ความสามารถในการเป็นพาหะนำเชื้อไวรัสชิคุนกุนยาและความสัมพันธ์ของเชื้อแบคทีเรียในทางเดินอาหารส่วนกลางของยุง *Aedes albopictus*

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จุฬาลงกรณ์มหาวิทยาลัย
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VECTOR COMPETENCE AND CORRELATION OF MIDGUT MICROBIOTA OF *AEDES ALBOPICTUS* FOR CHIKUNGUNYA VIRUS

Miss Ranida Tuanudom

A Dissertation Submitted in Partial Fulfillment of the Requirements
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รนิดา ต่วนอุดม : ความสามารถในการเป็นพาหะนำเชื้อไวรัสชิคุนกุนยาและความสัมพันธ์ของเชื้อแบคทีเรียในทางเดินอาหารส่วนกลางของยุง *Aedes albopictus* (VECTOR COMPETENCE AND CORRELATION OF MIDGUT MICROBIOTA OF *AEDES ALBOPICTUS* FOR CHIKUNGUNYA VIRUS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร. สนธยา เตียวศิริทรัพย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. น.สพ. ดร. ชาญณรงค์ รอดคำ, หน้า.

ไวรัสชิคุนกุนยาเป็นโรคติดเชื้อที่เกิดการอุบัติใหม่และอุบัติซ้ำแพร่กระจายไปทั่วโลกโดยมีแมลงพาหะนำเชื้อคือ ยุงลายสวน *Aedes albopictus* และยุงลายบ้าน *Aedes aegypti* และการศึกษาที่ผ่านมาพบว่าการเกิดโรคอุบัติซ้ำที่รุนแรงทั่วโลกเมื่อปี 2007 นั้นเกิดจากการที่ยุงลายสวนชนิด *Ae. albopictus* มีความไวในการเป็นพาหะของเชื้อไวรัสที่มีการเปลี่ยนแปลงพันธุกรรมและเกิดความรุนแรงขึ้น นอกจากนี้ยังสนใจถึงความสัมพันธ์ของแบคทีเรียในทางเดินอาหารส่วนกลางของยุงต่อการติดเชื้อไวรัส ดังนั้นการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาความสามารถในการเป็นพาหะนำเชื้อไวรัสชิคุนกุนยาของยุงลายสวนชนิด *Ae. albopictus* และศึกษาหาความสัมพันธ์ของเชื้อแบคทีเรียต่อการติดเชื้อไวรัส เชื้อไวรัสชิคุนกุนยาที่ใช้ในการศึกษานี้เป็นไวรัสที่แยกมาจากผู้ป่วยที่ติดเชื้อในปี 2010 ในประเทศไทย นำเชื้อไวรัสที่เพิ่มจำนวนไว้มาผสมกับเลือดแกะเพื่อให้ยุง *Ae. albopictus* กิน โดยแบ่งเป็น 6 กลุ่มตามปริมาณไวรัสคือ 10^6, 10^5, 10^4,10^3, 10^2 CID_{50}/ml และกลุ่มที่กินเลือดไม่ผสมไวรัส หลังจากให้ยุงกินเลือดและเลี้ยงต่อจนถึงวันที่ 14 จึงทำการแยกส่วนของปีกและขาเพื่อหากระจายตัวของเชื้อผ่านน้ำเลือดของยุง เก็บส่วนของน้ำลายเพื่อหาความสามารถในการแพร่เชื้อ และเก็บส่วนลำตัวเพื่อหาการติดเชื้อในตัวยุง นอกจากนี้แยกเก็บส่วนของทางเดินอาหารส่วนกลางของยุงเพื่อนำไปทำการศึกษาหาความสัมพันธ์ของแบคทีเรียในทางเดินอาหารส่วนกลางและการติดเชื้อไวรัสในยุง นำส่วนต่างๆของยุงไปเพาะเชื้อในเซลล์เพาะเลี้ยงและย้อมเซลล์เพื่อตรวจหาการติดเชื้อ อัตราร้อยละของการติดเชื้อไวรัสที่ตรวจได้คือ 83.3, 90, 100, 100 และ100 ส่วนอัตราร้อยละของการกระจายตัวของเชื้อคือ 70.8, 86.7, 100, 90 และ 98 และอัตราร้อยละของการถ่ายทอดเชื้อคือ 41.6, 70, 100, 90 และ 82.4 เมื่อได้รับไวรัสในปริมาณ 10^2, 10^3, 10^4,10^5 และ 10^6 CID_{50}/ml ตามลำดับ ผลการศึกษาบ่งชี้ว่ายุงลายสวนชนิด *Ae. albopictus* มีความไวต่อการเชื้อไวรัสและมีประสิทธิภาพในการเป็นพาหะของเชื้อไรรัสชิคุนกุนยา ในส่วนของผลการแยกเชื้อแบคทีเรียในทางเดินอาหารส่วนกลางของยุงนั้นได้ทำการเพาะแยกด้วยวิธีการเพาะเลี้ยงในอาหารเลี้ยงเชื้อ และคัดแยกแบคทีเรียไปเพิ่มจำนวนและทำการจำแนกชนิดของแบคทีเรียด้วยวิธี RT-PCR โดยศึกษา 16s rRNA จากนั้นแบคทีเรียที่แยกได้จะถูกนำไปเทียบกับฐานข้อมูลใน Gene bank ว่ามีความใกล้เคียงกับชนิดใดบ้างและพบว่า แบคทีเรียในสกุล *Micrococcus* เป็นเชื้อที่ถูกแยกได้มากที่สุดในกลุ่มยุงที่ติดเชื้อเมื่อได้รับไวรัสในปริมาณ 10^2 CID_{50}/ml และยังพบว่ามีความแตกต่างอย่างมีนัยสำคัญ ($P< 0.04$) เมื่อเทียบกับกลุ่มที่ไม่ติดเชื้อในปริมาณเดียวกัน นอกจากนี้ยังพบแบคทีเรียในสกุล *Stapphylococcus* เป็นเชื้อที่พบมากที่สุดในยุงกลุ่มที่เพาะเลี้ยงในห้องปฏิบัติการและยังพบว่ามีความแตกต่างอย่างมีนัยสำคัญ ($P< 0.0007$) เมื่อเทียบกับกลุ่มยุงที่เก็บจากพื้นที่ อย่างไรก็ตามการศึกษานี้ไม่พบความสัมพันธ์ของแบคทีเรียในทางเดินอาหารส่วนกลางของยุงลายสวนและการติดเชื้อไวรัสชิคุนกุนยาอย่างชัดเจน

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สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต ....

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KEYWORDS: TRANSMISSION / DISSEMINATION / INFECTION / MICROBIOTA / SUSCEPTIBILITY

RANIDA TUANUDOM: VECTOR COMPETENCE AND CORRELATION OF MIDGUT MICROBIOTA OF *AEDES ALBOPICTUS* FOR CHIKUNGUNYA VIRUS. ADVISOR: ASSOC. PROF. SONTHAYA TIAWSIRISUP, D.V.M., Ph.D., CO-ADVISOR: ASST. PROF. CHANNARONG RODKHUM, D.V.M., Ph.D., pp.

Chikungunya virus (CHIKV) is an important mosquito-borne virus and transmission cycle of this virus involves mosquito vectors (*Aedes albopictus* and *Aedes aegypti*) and infected vertebrate hosts. The recently studies found that CHIKV outbreak in 2007 have been *Ae. albopictus* as an important vector which it was susceptible to genetic variation of CHIKV and induce to virus virulence. However, the study about vector competence for CHIKV in Thailand is limited. Moreover, the previous study indicates the impact of midgut microbiota of mosquito to viral infection. This study was conducted to examine the effects of CHIKV titers in blood meals on vector competence of *Aedes albopictus* (Diptera: Culicidae) and to investigate the correlation of midgut microbiota of *Ae. albopictus*. Six groups of *Ae. albopictus* were allowed to feed on different levels of CHIKV in the blood meals which were 10^6, 10^5, 10^4,10^3, 10^2 CID_{50}/ml of CHIKV and the negative blood meal group. Body, legs and wings, and saliva samples from blood-fed mosquitoes were assayed for the presence of CHIKV by using immunocytochemistry staining on day 14 post blood feeding. Percent virus infection, dissemination, and transmission is defined as percent of blood-fed mosquitoes with virus in their bodies, legs and wings, and saliva, respectively. The percent infections were 83.3, 90, 100, 100, and 100%, the percent disseminations were 70.8, 86.7, 100, 90, and 98%, and the percent transmissions were 41.6, 70, 100, 90, and 82.4% after having been fed on 10^2, 10^3, 10^4, 10^5, 10^6 CID_{50}/ml of CHIKV, respectively. This study suggested that *Ae. albopictus* are susceptible for CHIKV infection and efficient vectors for CHIKV transmission, and CHIKV titers in blood meals have effects on virus infection, dissemination, and transmission in *Ae. albopictus* or vector competence of this mosquito. For the bacterial isolation and identification, the midguts were cultured and 16s rRNA gene were analyzed followed by blast to gene bank database. The dominating bacterial genus was *Micrococcus* in infected mosquitoes after fed on CHIKV 10^2 CID_{50}/ml, and it was significantly difference from the non-infected mosquitoes ($P<0.04$). In addition, *Staphylococcus* was the dominating bacterial genus in laboratory mosquitoes and it was significantly difference from field mosquitoes ($P<0.0007$). The correlation between midgut microbiota and CHIKV infection was not clearly indicated from this study.

Field of Study: Biomedical Sciences — Student's Signature ....
Academic Year: 2017 — Advisor's Signature ....
Co-Advisor's Signature ....

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LIST OF ABBREVIATIONS

°C	degree Celsius
µl	microliter
Ae.	Aedes
An.	Anopheles
ATP	adenosine triphosphate
bp	base pair
Bti	Bacillus thuringiensis serovar israelensis
CHIKV	Chukungunya virus
CID50	the 50% cytophatic infectious dose
CPE	cytopathic effect
DENV	Dengue Virus
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleoside triphosphate
DPI	days post infection
E. cloacae	Enterobacter cloacae
ECSA	East/Central/South African
FBS	fetal bovine serum
hr	hour
hrs	hours
ICC	Immunocytochemistry assay
IOL	Indian Ocean lineage
kb	kilo base pair

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LACV	La Crosse virus
MEM	modified Eagle's medium
min	minute
ml	milimeter
mM	Millimolar
Mosq	mosquito
nt	nucleotide
ONNV	O'nyong nyong virus
ORF	open reading frames
PBF	post blood feeding
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribosomal nucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
S.odorifera	Serratia odorifera
sec	second
spp	species
TCID50	the 50% tissue culture infectious dose
TSA	tryptose soya agar
TSB	tryptose soya broth
UV	ultraviolet
v/v	volumn by volumn
WNV	West Nile Virus

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CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Chikungunya virus (CHIKV) is a re-emerging mosquito borne virus, which can cause acute illness along with symptoms such as high fever, headache, nausea, vomiting, rash, and severe joint pain (1). The unique clinical sign of the chikungunya associated diseases is generalized arthralgia, which may continue for months or years (2). Over the past several years, CHIKV has been identified as causing health care problems. Its emergence and re-emergence has been widely reported from countries such as China, France, Malaysia, India and Thailand (3, 4). CHIKV can induce arthralgia that is more severe and more generalized than that which occurs by the dengue virus (2, 5-7). The CHIKV originated in Africa, where transmission from wild forest populations of the Aedes species to non-human primates was found to be prevalent (1).

