

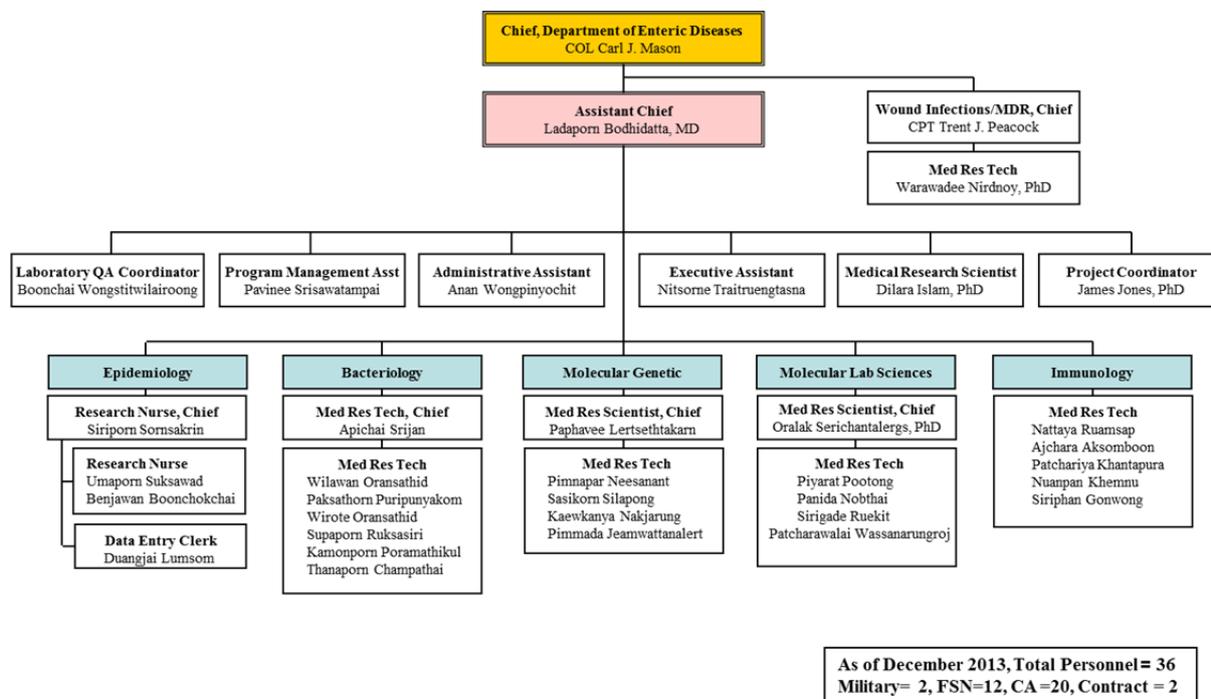


DEPARTMENT OF ENTERIC DISEASES

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
 Dr. James Jones – Project Coordinator
 Pavinee Srisawatampai – Program Management Assistant
 Anan Wongpinyochit – Administrative Assistant
 Nitsorne Traitruengtasna – Executive Assistant
 Boonchai Wongstitwilairoong – Laboratory Quality Assurance

Wound Infections and Multidrug Resistance

CPT Trent J. Peacock – Chief, Wound Infections MDR
 Warawadee Nirdnoy, PhD – Medical Research Technician

Epidemiology Section

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

Bacteriology Section

Apichai Srijan – Supervisor
 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
Wirote Oransathid – Medical Research Technician

Molecular Genetic

Paphavee Ketwalha, PhD – Supervisor
Pimnapar Neesanant, PhD – 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
Patcharawalai Wassanarungroj – Medical Research Technician
Piyarat Pootong – Medical Research Technician
Panida Nobthai – Medical Research Technician
Sirigade Ruekit – Medical Research Technician

Immunology Section

Nattaya Ruamsap – Supervisor
Dilara Islam, PhD. – Consulting Research Scientist
Ajchara Eksomboon – Medical Research Technician
Patchariya Khantapura – Medical Research Technician
Nuanpan Khemnu – Medical Research Technician
Siriphan Gonwong – Medical Research Technician

IN-HOUSE TRAINING PROGRAMS AND OUTSIDE TRAINING OF PERSONNEL

In-House Training Provided by Department

- Enteroaggregative *E.coli* (EAEC) PCR Workshop for Medical Laboratory Technicians from USAMRU-Kenya. 22-26 July 2013 (2 trained)

