During CY11, upgrades of facilities continued with the completion of renovation on the 5th floor of the Main Research Building. Renovate the 6th floor began with an estimated completion date of July 2012. Also, an insectary lab ventilation upgrade project began. These projects are extremely important to the ongoing research at AFRIMS where the buildings are more than 40 years old. Other upgrades to facilities that were completed in CY11 included motor pool repainting and re-roofing.

CY11 saw the implementation of a new purchase system through the Department of State which greatly impacted how the Department of Logistics purchases equipment and supplies for research. The Integrated Logistics Management System facilitates the process of purchase request with a quicker turnaround time for the request approval.

Continuing past trends of high safety standards and excellent work, the motor pool supported over 2,500 missions, driving over 1 million miles in support of AFRIMS research projects and VIP support.

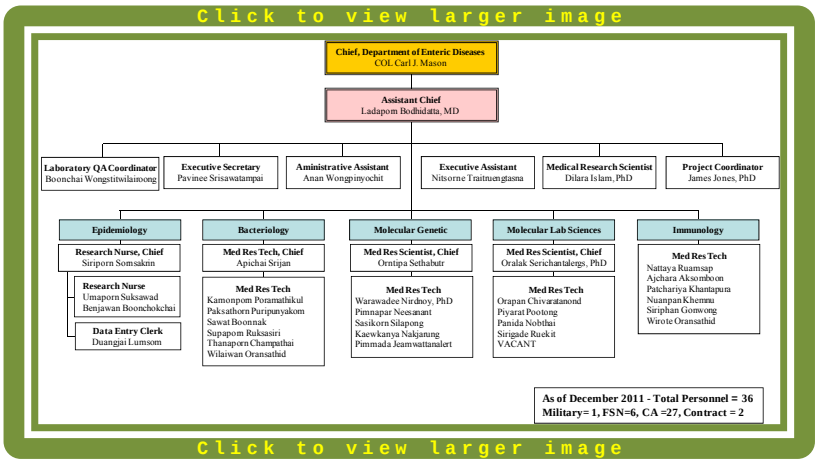
CY11 ended with the Thailand floods which lasted 2-3 months. Mission-essential equipment were moved to higher ground, and all the specimens from our freezer farm located in northern Bangkok were moved. With the untiring efforts of civilian and military staff, and their round the clock work, loss of equipment and supplies was minimized.

DEPARTMENT OF ENTERIC DISEASES

DEPARTMENT MISSION

Develop and evaluate interventions to diagnose, treat and prevent diarrheal disease.

PERSONNEL



Management & Administration

COL Carl J. Mason – Department Chief
Dr. Ladaporn Bodhidatta – Assistant Department Chief
Pavinee Srisawatampai – Executive Secretary
Anan Wongpinyochit – Administrative Assistant
Nitsorne Traitruengtasna – Executive Assistant

Epidemiology Section
Siriporn Sornsakrin – Supervisor
Umaporn Suksawad – Research Nurse (Study Coordinator)
Benjawan Boonchokchai – Research Nurse
Duangjai Lumson – Data Entry Clerk

Bacteriology Section
Apichai Srijan – Supervisor
Sawat Boonnak – Medical Research Technician
Boonchai Wongstitwilairoong – Medical Research Technician
Paksathorn Puripunyakom – Medical Research Technician
Wilawan Oransathid – Medical Research Technician
Supaporn Ruksasiri – Medical Research Technician
Kamonporn Poramathikul – Medical Research Technician
Thanaporn Champathai – Medical Research Technician

Molecular Genetic
Orntipa Sethabutr – Supervisor
Warawadee Nirdnoy, PhD. – Medical Research Technician
Pimmnapar Neesanant – Medical Research Technician
Sasikorn Silapong – Medical Research Technician
Kaewkanya Nakjarung – Medical Research Technician
Pimmada Jeamwattanalert – Medical Research Technician

Molecular Lab Sciences
Oralak Serichantalergs, PhD – Supervisor
Orapan Chivaratanond – Medical Research Technician
Piyarat Pootong – Medical Research Technician
Panida Nobthai – Medical Research Technician
Sirigade Ruekit – Medical Research Technician

Immunology Section
Dilara Islam, PhD. – Research Scientist
Ajchara Eksomboon – Medical Research Technician
Nattaya Ruamsap – Medical Research Technician
Patchariya Khantapura – Medical Research Technician
Nuanpan Khemnu – Medical Research Technician
Siriphan Gonwong – Medical Research Technician
Wirote Oransathid – Medical Research Technician

IN-HOUSE TRAINING PROGRAMS AND OUTSIDE TRAINING OF PERSONNEL

In-House Training Provided by Department

• Training for Collaborators from Tajikistan on Advanced PCR and Microbiology Laboratory Technique. January 2011.