For the previous epidemic of CHIKV in Africa, the major vector was identified as Aedes aegypti (Ae. aegypti) and it was then found that Aedes albopictus (Ae. albopictus) was the second major vector as well as the other Aedes spp. such as Ae. furcifer, Ae. taylori, Ae. luteocephalus, Ae. africanus, and Ae. neoafricanus (8). The recent outbreaks (2004-2005) in East Africa and the Comoros were also shown that Ae. aegypti was the potential vector of CHIKV (9). However, the outbreaks that occurred in the Reunion Islands (France), where there were different ecological environments, where the Ae. aegypti mosquito was rare or in limited numbers and Ae. albopictus mosquito was the predominant species. In addition, a mutation involving the substitution of A226V in the E1 glycoprotein was identified in CHIKV and the virus had spread into Madagascar and Mayotte, where populations of both Ae. aegypti and Ae. albopictus are present (2). The epidemic that was associated with CHIKV in areas of Asia occurred predominantly in urban areas and in particular in those areas where dengue is endemic and mostly transmitted by Ae. aegypti mosquitoes (10).

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The recent outbreaks of CHIKV in the Indian Ocean islands, Asia, Africa, and Europe, Ae. albopictus was identified as the potential vector for the transmission of CHIKV. The virus has now been identified in non-dengue endemic rural areas (2, 6, 11). These outbreaks including that in southern Thailand were due to a variant of CHIKV where there had been a substitution of A226V in the E1 glycoprotein (E1-226V) (10). This mutation demonstrated the unusual transmission of CHIKV of Ae. albopictus (2, 12). Moreover, the outbreaks during the epidemics of 2005-2006 have been identified as being due to the E1-A226V mutation, which modifies vector specificity, permitting the virus to capably adapt to replicating in Ae. albopictus and presumably leading to the extent of the outbreak (6). The study in France showed that the midgut barrier had a role to play in selecting the novel arbovirus variant (E1- A226V) to present at low levels for the dissemination in Ae. albopictus and could be the reason for the E1-A226V variant quickly emerging as soon as Ae. albopictus became the competent vector (13). This may increase the risk for CHIKV transmission by extending the geographic range of Ae. albopictus. Ae. albopictus has also colonized in both tropical and temperate countries. Arboviruses infect the mosquito midgut following ingestion of a viremic blood, replicate within the midgut, disseminate to the salivary glands, and emerge into saliva in order to be transmitted once the mosquito bites a host. The midgut and salivary glands act as barriers to virus infection and thus the mosquito escapes infection (14). Mosquitoes must salivate during blood feeding as their saliva contains different substances, which counteract the host’s normal hemostatic response. The result is that the various components of the saliva prevent blood coagulation and enhance vasodilatation during feeding. The components of saliva however, may differ from one species to another. It has also been reported that the saliva response is able to enhance viral infections (15). Vector competence is the intrinsic ability of insect vectors to acquire, maintain, and transmit a pathogen (16). There are many factors that obstruct infection and thus the dissemination, and transmission of arboviruses through mosquito vectors. Understanding of these mechanisms is important for creating more secure vaccines and innovative strategies to control pathogen transmission. The present study is based upon understanding the transmission potential of CHIKV in Ae. albopictus in Thailand. The critical importance

of this research is the testing of the lowest level of CHIKV (the outbreak strain in Thailand in particular) that can be ingested by Ae. albopictus. This will be achieved through qualitative and quantitative measurement of the viral infection in the body, hemolymph, and salivary glands of infected mosquitoes.

In the last few years, there have been many studies focused on the role of bacterial communities on the fitness and the competence of various insect vectors on the transmission of pathogens (8, 17-20). These studies lead to the potential utilization of microorganism as biopesticides to eliminate mosquitoes as an alternative for the current chemical pesticides (21). However, biopesticides such as Bacillus thuringiensis serovar israelensis (Bti) and Bacillus sphaericus have been of limited utility in mosquito eradication (22). Presently, there is a lack of knowledge of the microorganism diversity that occurs within insect hosts. A better understanding of the mechanisms that are involved in the process of pathogen propagation and the maintenance of these microorganism in the insect host are required. Therefore, the isolation and/or development of a bacterial strain where the mosquito midgut activity is not resistant to a specific viral ingestion could provide an alternative pathway for mosquito control. This study will provide the information about the relation between the midgut microbiota of Ae. albopictus and CHIKV infection.

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1.2 Research question

1) What are the correlations of levels of Chikungunya virus in blood meals and laboratory strain *Aedes albopictus* infection, dissemination, and transmission?
2) What are the correlations of bacteria in laboratory strain *Aedes albopictus* midgut and Chikungunya virus infection?
3) What are the differences of the midgut bacteria between field and laboratory strain *Aedes albopictus*?

1.3 Objectives

1) To examine the correlations of levels of Chikungunya virus in blood meals and *Aedes albopictus* infection, dissemination, and transmission
2) To examine the correlations of bacteria in *Aedes albopictus* midgut and Chikungunya virus infection
3) To examine the bacterial present in the midgut of field and laboratory strain of *Aedes albopictus*

1.4 Hypothesis

1) The levels of Chikungunya virus in blood meals affecting *Aedes albopictus* infection, dissemination, and transmission.
2) Bacteria in *Aedes albopictus* midgut affecting Chikungunya virus infection?
3) There are the differences of the bacteria present in the midgut of field and laboratory strain *Aedes albopictus*.

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CHAPTER II
LITERATURE REVIEWS

2.1 Background of Chikungunya virus: the structure, classification, and properties

Chikungunya virus (CHIKV) is a member of the Alphavirus genus, belonging to the family Togaviridae. The CHIKV nucleocapsid contains a single strand, a positive sense RNA virus, genome of about 11.8 kb, and two open reading frames (ORF) encoding the non-structural proteins (nsP1-nsP2-nsP3-nsP4), and the structural polyprotein (C-E3-E2-6K-E1) (23). The ORF is located at the 5' end of the genome encodes. The nonstructural proteins responsible for cytoplasmic RNA replication and modulation of cellular anti-viral response are in the host cells. The second ORF encodes the structural proteins (C, E1, E2), forming the viral nucleocapcid and envelope (24). CHIKV was initially isolated from the serum of febrile humans in Tanganyika (Tanzania) in 1953 (25) It has been the cause of several outbreaks of viral infection in Asia, Africa, and Indian Ocean islands, and these have raised public health concerns (2, 3, 26, 27).

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The first outbreak of CHIKV in Asia was reported from Bangkok in 1958 followed by a spread of outbreaks in Cambodia, Vietnam, Malaysia, and Taiwan and the rest of Asia in the later years (28). CHIKV infection is diagnosed by sudden onset of high fever, headache, rashes, arthralgia, and myalgia (1). This disease has typical clinical signs that come under the term of poly-arthralgia that become manifest by very painful symptoms that affect the joints and these may persist for several months extending to years (2). Originally, there was sylvatic cycle transmission between non-human primates and forest-habitat Aedes mosquitoes which is similar to that of the cycle of the yellow fever virus. The recent outbreaks have been associated with the urban Aedes mosquitoes (possible Ae. aegypti and Ae. albopictus) in which the direct human-

to-human transmission resembling that of the dengue virus and characterized by the absence of an animal reservoir (29, 30).

During the outbreaks of 2004-2009, it was demonstrated that a specific change at the 226 position of the E1 protein had taken place with the substitution of alanine with valine (E1: A226V). This had an important role in inducing the re-emergence of CHIKV in the French island of Reunion, India, and Thailand (2, 6, 31). The phylogenetic analysis of the CHIKV using the partial nucleotide sequences of the E1 gene resulted in it being classified into three major phylogroups. The three genotypes were the West African, Asian, and East/Central/South African (ECSA) (2, 3). Later, the new Indian Ocean genotype developed from the ECSA genotype was classified (30). The Indian Ocean genotype outbreaks originated from Kenya in 2004 and spread into the Indian Ocean islands, India, and Southeast Asia (30, 32).

The E1: A226V protein mutation, the important evolution of CHIKV, was found to be more frequent in coastal areas around the Indian Ocean islands. This mutation has been proved to cause vector alteration from Ae. aegypti to Ae. albopictus mosquitoes by laboratory evidence (11, 12). *Ae. albopictus* was the major vector for CHIKV transmission in the coastal areas of the Indian Ocean islands. The increased transmissibility of the virus and the higher epidemic potential may be enhanced by this mutation (2, 6, 11, 31).

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2.2 *Aedes albopictus*: Biology and the potential as a transmission vector

Aedes albopictus (Skuse), commonly known as the Asian tiger mosquito, is a mosquito that acts as the potential disease vector for the transmission of many viral pathogens which include the yellow fever, the dengue fever, and chikungunya fever virus. *Ae. albopictus* belongs to the family Culicidae of the order Diptera. The characteristic bold black scales with silver white bands on the palps and tarsi of the *Ae. albopictus* mosquito makes identification of the mosquito relatively easy. Of note

is the black with a distinguishing white stripe down the center of the scutum beginning at the dorsal surface of the head and continuing along the thorax (33). This species originates from the wild in Southeast Asia. For Thailand, *Ae. albopictus* has also prevalence in restricted areas in the south of the country (34). It does not exhibit any specific ecological specialization and has succeeded in colonizing temperate zones such as the United States of America and Europe. It is currently spreading throughout the African continent (35). *Ae. albopictus* overwinters in the egg stage in the temperate climates but is active throughout the year in tropical and subtropical habitats (33). This species has been shown to have both distinct cold tolerant and tropical strains (36).

It is a competent vector for various viruses in the natural and laboratory environment. Viruses which belong to Flaviviridae (genus *Flavivirus*), Togaviridae (genus *Alphavirus*), Bunyaviridae (genus *Bunyavirus* and *Phlebovirus*), Reoviridae (genus *Orbivirus*) and Nodaviridae (genus *Picornavirus*) are all known to be involved with the life cycle of *Ae. albopictus* (29, 37). Previously, it was considered that *Ae. albopictus* was an exophagic mosquito which preferred to bite in the early morning and late afternoon. However, many irregularities have been observed and studied showing a dependence on the season, region, host availability, and the natural human habitat (37). Even though this mosquito tends to favor mammalian hosts for feeding, it is known that the female can feed upon almost any group of vertebrates from cold to warm-blooded animals, including reptiles, birds, and amphibians (38)

The recent CHIKV outbreaks in several countries including the Indian Ocean islands, Kerala state in India, Gabon, Italy, and southern Thailand have reported *Ae. albopictus* as the potential vector for viral transmission during the outbreaks (31, 39, 40). These outbreaks involved the variant possessing the E1: A226V mutation, which is known to improve the competence of the salivary gland of *Ae. albopictus* mosquitoes to become infected and thus increases the capability of the mosquitoes to transmit the virus to another host (6). There are no vaccines or effective drugs available to provide the protection against CHIKV infection. Consequently, the major healthcare strategy for arresting and controlling the disease is upon the eradicating the

vector that is responsible for the virus transmission (41). Therefore, this study focuses upon the competence of *Ae. albopictus* for CHIKV by investigating the low-level limit of the virus on viral transmission.

2.3 Viral transmission: Ingestion, midgut and saliva gland

During the mosquito's intake of a blood meal from a viraemic hos, the virus passes into the lumen or the hind part of the midgut of the mosquito bypassing any gut diverticulum on the way ensuring that viral ingestion has occurred. The virus then has to enter the body of the mosquito host before it is inactivated by the antagonistic factors in the gut lumen or before it is excreted (16). Normally, the oral transmission must reach the salivary glands, with or without secondary amplification in other susceptible tissues, growing in them and then being released with the saliva into the salivary ducts where it is then available to infect a second vertebrate host following a feeding session.