Outside Training Received or Provided by Department

- Laboratory Methods for Identification of Enteric Pathogens. Kathmandu, Nepal. 22-25 April 2013
- One Health Concept. University of Florida, U.S.A. 8-27 May 2013
- Laboratory Training for Microbiology Laboratory Technicians from CIWEC Clinic. Kathmandu, Nepal. 10-13 June 2013
- Laboratory Methods for Identification of Enteric Pathogens. Thimphu, Bhutan. 16-17 July 2013
- Essential GCP for the New Coming Investigator Conducted by Army Medical Research Development Committee and Pharmaceutical Research and Manufacturer Association. Phramongkutklao Hospital, Bangkok, Thailand. 13 August 2013
- Laboratory Training for Microbiology Laboratory Technicians from Public Health Laboratory. Ministry of Health, Bhutan. 8-13 September 2013
- 17th International Workshop on Campylobacter, Helicobacter and Related Organisms. University of Aberdeen, United Kingdom. 15-19 September 2013
- Fundamentals of International Clinical Research Training (NIAID-NIH, International Clinical Studies Support Center). Bangkok, Thailand. 15-20 September 2013
- Dimagi CommCare Workshop. Bangkok, Thailand. 30 September-1 October 2013



AWARDS

Department of Enteric Diseases staff received no awards for work at WRAIR in CY2013.

ACCOMPLISHMENTS

1. Surveillance for Epidemiology of Diarrhea and Post-Infectious Sequelae in Travelers to Thailand

A prospective case-control study to determine the etiology of traveler's diarrhea and to describe development of post-infectious sequelae and chronic intestinal symptoms after the diarrhea episodes is ongoing. The study has been conducted in travelers who are adult residents of North America, Europe, Australia, New Zealand, Japan, Taiwan and South Korea with and without diarrhea at Bumrungrad International Hospital, Bangkok, Thailand. The enrollment was started in Jan 2012. An amendment to extend subject enrollment for one more year has been approved on 31 July 2013. After obtaining written informed consent, a stool specimen was collected, processed and examined for enteric pathogens by standard microbiology, ELISA or PCR at AFRIMS. A short questionnaire regarding demographic and clinical data was collected. Internet-based surveys at 7 days; 3, 6 and 12 months after enrollment have been conducted to address occurrence of post-infectious sequelae and chronic intestinal symptoms.

During this reporting period, we enrolled 59 subjects with acute diarrhea and 60 non-diarrhea controls. Major pathogens found among diarrhea cases were *Campylobacter*, norovirus, *Salmonella* and major pathogens found among non-diarrhea controls were Enteropathogenic *E.coli*, *Aeromonas*, and *Salmonella*. No enteric pathogens were identified in about 75% of non-diarrhea controls. The antimicrobial susceptibility pattern of bacterial isolates from this study revealed high resistance of *Campylobacter* isolates against nalidixic acid and ciprofloxacin.

2. Surveillance for Diarrhea Etiologic Agents at the Public Health Laboratory in Stool Samples from Children Attending Jigme Dorji Wangchuk National Referral Hospital (JDWRH), Thimphu, Bhutan

As planned, expansion of the diarrhea surveillance effort and improvement of reporting capabilities to multiple sites in Bhutan particularly in sites close to India and China has been conducted. A hospital based surveillance of enteric pathogens and antimicrobial resistance bacteria among children aged 3 months to 5 years with acute diarrhea and non-diarrhea controls at the Jigmi Dorji Wangchuk National Referral Hospital, Thimphu, and 3 regional hospitals in Mongar, Phuntsholing and Gelephu, Bhutan. Enrollment and sample collection was started on 23 July 2013. Stool specimens have been collected, processed and examined at the Public Health Laboratory (PHL), Thimphu, Bhutan and subsequently confirmed at AFRIMS. The Department of Enteric Diseases, AFRIMS has continued providing consultation and training to the local microbiologists on isolation and identification of enteropathogens.

During this reporting period, 409 cases with acute diarrhea and 366 non-diarrhea controls were enrolled. Rotavirus, norovirus, and *Shigella* were major pathogens found among diarrhea cases and infrequently found in controls. Enterotoxigenic *E.coli* was detected in both cases and controls in about 30% of each. The antimicrobial susceptibility pattern of bacterial isolates in Bhutan revealed a high proportion of *Shigella* isolates resistant to nalidixic acid, TMP-SXT and ciprofloxacin. Resistances to nalidixic acid and ciprofloxacin among *Campylobacter* isolates were also observed.