• Training for Infectious Diseases Fellows from Siriraj Hospital on Laboratory Methods for Detection, Isolation, Identification and Antimicrobial Susceptibility Testing of Enteric Pathogens from Stool Specimens. March 2011.

• Laboratory Training for Collaborators from Kathmandu, Nepal on the Detection of Norovirus by PCR Using RotorGene3000. March 2011.

• Laboratory Training for Collaborators from Kathmandu, Nepal on ESBLs Detection by Disk Test and PCR. April 2011.

• Laboratory Training for 4th Year Students from Department of Biology, Faculty of Science, Srinakharinwirot University. 1 April-14 May 2011.

• Laboratory Training for a Naval Preventive Medicine Unit-Kuwait Technician on Norovirus Screening Using Real Time RT-PCR. September 2011.

• Laboratory Training for a Naval Preventive Medicine Unit-Kuwait Technician on Multiplex PCR for *E.coli*, Capsular Genotyping of *C. jejuni*, PFGE, *V.cholerae* Genetic Arrays. September 2011.

• Laboratory Training on Methods for Detection, Isolation, Identification and Anti-microbial Susceptibility Testing of Enteric Pathogens from Stool Specimens for a Regional Medical Technologist, U.S. Embassy New Delhi, India. September 2011.

Outside Training Received or Provided by Department

• Laboratory Training for WARUN Technicians. Kathmandu, Nepal. 14-19 March 2011, 25-29 July 2011, 17-20 October 2011, 15-17 November 2011.

• Training on Identification and Serotyping of *Salmonella*, *Shigella* and *Vibrio* spp. The Public Health Laboratory, Bhutan for Bhutanese Technician. 27 February-March 2011, 21-24 November 2011.

• Calicinet Training provided by the CDC in Atlanta, GA, U.S.A. May 2011.

• Emerging Infectious Disease Research Course, University of Florida, Florida, U.S.A. May 2011.

• International Research in Infectious Diseases Conference, Washington DC, U.S.A. 31 May-2 June 2011.

• Keystone Symposia, Vaccine for Enteric Diseases. Cannes, France. September 2011.

• Keystone Symposia, Malnutrition, Gut-Microbial Interactions and Mucosal Immunity to Vaccines. New Delhi, India. November 2011.

• 60th American Society of Tropical Medicine and Hygiene Conference. Philadelphia, U.S.A. December 2011.

AWARDS

Department of Enteric Diseases staff received no awards for work at WRAIR in CY2011.

ACCOMPLISHMENTS

1. Surveillance for Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand: This study was completed. Over 4,000 stool samples were collected from diarrhea cases and non-diarrhea controls in Bangkok and four regional sites (Chiangrai, Phitsanulok, Nakon Ratchasima, Suratthani). Rotavirus and *campylobacter* were identified as the leading causes of acute diarrhea in this study. Genotyping of rotavirus positive samples was targeted on VP7 gene (G genotype) and VP4 gene (P genotype) by conventional PCR or sequencing. Rotavirus was detected in 26% of diarrhea samples. From all four sites, G1P[8] was the prominent genotype (31-47%), followed by genotype G2P[4] (21-31%). However, atypical genotypes such as G12P[8] and G9P[8] were also detected in lower percentage in all four regions. Norovirus genotyping was also tested and genotype II was predominant in over 95% at all sites.

2. Surveillance for Diarrhea Etiologic Agents at the Public Health Laboratory in Stool Samples from Children Attending Jigme Dorji Wangchuk National Referral Hospital (JDWNRH), Thimphu, Bhutan. A human use protocol to conduct a 2-year prospective surveillance of enteric pathogens and to monitor antimicrobial resistance in children aged 3 months to 5 years of age in Bhutan was completed and approved for implementation. Training on surveillance, informed consenting, enrollment, specimen collection, packing and shipping of biological samples was provided by AFRIMS staff to the site study staff in Bhutan prior to the start. A microbiology laboratory at the Public Health Laboratory (PHL) has been upgraded and training on media preparation, quality control, specimen processing, microbiological identification of enteric bacteria was also provided by AFRIMS microbiologists. Study enrollment started in March 2011 at Jigme Dorji Wangchuk National Referral Hospital, Thimphu, Bhutan. Enteric bacteria are initially identified at PHL by local staff and subsequently confirmed at AFRIMS.