There are three traditional methods for the estimation of potential arbovirus infection by mosquitoes: 1) intrathoracic inoculation, 2) oral exposure by using an artificial blood meal, or 3) oral exposure by feeding on a viremic vertebrate host. These methods have their advantages and disadvantages; although several studies have been able to demonstrate that the infection by artificial feeding from a prepared solution that mimics natural blood meal is good model for evaluating viral infection and transmission (14, 42).

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The virus initially infects the cells of the midgut, followed by fat body cells, neural tissue and salivary gland cells where it becomes available for transmission to a suitable host. The insect midgut consists of a single layer of columnar epithelial cells on the basal lamina on the abluminal or haemocoel side. After the intake of a blood meal the cells in the hind part of the midgut become flattened into squamous forms and begin the process of blood-meal digestion, which includes osmoregulation,

secretion of digestive enzymes and the transport of blood-meal nutrients across the mesenteronal epithelium into the haemocoel (43, 44). This study will therefore, concentrate on investigating the lowest dose of CHIKV required by *Ae. albopictus* through artificial feeding for potential transmission, Once the mosquitoes have been fed then the midgut, salivary glands and saliva including the hemolymph will be collected for analysis..

2.4 Bacteria in mosquito midgut

2.4.1 The effect of bacteria in mosquito midgut

Over the last few years, many studies have focused on the effect that microorganisms in the midgut have on the effectiveness of insects as competent vectors of pathogens. It has been shown that microorganisms can lead to insect host adaptations, including the point of vector control (17-20, 45) The bacteria found within the gut of many mosquito species have been the subject of study of both laboratory and field strains of mosquito populations(17, 20, 45). The more recent studies have shown that these bacteria seem to activate the mosquito immune system and thus indirectly improve protection against malaria parasites (46). The innate immunity is the immediate response of invertebrates for their protection against foreign substances and pathogens. In insects, it depends on both humoral and cellular responses that are mediated via certain recognizing receptors and activation of several signaling pathways. Fat body and hemocytes are the origins for the production and secretion of antimicrobial agents and activators/regulators of cellular response, while cell mediated immunity in insects is performed by hemocytes (47).

Ramirez, et al. (2012) found that certain field-derived bacterial isolates of the mosquito midgut exert a harmful effect on dengue virus infection. The effect is at least partly demonstrated through the action of the mosquito immune system, which

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is activated by microbes. Conversely, dengue virus infection induces immune responses in the mosquito midgut tissue that act against the natural mosquito midgut microbiota. (48).

A recent study investigated that the interaction of microbial flora of larvae and adult *Ae. aegypti* midgut is complex and is dominated by Gram negative proteobacteria. *Serratia odorifera* was found to be stably associated in the midgut of field collected and laboratory reared larvae and adult females. The potential influence of this sustainable gut microbe on dengue-2 susceptibility of this vector was evaluated by co-feeding *S. odorifera* with dengue-2 to adult *Ae. aegypti* females (free of gut flora). The observations revealed that the viral susceptibility of these *Aedes* females enhanced significantly as compared to sole dengue-2 fed and other gut inhabitants such as *Microbacterium oxydans* co-fed females. It was postulated that the dengue-2 susceptibility of *Ae. aegypti* females was due to the blocking of the prohibiting molecule present on the midgut surface of these females by polypeptides of the gut inhabitant *S. odorifera* (49).

Previous studies have attempted to demonstrate the efficacy of using biopesticides to control the mosquito vector. The biopesticides, *Bacillus thuringiensis* serovar *israelensis* (Bti) and *Bacillus aphaericus* were used in a worldwide study to control the populations of *Aedes*, *Culex*, and *Anopheles* larvae but it was not successful for adult mosquitoes (21). Later, the bacterium *Wolbachia* was a candidate to restrict the transmission and spread of arboviruses by using a symbiosis-based control (50).

Oliveira, et al. (2011) found that the presence of bacteria in the midgut of mosquitoes antagonizes infectious agents, such as Dengue and Plasmodium, acting as a negative factor in the vectorial competence of the mosquito. They demonstrated that toxic reactive oxygen species (ROS) generated by epithelial cells control bacterial growth in the midgut of *Aedes aegypti*, the vector of Yellow fever and Dengue viruses. ROS levels are inversely correlated with the presence of bacteria in the midgut and

therefore they investigated if ROS are involved in fighting bacterial infection in the gut. So, when bacterial in the midgut involves in ROS therefore it may induce/reduce virus infection in a mosquitoes midgut (51).

Zouache, et al. (2012) demonstrated that the whole microorganism community and their mutual interactions may impact increased CHIKV in *Ae. aegypti*. They also suggested that the multi-interactions between a community of genetic components from the hosts and their symbionts might have an impact at the population and community levels because of local adaptations arising from a changing environment, new colonizations due to invasion, and to the evolution of the species. (52).

2.4.2 The bacterial identification by using 16s rRNA gene

The use of 16S rRNA gene PCR as a tool for identification of bacteria is possible because the 16S rRNA gene is present in all bacteria (53). The reasons for using this gene is because the 16S rRNA gene consists of highly conserved nucleotide sequences, interspersed with variable regions that are genus or species specific. PCR primers targeting the conserved regions of rRNA amplify variable sequences of the rRNA gene. Bacteria can be identified by nucleotide sequence analysis of the PCR product followed by comparison of this sequence with known sequences stored in a data base (54).

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In general, prokaryotic ribosomes contain three rRNA molecules: 5S (∼120 nt), 16S (∼1.5 kb), and 23S (∼2.9 kb). The 16S rRNA is a part of the small 30S ribosomal subunit which is the site of codon–anticodon interaction, and there are altogether about 21 different proteins. The 3′terminus of 16S rRNA is known to interact within the initiation region of mRNA via the Shine–Dalgarno sequence. The 5S and 23S rRNAs are composed of the large 50S ribosomal subunit including the 36 proteins that are thought to be involved in the regulation of translation accuracy. The 23S rRNA has been proposed to bind the 3′terminus (–CCA) of tRNA in the ribosomal exit site (E-site)

and to promote actively translocation of tRNA from the P-site (53). The use of 16S rRNA gene sequences for the classification and identification of prokaryotes is mostly dependent on a comparison with data from a database of known sequences. Nowadays, the sequences of type strains of ~99% of prokaryotic species with validly published names are available in public databases, indicating the extent of information available for the identification of unknown *Bacteria* and *Archaea* (55).

Therefore, the bacteria in the mosquito midgut were examined and identified by using 16S rRNA tests relating to the CHIKV infection, and the different responses between laboratory and field strains of *Ae. albopictus* in this study.

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CHAPTER III

MATERIALS AND METHODS

The experimental phase of this study was divided into 4 stages. These were
3.1 Chikungunya virus propagation and titration in Vero cell;
3.2 Chikungunya virus infection, dissemination, and transmission in *Aedes albopictus;*
3.3 The correlation of Chikungunya virus infection with mosquito midgut bacteria;
3.4 Bacteria isolation and identification from field and laboratory strain of *Aedes albopictus*. The experimental flowchart showed in figure 1.

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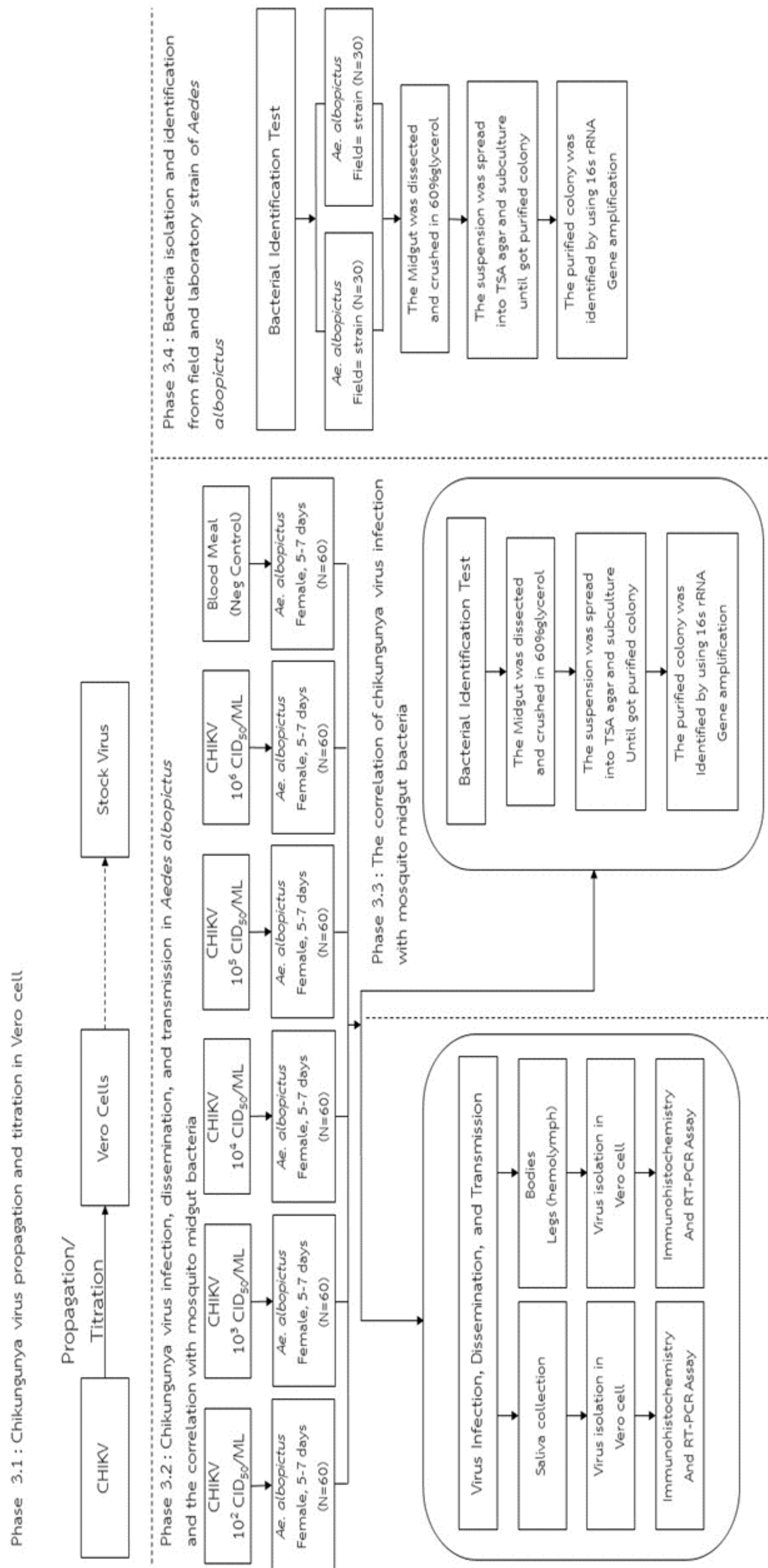

Figure: 1 Experimental outline

3.1 Chikungunya virus propagation and titration

3.1.1 Viruses and cells maintenance

Chikungunya virus (CHIKV) isolated from an infected patient during outbreak in 2008 to 2009 in Thailand was used in this study (28). The genotype was confirmed by sequence analysis, and phylogenetic analysis found that the isolate clustered within the Indian Ocean lineage. This isolated CHIKV was kindly provided by Prof. Dr. Yong Poovorawan (The Center of Excellence in Clinical Virology, Chulalongkorn University, CU Centenary Academic Development Project and King Chulalongkorn Memorial Hospital). CHIKV was propagated in the African Green Monkey Kidney, Vero cells and then stored under liquid nitrogen until required. The CHIKV stocks were titrated by the Reed and Muench method (1938) (56) to the 50% tissue culture infectious dose ($TCID_{50}$). All of work and handling of these viruses was performed in biosafety level 2 containment facilities. The Vero cells were maintained in modified Eagle's medium (MEM) (GIBCO, USA) containing 5% fetal bovine serum (FBS) (GIBCO, USA) at 37°C with 5% CO_2 using the standard culture procedure.

