 days after the first dose. The parasite recrudesced (relapsed) in each monkey within 14 and 16 days after the first dose of chloroquine or at 28 and 30 days after the inoculation respectively.

The testing compound, WR182393, at this dosage (30 mg/kg) showed partial to complete radical curative activity. It completely cured one monkey (DA867) and delayed the relapse in two monkeys (DA860 and DA870) to 69 and 94 days after the first dose of chloroquine or to 83 and 108 days after the inoculation respectively.

Experiment 2 (causal prophylactic test): The 2 control monkeys (DA889 and DA891) became parasitemic within 10 and 13 days after sporozoite inoculation respectively. The parasite presented in 2 monkeys at 47 and 75 days after the inoculation. One monkey (DA880) received WR283193 at 10 mg/kg and the other monkey (DA888) received WR182393 at 30 mg/kg.

The follow-up blood smears of the other 8 monkeys and the recent results on 13 Dec 01 have been negative. Blood smears were continued until day 100 after the last dose of drug (15 Dec 01).

The complete results are pending. All three compounds most likely contain exoerythrocytic activity to prevent development of malaria in the rhesus monkey. WR182393, at 30 mg/kg, delayed malaria infestation in one monkey and completely prevented it in the other monkey. WR283193 completely prevented malaria development in the two monkeys at 30 mg/kg. At 10 mg/kg, it delayed malaria infestation in one monkey and completely prevented it in the other monkey. WR283193 is more effective than WR182393 when given at the same dose (30 mg/kg). WR283205 at both 30 and 10 mg/kg is the most effective among the three compounds to completely prevent malaria.

5. References:

None

1b. A Plasmodium berghei-Mouse Model for Screening Antimalarial Drugs

1. Introduction: The mouse model for screening new candidate antimalarial drugs has been used for over 30 years and is very effective for making comparisons between drugs. This test is rapid and makes reliable predictions of how drugs will work in higher mammalian hosts, including humans. The increasing prevalence of multidrug resistance (MDR) malaria caused by *Plasmodium falciparum* (Pf) and the recent emergence of chloroquine resistant malaria caused by *Plasmodium vivax* (Pv) portends increased difficulties for the development of prophylactic or therapeutic antimalarial drugs. In parts of southeast Asia there currently are strains of Pf that are clinically resistant to all US approved antimalarial drugs, including chloroquine, mefloquine, quinine, halofantrine, and pyrimethamine plus sulfadoxine (Looareesuwan et al., 1992). The decreased clinical efficacy of these drugs has been consistent with increased resistance as measured by in vitro drug susceptibility assays (Wongsrichanalai et al., 1992).

Although the emergence and spread of multiple drug resistance has not been as rapid in other endemic areas, such as Africa and South America, recent history suggests that antimalarial drug resistance that begins in southeast Asia will later spread to other endemic areas. For example, chloroquine resistance was observed first in Colombia and along the Thai-Cambodian border in 1960. The chloroquine resistant genotype spread rapidly throughout southeast and southwest Asia and was first described in east Africa in 1979. In less than a decade, chloroquine resistance spread across the African continent to most of the holoendemic foci in west Africa. Clearly the spread of potentially untreatable falciparum malaria will impact on US military operations.

The developers of antimalarial drugs have long used rodent, avian, and simian animal models to conduct preclinical testing. The choice of an animal model is based upon technical objectives and practical considerations. The most widely used and best developed of these are mouse malaria models that use *P. berghei*, *P. yoelii*, or *P. chabaudi* in a variety of outbred or inbred mouse strains (Peters, 1990). These mouse models have the practical advantages of ease of manipulation of experimental conditions, genetic homogeneity of parasite and host species, and reproducibility of results with large numbers of animals, which are readily available at a relatively low cost.

The Rane test, a model that uses mortality of *P. berghei* to outbred Swiss mice as an endpoint, was used from 1964 until 1993 as the primary screening system for the Antimalarial Drug Development program at the Division of Experimental Therapeutics, Walter Reed Army Institute of Research. Briefly this simple screening procedure is based on the ability of a standard inoculum of *P. berghei* to produce a fulminant infection in mice within six days; survival of treated mice of more than twice that of the controls was considered evidence of activity. This program was successful as shown by the identification and development of mefloquine and halofantrine for treatment of chloroquine resistant falciparum malaria and for current efforts to develop an 8-aminoquinoline analog (WR238605) of primaquine for chemoprophylaxis and radical cure.

The current paradigm for preclinical testing is to use in vitro susceptibility testing of *P. falciparum* clones as a primary screen, followed by secondary screening in a mouse malaria model. This system takes advantage of the strengths of both models. The in vitro assay provides a rapid, quantitative screen to identify compounds active against sensitive and MDR Pf isolates, enables the conduct of structure-activity studies, and demonstrates patterns of cross-resistance (or lack thereof) with existing drugs, thus reducing the number of animals required for screening antimalarial drugs. A secondary screen using the mouse allows for confirmation of activity *in vivo,* provides insight into drug metabolism and toxicity, gives bioavailability estimates for various routes of administration, and allows for optimization of dosage regimens. Selected compounds identified in these test systems may then be subjected to tertiary testing in simian models for therapeutic or prophylactic indications. Compounds that successfully pass this gauntlet of efficacy testing are then transitioned to preclinical pharmacology and toxicology evaluation prior to Phase I clinical trials in humans.

2. Objective:

To evaluate potential antimalarial chemotherapeutic agents in the *P. berghei* ICR mouse - 'Thompson Test' model at AFRIMS. Utilize this *in vivo* efficacy model to screen compounds in order to yield candidates that warrant further development. Specifically, use this model to screen the most promising of the tryptanthrin, biguanide and antifolate classes of compounds.