3. Surveillance for Epidemiology of Diarrhea and Post-Infectious Sequelae in Travelers to Nepal

A 2-year prospective case-control study to determine the etiology of traveler's diarrhea and to describe development of post-infectious sequelae and chronic intestinal symptoms after the diarrhea episodes has been implemented in November 2012. The study has been conducted at the CIWEC Travel Medicine clinic in Kathmandu, Nepal. After obtaining written informed consent, a stool specimen was collected, processed and examined for enteric pathogens. Specimen processing, microscopic examination and microbiology work has been conducted in the CIWEC clinic laboratory in Nepal with confirmation and further characterization of organisms at AFRIMS in Thailand. A short questionnaire regarding demographic and clinical data was collected. Internet-based surveys at 7 days; 3, 6 and 12 months after enrollment have been conducted to address occurrence of post-infectious sequelae and chronic intestinal symptoms. Amendment to revise one of the overly restricted inclusion criteria has been approved for implementation since 31 July 2013.

In CY13, 218 cases and 124 non-diarrhea controls were enrolled. *Campylobacter*, Enterotoxigenic *E.coli* (ETEC), and rotavirus (7%) were commonly identified among travelers with diarrhea but not among controls. Enteropathogenic *E.coli* (EPEC) and Enteroaggregative *E.coli* (EAagg) were similarly found in both cases and controls.

4. Evaluation of Safety, Immunogenicity and Efficacy of an Oral Trivalent Inactivated Whole Cell *Shigella* Vaccine in Healthy Thai Adults

An evaluation of a trivalent inactivated whole cell *Shigella* vaccine containing three major serotypes of *Shigella* (*S.sonnei*, *S.flexneri 2a* and *S.flexneri 3a*) has been initiated during CY12. A clinical trial protocol and regulatory documents are under a review process. The study is planned for implementation in late CY14 in collaboration with the Vaccine Trial Centre, Mahidol University in Bangkok, Thailand.

5. Continued Evaluation of Multiplex PCR for Capsular Genotyping of *Campylobacter jejuni* Isolates and Distribution and Heterogeneity of Capsule Genotypes of *C. coli* Isolates from Thailand and Southeast Asia

In CY13, more primers specific for *C. jejuni* Penner serotypes HS63 and HS33& HS35 (Alpha set), HS62 (Gamma set), and HS 38, HS40, HS52 and HS55 (Delta set) have been received from NMRC between January and March 2013. These new primers sets were included with the previous primer sets in CY12 and thus made up of a total of 38 primer sets (Alpha set, HS2, 3, 4, 6, 10, 15, 41, 53, 19, 63, 33; Beta set, HS1, 17, 8486, 23, 42, 31, 12, 21,27, 57; Gamma set, HS37, 22, lpxA, 44, 9, 18, 29, 45, 62; and Delta set, HS60, 32, 58, 11, 40, 52, 55, 38). The primers were re-evaluated with DNA extracts from *C. jejuni* isolates from AFRIMS studies. The DNA extracts included 1013 *C. jejuni* isolates from previous studies (331 isolates from Bumrungrad Hospital 2001-2002 and Cobra Gold 1998-2003, 469 stains from Thailand multi-center diarrheal surveillance study 2004-2006, Nepal diarrheal surveillance study 2001, 2007-08 and Cambodia diarrheal surveillance study 2004, and 213 strains from Thailand surveillance study from 2008-2010) and 53 recent studies (Bumrungrad Hospital 2011, Bhutan 2011, Nepal CIWEC 2012, Nepal MAL-ED 2012). The three most common Penner HS 2 (14.4%), HS 8 or HS17 (9.9%) and HS 4cpx A+B or HS62 (9.0%) were detected by multiplex PCR and accounted for 32.4% for 1013 *C. jejuni* isolates. Results obtained with 53 recent *C. jejuni* isolates showed that HS 8 or HS17 (15.1%), HS 2 (13.2%) and HS 1 or HS1/44 (11.3%) were the most three common Penner serotypes from Southeast Asia. The four multiplex PCR sets (ALPHA, BETA, GAMMA, and DELTA) identified most of *C. jejuni* Penner serotypes. The untyped *C. jejuni* isolates decreased to 2% (as previously detected ~ 5%).



In CY13, AFRIMS received 4 primer sets for detection of Penner serotypes HS61, HS51, HS30 and HS14 in *C. coli* isolates by singleplex PCR. These primers were evaluated with 185 *C. coli* from archived isolates from 1998-2010 (Thailand 2001-2010 (n = 101), Cobra Gold Exercises 1998-2004 (n = 27), Cambodia 2004 (n = 8), and Nepal 2001, 2007-2008 (n = 49). The results demonstrated that only 15% (27/185) of *C. coli* were typed. Penner serotypes HS30 (10/185), HS61 (11/185) and HS51 (6/185) were detected from these *C. coli* isolates.