3.1.2 Viruses propagation and titration

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The Vero cells were re-plated every 4-5 days in a T25 flask until the cell stable. The CHIKV sample was propagated in Vero cell for 3 – 4 days in T25-flasks and freeze-thawed on day 5. Cells suspension were centrifuged 3,000 rpm, 10 mins, and the supernatant was collected for use as stocks virus. The CHIKV stock was diluted 10-fold dilution and titrated on Vero cell monolayers (70% confluent) in 96-well plates. These were inoculated with 100 µl/well of diluted virus stock and the plates were then incubated for 5 days. The plates were examined for the cytopathic effect (CPE), which was then confirmed by using the Immunocytochemistry (ICC) assay. The CHIKV stocks that were used in this study were those where the calculated titer was 10^7 CID_{50}/ml.

3.1.3 Immunocytochemistry assay (ICC)

Titration of the CHIKV stocks and viral infected-mosquito suspensions were performed using the ICC method (57). The CHIKV, Vero cell monolayers (70% confluent) in 96-well plates were inoculated with 100 µl/well of virus dilution and the plates were incubated for 7 days. The plates were examined for the cytopathic effect (CPE), which was then confirmed by using the ICC assay. Before staining, the cells were fixed with 4% formalin and washed with 0.5% Tween-20 in phosphate buffered saline (PBS). The cells were incubated for 1 hr with mouse monoclonal anti-Chikungunya antibody (Abcam, Cambridge, United Kingdom). After washing, the cells were incubated for 1 hr with the rabbit anti-mouse IgG conjugated horseradish peroxidase (Dako Cytomation, Carpinteria, California). The color was developed using a chromogen aminoethyl carbazole substrate (Sigma, United States). The infected cells, those showing a red color in the well, were recorded to calculate 50% tissue culture infectivity dose ($TCID_{50}$) of the virus (56).

3.2 Chikungunya virus infection, dissemination, and transmission in *Aedes albopictus*

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3.2.1 Mosquitoes maintenance

The laboratory strain of *Aedes albopictus* that had been maintained under laboratory conditions for several years, was used in this study. All mosquitoes were maintained under controlled environmental conditions at 28 ± 5 ^{o}C and 80 ± 5% relative humidity with a 12:12 hr photoperiod and were fed with a 10% sucrose solution. Mosquitoes were starved of the sucrose solution for 24 hrs before being allowed to feed on artificial blood meals.

3.2.2 The CHIKV infection to *Aedes albopictus*

The mosquitoes were divided into 6 groups for the various blood meal CHIKV titer. The virus titers used were 10^6 CID_{50}/ml (n=60), 10^5 CID_{50}/ml (n=60), 10^4 CID_{50}/ml (n=60), 10^3 CID_{50}/ml (n=60), 10^2 CID_{50}/ml (n=60) as well as a blood meal negative control group. The blood meals contained the viral stocks derived and diluted from the Vero cells (10^6, 10^5, 10^4, 10^3, 10^2 CID_{50}/ml), and then mixed with 20% fetal bovine serum, 1% sucrose, 70% (v/v) packed sheep erythrocytes (from the Department of Animal Husbandry, Faculty of Veterinary Science, Chulalongkorn University), and 3 mM ATP (as a phago-stimulant) (6, 42). Artificial blood meals were contained within porcine intestinal membranes to mimic animal skin and warmed to 37 ^{o}C using a glass bottle Hemotek feeder. The feeder was then placed on the screened lids of cartons (58). Mosquitoes were allowed to feed for 45 min, and the engorged females were maintained for 14 days as showed in figure 2.

Figure: 2 The process for CHIKV infection to mosquitoes

After extrinsic incubation, the infected mosquitoes were anesthetized at -20 °C, after which the legs and wings were removed. The proboscis was inserted into a 20 µl sterile pipet tip containing 5% sucrose solution in MEM; with 20% FBS to induce salivation for 20 min for saliva collection (59). Each saliva sample was transferred into a separate tube containing 200 µl of 10 % FBS in MEM (cold condition) as shown in figure 3. The midgut was dissected under sterile conditions for the processing of bacterial identification. (shown as figure 4)

Figure: 3 The saliva collection

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Figure: 4 The midgut dissection

The bodies and legs of infected mosquitoes were crushed separately in individual tubes containing 500 µl, 10 % FBS in MEM (cold condition) (shown as figure 5). These preparations were passed into 96 well plates containing Vero cell monolayers. Cell cultures were observed for CPE for up to 7 days and assays verified by immunocytochemistry (ICC) and polymerase chain reaction (PCR). These processes were carried out in order to determine the presence or absence of the virus (6, 58, 59). CHIKV that was present in the mosquito body, legs, and saliva indicated the virus infection, dissemination, and transmission, respectively.

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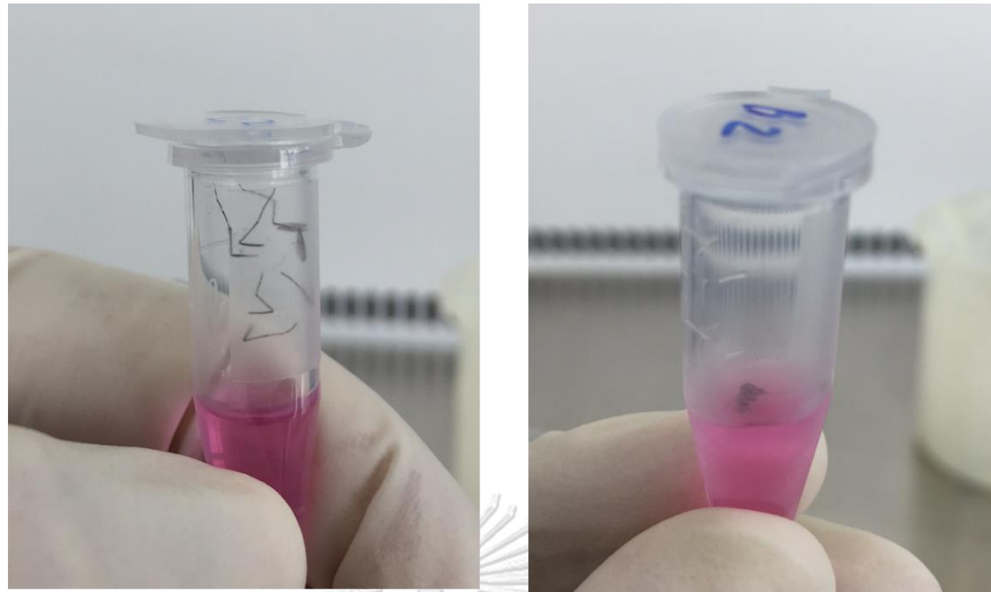

Figure: 5 The legs, wigs, and body collection for cell culture

3.2.3 Molecular assay for Chikungunya virus identification

3.2.3.1 Viral nucleic acid extraction

Viral nucleic acid was extracted from an individual cell culture medium by using the viral nucleic acid extraction kit II (Geneaid, Taiwan) and was used in accordance with the manufacturer's recommendation, and each were kept at -80 °C until time was available to test the reverse transcription polymerase chain reaction (RT-PCR).

3.2.3.2 Reverse transcription polymerase chain reaction (RT-PCR)

Each extracted viral nucleic acid sample was tested for CHIKV by using reverse transcription polymerase chain reaction (RT-PCR) according to Naresh Kumar et al. (2007) (40) and Theamboonlers et al. (2009) (27) with the modification suggested by Tiawsirisup et al., 2012 (60).

The primers were DVRChk-R 5'GGGCGGGTAGTCCATGTTGTAGA3' and DVRChk-F 5'ACCGGCGTCTACCCATTCATGT3' (40). The primer pair was chosen in order to amplify the E1 gene of CHIKV. RT-PCRs were performed in 25 μl-reactions. One and a half μl of RNA was mixed with 12.5 μl of 2X-master mix (0.4 mM dNTP, 3.2 mM $MgSO_4$) (Invitrogen, Carlsbad, CA), 1 μl of forward and reverse primer (10 μM), 1 μl of SuperScript III RT/Platinum Taq Mix (Invitrogen, Carlsbad, CA), and 8 μl of ultrapure water (Invitrogen, Carlsbad, CA). After the reverse transcription step at 48°C for 30 min and the initial PCR activation step at 94°C for 5 min, the amplification was carried out for 35 cycles with the following temperature cycling parameters: 94°C for 45 sec of denaturation, 56°C for 45 sec of annealing, and 72°C for 1 min of extension. The final amplification cycle included an addition of 7 min extension at 72°C. RNA was amplified by using thermocycler (Perkin Elmer Cetus 9600, Perkin Elmer, Waltham, MA). The PCR product was mixed with 6 μl of loading buffer (BlueJuiceTM Gel Loading Buffer, Invitrogen, Carlsbad, CA) and analyzed in 1.5% agarose gel (UltraPure™, Invitrogen, Carlsbad, CA) with expected 330 base pair band as showed in figure 6.

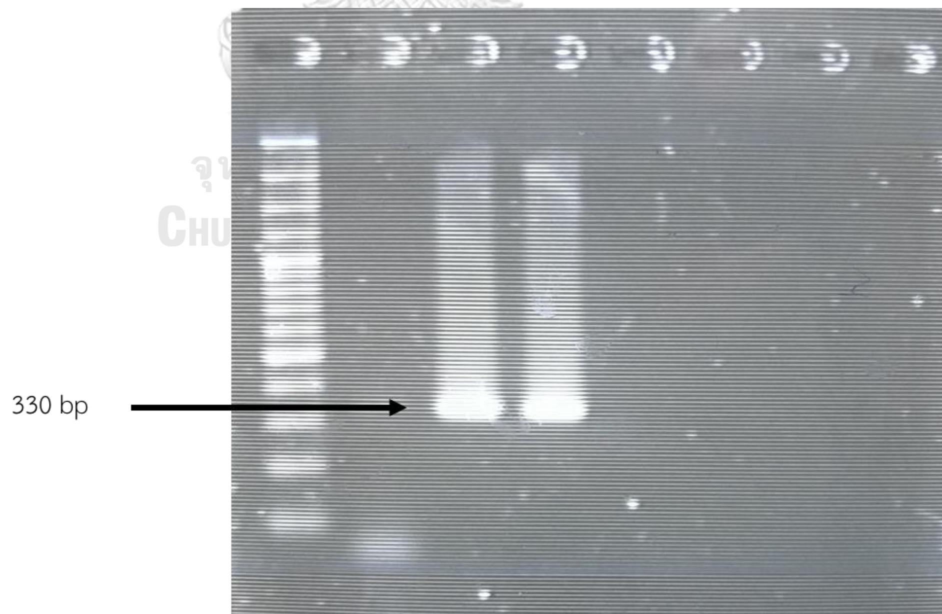

Figure: 6 The expected E1-CHIKV gene; 330 base pair

3.2.4 Statistical analysis:

Differences in percent infection, dissemination, and transmission among different levels of virus in the blood meal which were 10^2, 10^3, 10^4, 10^5, and 10^6 CID_{50}/ml of CHIKV were compared by Student's t-test.