During this reporting period 460 subjects (235 cases with acute diarrhea and 225 non-diarrhea controls) have been enrolled. Rotavirus (15%) and *shigella* (9%) are major pathogens found among diarrhea cases but not frequently found in controls. Serotypes distribution of *shigella* isolates are *S. sonnei* (62%) followed by *S.flexneri* (15%) and *S.boydii* (15%). Enteroaggregative *E.coli* was detected in both cases (25%) and controls (31%). The antimicrobial susceptibility pattern of bacterial isolates in Bhutan reveals a high proportion of *shigella* isolates resistant to nalidixic acid (94%), TMP-SXT (89%) and ciprofloxacin (56%). Resistance to ciprofloxacin among *campylobacter* isolates was observed in 57% of the isolates.

This is the first significant effort to establish comprehensive diarrhea microbiology capabilities in Bhutan. This project suggests that even in relatively remote areas, antibiotic resistance is a concern and warrants continued surveillance. We plan to extend this surveillance effort and improve reporting capabilities to multiple sites in Bhutan particularly in sites close to India and China.

3. Development and Standardization of Real-Time PCR Assays for Detection and Characterization of Enteric Pathogens: Probes and primer sets have been developed and evaluated for the enteric pathogens *Shigella*, ETEC, *Campylobacter*, Cryptosporidia, *Cyclospora*, Norovirus, Sapovirus, Rotavirus, Salivirus, and Astrovirus. During CY2011, over 2,000 frozen clinical stool samples received from multiple study sites in Thailand were tested by a real-time reverse transcriptase PCR for norovirus, sapovirus, rotavirus and astrovirus.

For JBAIDS platform transition, freeze-dried reagents for *Shigella*, LT-ETEC, STIa-ETEC, STIb-ETEC and *Cryptosporidium* were formulated. Detection of norovirus GI and GII genotypes with wet reagents was also completed with formulation of freeze dried reagents for norovirus is pending. However, FDA registration of the JBAIDS platform for enteric pathogens will not pursued further due to insufficient of funds for GMP lot production of freeze dried reagents.

4. Evaluation of the Next Generation Diagnostic Platforms for Enteric Pathogens: As part of a network to evaluate the next generation diagnostic platforms, Luminex, Real-time multiplex PCR and Taqman low density array (TLDA) for the identification of enteric pathogens were evaluated in collaboration with the University of Virginia (UVA). We successfully completed a site verification for Luminex and started testing of clinical samples for viral panel. Bacterial panel samples have been delivered and are in process to be tested with Luminex method. Site verification and testing of clinical samples for viral panel using RotorGene is ongoing. TLDA is awaiting the development of SOP by UVA.

Surveillance of Respiratory Pathogens in Patients Attending Royal Thai Army Hospitals: This study is ongoing with eight active surveillance sites. Of a total of 3,565 volunteers, 657 (18%) were positive by on site rapid testing (482 influenza A, 171 influenza B and 4 both influenza A and B). More comprehensive PCR testing at AFRIMS on an initial 3,198 samples found 790 samples (25%) positive (109 influenza A/H1, 251 influenza A/SW H1, 223 influenza A/H3, 24 unsubtyped influenza A and 183 influenza B). Detection of causative agents for respiratory diseases using MassTaq PCR revealed 322 of initial 926 samples (35%) were infected by viruses (adenovirus, corinavirus-OC43, enterovirus, hMPV, influenza A, -B, RSV-A, -B, and PIV 4 subtypes) and bacteria (*M. pneumoniae*, *H. influenzae* and *S. pneumoniae*). Ninety-three local influenza A/H1 isolates were successfully established in cell culture. Genetic characterization of those isolates is ongoing.

5. Population-Based Sero-Prevalence Survey of Human Infection with Avian Influenza (H5N1) in Vietnam: This study was completed in CY2011. We conducted the study in 3 provinces in Vietnam. A total of 9,564 blood samples were collected (HaTay - 4,197, Thua-Thien Hue - 2,023 and TienGiang - 3,344) with associated information to determine risk factors of exposure e.g., age, sex, exposure to poultry, etc. Antigens for hemagglutination inhibition assay (HI) assay were beta-propiolactone inactivated for six different Influenza A viruses (3 H5N1 and 3 H5N3), which were based on available historical viruses that circulated in the region. There were 107 positive samples from 9,564 samples (1.1%) assayed by HI. Nine of the 107 positive samples were cross reactive for 1 or more viruses. The majority of "positive" HI reactions were to HK97 and VN1203, the strains known to have infected humans in significant numbers. Micronutralization assay (MN) was performed on 107 HI positive samples and randomly selected 3% of the negative samples (approximately 300 samples). Analysis of MN data is in progress.