3. Methods:

Candidate drugs are tested in mice using standard procedures established in the mouse malaria model (AFRIMS protocol 00-01). Typically in this test, 6-16 groups of 8 female mice are inoculated intraperitoneally (IP) with *P. berghei*-infected erythrocytes and then treated with candidate drugs to determine the antimalarial activity. Infected erythrocytes are provided

from donor mice, inoculated (IP) with stored frozen infected blood 8 days prior to the start date of the individual experiment. Each mouse is identified individually by ear notch codes. On experimental day 0, the donor mice are exsanguinated via cardiac puncture, the blood pooled, a parasitemia determined and the pooled blood diluted with normal mice serum to a concentration of 1 X 106 P. berghei-infected erythrocytes per inoculum. The groups of experimental and control mice are inoculated with parasitized blood on day 0. On days 3, 4, and 5 these mice are then treated with either the candidate antimalarial drug or with vehicle without drug (negative control), to serve as the negative control. The drugs are administered orally (PO) and/or subcutaneously (SC) up to three times a day, based on the drug's individual and unique pharmacodynamics, as determined by ET-WRAIR. Each experimental group will receive a different dose level, with up to five different dose groups per compound. Blood films are taken on the third and sixth days post-infection, stained with Giemsa stain, examined by light microscopy, and the percent parasitemia determined. Blood smears are taken at weekly intervals through day 60. Daily observation of mice will determine the number of mice dead and alive. Surviving mice are followed to 60 days post-infection; all mice with negative smears at 60 days are considered cured. Weights of live animals are also determined on days 0, 3, 6, and weekly thereafter. Observational data are recorded twice daily. Thick and thin blood smears are Giemsa stained, the slides are examined by light microscopy for the detection of *P. berghei* infected erythrocytes and the percent parasitemia determined (per 500 erythrocytes counted). All dead mice are necropsied to confirm death from malaria infection. Tissues are submitted for histopathological diagnosis if usual lesions are detected.

Drugs used in this protocol are candidate antimalarial drugs, chemotherapeutic agents, or standard antimalarial drugs. All of the compounds are provided to the AFRIMS-DVM by the WRAIR-ET. Drugs are dissolved in an appropriate vehicle, as determined by ET-WRAIR, such as 0.5% hydroxyethylcellulose (HEC), 0.5% HEC: 0.1% Tween 80 (HECT), Cremophor EL, peanut oil, or DMSO (<10% v/v) prior to administration. Solubility problems are noted. The selection of drugs to be tested, the dosing regimens to be used, and the prioritization of the experiments is the responsibility of the WRAIR-ET.

4. Results:

Nine compounds were provided to the AFRIMS-DVM by the WRAIR-ET for testing. These compounds were tested in 2 experiments (B0048 and B0049).

The total number of animals used in two experiments including infected donors, vehicle controls and experimental mice was:

Experiment B0048:

1 compound (BP07829), 2 routes (PO and SC), at 5 dosages (64, 16, 4, 1, 0.25 mg/kg) [6 + 8 + 80] = 94 mice

Experiment B0049:

8 compounds (BP20037, BP20055, BP20073, BP20019, BP20082, BP20028, BP20064 and BP20046), 1 route (PO) at one dosage (16 mg/kg) [4 + 8 + 64] = 76 mice

Reduction of future numbers of mice used as serum donor was evaluated by an approved amendment to the original protocol to add 2 groups of mice inoculated with infected

erythrocytes diluted in phosphate buffered saline (PBS). So, 16 mice each were inoculated as PBS controls in both experiments. Excess mice (836) produced but not used were euthanized and exsanguinated and serum was saved for future use. Additionally, 20 mice were issued as infected donors for cryopreservation of the infected erythrocytes for future use. Grand total of mice used in this project = 1,058

Drug Preparation: No solubility problems were indicated. Experiment 48: Drugs were dissolved in 0.5% HEC: 0.1% Tween 80 (HECT) or undiluted DMSO prior to administration. Experiment 49: Drugs were dissolved in 10% DMSO prior to administration.

Acute toxicity and other deaths: none

Blood Smear Results and Death Counts:

All mice were positive on day 3 as usual. All control mice and study mice showed high parasite count on day 6 (mean parasitemia >40%). All control mice died between days 7 to 10 (Experiment 48) and days 6 to 9 (Experiment 49). All study mice died between days 6 to 8 (Experiment 48) and days 6 to 10 (Experiment 49).

Necropsy Results: All experimental mice showed typical gross lesions such as dark gray liver, dark spleen, and pale emaciated carcass (like control mice group 1), which indicated fatal malaria infection. No tissues were submitted for histopathology.

Conclusions: All 9 testing compounds showed no therapeutic efficacy for the treatment of mice malaria. Toxicity of the compounds was not detected at the dosages used in the two experiments.

5. References:

None.

1c. Surveillance of Emerging and Re-emerging Flavivirus Infections in Sangkhlaburi District By Using Sentinel Animals

1. Introduction:

The Department of Immunology and Medicine, Armed Forces Research Institute of Medical Sciences (USAMC-AFRIMS) has an active surveillance program for emerging infectious diseases in Sangkhlaburi District, Kanchanaburi Province, Thailand. Part of this project involves following a cohort of village volunteers with serial (before and after each rainy season) serological assessment for mild and inapparent arthropod-borne infections. Seroepidemiological surveys of over 400 residents of Viakadee and Mong Satur villages in 1999-2000 suggested high prevalence of antibodies specific for previous flavivirus infection, with peak transmission during the rainy season. These antibodies were specific for Japanese encephalitis (JE), West Nile (WN) and dengue viruses.

2. Objective:

To expand surveillance for flavivirus infections using sentinel animals (swine and poultry) at the two villages in Sangkhlaburi District, Kanchanaburi Province, in coordination with ongoing human epidemiological and insect vector assessments. Using sentinel animals, this

study will increase our understanding of the ecology of these viruses and their potential as a disease threat to U.S. military forces, travelers and persons living in high risk areas.

3. Methods:

20 animal shelters (sites) were placed in two target villages (Monsatau and Viakadi). Each site maintained 4 pigs, 5 chickens and 5 ducks. A total of 80 pigs, 100 chickens and ducks were used in the first experiment. The animal use protocol was designed to allow up to 3 experiments to compare the disease incidence between dry and rainy seasons.