3.2.5 Sequencing and phylogenetic analysis

All partial 16s rRNA gene sequence assembly and analysis were carried out by using Lasergene package version 5.03 (DNASTAR, Inc., Madison, Wis. USA). The sequence obtained in our study were compared with GenBank data base using the BLAST algorithm (https://www.ncbi.nlm.nih.gov/BLAST). The homologous sequences were retrived from the GenBank, and aliged using ClustalW program. Phylogenetic was determined by tree reconstructed using Neighbor-Joining method (Kimura-2 parameter for distance calculation), incorporated in MEGA 7.0.26 package. Robustness of the phylogenetic tree was examined through 1000 bootstrap replicates, and the consensus tree was used for analysis. All of the sequences have been submitted to the NCBI (National Centre for Biotechnology and Information) GenBank sequence database. The accession numbers are the following; (Submission number, SUB3724128: MG996794 - MG996888), (Submission number, SUB3733025: MG997080 - MG997092), (Submission number: SUB3782911 MH050409 - MH050425), (Submission number: SUB3782990 MH050699 - MH050738).

3.3 The correlation of Chikungunya virus infection with mosquito midgut bacteria

Dissected mosquito midgut from a previous study was processed for bacterial isolation and identification. The midgut contents were suspended in 300 µl of 60% glycerol and a 100 µl aliquot of the suspension was spread on tryptose soya agar (TSA) supplemented with 5% sheep blood and incubated at 37°C for 24 hrs. The resulting bacterial colonies were grouped; based on their colony morphology. Bacterial colonies that are morphologically distinct were selected and subcultured on TSA plates until a pure culture was obtained and then subjected for further analysis. Then pure bacterial isolates from mosquito midguts were subcultured in 2 ml of tryptose soya broth (TSB) at 37°C for 24 hrs. Cell pellets were suspended in distilled water and lysed using repeated cycles of freezing and thawing. The bacteria DNA was extracted by using a boiling method. Complete 16S rRNA gene (approx. 1.5 kb size) were amplified from the extracted DNA of the isolates as described by Djadid et al., 2011 (61) using eubacteria specific primers

16s Forward 5'– AGT TTG ATC CTG GCT CAG – 3' and

16s Reverse 5' –GCT ACC TTG TTA CGA CTT C-3'.

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This study has use another primer as an alternative method, following Marchesi et al., 1998 (62), to amplify approximately 1,300 bp

Forward primer 63f 5'-CAG GCC TAA CAC ATG CAA GTC-3' and

Reverse primer 1387r 5' –GGG CGG WGT GTA CAA GGC-3'

Amplification of the 16S rRNA gene was confirmed by gel electrophoresis using 1.5% agarose and was stained with SYBR® Safe DNA gel stain (Invitrogen, California), and the bands were visualized by UV transillumination shown in figure 7. The amplified fragments were purified by Gel/PCR DNA Fracments Extraction kit (Geneaid, Taiwan). The resulting PCR fragments were excised from the gel and sequenced by First BASE

Laboratories (Singapore). BLAST searches on NCBI (www.ncbi.nlm.nih.gov/BLAST/) were used to search for close evolutionary-related sequences in the GenBank database.

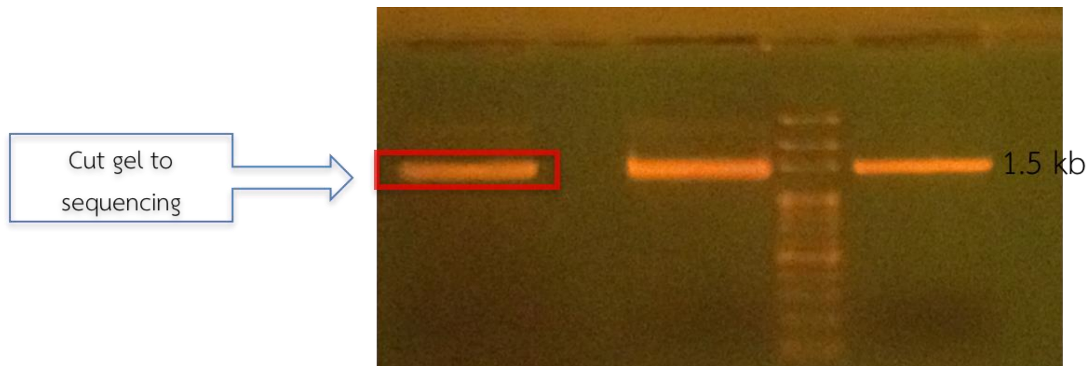

Figure: 7 The expected 16s rRNA-gene; 1500 base pair

3.4 Bacteria isolation and identification from field and laboratory strain of *Aedes albopictus*

3.4.1 Bacteria isolation and identification from laboratory strain of *Aedes albopictus*

Laboratory strains of female *Aedes albopictus* mosquitoes were anesthetized at -20 °C and the dissections were done under sterile conditions after surface sterilization with 70% ethanol for 5 min. The mosquito was washed in PBS twice before further processing of the midgut isolates and subsequent bacterial cultivation. The midgut was dissected and isolated in order to identify the contents by bacterial subculture and molecular tests. The midgut contents were suspended in 300 µl of 60% glycerol and a 100 µl aliquot of the suspension was spread on tryptose soya agar (TSA) supplemented with 5% sheep blood and incubated at 37°C for 24 hrs. The resulting bacterial colonies were grouped; based on their colony morphology. Bacterial colonies that are morphologically distinct were selected and subcultured on TSA plates until a pure culture was obtained so that further analysis might be undertaken. Pure bacterial isolates from mosquito midguts were subcultured in 2 ml of tryptose

soya broth (TSB) at 37°C for 24 hrs. Cell pellets were suspended in distilled water and lysed using repeated cycles of freezing and thawing. The bacteria DNA was extracted by using the boiling method. Complete 16S rRNA gene (Approx. 1.5 kb size) were amplified from the extracted DNA of the isolates as described by Djadid et al., 2011 (61) using eubacteria specific primers

16s Forward 5'– AGT TTG ATC CTG GCT CAG – 3' and

16s Reverse 5' –GCT ACC TTG TTA CGA CTT C-3'.

This study also used another primer as an alternative method, following Marchesi et al., 1998 (62), to amplify approximately 1,300 bp

Forward primer 63f 5'-CAG GCC TAA CAC ATG CAA GTC-3' and

Reverse primer 1387r 5' –GGG CGG WGT GTA CAA GGC-3'

Amplification of the 16S rRNA gene was confirmed by gel electrophoresis using 1.5% agarose and was stained with SYBR® Safe DNA gel stain (Invitrogen, California), and the bands were visualized by UV transillumination. The amplified fragments were purified by Gel/PCR DNA Fracments Extraction kit (Geneaid, Taiwan). The resulting PCR fragments were excised from the gel and sequenced by First BASE Laboratories (Singapore). BLAST searches on NCBI (www.ncbi.nlm.nih.gov/BLAST/) were used to search for close evolutionary-related sequences in the GenBank database.

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3.4.2 Bacteria isolation and identification from field strain of *Aedes albopictus*

The field area that was chosen and described in previous reports have been the re-emergence of Chikungunya fever in Yala, Chumphon, and Shigha Buri province (63).

CHAPTER IV
RESULTS

4.1 Chikungunya virus infection in mosquitoes

4.1.1 Mosquito infection, dissemination, and transmission

The vector competence of *Aedes albopictus* for the Chikungunya virus (CHIKV) and the effects of CHIKV titers in blood meal on virus infection, dissemination, and transmission in *Ae. albopictus* were examined in this study. The percentage of infection is defined as the percentage of blood-fed mosquitoes with virus in their bodies, percentage dissemination is defined as the percentage of blood-fed mosquitoes with virus in hemocoel as indicated by detecting virus in their legs and wings, and the percentage transmission is defined as the percentage of blood-fed mosquitoes with virus in their saliva. Five groups of *Ae. albopictus* were allowed to feed on different levels of Thailand 2010 strain CHIKV in the blood meal; these were 10^2, 10^3, 10^4, 10^5, or 10^6 CID_{50}/ml of CHIKV. On day 14 post blood feeding (PBF), the body, leg and wing, and saliva samples from the blood-fed mosquitoes were assayed for the presence of CHIKV through immunocytochemistry (ICC) staining as indicated by a red brown color in the cells (Figure 8). Culture media from the infected cells were also confirmed by the reverse transcription polymerase chain reaction (RT-PCR).

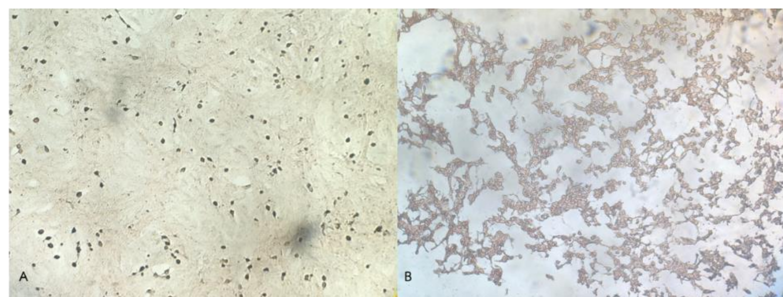

Figure: 8 (A): Normal infected MEM Vero cell, (B): CHIKV 10^6 CID_{50}/ml infected Vero cell

4.1.1.1 Mosquito infection

The percentage of CHIKV infection in *Ae. albopictus* was 100% after being fed on 10^4, 10^5, and 10^6 CID_{50}/ml of CHIKV. The percentage of infection in *Ae. Albopictus* was 83.3% and increased to 90% after being fed on 10^2 and 10^3 CID_{50}/ml of CHIKV, respectively. However, there was no significant difference in the percentage of infection between these latter two CHIKV levels.

Table : 1 Percent Infection of Chikungunya virus (CHIKV) in *Aedes albopictus* at 14 days after feeding on CHIKV infected blood meal

CHIKV titer in mosquito blood meal (log_{10} CID_{50}/ml)	No. of tested mosquitoes	Percent infection* (± SE**)
2	24	83.3 ± 7.8^{1}
3	30	90 ± 5.6^{1}
4	30	100^{2}
5	30	100^{2}
6	30	100^{2}

*Values within each category that have a numerical superscript letter in common indicate no statistically significant differences.

**SE = Standard Deviation

4.1.1.2 Mosquito dissemination

The percentage of CHIKV dissemination in *Ae. albopictus* was 70.8% after being fed on 10^2 CID_{50}/ml of CHIKV, and was 86.7, 100, 90, and 98% respectively after blood meals with the titers of 10^3, 10^4, 10^5, and 10^6 CID_{50}/ml of CHIKV, respectively. However, there was no significant difference in the percentage of CHIKV dissemination among the virus titers of 10^3 CID_{50}/ml of CHIKV and higher.

Table: 2 Percent dissemination of Chikungunya virus (CHIKV) in *Aedes albopictus* at 14 days after feeding on CHIKV infected blood meal

CHIKV titer in mosquito blood meal ($\log_{10}$ CID_{50}/ml)	No. of tested mosquitoes	Percent dissemination* (± SE**)
2	24	70.8 ± 9.5^{1}
3	30	86.7 ± 6.3^{2}
4	30	100^{2}
5	30	90 ± 5.6^{2}
6	30	98 ± 2.0^{2}

*Values within each category that have a numerical superscript letter in common indicate no statistically significant differences.

**SE = Standard Deviation

4.1.1.3 Mosquito transmission

The percentage of CHIKV transmission in *Ae. albopictus* was 41.6% after being fed on 10^2 CID_{50}/ml of CHIKV, and was 70, 100, 90, and 82.4% after blood meals with the titers of 10^3, 10^4, 10^5, and 10^6 CID_{50}/ml of CHIKV, respectively. There were significant differences in the percentage of CHIKV transmission among the different virus titers of CHIKV (Table 3). The lowest percent transmission was 41.6% and the highest percent transmission was 100% after being fed on 10^2 and 10^4 CID_{50}/ml of CHIKV. The percentage of transmission after being fed on 10^2 CID_{50}/ml, was significantly different from that after being fed on 10^3, 10^4, 10^5, and 10^6 CID_{50}/ml, while the percent transmission after being fed on 10^4 CID_{50}/ml was significantly different from that after being fed on 10^2, 10^3, and 10^6 CID_{50}/ml of CHIKV.