We plan to randomly select samples for those representing high and low risk populations and evaluate specific antibody responses by nucleoprotein assay, neuraminidase assay, and virus neutralization assay using H5pp (H5 viral pseudo particles).

6. Capsule Genotypes of 213 *C. jejuni* Isolated from Children with Diarrhea in a Multicenter Surveillance Study: Thailand in 2008-2011 were amplified by two multiplex PCR reactions (alpha and beta sets). In this study, six most common capsules in human *C. jejunj* isolates were O:2 (20%), O:17 (13%), O:3 (9%), O:1 (7%), O:4,8486 (7%), and O:4 (5%). These six capsular types were similar to findings reported in CY2010 with the exception of O:5 which was not detected in this study. Approximately 15% of the isolates contain untypable capsule genotypes and will be further identified for capsular sequences.

The capsule of one predominant genotype (O:9,37) was selected and amplified by PCR with primers specific for capsular region(KpsF-KpsC). Amplified products of capsule loci have been sequenced by shot gun assay and approximately 800 clones were detected. Cloning sequences alignment from these clones suggested a complete capsular sequences of approximately 30 kb size. The comparison of capsule sequences of KpsF-KpsC will be continued in CY2012.

Eleven capsular polysaccharide of common *C. jejuni* isolates (O:1, O:2, O:3, O:4, O:4,8486, O:10, O:15, O:7, O:5, O:37, and O:9,37) in Thailand were extracted and run electrophoresis by SDS-PAGE. These capsule polysaccharide extracts were transferred on nitrocellulose by western blot assay. The blotting membrane was reacted with specific rabbit antiserum immunized with *C. jejuni* of selected Penner's serotype. Cross reactivity of rabbit antiserum in different capsular genotypes was observed after immunodetection. These assays will be expanded to perform with cross reactivity in human paired sera obtained from patients with *campylobacter* diarrhea in CY2012.

7. Detection of Extended-Spectrum and AmpC Beta-Lactamase in ETEC Isolates in Thailand, Nepal, Vietnam, Cambodia, Uzbekistan and Kenya: Among 377 ETEC strains isolated from human stool samples collected during 2001- 2010 from those countries, a total of six (1.6%) ESBL-producing ETEC isolates were detected in this study, 5 out of 78 ETEC strains from Nepal, and 1 out of 213 ETEC strains from Thailand. They were selected and phenotypically confirmed by Double Disks Synergy Test (DDST), Combination Disk Test, and detected for Class C β-lactamase production. No ESBL-producing ETEC strain was detected in samples from Cambodia, Kenya, Vietnam, and Uzbekistan. Real-time PCR was useful for the molecular typing of extended-spectrum β-lactamases (ESBLs) of ETEC. Four ETEC strains contain ESBL gene encoding for CTX-M-I and one ETEC strain contains a gene encoding for CTX-M-IV. TEM encoding gene was identified in an ETEC strain that harbors CTX-M-IV encoding gene and in another two ETEC strains that also harbor CTX-M-I encoding gene. One ETEC strain was identified to harbor an ESBL gene encoding in a plasmid-mediated Class C ESBL; LAT. All six ETEC strains were found to carry a chromosomal mediated Class C gene; AmpC. All of ESBL-producing ETEC isolates showed resistance to cefotaxime with the MICs in the range of 256-1,024 µg/mL and were effectively inhibited by clavulanic acid. Sequencing analysis of all 6 ESBL genes has confirmed the types of β-lactamase as identified by real-time and conventional PCR. Comparison of amino acid sequences of CTX- M-I, CTX-M-IV, TEM, and LAT-β-lactamases found in the six ETEC strains reveal 100% identity to the originally identified β-lactamase from *E. coli* strains. The conjugation results in this study revealed that plasmids play an important role in the spreading of β-lactam antibiotic resistance from Enterotoxigenic *E. coli* to other *E. coli* strains. All of ESBLs genes present in six ETEC strains were completely transferred to a recipient *E. coli* strain DH5α by conjugation. The MICs against cefotaxime and ceftazidime of each transconjugant obtained have been determined.