Surveillance Methods: Flavivirus antibody-seronegative animals (pigs, chickens and ducks) were randomly placed at the 20 sites and maintained for 8 weeks under routine husbandry conditions. Serial blood samples were collected.

Laboratory Methods: Detection of antibody to JE/WNV viruses: Screening by IgM capture EIA to detect IgM antibody; hemagglutination inhibition assay (HAI); those that are negative by HAI are confirmed by a plaque reduction neutralization test (PRNT). If the screening period is less than 3 weeks, then the HAI and PRNT will be done simultaneously. Dilutions of sera are incubated with 50-100 plaque-forming units (pfu) of JE virus. The virus-antibody mixture is inoculated onto Vero cell monolayers in culture. After 6 days of incubation at 35° C, the monolayers are stained with neutral red to visualize the virus-induced plaques. The dilution of serum that gives a 50% reduction in the number of input virus plaques, determined by probit analysis, is recorded as the $PRNT_{50}$ titer. PCR using primers specific for dengue, JE and WNV are performed on acute IgM positive sera as well as viral isolation into *Toxorhynchities* mosquitoes and C6/36 cell culture, if positive.

Specimen Collection: The animals were bled on the day of arrival to the area (day 0), day 4, day 7, and weekly for the next 7 weeks. At each bleeding, 3 ml blood was taken from pigs and 0.4 ml of blood was taken from chickens and ducks.

4. Results:

Some HAI antibodies to JE and WN were available but not yet completed. Most pigs and ducks seroconverted to a flavivirus between 14 and 28 days post-inoculation. Between the two villages, more animals in Viakadi were positive than in Monsatua. Ducks were surprisingly good sentinel animals. Very few chickens showed seroconversion. Other results are pending.

5. References:

None.

E. Department of Virology, AFRIMSFY01 Research Accomplishments

1a. Febrile disease surveillance, Kathmandu, Nepal

1. Staff:

Gaye Ruble, D.V.M., M.P.H., Timothy P. Endy, M.D., M.P.H., Ananda Nisalak, M.D., Khin Saw Aye Myint, M.D., Robert M. Scott, M.D., Mrigendra Shrestha, M.D., and Mammen P. Mammen, Jr., M.D.

2. Introduction:

Kathmandu is the commercial center and capital of Nepal. It is geographically unique in being at the crossroads of commerce between India and Nepal as well as a tourist center for travelers from China, Japan, Europe and the Americas. A wide number of diseases are known to occur in Nepal especially in the plain areas to include leishmaniasis, dengue, scrub typhus, malaria, leprosy, typhoid fever, antibiotic resistant enteric organisms, and others. While the incidences of these diseases appears to be increasing, there is no organized national surveillance program in Nepal to monitor for febrile illnesses or emerging infectious diseases.

3. Objective:

- To determine if the etiology of pyrexia in travelers or the indigenous population is due to potential emerging diseases of interest such as scrub typhus, dengue, Japanese encephalitis, leptospirosis, or other pathogens such as ehrlichia.
- To validate prototype test kits for diagnosis of arthropod-borne and zoonotic diseases, with potential application to enhance diagnostic surveillance capabilities.

4. Methods:

Collaborators:

- CIWEC International Clinic
- Patan and Teku Hospitals
- Shree Birendra Hospital of the Royal Nepal Army (RNA)

AFRIMS has collaborated with Environmental Health Project (EHP) and Nepal Ministry of Health (MOH) on the seroprevalence study of flaviviruses in the Terai. 2573 HAIs were done on DEN, JE, WNV, and CHIK (1986 samples for MVE) at the Department of Virology with 2 personnel working full time for nearly 4 weeks.

5. Results:

A brief summary of the results is as follows (Table 1):

- There seemed to be a clustering effect of all 7 chikungunya cases
- There were 30 samples purely positive for WNV (1%)

- There were only 9 samples (0.35%) purely positive for JE, the truer number may be reflected in more broadly cross-reactive results across multiple flaviviruses
- There were 19 samples purely positive for MVE (1%)
- Although cross- reactivity was seen with flaviviruses, these results may lend themselves to speculation that MVE, WNV and CHIK may be at least focally present in addition to JE
- The paucity of purely dengue results suggest against this being a problem in the Terai
- It is very important that these results not be over-stated as HAI testing is only suggestive and not conclusive

We have recommended to EHP that they seek further historical clinical correlation, preferably with attempts to minimize bias in the questioning. This would enable assessments of the plausability that WNV, MVE, CHIK viruses are present in the Terai. To make the greatest impact, one may want to target the positive serologies that correlate with the greatest clinical illness. This study could set the stage for further diagnostic testing, preferably targeting virus isolation either from mosquitoes or preferably patients. AFRIMS Virology Department will provide continued support to EHP.

Through its Virology field station, WARUN, AFRIMS has supported fever surveillance at three hospitals in Kathmandu. We have screened sera samples from febrile patients for a number of infectious diseases, including dengue, JE, leptospirosis, scrub typhus, spotted fever, and ehrlichia (TABLE 2). A total of 113 specimens from patients with encephalitis were received and 66 (58%) were tested positive for JE mainly from Patan and Shree Birendra Hospitals. Prior to this study, we are not aware of evidence to suggest that JE is endemic to KTH Valley. This may lead to formal request of CINCPAC to offer JE vaccination to RNA. Further epidemiologic characterization of JE in Kathmandu Valley should be the goal for FY02.

60 dipstick results were compiled from the fever surveillance study. There was good correlation between Dipstick Assay for leptospirosis and the IgM ELISA as 85.7% of the dipstick positives was confirmed by ELISA. A panel of assays including EIA and HEV PCR were done on approximately 100 cases with febrile jaundice. Hepatitis E was the leading cause of acute hepatitis in Kathmandu (39% of cases)

6. References:

Woods CW et al. Etiology of Febrile Illness in Urban Nepal. Suppl. to the American Journal of Tropical Medicine and Hygiene (2001) 65(3):149.

