SHORT TERM SCIENTIFIC MISSION (STSM) SCIENTIFIC REPORT

This report is submitted for approval by the STSM applicant to the STSM coordinator

Action number: CA17108 - Aedes Invasive Mosquitoes
STSM title: Morphological and Molecular Characterization of Invasive and Native Mosquito Species and Screening of Mosquito-Borne Arboviruses
STSM start and end date: 01/09/2020 to 26/09/2020
Grantee name: Dr. Leila Tajedin, University of South-Eastern Norway (USN), Norway

PURPOSE OF THE STSM:

The purposes of this STSM were as follows:

1. To acquire training in following techniques and methods: morphological and molecular identification of mosquito species; arbovirus screening in mosquito vector samples; modelling of spatio-temporal abundance and distribution of mosquitoes and arboviruses; and real time PCR data analysis. The skills and knowledge will be exchanged with interested students, researchers and public in Norway to create public interest and awareness. Most importantly, the knowledge will be utilized by the University of South-Eastern Norway to initiate projects on entomological surveillance of mosquito species and mosquito borne arboviruses in Norway.
2. To strengthen long-term research collaboration between USN, Norway and Umeå University, Sweden in programs related to mosquito surveillance and arbovirus screening in Norway and Sweden. Norway and Sweden share borders and also face common challenges posed by mosquitoes and mosquito borne arboviruses (i.e. Aedes communis a key vector of the endemic inkoo virus). Norwegian research institutions and scientific community in the field of mosquito borne disease epidemiology can benefit a lot by enhancing regional cooperation with Magnus Evander's research group at Umeå university – prominent and actively involved in mosquito vector and arbovirus research in Sweden and other parts of the world. Additionally, it was a great opportunity to seek new research contacts in Sweden and look for avenues to collaborate in research projects of common interest.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

I got acquainted with techniques and methods associated with STSM objectives which are mentioned below:

1. **Mosquito sampling methods and designing sampling procedures (weeks 2 and 4).**
   Theory sessions: Lectures on mosquito sampling methods and how to design sampling procedures were delivered by Dr. Olivia Wesula Lwande and Prof. Joacim Rocklöv. Following were discussed: A) surveillance and monitoring tools including various traps, B) biology of vector species, C) Mapping of breeding sites, mapping of adult mosquitoes and arboviruses D) process based modeling, statistical and niche modeling.

Practical session: Mock presentation was given on how to assemble different types of traps such Mosquito Magnet® trap, gravid trap, oviposition trap, CDC light trap-CO_2 baited, BG-Sentinel® trap with chemical lure and Improved Prokopack Aspirator (for trapping indoor mosquitoes). The CDC light traps (CO_2 baited) and BG-Sentinel® traps (having chemical BG-lure) were set in the field around the campus for adult mosquito collection. Two sites in the Umeå region known to contain several natural bird and wild animal habitats were visited to study the type of vegetation and identify suitable sampling sites.

2. **Morphological identification of various mosquito species (week 3-4).**
   Theory sessions: Prof. Anders Lindström, the renown entomologist in Sweden (from the Swedish National Veterinary Institute) delivered a lecture on mosquito species types, morphology, species distribution, vector biology and vector competence. He particularly focused on mosquitoes in Sweden and that on invasive Aedes species found in Europe. In addition, a theoretical lecture on basic taxonomy and morphology based identification of mosquitoes was given by Dr. Olivia Wesula Lwandes.
   Practical sessions: To practice morphological identification of mosquitoes, several mosquito specimens belonging to various genera including Anopheles, Aedes, Culex and Culiseta were provided to me. Morphological identification of mosquito specimens (from Evander's projects) was done under stereomicroscope upto species level using Illustrated Key to the Mosquitoes of Fennoscandia: Finland, Sweden, Denmark, Norway (Stojanovich, C.J. 1995).
3. **Genetic barcoding technique for characterization of vector species (week 3).**
   Practical session: For molecular characterization of mosquito vector species, samples were subjected to DNA extraction and barcoding and is described as follows. Each individual mosquito was homogenized in a 2 ml micro tube with cap (Sarstedt, Nümbrecht, Germany) containing three 2 mm steel beads (AB Nino Lab, Upplands Väsby, Sweden) and 350 µl of 1x sterile filtered Dulbecco's Modified Eagles Media (DMEM) (Sigma-Aldrich, St Louis, MO, US) with 2% HEPES (Fisher Scientific, Fair Lawn, NJ, US). Homogenisation was performed using FastPreps 120 (Q-BIOgene, Irvine, CA, US) at 6.5 m/s for 20 s. Then 30 µl of homogenized mosquito was mixed with 70 µl PBS and DNA was extracted using NucleoSpin DNA Insect (Macherey-Nagel, Germany) according to the manufacturer's instructions. Conventional PCR was done to identify mosquito species using the extracted DNA as template and universal DNA primers for PCR amplification of a 710-bp fragment of the mitochondrial cytochrome c oxidase subunit I gene (Folmer, et al. 1994). Subsequently, PCR clean-up of COI amplicon of mosquito samples was done using ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher) according to the manufacturer's instructions. Treated PCR product was sent for sequencing to Eurofin. The sequenced results were matched in NCBI nucleotide database using the online BLAST program and the corresponding mosquito species were identified.
4. **Screening of arboviruses using various molecular methods and cell culture virus isolation (week 1-4).**
   Theory session: Lecture on mosquito-borne arboviruses by Olivia Wesula Lwande.
   Practical session:
   1) Molecular method: Total RNA extraction from pooled mosquitoes following homogenization was done using QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (Qiagen). The extracted RNA from pools were subjected to nucleic acid amplification based detection methods to screen major mosquito-borne viruses mainly: Alphviruses, Flaviviruses and Orthobunyaviruses. **A) One-step RT-PCR** using Invitrogen SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase and specific primers, **B) Two-step RT-PCR** (1st step is cDNA synthesis using Thermo Scientific RevertAid