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Table: 3 Percent transmission of Chikungunya virus (CHIKV) in *Aedes albopictus* at 14 days after feeding on CHIKV infected blood meal

CHIKV titer in mosquito blood meal (log_{10} CID_{50}/ml)	No. of tested mosquitoes	Percent transmission* (± SE**)
2	24	41.6 ± 10.3^{1}
3	30	70 ± 8.5^{2}
4	30	100^{3}
5	30	$90 \pm 5.6^{3,4}$
6	30	$82.4 \pm 5.4^{2,4}$

*Values within each category that have a numerical superscript letter in common indicate no statistically significant differences.

**SE = Standard Deviation

4.2 Microbiota identification in CHIKV infected mosquito midgut

4.2.1 Bacterial genera identification after being fed on CHIKV infected blood meal and non-infected blood meal.

One group of female *Ae. albopictus* was fed on a negative blood meal whilst other five groups of female *Ae. albopictus* were fed on CHIKV infected blood meal with varying doses. From the sample in 4.1, the CHIKV infected female's midguts were analyzed for bacteria isolation and identification. The purified bacteria isolated from each group was propagated in TSB and identified by 16s rRNA gene amplification. The sequence analysis showed that the bacterial species were agreed with the NCBI data base. The correlation of CHIKV with bacterial identification were compared between the infected mosquito and the non-infected mosquito of each dose group.

Bacterial identified from the *Ae. albopictus* midgut after having been fed varying doses of CHIKV are shown in Table 4. We selected 100 bacterial isolates for 16s rRNA gene sequence-based identification. From all the categories of individuals, we could identify 48 distanct bacterial species from 18 bacterial genera which belonged to four major phyla namrly, Actinobacteria Fimicutes, and Proteobacteria, with abroad range being present in each dose group (Table 4). The results from the bacterial isolation found that there were the organism gram-negative, and gram-positive genera in both infected and non-infected in all five CHIKV dose. But there was no significant difference between all of CHIKV infected groups. Although, the gram-positive genera were dominate in almost CHIKV infected dose, the gram-negative genera also were found in infected of CHIKV infected groups. In addition, bacterial isolation were varieties genera in each CHIKV infected dose. However, the isolated bacterial genera were dominated by *Micrococcus* spp. in infected mosquito that were fed on 10^2 CID_{50}/ml of CHIKV. These were also significant differences between infected and non-infected mosquitoes group after being fed on 10^2 CID_{50}/ml of CHIKV ($P< 0.05$), while the other bacterial genera are not difference.

A diversity of bacterial genera was found in both the infected and non-infected mosquitoes that were fed on 10^3 CID_{50}/ml of CHIKV. For the infected mosquitoes, the identified bacterial genera were dominated *Micrococcus* spp., followed by *Staphylococcus* spp., and *Bacillus* spp., respectively. The relevant percentage were 71.43%, 38.10%, and 14.29%, respectively. Whilst the main bacterial genera in the non-infected mosquitoes were *Staphylococcus* spp. and *Micrococcus* spp., with the percentage being 33.33% and 22.22%, respectively.

For the bacterial identification in infected mosquitoes after being fed on 10^4 CID_{50}/ml of CHIKV were not compared with the non-infected mosquito because the percentage of transmission are 100% in this group. Which the bacterial genera were dominated *Bacillus* spp., followed by *Staphylococcus* spp.

There were 40.74% of *Micrococcus* spp. found in infected mosquito group after being fed on 10^5 CID_{50}/ml of CHIKV. While *Staphylococcus* spp. had higher bacterial midgut percentage (66.67%) in non-infected mosquitoes group. For the bacterial midgut in the infected mosquito group after being fed on 10^6 CID_{50}/ml of CHIKV were also dominated by *Micrococcus* spp., followed by *Bacillus* spp., *Brachybacterium* spp., and *Staphylococcus* spp., respectively. The relevant percentage were 16%, 12%, and 8%, respectively. However, the bacterial genera were unidentified in non-infected mosquito group.

A total of 37 phylotypes were observed with 99% similarity values as cut off. The 16s rRNA gene sequence from a variety of phylogenetic groups are shown in figure 9. The majority of the cultured isolates from CHIKV infected and non-infected *Ae. albopictus* were found to belonging Actinobacteria phylum. Distinct genera were *Micrococcus*, *Actinomyces,* *Brachybacterium*, *Brevibacterium*, *Corynebacterium, Kocuria*, *Streptomyces*, and *Sinomonas.* Firmicutes represented second abundant phylotypes containing *Bacillus, Staphylococcus*, *Paenibacillus*, and *Streptococcus*.

For the percentage of bacterial species identification compared per mosquito in each group were shown on the topic 4.2.2, 4.2.3, 4.2.4, 4.2.5, 4.2.6, 4.2.7, and 4.2.8 respectively.

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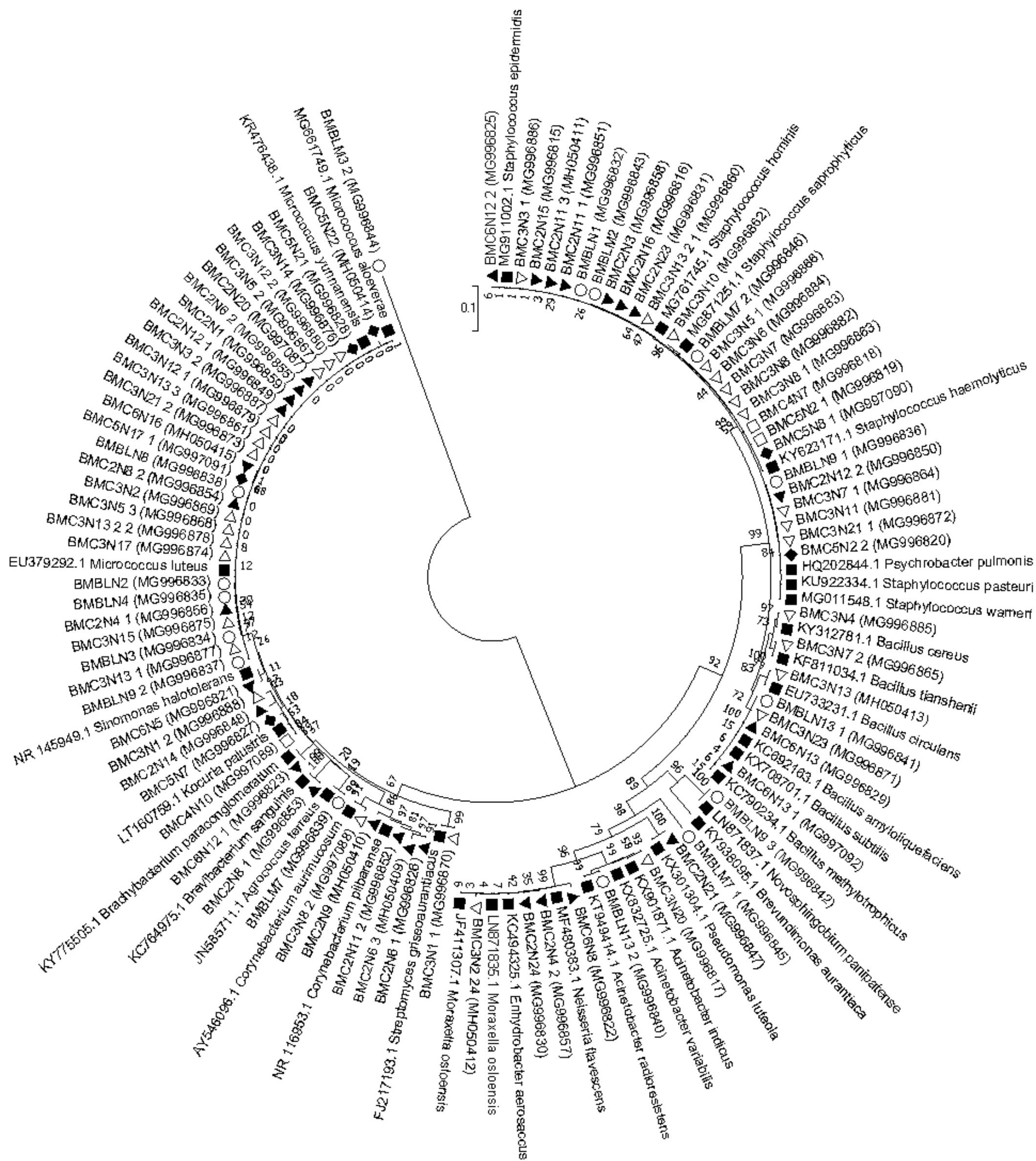

Figure: 9 Phylogenetic tree constructed for partial 16S rRNA gene of isolates cultured from CHIKV infected and non-infected *Ae. albopictus*

Bootstrap values are given at nodes. Entries with black square represent reference names and accession numbers (in parentheses). Entres from this study are represented as: strain number, accession number (inparenteses). (■ as reference names ○ as non-infected blood meal group, ▲ as CHIKV infected 10^2 CID_{50}/ml group, △ as CHIKV infected 10^3 CID_{50}/ml group, □ as CHIKV infected 10^4 CID_{50}/ml group, ◆ as CHIKV infected 10^5 CID_{50}/ml group, ▼ as CHIKV infected 10^6 CID_{50}/ml group)

Table: 4 The percentage of identified bacterial genera from Aedes albopictus midgut after being fed on different levels of chikungunya virus infected blood meal.

Bacterial Phylum	Genus (spp.) and Gram	10^6 CID_{50}/ml			10^5 CID_{50}/ml			10^4 CID_{50}/ml	10^3 CID_{50}/ml			10^2 CID_{50}/ml		
		Saliva (+) (n=25)	Saliva (-) (n=5)	P value	Saliva (+) (n=27)	Saliva (-) (n=3)	P value	saliva (+) (n=30)	Saliva (+) (n=21)	Saliva (-) (n=9)	P value	Saliva (+) (n=10)	Saliva (-) (n=14)	P value
Actinobacteria	*Actinomyces* (+)	4.00	0.00	0.66	-	-	-	-	-	-	-	-	-	-
	Brachybacterium (-)	8.00	0.00	0.51	-	-	-	-	-	-	-	-	-	-
	Brevibacterium (+)	-	-	-	-	-	-	3.33	-	-	-	10.00	0.00	0.25
	Corynebacterium (+)	-	-	-	3.70	0.00	0.75	-	0.00	11.11	0.13	30.00	0.00	0.09
	Kocuria (-)	-	-	-	3.70	0.00	0.75	3.33	4.76	0.00	0.52	0.00	7.14	0.41
	Micrococcus (+)	16.00	0.00	0.35	40.74	0.00	0.25	3.33	71.43	22.22	0.14	70.00	28.57	**0.04***
	Sinomonas (+)	4.00	0.00	0.66	-	-	-	-	-	-	-	-	-	-
	Streptomyces (+)	4.00	0.00	0.66	-	-	-	-	0.00	11.11	0.13	-	-	-
Firmicutes	*Bacillus* (+)	12.00	0.00	0.43	-	-	-	10.00	14.29	11.11	0.82	-	-	-
	Paenibacillus (-)	4.00	0.00	0.66	-	-	-	3.33	-	-	-	-	-	-
	Staphylococcus (+)	8.00	0.00	0.53	3.70	66.67	**0.05**	6.67	38.10	33.33	0.81	60.00	21.43	0.16
	Streptococcus (+)	-	-	-	3.70	0.00	0.75	3.33	-	-	-	-	-	-
Proteobacteria	*Acinetobacter* (-)	-	-	-	-	-	-	-	4.76	0.00	0.52	-	-	-
	Brevundimonas (-)	-	-	-	-	-	-	3.33	-	-	-	-	-	-
	Enhydrobacter (-)	-	-	-	-	-	-	-	-	-	-	10.00	0.00	0.25
	Moraxella (-)	4.00	0.00	0.66	7.41	0.00	0.64	-	0.00	11.11	0.13	0.00	7.14	0.41
	Pseudomonas (-)	-	-	-	-	-	-	-	-	-	-	0.00	7.14	0.41
	Psychrobacter (-)	-	-	-	-	-	-	-	-	-	-	0.00	7.14	0.41

*P ≤ 0.05 indicated significant difference between the infected and non-infected mosquitoes in each group as determined by Student-t test.