TABLE 1 : SEROPREVALENCE STUDY FROM THE TERAI (EHP)

	PURELY POSITIVE (%)	TOTAL (%)
Dengue	1 (0.04 %)	148 (5.75 %)
Japanese Encephalitis	9 (0.35 %)	63 (2.45 %)
West Nile	30 (1.17 %)	127 (4.94 %)
Chikungunya	7 (0.27 %)	7 (0.27 %)
Murray Valley Encephalitis	19 (0.96 %)	94 (4.73 %)

TABLE 2 : FEVER SURVEILLANCE IN NEPAL

TYPE OF ASSAY	MARKER	TOTAL # OF SAMPLES	# OF POSITIVE SAMPLES
Dip-S-Ticks	Leptospira	60	7 (11.7 %)
	Sennets	60	29 (48.3 %)
	Typhi	60	40 (66.7 %)
	Chaffeen	60	39 (65.0 %)
	Scrub Typhus	60	18 (30.0 %)
ELISA	Leptospira IgM EIA	58	15 (25.9 %)
	Scrub typhus IgM EIA	28	1 (3.6 %)
	Spotted Fever IgM EIA	13	0 (0.0 %)
HEPATITIS	Anti-HAV IgM	107	6 (5.6 %)
PANEL	HBsAg	90	1 (1.1 %)
	Anti-HBc IgM	107	1 (0.9 %)
	Anti-HCV	107	1 (0.9 %)
	Anti-HEV IgM	112	44 (39.3 %)
	Anti-HEV Ig	112	44 (39.3 %)
	HEV PCR	109	34 (31.2 %)

1b. Hospital-based EID surveillance, Kamphaeng Phet, Thailand

1. Staff:

Gaye Ruble, D.V.M., M.P.H., Timothy P. Endy, M.D., M.P.H., Khin Saw Aye Myint, M.D., Ananda Nisalak, M.D., and Mammen P. Mammen Jr., M.D.

2. Introduction:

Emerging infectious diseases are those that have increased in prevalence or threaten to do so because of biological or ecological reasons. Systematic surveillance may allow for early detection and intervention of infectious diseases prior to becoming pandemics. Kamphaeng Phet Provincial Hospital located in northwestern Thailand serves both urban and rural communities with a large population of hilltribe Thais and Burmese. This unique environment offers an opportunity to study and identify emerging diseases with potential impact on regional and national health issues.

3. Objective:

To monitor for diseases of interest and of potential health impact

- To expand infrastructure by renovation of a 2-story building to increase laboratory space
- To reassess diagnostic capabilities for selected clinical syndromes and introduce on-the-ground PCR capability (DEN, JE, WNV, HCV)
- To shift responsibility from physicians to KPP hospital nurses given the inordinate clinical demands on the physicians

4. Methods:

Participants:

Saroj Suntayakorn, M.D., Deputy Director, Kamphaeng Phet Provincial Hospital

This is a hospital-based study to monitor febrile syndromes and is unrelated to dengue projects funded by NIH and midrp. During the period of October 2000 through September 2001, KPPH and AFRIMS' staff reviewed inpatient records from eight wards, six adult medicine wards, two pediatric wards, and the emergency room. Records were reviewed by AFRIMS nurses every weekday for cases that presented with one of the following clinical syndromes- DEN, JE, DLI, ELC, MAL, HF, HEP, RF or USD. All case definitions include fever or history of fever $\geq$ 38°C:

Dengue-like illness (DLI)—

Clinical diagnosis of dengue or presentation of fever, rash or shock, or a clinical diagnosis of scrub typhus, leptospirosis, typhoid fever, chikungunya, or viral syndrome.

Encephalitis-like complex (ELC)—

Clinical diagnosis of encephalitis or presentation of fever, headache, mental status changes, seizures, neck stiffness without a bacterial cause.

Malaria (MAL)—

Clinical diagnosis of malaria or laboratory evidence of malaria.

Hemorrhagic fever/sepsis syndrome (HF)—

Clinical diagnosis of hemorrhagic fever or presentation of fever, bruising, bleeding from any orifice, petechiae or a clinical diagnosis of bacterial sepsis.

Hepatitis (HEP)—

Clinical diagnosis of jaundice or hepatitis or laboratory evidence of two times elevation of ALT or AST.

Respiratory fever (RF)—

Clinical diagnosis of respiratory infection or a specific diagnosis of pneumonia or pneumonitis.

Unexplained sudden death (USD)—

Death within 48 hours of admission.

5. Results:

Nearly 2000 cases presented with one of the clinical syndromes listed above. The clinical syndrome numbers are as follows:

CLINICAL SYNDROME	NO.CASES	MEAN AGE	MINIMUM	MAXIMUM
DLI	241	21 yr.	1 mo.	88 yr.
ELC	27	23 yr.	4 mo.	71 yr.
MAL	127	31 yr.	3 yr.	76 yr.
HF	49	22 yr.	1 mo.	95 yr.
HEP	19	22 yr.	1 mo.	47 yr.
RF	262	3 yr.	1 mo.	30 yr.
USD	3	21 yr.	15 yr.	27 yr.

At the physician's discretion, acute and convalescent sera were collected from patients. The sera were used to test for dengue or JEV (for patients presenting with DLI or ELC, respectively), or hepatitis A, B, C, E, for patients presenting with febrile jaundice. The sera can also be screened, using a rapid dip-stick, for leptospirosis, scrub typhus, typhoid fever, *Ehrlichia sennetsu* and *E. chafeensis*. All those positive for leptospirosis by Dipsticks were confirmed using and IgM ELISA. Also IgM ELISA was done in all suspected cases when the hospital ran out of Dipsticks. There were 58 cases of leptospirosis confirmed by EIA in this population (TABLE 3).

An interesting and important finding was the large number of adult dengue cases admitted to the Kamphaeng Phet Provincial Hospital especially the young adult group (Table 5). Dengue, usually considered a pediatric disease, caused many adult cases in Kamphaeng Phet. This likely reflects the high DV transmission seen in 2001 throughout Thailand. As AFRIMS Virology has gained sequencing technology, efforts will be made to compare sequences if Dengue viruses isolated from adults and children at KPP Provincial Hospital in 2001 and correlate these sequences with disease severity.