RT Kit and second step is conventional PCR using Thermo Scientific Phusion Green Hot Start II High-FidelityPCR Master Mix and specific primers). The PCR products were separated in agarose gels using gel electrophoresis technique to detect the positive virus samples depending on the expected fragment size using a low range ladder (Thermo Fisher Scientific) including the respective virus controls representing each genus. The PCR samples identified as positive by gel analysis were sent for sequencing to identify the taxonomy of virus species. Later in order to identify the individual mosquitoes that contributed to the virus in the virus-positive pooled samples - RNA of individual mosquitoes (50 individual homogenized mosquito samples that comprised virus positive pools) were extracted and subjected to two-step RT-PCR followed by gel analysis to screen positive PCR amplified samples which were then subsequently sent for sequencing to identify virus species. In addition, **C) Melt Analysis of Mismatch Amplification Assays (Melt-MAMA)**, was used to differentiate between O'nyong-nyong virus (ONNV) and chikungunya virus (CHIKV) (both of which are closely related mosquito-borne Alphaviruses that are difficult to distinguish serologically) in mosquito samples and human sera (100 samples were analyzed).

Table 1: List of specific forward and reverse primers used in RT-PCR.

Target Virus family	**Primers**	**Sequence (5′-3′)**	***PCR product (bp)***	***Reference***
Flavivirus	FUI	TACAACATGATGGGAAAGCGAGAGAA	265	Li-Jung Chien, et al. (2006)
	CFD2	GTGTCCCAGCCGGCGGTGTCATCAGC		
Orthobunyavirus	BCS82C	ATGACTGAGTTGGAGTTTCATGATGTCGC	251	Kuno G. et al. (1996)
	BCS332V	TGTTCCTGTTGCCAGGAAAAT		
Alphavirus	Vir 2052 F	TGGCGCTATGATGAAATCTGGAATGTT	138	Eshoo et al. (2007)
	Vir 2052 R	TAC GAT GTT GTC GTC GCC GAT GAA		

2) Cell culture for virus isolation: For isolation of virus using cell culture method, following steps were done, (A) propagation of four cell cultures from frozen stocks comprising mammalian cells (VeroB4) and insect cells obtained from Ae. albopictus and Cx. quinquefasciatus (C6/36, C710 and Hsu), (B) daily maintenance of the cell cultures (passaging/splitting when confluent), (C) freezing uninfected cells for future use, (D) infecting Vero-B4 and C6/36 cells with homogenized pooled mosquito samples from field (E) Daily monitoring and recording of any visible cytopathic effects (CPE) in host cells that include: rounding, detachment (plaques), clumping, cell fusion (syncytium formation) and inclusion body formation, (F) harvesting the infected Vero-B4 and C6/36 with cytopathic effect and storage at -80 ºC for further virus isolation and RNA extraction procedures.

Plaque assay for quantification of o'nyong'nyong virus (ONNV) (source of virus CDC) was carried out in Biosafety Level (BSL) 2 laboratory. Vero-B4 cells were infected with virus stock serially diluted in maintenance medium, at a range of 10 to 10^{-7}. Cells were covered with an overlay medium (CMC) to immobilize viruses and avoid cross-contamination among plaques. The assay was followed by cell fixation, staining and enumeration of plaques forming unit per mL (pfu/mL).

5. **Basic knowledge on nanopore sequencing techniques to detect and identify arboviruses from mosquitoes (week 4).**

Theory session: A very informative lecture was given by Dr. Jonas Naslund from Swedish Defence Research Agency (FHI) on Nanopore sequencing technology, its application in arbovirus screening and microbiota and the bottlenecks of this technology. MinION flow cell is

the only portable device capable of doing real time sequencing of DNA and RNA. For metagenomic detection of mosquito-borne arboviruses using MinION, the following steps are followed: 1) RNA extraction from mosquito sample, 2) cDNA preparation, 3) sequencing on the MinION and 4) bioinformatic analysis of the MinION reads.