In CY13, PFGE and MLST assays were completed in 311 *C. jejuni* isolates from travelers' diarrhea (Bumrungrad Hospital 2001-2002 and Cobra Gold 1998-2003) and 211 *C. jejuni* isolates from indigenous population (Thailand surveillance study from 2008-2010). The PFGE, MLST results were analyzed using BioNumerics software and compared to input data for Penner serotypes by multiplex PCR. A clonal relationship of PFGE, MLST and Penner serotypes was seen in isolates from Travelers' diarrhea. Some specific ST types from MLST were related to specific Penner serotypes for example in HS3cpx-MLST 5, HS 37-MLST 51.

6. 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 (seropositive) of measles antibodies was 77%, Leptospirosis was 23%, Hepatitis E. was 13%, Scrub typhus was 12%, Spotted fever group was 3% and Murine typhus was 6%. 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. The current status is on manuscript writing for seroprevalence of Measles and Hepatitis E.

7. Defining the Therapeutic Efficacy of Polypropyletherimine Dendrimer Glucosamine (PETM-DG), a Non-Antibiotic Based Drug, in Rhesus Monkeys after *Shigella dysenteriae* 1 Infection

The GLP study is May 2013. The primary objective of this protocol was to evaluate the efficacy of PETIM-DG as a useful new therapeutic medicine in Rhesus monkeys: i) for safety in rhesus monkeys after oral administration, by monitoring the presence and severity of clinical signs, (ii) protective/therapeutic efficacy by challenging PETIM-DG treated monkeys with *S. dysenteriae* 1 1617 strain, (iii) measuring the cytokine mediated inflammatory responses in the ileum, jejunum, colon and rectum, (iv) histopathological outcome in the ileum, jejunum, colon and rectum and (v) obtaining Rhesus monkey data for a novel therapeutic approach for *S. dysenteriae* 1 infection to support an "Investigational New Drug Application". There were DG treatment group and a Control Group. The study will be performed in three phases, 4 monkeys in each phase with 2 monkeys per group. Monkeys will be randomly assigned, regardless of sex, to the DG Group or Control Group. The DG-Gr. Monkeys were given prophylactic DG (6 mg/kg body weight) on study days 0, 1, 2, 3 and 4 by intragastric administration of the 20 ml DG solution by a naso-gastric tube, after administration of 20 ml bicarbonate buffer. The control group received a placebo (20 ml sterile water) by intragastric administration via naso-gastric tube. On study day 00 (4 ± 1 hour after DG treatment or placebo given), the DG treatment group and control monkeys were challenged by intragastric administration with wild type strain *S. dysenteriae* 1 1617, via naso-gastric tube, with a dose of 2×10^9 CFU in 20 ml of sterile PBS, after administration of 20 ml bicarbonate buffer. This study was conducted to evaluate whether PETIM-DG can prevent gut wall tissue damage in rhesus monkeys infected with wild type *Shigella dysenteriae* 1 1617 strain and without the use of antibiotics.



Real-time PCR analysis support was provided to detect *Shigella* from stool samples during the pre-infected and infection periods. Evaluation of cytokines post-infection from tissues samples will be performed using real-time reverse transcription (RT)-PCR. Method validation to show that real-time RT-PCR is suitable for evaluation of cytokines is completed.

For currently status of the study, animal part has been completed. The preliminary results suggest PETIM-DG may have clinical utility in the management of the mucosal damage produced by both gastrointestinal infection and inflammation.

For laboratory assays;

- **ELISA:** The assay was conducted for measuring IgA, IgG and IgM antibody titers against *S. dysenteriae* 1 LPS and Invaplex antigens. The data is under analysis process.

- **Real-time reverse transcription (RT) PCR assay:** Method validation of RT-PCR is currently being performed. Once the validation is completed, the RT-PCR assay will be performed on study tissue samples.

- **Histopathologic examination:** Tissue sections were processed by histologic method and stained with hematoxylin and eosin. The data is under analysis process.

- **FACS analysis of intracellular cytokines with phenotype of T cells:** SOP of FACS analysis is under reviewed by Quality Assurance Unit. Once the SOP is approved, flow cytometry assay will be performed.

8. Development, Optimization and Standardization of Real-Time PCR Assays for Detection and Characterization of Enteric Pathogens

The focus of development and optimization has been on salivirus, a newly discovered pathogen. Assay development to detect salivirus has been previously attempted in the laboratory but was not successful. For the current attempt, stool samples known to contain salivirus were obtained and are being used in positive control development. The development of positive control has produced promising results and confirmation of results will allow us to proceed with the optimization of the detection assay. Archived frozen stool samples that were identified to be pathogen negative will be screened for salivirus using the optimized detection assay.