An unexpected finding in this population was seroconversion by five patients to a *Bartonella* sp. never before previously described in Thailand (submitted for publication). *Bartonella elizabethae* was isolated originally from a patient with endocarditis in Massachusetts, USA. Recently, a large number of microorganisms, genetically identical or closely related to *B. elizabethae* were found among domestic rats of the genus *Rattus*. A hypothesis of an Asian origin for *B. elizabethae* was proposed and seroprevalence studies for *Rattus* species was done in south China and human seroreactivity to Bartonella species in Thailand. Bartonella microorganisms were isolated from 27 of 64 (42%) rats tested from Yunnan. DNA sequence analyses indicated that 12 of these Bartonella isolates represented unique genotypic variants. Twenty-six of the 27 cultures were placed along with *B. elizabethae* and other rat-associated isolates from Europe and the Americas, on a single branch of a phylogenic tree that contained representatives of all known *Bartonella* genotypes. Elevated anti-*B. elizabethae* IgM titers were demonstrated in 5 of 14 acute serum samples from Thailand who experienced fevers of unknown origin. Convalescent sera were available from 10 of the above patients; elevated anti-*B. elizabethae* IgG titers were demonstrated by IFA for 3 of these samples. Two of the 10 paired serum samples exhibited seroconversion with 4-fold decreases of IgM titers and 4-fold increases of IgG titers. Further studies are being considered to attempt isolation of this organism from patient blood and to better characterize the disease clinically.

6. References:

Kozik CA et al. Hepatitis B virus infection in Thai children. Trop Med Int Health (2000) 5 (9):633-639.

Endy TP et al. Determinants of dengue disease severity and transmission in a prospective study of primary school children in Kamphaeng Phet, Thailand. Suppl. to the American Journal of Tropical Medicine and Hygiene (2000) 62(3):145-146.

TABLE 3: FEVER SURVEILLANCE IN KAMPHAENG PHET

TYPE OF ASSAY	MARKER	TOTAL # OF SAMPLES	# OF POSITIVE SAMPLES
Dip-S-Ticks	Leptospira	113	54 (47.8 %)
	Sennets	113	20 (17.7 %)
	Typhi	113	73 (64.6 %)
	Chaffeen	113	45 (39.8 %)
	Scrub Typhus	113	0 (0.0 %)
ELISA	Leptospira IgM EIA	74	58 (78.3 %)
HEPATITIS	Anti-HAV IgM	13	0 (0.0 %)
PANEL	HBsAg	13	8 (61.5 %)
	Anti-HBc IgM	13	4 (30.8 %)
	Anti-HCV	13	0 (0.0 %)
	Anti-HEV IgM	13	0 (0.0 %)
	Anti-HEV Ig	13	0 (0.0 %)

TABLE 4: NUMBER OF DENGUE AND JE FROM KAMPHAENG PHET

TOTAL # OF CASES	# OF POSITIVE CASES	
	JE	DENGUE
1879	4 (0.2 %)	1417 (75.4 %)

TABLE 5: ADULT DENGUE ADMITTED AT KAMPHAENG PHET HOSPITAL

AGE	TOTAL # OF CASES	SEROLOGICAL DENGUE
< 20	65	57 (87.7 %)
20 – 29	93	63 (67.7 %)
30 – 39	28	15 (53.6 %)
40 – 49	10	6 (60.0 %)
50 +	7	3 (42.9 %)
TOTAL	203	144 (70.9%)

1c. Influenza surveillance

1. Staff:

Gaye Ruble, D.V.M., M.P.H., Timothy P. Endy, M.D., M.P.H., Khin Saw Aye Myint, M.D., Gary Penner, M.D., R. Scott Miller, M.D., Chansuda Wonsrichanalai, M.D., Dr.P.H., Robert M. Scott, M.D., Mrigendra Shrestha, M.D., and Mammen P. Mammen, Jr., M.D.

2. Introduction:

Influenza is an important cause of morbidity and mortality in young and old adults. Continuous surveillance and viral isolation of influenza viruses provide important information for annual vaccine formulation. In addition, continuous surveillance and viral isolation will be able to detect new and emerging influenza strains with potential for high mortality and worldwide spread.

3. Objective:

- To provide influenza viral isolates from countries in South Asia as part of the global surveillance network for influenza, " Project Gargle".
- To evaluate rapid diagnostics at select sentinel sites, in an attempt to validate these tests, increase sample submission, and improve patient care.
- To expand network to include U.S. Embassies of Southeast Asia.

4. Methods:

Participants/Sentinel sites:

Gary Penner, M.D., US Embassy Medical Unit, Bangkok, Thailand

Phil McDaniel, M.D., Kwai River Christian Hospital, Sangkhlaburi, Thailand

Saroj Suntayakorn, M.D., Kamphaeng Phet Provincial Hospital, Kamphaeng Phet, Thailand

Prativa Pandy, M.D., CIWEC, Kathmandu, Nepal

Samples are collected from patients with clinically suspected influenza infection (case definition includes fever or history of fever $\geq$38°C and two or more of the following symptoms: cough, sore throat, coryza, muscle aches, malaise/fatigue, or headache). Clinical history forms, which include basic demographic information and clinical signs, and nasopharyngeal swabs will be collected and placed in viral media and stored at -70°C. All specimens are shipped on dry ice to AFRIMS, which will then ship the samples to Armstrong Laboratory, Brooks AFB. Rapid diagnostics are being field tested at the above four sites.