8. Detection of Extended-Spectrum and AmpC Beta-Lactamase in *E.coli* isolates from Urine Samples from National Public Health Laboratory, Nepal. Of 56 ESBL *E.coli* isolated from 55 urine specimens and one pus specimen during 2009-2011 in Nepal, 49 were confirmed ESBL positive *E.coli*. Isolates were tested by phenotypic tests (Double Disc Synergy Test, Combination Test, Class C Beta-Lactamases, and MIC determination assays) and molecular typing of β-lactamases using real time PCR and conventional PCR assays to detect β-lactamase genes encoding TEM, SHV, CTX-M-I, CTX-M-II, CTX-M-III, CTX-M-IV, CTX-M-V, AmpC, and plasmid encoding Class C ESBLs, including MOX, LAT, DHA, ACC, MIR and FOX. All 49 isolates were ESBL positive and Class C Beta-Lactamases negative. The β-lactamase genes found in all 49 *E. coli* strains, CTX-M-I in 48 isolates, CTX-M-IV in one isolate, and TEM in 26 isolates. AmpC gene was found in all isolates.

9. Prevalence of Antibodies to Infectious Diseases of Public Health Importance among Recruits in the Royal Thai Army: A total of 7,760 serum samples were tested to measure prevalence of antibodies to infectious diseases of public health importance by ELISA. The 6 antigens are for: i) Measles, ii) Leptospirosis, iii) Hepatitis E, iv) Scrub typhus, v) Spotted fever group and vi) Murine typhus. The overall unadjusted seroprevalence of protective levels (seropositive) of measles antibodies was 77%, Leptospirosis was 23% and Hepatitis E was 13%. For Scrub typhus, Spotted fever group and Murine typhus the data analysis is ongoing. Data will be analyzed in correlation with demographic information. A geographic information system (GIS) databases will be applied to better understand the spatial distribution of these infectious diseases in different regions of Thailand.

10. Safety, Immunogenicity and Efficacy Studies of WRSS1, a Live Attenuated *Shigella sonnei* Vaccine Candidate, in Healthy Thai Adults: This study was completed in September 2011. In summary, a single dose of 10^4 CFU of WRSS1 is safe. There is no significantly different between the occurrence of related adverse events (AEs) among vaccinees [5/13 (38%)] and placebo controls [4/6 (66%)]. Related AEs included abdominal pain, feverish, tenesmus, headache, malaise, loose stool (not meet diarrhea definition). Most of the related AEs severity was graded as mild. The WRSS1 shows limited vaccine uptake and immunogenicity. Excretion of the vaccine strain was detected among 3/13 (23%) of vaccinees by culture and 9/13 (69%) by PCR. The vaccine strain was not detected in any of the placebo controls by both culture and PCR. 3/13 (23%) and 5/13 (38.5%) of vaccinees had IgG and IgA seroconversion, respectively. All placebo recipients showed no seroconversion.

The WRSS1 vaccine efficacy was determined by challenge 10 vaccinees and 10 naïve controls with 1,600 CFU of *Shigella sonnei* wild type strain 53G. Vaccine efficacy was determined by blinded external review of clinical records for *shigella* related illness. The 3/10 (30%) of vaccinees and 6/10 (60%) of controls were determined to have *shigella* related illness. Vaccine efficacy was calculated as 50%. A single dose of WRSS1 had limited efficacy in this population of Thai adults. Consideration might be given to an escalating-dose multi-dose trial.