9. Evaluation of the Next Generation Diagnostic Platforms for Enteric Pathogens

In collaboration with the University of Virginia (UVa) as part of a network to evaluate the next generation diagnostic platforms: Luminex, real-time multiplex PCR, and TaqMan[®] Array Card (TAC) for the identification of enteric pathogens, the department continued to the next phase of the project and successfully evaluated performance of TAC on clinical diarrhea stool samples. Currently, we are on the last phase of the project applying TAC to study the impact of enteropathogens on diarrhea burden and growth on birth-cohort study.

10. Detection of Extended-Spectrum and AmpC Beta-Lactamase in Diarrheagenic *E.coli* and *Shigella* Isolates in Bhutan

In CY13, a total of 575 Diarrheagenic *E. coli* (DEC) and 87 *Shigella* spp. isolated were screened by disk diffusion assay for Extend-Spectrum β -lactamases (ESBLs) producing strains and confirmed by combination disk diffusion assay. The boronic acid assay was performed on these ESBLs strains for plasmid-mediated AmpC β -lactamases phenotypes, respectively. Total of 144 DEC isolates and 2 *Shigella* isolates was phenotypically confirmed as ESBLs isolates by combination disk diffusion assay. The result showed that 146 ESBLs isolates accounted for 25.0 % (144/575) and 2.3% (2/87) among DEC and *Shigella* isolates, respectively. Boronic acid assay were detected in 10 of 146 ESBLs isolates, thus they were phenotypically identified as AmpC β -lactamases strains. All 146 ESBLs producing strains were further characterized by 5 multiplex PCR sets and 2 singleplex PCR sets for detection of β -lactamases genes. These



multiplex PCR/singleplex PCR identify genes encoding for ESBLs Class A β -lactamases (TEM, SHV, OXA, and CTX-M), AmpC β -lactamases (ACC, FOX, MOX, DHA, CIT, and EBC), and Minor ESBLs (GES, PER, VEB, OXA-48 like, IMP, VIM, and KPC). The PCR results showed that 98.6% (142/146) of ESBLs strains were positive for gene(s) coding for β -lactamases. By PCR assays, genes coding for ESBLs [CTX-M group1 (84.8%), TEM (34.5%), OXA (23.5%), SHV (6.9%), CTX-M-Group 9 (0.7%)] and genes for AmpC β -lactamases [DHA (3.5%) and CIT (0.7%)] were detected from 146 ESBLs phenotypes isolates. None of 146 ESBLs isolates harbored gene coding in the Minor β -lactamases ESBLs. By direct sequencing of amplified products and amino acid alignment, all CTX-M-ESBLs belonged to CTX-M-15 and CTX-Group 9 was CTX-M-14. All TEM type ESBLs belonged to TEM-1 including all SHV-type ESBLs was SHV-12. All OXA genes were OXA-1. The DHA gene detected in five ESBLs isolates were DHA-1 and suggested AmpC β -lactamases genotype whereas 10/146 AmpC β -lactamases were detected phenotypically. One positive CIT gene isolate was CMY-42. The three most frequent β -lactamases genotypes identified in 144 DEC and 2 *Shigella* spp. in this study were CTX-M-15, TEM-1+ CTX-M-15, CTX-M-15+OXA, which accounted more than 75% of all ESBLs isolates.

In conclusion, our data showed high prevalence of CTX-M-15 among ESBLs producing strains from DEC/*Shigella* isolates from children diarrhea in Bhutan. The plasmid mediated AmpC β -lactamases gene were detected infrequently (<5%) and the absence of minor ESBLs genes among DEC/*Shigella* isolates suggested that no new variant ESBLs in the enteric *E. coli* and *Shigella* in Bhutan.

11. 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 three 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. Microneutralization assay (MN) was performed on 107 HI positive samples and randomly selected 3% of the negative samples (approximately 300 samples). The remaining study samples are maintained in -80 F freezers at the National Institute of Hygiene and Epidemiology in Hanoi. We have prepared an additional protocol, currently under review, to further examine avian influenza virus (H5N1) exposure in 6,154 samples. An influenza A IgG ELISA and an H5N1 ELISA will be utilized as test assays. The study will be initiated in 2014.