4.2.2 Bacteria species identification from *Ae. albopictus* midgut after being fed on negative blood meal (n=30)

The bacterial species that were identified from *Ae. albopictus* midgut after being fed negative blood meal were summarized in Table 5. A total of 11 bacterial species were identified in the study and showed that the dominating species was *Micrococcus luteus*, followed by *Micrococcus yunnanensis,* and *Acinetobacter radioresistens*. The most identified bacterial species were belonged to Actinobacteria phylum. Moreover, the results showed that there were only two bacterial species found in this group which were *Agrococcus terreus* and *Bacillus amyloliquefaciens*.

Table: 5 The percentage of bacterial species found in *Aedes albopictus* midgut after being fed on negative blood meal.

Bacterial Phylum	Closest related bacterial species**	Percent infected mosquitoes*
Actinobacteria	*Agrococcus terreus*#	4.17
	Janibacter indicus	4.17
	Micrococcus luteus	12.50
	Micrococcus yunnanensis	8.33
Firmicutes	*Bacillus amyloliquefaciens*#	4.17
	Staphylococcus hominis	4.17
	Staphylococcus cohnii	4.17
	Staphylococcus pasteuri	4.17
Proteobacteria	*Acinetobacter radioresistens*	8.33
	Neisseria perflava	4.17
	Novosphingobium panipatens	4.17

Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

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4.2.3 Bacterial identification from *Aedes albopictus* midgut after being fed on CHIKV 10^6 CID_{50}/ml (n=30)

The bacterial species that were identified in *Ae. albopictus* midgut after being fed on CHIKV 10^6 CID_{50}/ml were summarized in Table 6. A total of 13 bacterial species were identified and the dominating species was *Micrococcus luteus*, followed by *Bacillus megaterium*. In addition, there were eight other species that were identified only in this particular group but the abundance of these was significantly less. However, the identified bacterial species were belonged to Actinobacteria phylum and Firmicutes phylum, which only one species, *Moraxella osloensis,* belonging to Proteobacteria phylum. The correlation of CHIKV and bacterial identification between infected and non-infected mosquito cannot be shown because there was no identified bacteria in the non-infected mosquito group.

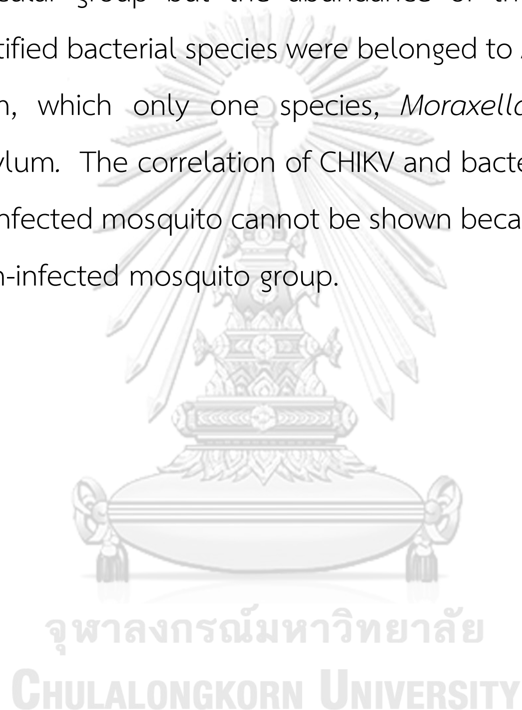
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Table: 6 The percentage of bacterial species found in *Aedes albopictus* midgut after being fed on 10^6 CID_{50}/ml chikungunya virus (CHIKV)

Bacterial Phylum	Closest related bacterial species**	Percent infected mosquitoes* (positive CHIKV)	Percent infected mosquitoes (negative CHIKV)
Actinobacteria	*Actinomyces naeslundii*[#]	4.00	0
	Brachybacterium nesterenkovii	4.00	0
	Brachybacterium paraconglomeratum[#]	4.00	0
	Micrococcus aloeverae[#]	4.00	0
	Micrococcus luteus	12.00	0
	Sinomonas halotolerans	4.00	0
	Streptomyces pseudogriseolus[#]	6.25	4.00
Firmicutes	*Bacillus megaterium*[#]	8.00	0
	Bacillus subtilis	4.00	0
	Paenibacillus timonensis[#]	4.00	0
	Staphylococcus epidermidis	4.00	0
	Staphylococcus hominis	4.00	0
Proteobacteria	*Moraxella osloensis*	4.00	0

[#] Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

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4.2.4 Bacterial identification from *Aedes albopictus* midgut after being fed on CHIKV 10^5 CID_{50}/ml (n=30)

The bacterial species that were identified in *Ae. albopictus* midgut after being fed on CHIKV 10^5 CID_{50}/ml were summarized in Table 7. The dominating bacterial species found in infected mosquitoes were *Micrococcus luteus,* followed by *Micrococcus yunnanensis*, and *Moraxella osloensis.* The bacterial species found only in this group was *Corynebacterium ihumii*. While the dominating bacterial species in non-infected mosquitoes were *Staphylococcus haemolyticus* and *Staphylococcus warneri.* In addition, the correlation of CHIKV with bacteria species are not significantly different between infected and non-infected mosquitoes. However, the most bacterial species were belonged to Actinobacteria phylum and Firmicutes phylum, but there was only one species, *Moraxella osloensis*, belonged to Proteobacteria phylum.

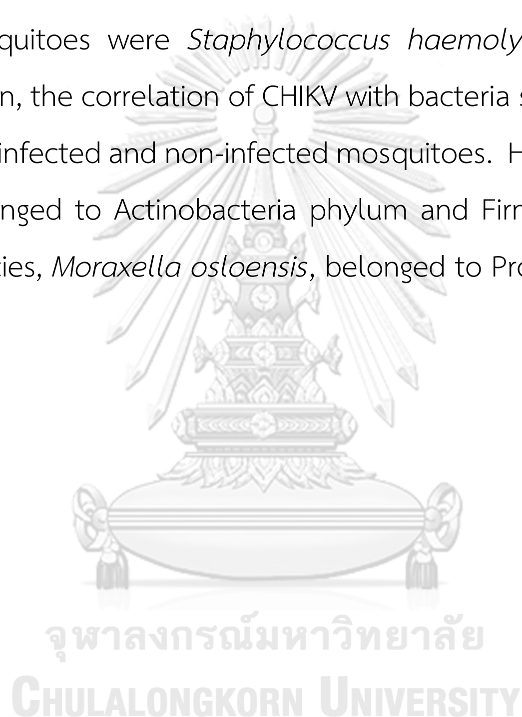
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Table: 7 The percentage of bacterial species found in Aedes albopictus midgut after being fed on 10^5 CID_{50}/ml chikungunya virus (CHIKV)

Bacterial Phylum	Closest related bacterial species**	Percent infected mosquitoes* (positive CHIKV)	Percent infected mosquitoes (negative CHIKV)
Actinobacteria	*Corynebacterium ihumii*[#]	3.70	0
	Kocuria palustris	3.70	0
	Micrococcus luteus	29.63	0
	Micrococcus yunnanensis	11.11	0
Firmicutes	*Staphylococcus cohnii*	3.70	0
	Staphylococcus haemolyticus	0	33.33
	Staphylococcus warneri	0	33.33
	Streptococcus mitis	3.70	0
Proteobacteria	*Moraxella osloensis*	7.41	0

[#] Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

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4.2.5 Bacterial identification from *Aedes albopictus* midgut after being fed on CHIKV 10^4 CID_{50}/ml (n=30)

The bacterial species were identified from *Ae. albopictus* midgut after being fed on CHIKV 10^6 CID_{50}/ml are summarized in Table 8. A total of 11 bacterial species were identified and there was no dominating species because the percentage were equal. However, the most of bacterial species being Firmicutes phylum and there is only one species, *Brevundimonas diminuta*, belong to Proteobacteria phylum. In addition, the correlation of CHIKV infection and bacteria species compare between infected and non-infected mosquitoes cannot report because the percentage of transmission were 100% in this group.

Table: 8 The percentage of bacterial species found in *Aedes albopictus* midgut after being fed on 10^4 CID_{50}/ml chikungunya virus (CHIKV)

Bacterial phylum	Closest related bacterial species**	Percent infected mosquitoes* (positive CHIKV)
Actinobacteria	*Brevibacterium casei*#	3.33
	Kocuria palustris	3.33
	Micrococcus luteus	3.33
Firmicutes	*Bacillus aquimaris*#	3.33
	Bacillus cereus	3.33
	Bacillus clausii#	3.33
	Paenibacillus lautus#	3.33
	Staphylococcus epidermidis	3.33
	Staphylococcus haemolyticus	3.33
	Streptococcus mitis	3.33
Proteobacteria	*Brevundimonas diminuta*#	3.33

Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

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4.2.6 Bacterial identification from *Aedes albopictus* midgut after being fed on CHIKV 10^3 CID_{50}/ml (n=30)

The bacterial species were identified in *Ae. albopictus* midgut after being fed on CHIKV 10^3 CID_{50}/ml were summarized in Table 9. The dominating bacterial species identified in the infected mosquitoes were *Micrococcus luteus,* followed by *Micrococcus yunnanensis*, and *Staphylococcus haemolyticus,* respectively. While the dominating bacterial species found in non-infected mosquitoes were *Micrococcus yunnanensis* with the other five species being equal by percentage. In addition, the correlation of CHIKV with bacteria species indicated that there was no significant different between infected and non-infected mosquitoes.