6. **Basic knowledge on commonly employed statistical methods for distribution modelling studies of mosquito species and arboviruses (week 1 and 3).**
   Theory session: Two lectures were given by Prof. Joacim Rocklöv at the Department of Public Health and Clinical Medicine on following topics: 1) principles of infectious disease modeling, 2) modelling and correlation studies on distribution and spread of MVS with environmental variables, 3) descriptive data analysis to evaluate geographical variations (mean rainfall, mean temperature etc.), 4) ecological competition, influence of health system and surveillance capacity.
7. **Networking activities**
   I introduced the BioMEMS group of USN, Norway to the Department of Clinical Microbiology and also to Department of Public Health and Clinical Medicine, at Umeå University. I met with Prof. Joacim Rocklöv and Dr. Henrik Sjödin (Department of Public Health and Clinical Medicine, Section of Sustainable Health, Umeå University), Prof. Clas Ahlm (Section of Infection and Immunology, Umeå University), Dr. Goran Bucht and Dr. Jonas Naslund from Swedish Defence Research Agency (FOI). In addition, I visited the research group of Oliver Billker at Molecular Infection Medicine Sweden (MIMS), Umeå University. Billker's group has infrastructure for malaria research such as insectary for rearing malaria mosquito vectors. The research interests and potential options for future collaboration were discussed.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

1. Among the mosquito specimens provided for this training *Culex pipiens, Culex sp., Ae. punctor* and *Ae. Communis* were identified by the morphology based methods.
2. Mosquito samples for arbovirus screening had been provided by Prof. Evander's group associated with their projects in Sweden and Kenya, the results have not been published yet therefore I am not allowed to disclose the results of sequencing. The mosquito pools (each pool contained 10 individual mosquitoes) were screened for mosquito-borne arboviruses. In the first 32 pools previously subjective to culture, 5 were positive for Flaviviruses. The 50 individual corresponding to 5 virus-positive pools were screened by 2 step RT-PCR and 19 flavivirus positive mosquitoes were identified. The virus species was determined by sequencing and the mosquito specimens were identified by DNA barcoding and subsequent sequencing. Another 48 pools were screened where one pool was found to be positive for *Orthobunyavirus* and 45 pools were *Flavivirus* positive.
3. I was able to differentiate between ONNV and CHIKV using Melt Analysis of Mismatch Amplification Assays (Melt-MAMA). This project will be continued further and we will publish the results together.
4. Plaque assay for ONNV was repeated twice. The first experiment failed probably since the old overlay medium was used. In the second experiment plaques were visible by day 3 postinfection. The serial dilutions $>10^{-3}$ induced cell death. I was not able to determine the titer of the virus since the number of plaques obtained in serial dilutions ranging between 10^{-3} to 10^{-7} were too numerous to count.

5. All of the Vero-B4 and C6/36 cell culture plates which were infected with 5 different homogenized pooled mosquito samples (in duplicates) displayed cytopathic effects such as detachment (plaques) and clumping. The manifestation of CPE varied with time for each sample and also with the cell type.
6. In the mosquito sampling activity, CDC light trap captured only 1 male Culex mosquito while none was found in BG sentinel trap since mosquito activity during September is greatly reduced in Umeå.

The overall outcomes from this STSM:

1. I acquired extensive knowledge and practical skills in conventional vector and arboviruses surveillance practices and techniques.
2. I was able to build scientific network connections between USN, Norway and Department of Clinical Microbiology, Section of Virology, Umeå University.

FUTURE COLLABORATIONS (if applicable)

The BIOMEMS group at USN, Norway has already partnered Prof. Magnus Evander's group at Umeå university, Sweden in a project application that was submitted to Research council of Norway this year for developing an advanced system for surveillance of mosquito biodiversity and mosquito borne arboviral pathogens using molecular methods in Norway.
During the course of STSM in Evander's group, we had started a research project to develop a cost effective method to detect and differentiate between ONNV and CHIKV viruses and I intend to contribute to the project further.
In addition, we aim to analyse the spatio-temporal association between incidence of ONNV and malaria transmission.

Reference

1. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3: 294-299.
2. Li-Jung Chien, et al. (2006) Development of Real-Time Reverse Transcriptase PCR Assays To Detect and Serotype Dengue Viruses, J Clin Microbiol, 44(4): 1295–1304.
3. Kuno G. Mitchell CJ. Chang GJ. Smith GC. Detecting bunyaviruses of the Bunyamwera and California serogroups by a PCR technique. J Clin Microbiol. 1996;34:1184–1188.
4. Eshoo MW, Whitehouse CA, Zoll ST, Massire C, Pennella TT, Blyn LB, et al. (2007) Direct broad-range detection of alphaviruses in mosquito extracts. Virology.368: 286–295.