5. Results:

AFRIMS submitted 75 samples from October 2000 through October 2001, 8 from US Embassy Bangkok, 42 from KRCH Sangkhlaburi, and 25 from CIWEC Kathmandu. The results of viral isolation from the January 01 shipment containing 63 samples (March 2000 – January 2001) is as follows:

SITE	TOTAL # OF SAMPLES	# POSITIVE SAMPLES	INFLUENZ A A	INFLUENZ A B	SEROTYPE
US Embassy BANGKOK	4	0	N/A	N/A	N/A
KRCH Sangkhlaburi	17	4	0	4	B/YAM-LIKE
CIWEC Kathmandu	22	14	13	1	A/H1N1, A/H3N2 B/YAM-LIKE
KPP Kamphengphet	20	2	2	0	A/H3N2

6. References:

Canas LC, et al. The Department of Defence Laboratory-Based Global Influenza Surveillance System. Military Medicine (2000) 165, Suppl. 2:052.

1d. Project: The Dengue Hemorrhagic Fever Project II: Continued Prospective Observational Studies of Children with Suspected Dengue

Armed Forces Research Institute of Medical Science, Queen Sirikit National Institute of Child Health, Ministry of Public Health, University of Massachusetts Medical Center Department of Transfusion Medicine, Siriraj Hospital, Mahidol University

1. Introduction:

The four known serotypes of dengue virus cause a spectrum of disease throughout the tropical and subtropical regions of the world affecting millions of people each year. Severity ranges from mild, or even asymptomatic, to fatal cases of dengue hemorrhagic/dengue shock syndrome. Dengue is a disease of military importance following only Malaria on the U.S. Army's priority list of diseases for which a vaccine or treatment should be developed. Dengue was a major cause of morbidity in Vietnam and is presently a serious problem for the United Nation Troops in Cambodia. There is no treatment for this potentially fatal illness. Supportive care centers around careful fluid management. No vaccine available. The need for improved intervention and vaccine prevention is obvious. The purpose of this study is to better define the pathophysiology of dengue disease to provide insights into methods of prevention and intervention.

2. Objective:

This study is comprised of distinct parts combined under human use protocol including:

a. A search for serum or plasma markers, identified early in the illness, that predict (or possibly cause) severe plasma leakage. This may allow identification of subgroups of patients that would benefit from early medical intervention.. This search will include:

(1) Measuring mediators of shock and capillary leak syndrome (e.g. platelet activating factor, prostaglandins)

(2) Determining cytokine levels (e.g. IL-1, IL-2, tumor necrosis factor)

(3) Detecting products of T cell activation (e.g. beta 2 microglobulin, soluble CD8)

(4) Defining the cellular immune response in dengue hemorrhagic fever/dengue shock syndrome due to primary and secondary infection by analyzing expression of T cell activation marker (e.g. receptors and determining monocytes/macrophage activation markers).

b. Use of dengue virus isolates to develop mouse model of dengue.

c. Sequencing portions of dengue genome from patients with mild dengue fever and those with severe dengue hemorrhagic fever/dengue shock syndrome to test a hypothesis that severity of disease is strain related.

d. Relating the proportion of dengue infected mononuclear cells to clinical outcome. Can the degree of viremia be related to outcome?

e. Measurement of neutralizing antibody elicited by primary infections, over an extended period (up to 3 years). Understanding wild-type responses will help to set realistic standards for vaccines.

f. Establish a registry of patients to retrospectively evaluate of class I or II allotypes to clinical outcome.

3. Methods:

This is a continuation of a prospective observational study of dengue disease in children. Child volunteers meeting a case definition with a high positive predictive value for dengue infection will be recruited from the outpatient department of the Queen Sirikit National Institute of Child Health in Bangkok, Thailand. Upon obtaining informed consent from a parent or guardian, research nurses will collect a blood specimen daily from study entry until 1 day after defervescence or onset of shock. Follow-up blood specimens will be collected from all volunteers on study day 9 for serologic diagnosis and from volunteers with confirmed dengue infection at 6 and 12 months after study entry and annually thereafter, for determination of neutralizing antibody and T-cell memory responses. Host responses in the early febrile phase of illness will be related to outcome. Plasma leakage will be quantified at the same time-point for each volunteer, i.e. day following defervescence. Volunteers with febrile illness other than dengue will serve as controls

4. Results:

Summary of publications that has resulted from the first four years of this study:

1. Dengue in the Early Febrile Phase: Viremia and Antibody Responses, DW Vaughn et al, JID 1997; 176:322-30. Results of 189 children enrolled in the DHF Project demonstrated that viremia was highly correlated with fever. It was demonstrated that all four serotypes of dengue virus caused DHF and that all dengue patients experienced viremia during the febrile phase and that as fever resolves, so does viremia.

2. Early Clinical and Laboratory Indicators of Acute Dengue Illness, S. Kalayanarooj, DW Vaughn et al. JID 1997;176:313-21. Results of 172 children in the DHF Project demonstrated that children with dengue were more likely than children with other diseases to report anorexia, nausea, and vomiting. Children with dengue were also more likely to have a positive tourniquet test, lower total white blood cell counts, lower absolute neutrophil and absolute monocyte counts and higher ALT and AST liver function levels.

3. Early Immune Activation in Acute Dengue Illness is Related to Development of Plasma Leakage and Disease Severity, S. Green, DW Vaughn et al. JID 1999;179:755-62. Results of this study demonstrated that mean levels of soluble YNF receptors were significantly higher in children who later developed DHF than in those with DF or nondengue febrile illness. In addition, there was an early elevation of the mean levels of soluble CD8 and soluble IL-2 receptor in children with DHF as compared to those with DF. This study demonstrated that early immune activation correlates with the severity of subsequent plasma leakage in DHF and that elevated levels of soluble TNF receptors may be useful to predict disease severity in dengue.

4. Rapid Diagnosis of Dengue Viremia by Reverse Transcriptase-Polymerase Chain Reaction Using 3'-Noncoding Region Universal Primers, T.M. Sudiro. H. Ishiko, S. Green et al. Am J Trop Hyg, 1997;56:424-29. Areverse transcriptase-polymerase chain reaction (RT-PCR) method was developed for the rapid diagnosis of dengue viremia. Using samples from the DHF project, this assay was demonstrated to be highly sensitive and specific foe detecting viremia in children with dengue (91.4% sensitivity and 95.4% specificity).