11. Evaluation of Live, Attenuated Oral *Shigella Dysenteriae* 1 Vaccine Candidates WRSd3 and WRSd5 in Rhesus Monkeys (*Macaca mulatta)*: The primary objectives of this study was the refinement of evaluation of WRSd3 and WRSd5 vaccine candidates : (i) for safety (ii) protective efficacy by challenging immunized monkeys with *S. dysenteriae* 1 1617 strain (iii) the immune responses and inflammatory responses in blood (iv) to rank the vaccine candidates by comparing the safety, protective efficacy, immune responses and inflammatory responses following immunization with WRSd3 and WRSd5 to obtain the best vaccine candidate for *S. dysenteriae* 1 for "Investigational New Drug Application". Twenty-five (25) monkeys were used for this GLP study. Both vaccine candidates were characterized by using: i) Slide agglutination test with *S. dysenteriae* 1-specific sera, ii) Invasion assay in HEp-2 cells, iii) PCR assay utilizing multiple primers, to confirm the presence or absence of genes and Stability Assay. A total of 25 monkeys were used for the study, 10 monkeys in each immunization group and 5 monkeys as control. The monkeys were immunized on study days 0, 3 and 6 by intragastric administration of the vaccine candidate at a dose of 2×10^{10} CFU. On study day 37 (30 days after last immunization or placebo), the immunized and control monkeys were challenged by intragastric administration with a dose of 2×10^9 CFU of the wild-type *S. dysenteriae* 1 1617 strain. Only after first immunization 5-6 monkeys vomited and 1 monkey immunized with WRSd3 had dysentery. After challenge among the immunized monkeys only 1 (WRSd3) had dysentery, where as 3 control monkeys had dysentery and 2 monkeys vomited. All immunized monkeys elicited immune responses both after immunization and as well as after challenge, whereas only 50% control monkeys elicited immune responses. In conclusion, both WRSd3 & WRSd5 vaccine candidates protected monkeys against challenge by wild-type *S. dysenteriae* 1 1617. Both WRSd3 & WRSd5 vaccine candidates are safe and immunogenic. Arbitrary ranking scored WRSd5 as # 1 vaccine candidate.

FUTURE PLANS AND STRATEGIES

- Continue surveillance of antimicrobial resistance of enteric pathogens and expand surveillance sites in Bhutan and other countries in the region.
- Continue to screen rotavirus, norovirus, and astrovirus from stool samples from various sites and characterize their genotype.

• Continue to collaborate with UVA on testing next generation molecular methods for the detection of enteric pathogens.

• Continue further study on the presence of ESBL genes and the resistant functions using site-specific mutagenesis. Explore for the presence of additional ESBL genes or other β-lactam antibiotic resistant mechanisms and roles of plasmid in spreading β-lactam antibiotic resistances.

• Continue to collaborate with NMRC to evaluate capsule genotypes of *C. jejuni* isolates by using new primer sets, especially in serotypes O:5 , O:19, O: 12, O:21, O:22, O:31 and O:37. Primer sets for these serotypes will be included in the multiplex PCR panel and re-evaluated with *C. jejuni* isolates at AFRIMS.

• Sequencing of other common capsule locus (O:11, O:40) of *C. jejuni* isolates in Thailand. Capsule locus will be amplified, sequenced, and analyzed by long-PCR reaction and short gun cloning or other next generation sequencing.

• Continue studies on pathogen discovery with UCSF and Washington University.

• Molecular characterization of enteroaggregative *E. coli* isolates from diarrhea cases/ controls in Thailand, Nepal and Cambodia.

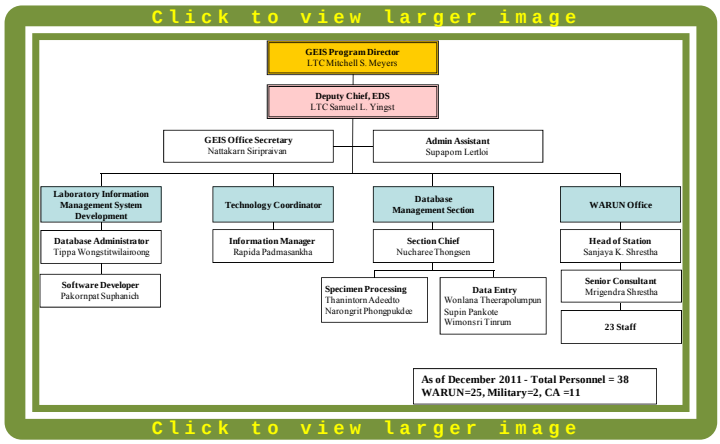
• Characterize antimicrobial resistances of febrile blood borne pathogens (*Salmonella typhi* and *Salmonella paratyphi A*) isolated in Nepal in 2009-2010.

• Study of epidemiology of travelers diarrhea and post-infectious sequelae is planned for implementation in CY2012.

• Study of cross reactivity of human sera of diarrhea patients with *campylobacter* infection to common capsular antigen extract in this geographical area. Evaluate higher dose (105) and multiple doses of WRSS1 *Shigella* vaccine.

DEPARTMENT OF EPIDEMIOLOGY AND DISEASE SURVEILLANCE (EDS)

DEPARTMENT MISSION

The mission of the Epidemiology and Disease Surveillance Department is to support AFRIMS and AFHSC-GEIS goals and objectives pertaining to bio-surveillance, epidemiology, and capacity building for emerging infectious disease outbreak detection and response in Southeast Asia.

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