12. Characterization of Enteroaggregative *E. coli* (EAEC) Isolated from Diarrhea in Bhutan, Cambodia, Kenya, Thailand, and Nepal

A total of 343 EAEC from Bhutan (178 cases, 165 controls), 63 EAEC from Nepal (36 cases, 27 controls), 30 EAEC from Thailand (15 cases, 15 controls), 49 EAEC from Cambodia (49 cases, 0 controls), and 100 EAEC from Kenya (62 cases, 38 controls) were characterized for adherence fimbria genes, serine protease autotransporter toxins (SPATES), and enterotoxin genes in order to define epidemiological potential virulence related strains in EAEC isolated from diarrhea cases/controls using singleplex/multiplex PCR reactions. The primers sets, designed to amplified products for common SPATEs genes; *sat*, *sigA*, *pet* (Class I SPATE), *pic*, *sepA* (Class II SPATE), including adherence fimbriae genes encoding from plasmid (*aggR*, *aggA*, *aafA*, *agg3A* and *agg4A*) and enterotoxin genes (*astA* and *set*) were used to tested with all EAEC isolates from the collections from Bhutan, Thailand, Nepal, Kenya, and Cambodia.



Of a total of 343 EAEC isolates from Bhutan (178 cases, 165 controls), adherence fimbria type IV (*agg4A*) was significantly detected from EAEC isolated from Bhutan. None of SPATES genes were significantly detected in case/controls from EAEC isolates. However, EAEC possessed genes two enterotoxins (*astA* and *set*) genes that showed more significance in cases than in controls ($p < .05$).

A total of 93 EAEC isolates including 63 EAEC isolates (36 cases, 27 controls) from travelers' diarrhea study at Bumrungrad International Hospital, Bangkok, Thailand and 30 EAEC isolates (15 cases, 15 controls) from CIWEC travelers' diarrhea study in Nepal were amplified for adherence fimbria genes, SPATES genes, as well as enterotoxin genes. None of adherence fimbria genes, SPATEs and enterotoxin genes were found statistically significance in EAEC isolates from cases and controls.

A total of 100 EAEC archived isolates (62 cases, 38 controls) from Diarrhea study conducting by USAMRU-K, Kenya has been tested for multiplex PCR and singleplex PCR for adherence fimbria, SPATEs and two enterotoxin genes. Only SPATE II (*pic*) gene was detected significantly in cases than in controls ($p < .05$) in EAEC isolates from Kenya, where as adherence fimbria gene type II (*aafA*) was significant ($p < .05$) among cases/controls from EAEC isolates from Kenya.

A total of 49 EAEC archived isolates from Diarrhea study conducting by NAMRU II has been tested at NAMRU-2 laboratory for multiplex PCR and singleplex PCR for adherence fimbria, SPATEs and two enterotoxin genes. All 49 EAEC isolates were obtained from cases so the significance of each gene could not be compared to asymptomatic controls.

In summary, combined all EAEC data suggested that adherence fimbria type II (*aafA*) and IV (*agg4A*) were more common in EAEC isolates from Kenya and Bhutan, respectively. Our previous data showed adherence fimbria type I (*aggA*) and type III (*agg3A*) were the two most common fimbria types in Thailand and Cambodia, respectively. None of SPATE genes were significant among EAEC isolates from all sites except *pic* gene in SPATE II in EAEC isolates from Kenya. Two enterotoxin genes (*astA* and *set*) previously described in EAEC and *Shigella* spp., respectively were also detected significantly in EAEC isolates from cases in Bhutan.

13. Evidence of Exposure to Human Influenza A Virus and Avian Influenza Virus (H5N1) among two High Risk Areas in Thailand

The primary objective of this study is to determine the evidence of exposure to human and avian influenza virus during H5N1 outbreaks in Royal Thai Army recruits from two high risk areas; Bangkok and Suphanburi Province during 3 periods (pre-, during and post- avian influenza outbreak) in Thailand. The two of geographic sites are selected based on risk factors of exposure to influenza viruses;

1) Bangkok Province represents a high risk area of human influenza exposure for different reasons, this urban center of over 10 million people is a political, cultural, economic and travel hub of Southeast Asia. The capital to the kingdom of Thailand, Bangkok is one of the most populated cities in Asia with more than eight million people live here but it is notoriously difficult to estimate Bangkok's population because of large undocumented immigration population. The community of overcrowded population, poor sanitation, largest consumption of food products, traffic and pollution may increase a threat in causing influenza pandemic which may easily spread throughout the country.