5. Molecular Evolution of Dengue Type 2 virus in Thailand, R. Rico-Hesse, L.M. Harrison, A Nisalak et al. Am J Trop Med Hyg, 1998;58:96-101. Genotyping of dengue 2 viruses from the DHF Project demonstrated that the phylogenetic tree of this virus reflect long – term evolutunary relationships among strains. This suggested that many different virus variants might circulate in Thailand. Also demonstrated that viruses belonging to two previously distinct genotyping groups have been isolated from both DF and DHF cases supporting the view that they arose from a common progenitor and that viruses associated with the potential to cause DHF segragate into one large genotypic group.

6. Predominance of HLA-Restricted Cytotoxic T-lymphocyte Responses to Serotype-Cross-Reactive epitopes on Nonstrutural Proteins Following Natural Secondary Dengue Virus Infection, A Mathew, S Green et al. J of Virol. 1998;72:3999-4004. Using perpheral blood mononuclear cells from the DHF Project, it was demonstrated that there was a predominance of HLA-restricted cytotoxic T-lymphocytes that reacted to serotype-cross-reactive epitopes on nonstructural protein of dengue virus following natural secondary dengue infection.

7. Dengue Viremia Titer, Antibody Response Pattern and Virus Serotype Correlate with Disease Severity, DW Vaughn, S Green, S Kalayanarooj, BL Innis, S Nimmannitya, S suntayakorn, TP Endy, B Raengsakulrach, AL Rothman, FA Ennis, A Nisalak. JID. 2000 Jan; 181 (1):2-9. Demonstration of the association of viral factors such as specific dengue serotypes with more severe dengue disease and that peak virus titer correlates with clinical disease severity.

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1e. Project: Prospective Study of Dengue Virus Transmission and Disease in Primary School Children.

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1. **Introduction:**

Current hypotheses suggest that DHF occurs as a result of a number of different factors including genetic susceptibility, viral virulence and previous dengue immune status. The pathogenesis of DHF is thought to be a result of a very intense immune response resulting in thrombocytopenia and plasma leakage. This is based on a number of studies of T-cell activation and cytokine responses in children with DHF. Our studies on The Dengue Hemorrhagic Fever

Project with Dengue patients at the Queen Sirikit National Institute of Child Health concluded that (1)T-lymphocytes are activated in both DHF and DF with higher levels of activation observed in DHF as compared to DF. CD8 T-lymphocyte activation was also observed in DHF but not seen in DF, (2) There are markers of immune activation that occur early in dengue infection which may predict the development of DHF. It is known that malnutrition impairs the immune response and that individuals with malnutrition are at risk foe bacterial, viral and parasitic infections. The role of nutrition and the development of symptomatic secondary dengue infections and DHF is less well described. However, the nutritional status of children and risk for DHF has been clinically observed.

Dengue is a threat to United States military forces deployed into areas which are endemic for dengue. The United States Army has directed that a vaccine be developed against dengue infection. To meet this directive, The Department of Virus Diseases at WRAIR and the Department of Virology, AFRIMS, has been involved in a collaborative effort to study the epidemiology of dengue and to produve a vaccine against dengue (STO-S). This propective study on dengue infection will provide valuable information on the immune responses to dengue infection and viral information on how potential dengue vaccines can be designed and be effective in providing immunity against infection. In addition, this prospective study is vital as a future working model for a phase III dengue vaccine trial. This study will provide the necessary infrastruture and study design for a vaccine trial when suitable dengue vaccine becomes available.

2. Objective:

Hypothesis Testing

1) Demonstrate that symptomatic secondary dengue is a result of early increased T-cell activation of dengue specific cross-reactive T-cells and measured by T-cell proliferation and cytotoxic T-cell lymphocyte assay.

2) Demonstrate that viral immune enhancement due to subneutralizing heterotypic antibodies (antibody dependant enhancement or ADE) after a primary dengue infection will predict the development of symptomatic secondary dengue.

3) Demonstrate that other factors which contribute to the development of symptomatic secondary dengue infection includes HLA type, nutritional indices, and immunoglobulin subclass responses.

Specific objectives:

The specific study objectives are to:

1) Develop a prospective cohort population of 2,000 school-aged volunteers at risk for dengue infection.

2) To develop a bank of plasma and PBMC samples from this population prior to the start of the dengue season.

3) Identify subgroups of individuals without previous exposure to dengue and individuals with previous evidence of dengue infection.

4) To follow this population prospectively for five years with scheduled sampling of plasma and T-cells as well as active acute illness case finding with serum sampling during acute dengue infection.

5) To identify individuals with dengue viremia without accompanying illness or loss of school days.

3. Method:

This is be a prospective school based study of 2,000 children which has been starting in January of 1988 and end in December 2002. The study cohort are drawn from approximately six grade schools (1st through 5th grade) Kamphaeng Phet Province. These schools locate near the project study laboratory located at the Kamphaeng Phet Provincial Hospital with previously established high rates of dengue. The project was discussed with teachers and students at all partcipating study schools in January of 1998. Students with parents or guardians'consent in grade 1 to grade 5 are entered into the study in January 1998 where baseline demographics, anthropometric measurement, plasma and T-cells are obtained. This full baseline evaluation will take place every January for the duration of the study (january 1999, January 2000, January 2001 and January 2002). Each new first grade class are enrolled and students are followed until they disenroll at the completion of sixth grade. Scheduled evaluation of the entire cohort for serum by venupuncture will be performed on June 1st,August 15th and November 1st of ever study year. Active case surveillance of the cohort schools, using greater than or equal to 1 day school absence or reported illness to the school health nurse, will start at the beginning of the dengue season on 1 June and continue until the end of the dengue season, 1 November of each year. Village Health works will track school abscences or school illness and make first contact with absent students. They will contact students on every day of school absence and complete a questionnaire on symptoms and obtain a temperature. For children with fever or a history of fever within seven days on any day of their school absence, venous blood will be obtained at the time of illness at the Public Health center by a Public Health nurse of that Public Health centre, and at 14 days by an AFRIMS Research nurse.

Routine Laboratory Studies: In January each year bank for HLA typing (only for new enrollees), serum and peripheral blood mononuclear cells for memory CD4+ and CD8+ T cell studies (all study participants each year). On patients with documented dengue seroconversion by HAI acute dengue infection ented dengue seroconversion by HAI or patients with acute dengue during active surveillance by EIA, HAI, PCR and visus isolation, the following are performed on pre-seroconversion and post-seroconversion sera and T-cells for Dengue specific IgG subclasses, dengue western blots for antibodies structural and non-structural dengue proteins, den1-4, JE PRNT titers, and cross-reactive dengue epitope T-cell studies.

4. Result:

Some conclusions of the study results:

Demographic factors does not appear to be a risk factor in dengue disease severity.

An important risk factor in determining dengue disease severity during secondary dengue infections in our study appears to be pre-existing flavivirus antibody.

Den-3 viral load during the first day of school absence is associated with more severe dengue disease.

Analysis of other laboratory results, and publications are being processed.

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III. APPENDICES: PERSONNEL ASSIGNED UNDER AGREEMENT

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1	Ms.	Bang-on	Kesdee
2	Mrs.	Sutthida	Srijan
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4	Mr.	Sompol	Boonnak
5	Ms.	Pattrapan	Jullasing
6	Mr.	Puwanai	Sangsri

Department of Logistics

7	Mr.	Nipat	Promchart
8	Mr.	Thongsuk	Munmuenpom
9	Mr.	Somchai	Putsang
10	Mr.	Mongkol	Puramas
11	Mr.	Surapol	Ogpai
12	Mr.	Sawadi	Boonnak
13	Mr.	Charan	Kajeechitr
14	Mr.	Thongchai	Duangkaew
15	Mr.	Boonthum	Jamjang
16	Mr.	Komson	Boonnak
17	Mr.	Somporn	Pinpo
18	Mr.	Chatchai	Sang-ngern
19	Mr.	Nirutti	Boonnak
20	Mr.	Panutat	Inthamattayakul
21	Mr.	Prasitchai	Krauysawat
22	Mr.	Yuthana	Seemat
23	Mr.	Suvit	Sukhitmongkolkul
24	Mr.	Sawet	Amnuay

Department of Veterinary Medicine

25	Mr.	Komdej	Kongsunarat
26	Mr.	Phongsak	Maneerat
27	Mr.	Suchin	Poolgird
28	Mr.	Thonglor	Dejkokao
29	Mr.	Sawang	Sripakdee
30	Mr.	Patcharaphon	Jaikla
31	Mr.	Samruay	Jecksang
32	Mr.	Dechmongkol	Onchompoo
33	Mr.	Manop	Pooyindee
34	Ms.	Choosri	Sangsri
35	Ms.	Siriwan	Korpiboonkij
36	Mr.	Charin	Keawjarat
37	Mr.	Srawuth	Komjalern
38	Ms.	Anchalee	Tungtang
39	Mr.	Surayuth	Srigaewin
40	Mr.	Somkid	Tosawong
41	Mr.	Manas	Gaewsurin
42	Mr.	Yongyut	Kongkaew
43	Mr.	Vittavat	Sangalee
44	Mr.	Mana	Saithasao
45	Mr.	Alongkorn	Hanrujirakomjohn
46	Ms.	Anchalee	Pothipote
47	Mr.	Bumrung	Jaikwang
48	Mr.	Sornchai	Junsuwan
49	Mrs.	Intira	Jareonwatanan
50	Mr.	Amnaj	Andang
51	Mr.	Suvit	Boonkali

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54	Ms.	Utaiwan	Kum-arb
55	Mrs.	Mali	Ittiverakul
56	Ms.	Amporn	Chalouydumrong
57	Ms.	Nillawan	Buathong
58	Mr.	Chaiyawat	Mathavarat
59	Mr.	Prasit	Sookto
60	Ms.	Nitima	Chanarat
61	Mrs.	Somchit	Tulyayon
62	Ms.	Noojcharin	Labhantakul
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66	Mrs.	Chuanpis	Ajanriyakhajom
67	Mrs.	Sumitda	Narupiti
68	Mrs.	Naowayubol	Nutkumhaeng
69	Mrs.	Vipa	Thirawuth
70	Ms.	Panor	Srisonggkram
71	Ms.	Nawarat	Charoensri
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73	Ms.	Patama	Permpanich
74	Mr.	Surind	Siriranond
75	Mr.	Somsak	Imlarp
76	Mr.	Pairote	Tararut
77	Mr.	Pongpun	Sawatwong

78	Mr.	Wichean	Sa-Nguansuk
79	Mr.	Prachakkra	Panthusiri
80	Mrs.	Pranom	Vangnai
81	Mr.	Thongchai	Khainkaew
82	Ms.	Warinda	Srikum
83	Ms.	Wallika	Kulthongkum
84	Mr.	Wanchai	Inpho
85	Mrs.	Pannarat	Chuakunabon
86	Dr.	Robert McNair Scott	
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103	Mr.	Sommai	Phomstaporn
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110	Mr.	Alongkot	Ponlawat
111	Mr.	Chalermpon	Kumpitak
112	Dr.	Rampa	Rattanrithikul
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114	Mr.	Glerkwich	Channoi
115	Ms.	Jaruwan	Tawong
116	Ms.	Ratchaneeporn	Jenwithisuk
117	Mr.	Somporn	Chanaimongkol
118	Mr.	Weeraphan	Kongtak
119	Mr.	Vajira	Auevanich
120	Mr.	Inkam	Inlao
121	MG.	Vichai	Sangkasuwan

Department of Enteric Diseases

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124	Ms.	Ovath	Thonglee
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127	Ms.	Anuchittada	Sirisriro
128	Ms.	Chittima	Pitarangsi
129	Ms.	Waraporn	Tripanichkul
130	Mr.	Boonchai	Wongstitwilairoong
131	Ms.	Ajchara	Aksomboon