2) Suphanburi Province represents the high risk area of H5N1 outbreaks where H5N1 human cases were detected; and it was counted for the second highest area of rice crop production representative of H5N1 spread from free grazing ducks. Suphanburi Province has 868,681 people in 195,270 households across 10 districts, the largest population of free-grazing



ducks and the most HPAI outbreaks since 2003. The preliminary analysis on HPAI distribution in Thailand indicated that Suphanburi Province accounted for nearly 50% of all outbreaks in ducks (outbreaks in ducks referred to all types of domestic ducks). Pre-existing sera samples collected as a part of the ongoing HIV-1 surveillance activity, from recruits entering the RTA will be used for this study. The phase of sample collected would be the onset of H5N1 outbreaks, which then will be divided into 3 periods; 1) during the 2001-2002 representing pre-pandemic period of H5N1 outbreak, 2) during the 2004-2005 representing pandemic period and 3) during the 2010-2011 representing post-pandemic period. The sera specimens will be evaluated for serologic evidence of influenza A virus exposure by detecting:

- a) NP Influenza antibody response determined by commercially ELISA kit.
- b) Antibodies specific to highly conserved regions of H5N1 influenza viruses by performing H5N1- ELISA.
- c) Subtype-specific antibodies against hemagglutinin (HA) of Influenza A H5 viruses by performing hemagglutination inhibition (HI) assay. The assay will be conducted with 6 of H5 influenza A virus strains; A/Duck/Hong Kong/820/80 (H5N3), A/Duck/Hokkaido/4/96 (H5N3), rg-A/Duck/Singapore/3/97 (H5N3), rg-A/Hong Kong/156/97 (H5N1), rg-A/Viet Nam/1203/04 (H5N1) and rg-A/Duck/Laos/3295/06 (H5N1)

For current status of the study; the protocol has been approved by Phramongkutklao Institutional Review Board. In the process of laboratory assays as mentioned below.

- 1) NP Influenza antibody response is being performed.
- 2) H5N1-SeroDetect ELISA is under testing trial assay condition.
- 3) HI assay was completed and data analysis is being performed.

14. Establishment of an ICR mouse (*Mus musculus*) Challenge Model for *Salmonella typhimurium* and Enterotoxigenic *Escherichia coli*, and Evaluation of Recombinant *S. typhimurium*ST Δ aroA/ Δ htrA CJ0113/PAL/cHMGB1 and Killed *Bacillus subtilis* 1A857 Vaccine Candidates in the ICR Mice Model

The study was approved by IACUC in October 2013 and animal part was started in November 2013. The objectives of this protocol are to: (i) establish a viable challenge model for *Salmonella typhimurium* (ST) and ETEC infection in ICR mice and to evaluate recombinant live double-attenuated *S. typhimurium* ST Δ aroA/ Δ htrA CJ0113/PAL/cHMGB1 and killed *B. subtilis* 1A857 vaccine candidates for, (ii) safety in mice after oral administration of single or double doses by monitoring the presence and severity of clinical signs after immunization, (iii) protective efficacy by challenging immunized mice with ST and ETEC strains, and (iv) evaluation of immune responses in blood and intestinal washes and inflammatory responses in gut tissue samples. The experimental design, mice will be divided into two experimental parts (part-1 and part-2). Mice in each part will be divided into several groups.

Part-1: Optimal challenge dose determination

The purpose is to determine optimal challenge dose of *S. typhimurium* (ATCC 14028) (ST) strain and ETEC H10407 strain. For part-1, there will be three groups for each challenge strain (total 6 groups), with 20 mice for each challenge group, and 5 control mice. Three days (at study day -3) prior to challenge (on study day 0) mice will be moved to ABSL3 containment from the breeding colony. All three challenge groups (for each challenge strain) will be challenged consecutively with 10^7 to 10^9 CFU of the challenge strain to determine the optimal challenge dose that causes moribund illness in mice (i.e.; inability to move, severe depression, reduced appetite, ruffled fur, lethargy, hunched posture, ataxia, tremors, severe diarrhea).



Part-2: Evaluation of vaccination (single or double-dose) in mice challenged with ST or ETEC

The vaccine candidates will be evaluated in this study are recombinant whole cell vaccine candidates, include: i) a live double-attenuated *Salmonella* vaccine construct: STΔaroA/ΔhtrACJ0113/PAL/cHMGB1, ii) a killed *Bacillus subtilis* 1A857 vaccine construct; and iii) the killed *B. subtilis* with adjuvant mannosylated chitosan. This adjuvant mannosylated chitosan has already been used widely including cancer immunotherapy. Groups of 5- to 7-week-old female ICR mice will be immunized intra gastrically via oral gavage with one or two doses of the vaccine strains. The mice will be immunized on days 0 and/or 7 and will be challenged with *Salmonella* and ETEC strains 28 days after the last immunization. There will be three control mice groups for each of the vaccine evaluations, with 45 mice in each group. PBS is the placebo for groups CON-Gr-2 and CON-Gr-3, and PBS plus the adjuvant mannosylated chitosan is the placebo for CON-Gr-4.

For currently status of the study, animal part is being conducted to determine the optimal dose of challenge strains for of *S. typhimurium* (ATCC 14028) (ST) strain and ETEC H10407 strain. Once the optimal dose is determined, experimental design for part-2 will be conducted to evaluate vaccination (single or double-dose) in mice challenged with ST or ETEC.

15. Antimicrobial Resistance Patterns and Molecular Characterization of *N. gonorrhoeae* Isolates in Bhutan

A study of antimicrobial resistance patterns and molecular characterization of *N. gonorrhoeae* (NG) isolates collected in the Jigmi Dorji Wangchuk National Referral Hospital (JDWRH), Thimphu, Bhutan has been conducted. Isolates kept at -70°C at Clinical Laboratory of JDWRH have been shipped to AFRIMS for further examination. Isolates are subcultured for a rapid identification confirmation and also detection of β-lactamase production by a commercially available API® NH (bioMérieux, Inc., Durham, NC, USA). Antimicrobial susceptibility testing of the confirmed isolates has been performed by using E-test method to determine Minimal inhibitory concentration (MIC) (μg/mL) against Penicillin, Tetracycline, Ciprofloxacin, Ceftriaxone, Cefixime, Azithromycin, and Spectinomycin.

During this period, 146 frozen NG isolates were shipped to AFRIMS. Confirmation of isolates and antimicrobial susceptibility testing by MIC has been completed on 36 isolates. Of these, all NG isolates show no resistance to Ceftriaxone, Cefixime and Spectinomycin. All except one isolate showed either resistances (28/36 or 78%) or intermediate susceptibility (7/36 or 19%) to ciprofloxacin. 24/36 (67%), 7/36 (19%), 5/36 (14%) were resistant, intermediate susceptible or susceptible to tetracycline, respectively. Twenty four isolates (67%) were found positive for beta lactamase production as tested by API NH. Selected NG isolates will be submitted for molecular characterization of resistance genes.

16. Clinic-Based Surveillance for Diarrhea Etiologic Agents in Children and Military Personnel in Battambang, Cambodia

A human use protocol for diarrheal disease surveillance in military population and children at the Military R5 and Battambang Regional hospitals, Battambang, Cambodia has been developed, reviewed and approved by AFRIMS scientific review committee and the local IRB (National Ethics Committee on Health Research, NECHR) and the WRAIR IRB. An approval from the United States Army Medical Research and Materiel Command, Office of Research Protections, Human Research Protections Office (USAMRMC ORP HRPO) is pending. Study site assessment, lab setup and staff training have been initiated. Study implementation is planned in the third quarter, CY14 pending final approval of the protocol.



FUTURE PLANS AND STRATEGIES

- Continue to conduct diarrhea etiology and antimicrobial resistance surveillance in military population and travelers.
 - Continue to maintain an archive of well characterized stool samples and enteric bacteria for future testing of newly emerged pathogens, diagnostic platforms or molecular evolution.
 - Continue to provide training, infrastructure and capability development to field laboratories as part of a collaboration on diarrhea surveillance.
 - Continue to screen for rotavirus, norovirus, and sapovirus 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 to develop and optimize detection assays to detect newly discovered pathogen.
 - Plan to complete laboratory assays for PETIM-DG monkey study.
 - Plan to complete laboratory assays for the study of “Evidence of exposure to human influenza A virus and avian influenza virus (H5N1) among two high risk areas in Thailand”.
 - Conduct the study of “Prevalence of antibodies against influenza A and avian influenza (H5N1) viruses in Vietnam”. The National Institute of Hygiene and Epidemiology in Vietnam has given IRB approval for the study. As soon as we receive a study participation agreement letter from the World Bank, the WRAIR HSRB will approve the study. Anticipate a May 2014 start.
 - Continue to collaborate with NMRC to evaluate capsule genotypes of *C. jejuni* and *E. coli* isolates by incorporated new available primer sets in singleplex/multiplex PCR assay.
 - Continue to collaborate with NMRC to subtype and characterize *C. jejuni* isolates of Penner HS4cpx, HS2, and HS1 for isolates lacking of LOS synthesis genes that mimic human ganglioside for further use in vaccine study by NMRC.
 - Continue to characterize archived *C. jejuni* isolates for Type 6 Secretion System (T6SS).
 - Continue to conduct animal part of the mice protocol to establish a viable challenge model for *Salmonella typhimurium* (ST) and ETEC infection and to evaluate recombinant live double-attenuated *S. typhimurium* ST Δ aroA/ Δ htrA CJ0113/PAL/cHMGB1 and killed *B. subtilis* 1A857 vaccine candidates.