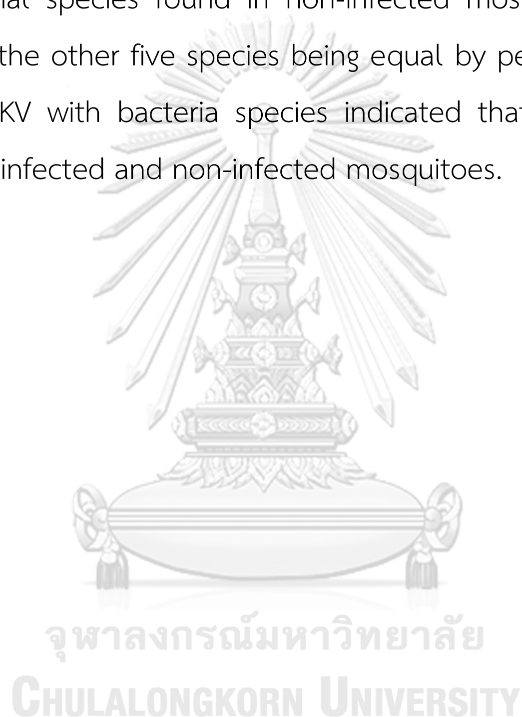

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Table: 9 The percentage of bacterial species found in *Aedes albopictus* midgut after being fed on 10^3 CID_{50}/ml chikungunya virus (CHIKV)

Bacterial phylum	Closest related bacterial species**	Percent infected mosquitoes* (positive CHIKV)	Percent infected mosquitoes (negative CHIKV)
Actinobacteria	*Corynebacterium aurimucosum*#	4.76	0
	Kocuria palustris	4.76	0
	Micrococcus luteus	42.86	0
	Micrococcus yunnanensis	28.57	22.22
	Streptomyces griseoaurantiacus#	0	11.11
Firmicutes	*Staphylococcus epidermidis*	4.76	0
	Staphylococcus haemolyticus	19.05	0
	Bacillus cereus	4.76	0
	Bacillus circulans#	4.76	0
	Bacillus methylotrophicus#	4.76	0
	Bacillus tianshenii#	4.76	0
	Staphylococcus hominis	0	11.11
	Staphylococcus pasteuri	0	11.11
	Staphylococcus saprophyticus#	0	11.11
Proteobacteria	*Moraxella osloensis*	0	11.11
	Acinetobacter indicus#	4.76	0

Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

4.2.7 Bacterial identification from *Aedes albopictus* midgut after being fed on CHIKV 10^2 CID_{50}/ml (n=24)

The bacterial species were identified in *Ae. albopictus* midgut after being fed on CHIKV 10^2 CID_{50}/ml were summarized in Table 10. A total of 13 bacterial species were identified and the dominating bacterial species in infected mosquitoes were *Micrococcus luteus,* followed by *Staphylococcus epidermidis* and *Corynebacterium pilbarense.* While the dominating bacterial species in non-infected mosquitoes were *Micrococcus luteus,* followed by *Staphylococcus epidermidis* and the other five species are equally percentage. In addition, the correlation of CHIKV with bacteria species was not significantly different between infected and non-infected mosquitoes.

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Table: 10 The percentage of bacterial species found in *Aedes albopictus* midgut after being fed on 10^2 CID_{50}/ml chikungunya virus (CHIKV)

Bacterial phylum	Closest related bacterial species**	Percent infected mosquitoes* (positive CHIKV)	Percent infected mosquitoes (negative CHIKV)
Actinobacteria	*Brevibacterium sanguinis*[#]	10.00	0
	Corynebacterium jeikeium[#]	10.00	0
	Corynebacterium pilbarense[#]	20.00	0
	Kocuria marina[#]	0	7.14
	Micrococcus luteus	60.00	21.43
	Micrococcus yunnanensis	10.00	7.14
Firmicutes	*Staphylococcus epidermidis*	20.00	14.29
	Staphylococcus haemolyticus	10.00	0
	Staphylococcus hominis	30.00	7.14
Proteobacteria	*Moraxella osloensis*	0	7.14
	Pseudomonas luteola	0	7.14
	Psychrobacter pulmonis[#]	0	7.14
	Enhydrobacter aerosaccus[#]	10.00	0

[#] Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

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4.3 Bacterial identification from laboratory and field-collected mosquitoes

4.3.1 Bacterial genera identification from laboratory-reared and field-collected *Aedes albopictus*

The midguts from one group of laboratory-reared female *Ae. albopictus* midguts and four groups of field-collected *Ae. albopictus* were dissected for bacterial isolation and identification. The purified bacteria isolation from each group were propagated in TSB and identified by 16s rRNA gene amplification. The sequence analysis indicated the bacterial species were closely related with NCBI data base and the percent bacterial identification per mosquito were compared.

Bacterial identified from the *Ae. albopictus* midgut after having been fed varying doses of CHIKV are shown in Table 4. We selected 65 bacterial isolates for 16s rRNA gene sequence-based identification. From all the categories of individuals, we could identify 53 distanct bacterial species from 31 bacterial genera which belonged to four major phyla namrly, Actinobacteria Fimicutes, and Proteobacteria, with abroad range being present in each dose group. The bacterial genera found in the laboratory-reared and filed collected *Ae. albopictus* midguts were summarized in Table 11. A total of 31 bacterial genera were identified of which the majority of organism were gram-negative genera. The bacterial genera dominating in the laboratory-reared *Ae. albopictus* were *Staphylococcus* spp., followed by *Micrococcus* spp. and *Microbacterium* spp. while *Rhizobium* spp. and *Agrobacterium* spp. were dominated species in field-collected *Ae. albopictus.* Interestingly, the study found that the percentage of *Staphylococcus* spp. were significantly different between laboratory-reared and field-collected *Ae. albopictus* ($P<0.0007$). In addition, the most identified bacterial genera were belonged to Proteobacteria phylum and there were only two genera, *Bacillus* spp. and *Staphylococcus* spp., belonging to Firmicutes phylum.

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A total of 53 phylotypes were observed with 99% similarity values as cut off. The 16s rRNA gene sequence from a variety of phylogenetic groups are shown in figure 10. The majority of the cultured isolates from laboratory-reared *Ae. albopictus* were found to belonging Proteobacteria phylum. Distinct genera were *Acinetobacter, Agrobacterium, Beijerinckia, Brevundimonas, Burkholderia, Candidatus Rhizobium, Chryseobacterium, Enhydrobacter, Enterobacter, Erwinia, Klebsiella, Massilia, Moraxella, Novosphingobium, Pandoraea, Pantoea, Pectobacterium, Providencia, Pseudomonas, Rahnella, Rhizobium, Serratia, and* Sphingomonas. Actinobacteria represented second abundant phylotypes containing *Actinomyces, Brachybacterium, Leucobacter, Microbacterium, Micrococcus, and Nocardioides.*

The bacterial species identified was shown in the percentage per species per mosquito, the details were presented in the topics discussed in section 4.3.2 and 4.3.3.

Figure: 10 Phylogenetic tree constructed for partial 16S rRNA gene of isolates cultured from laboratory-reared and field-collected *Ae. albopictus*

Bootstrap values are given at nodes. Entries with black square represent reference names and accession numbers (in parentheses). Entres from this study are represented as: strain number, accession number (inparenteses). (■ as reference names, □ as laboratory-reared *Ae. albopictus*, △ as field-collected from Chumphon, ▲ as field-collected from Yala)

Table: 11 The percentage of bacterial genera from laboratory-reared and field-collected *Aedes albopictus*

Bacterial phylum	Genus and gram stain	Percent infected in laboratory-reared mosquitoes (n=33)	Percent infected in field mosquitoes (n=80)	P-value
Actinobacteria	*Actinomyces* (+)	0	1.25	0.52
	Brachybacterium (+)	0	1.25	0.52
	Leucobacter (+)	3.03	0	0.12
	Microbacterium (+)	12.12	3.75	0.09
	Micrococcus (+)	21.21	6.25	0.13
	Nocardioides (+)	0	1.25	0.52
Firmicutes	*Bacillus* (+)	0	6.25	0.14
	Staphylococcus (+)	27.27	3.75	0.0007*
Proteobacteria	*Acinetobacter* (-)	3.03	5.00	0.65
	Agrobacterium (-)	12.12	7.50	0.44
	Beijerinckia (-)	0	1.25	0.52
	Brevundimonas (-)	0	1.25	0.52
	Burkholderia (-)	0	2.50	0.36
	Candidatus Rhizobium (-)	0	2.50	0.36
	Chryseobacterium (-)	0	2.50	0.36
	Enhydrobacter (-)	3.03	0	0.12
	Enterobacter (-)	0	6.25	0.14
	Erwinia (-)	0	1.25	0.52
	Klebsiella (-)	3.03	5.00	0.85
	Massilia (-)	0	1.25	0.52
	Moraxella (-)	0	1.25	0.52
	Novosphingobium (-)	0	1.25	0.52
	Pandoraea (-)	3.03	0	0.12
	Pantoea (-)	0	2.50	0.36
	Pectobacterium (-)	0	1.25	0.52
	Providencia (-)	0	3.75	0.26
	Pseudomonas (-)	6.06	3.75	0.59
	Rahnella (-)	0	1.25	0.52
	Rhizobium (-)	0	8.75	0.08
	Serratia (-)	0	1.25	0.52
	Sphingomonas (-)	0	2.50	0.36

*P ≤ 0.05 indicated significant difference between the laboratory-reared and field-collected mosquitoes as determined by Student-t test

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4.3.2 Bacterial species identification from laboratory-reared *Aedes albopictus* midguts (n=30)

The bacterial species which were identified from laboratory-reared *Ae. albopictus* midguts were summarized in Table 12. A total of 16 bacterial species were identified and the dominant bacterial species in laboratory-reared *Ae. albopictus* were *Micrococcus luteus,* followed by *Staphylococcus epidermidis*, and *Corynebacterium pilbarense.* While the dominant bacterial species in field-collected *Ae. albopictus* were *Micrococcus luteus,* followed by *Agrobacterium tumefaciens,* and *Staphylococcus epidermidis.* However, whilst the percentage of bacterial species was low, but the diversity of bacterial species was also found. In addition, the identified bacterial species were belonged to Actinobacteria, Firmicutes, and Proteobacteria phylum.

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Table: 12 The percentage of bacterial species identification from laboratory-reared *Aedes albopictus* (n=30)

Bacterial phylum	Closest related bacterial species**	Percent infected mosquitoes*
Actinobacteria	*Leucobacter chironomi*	3.03
	Microbacterium dextranolyticum	9.09
	Microbacterium laevaniformans	3.03
	Micrococcus luteus	15.15
	Micrococcus yunnanensis	6.06
Firmicutes	*Staphylococcus arlettae*	6.06
	Staphylococcus epidermidis	12.12
	Staphylococcus pasteuri	3.03
	Staphylococcus warneri	6.06
Proteobacteria	*Acinetobacter variabilis*	3.03
	Agrobacterium tumefaciens	12.12
	Enhydrobacter aerosaccus	3.03
	Klebsiella pneumoniae	3.03
	Pandoraea sputorum	3.03
	Pseudomonas aeruginosa	3.03
	Pseudomonas luteola	3.03

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

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4.3.3 Bacterial species identification in field-collected *Ae. albopictus* midgut

The mosquitoes were collected from Sigha Buri, Chumphon, and Yala province. These provinces were representative of the central, upper southern, and lower southern areas of Thailand, respectively.

4.3.3.1 Bacterial species identification from Aedes albopictus midguts collected from Sigha Buri province (n=10)

The bacterial species identified from *Ae. albopictus* midguts collected from Sigha Buri province were *Bacillus subtilis*, *Serratia marcescens, Staphylococcus haemolyticus*, and *Staphylococcus hominis* and were present equal percentages, the details were summarized in Table 13. In addition, the *Serratia marcescens* was identified only in this area.

Table: 13 The percentage of bacterial identification from *Aedes albopictus* midguts collected from Sigha Buri province (n=10)

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Bacterial phylum	Closest related bacterial species**	Percent infected mosquitoes*
Firmicutes	*Bacillus subtilis*	10.00
	Staphylococcus haemolyticus	10.00
	Staphylococcus hominis	10.00
Proteobacteria	*Serratia marcescens*#	10.00

Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

4.3.3.2 Bacterial species identification from Aedes albopictus midguts collected from Chumphon province (Muang district) (n=20)

The bacterial species that were identified in the midguts of *Ae. albopictus* from Chumphon province. Mosquitoes were collected was from two main areas in Chumphon; the firs area was Saphee sub-district, Muang district and the results were summarized in Table 14. The second area was Suan Nai Dam sub-district, Thung Tago district and the results were shown in Table 15. A total of 11 bacterial species were identified and the dominant bacterial species from *Ae. albopictus* collected from Muang district were *Enterobacter cloacae.* Eight bacterial species were identified from *Ae. albopictus* collected from Muang district only and the equal percentage was found. However, the percentage of bacterial species was not high, but the diversity of bacterial species was also revealed. In addition, there were many bacterial species that were found only in this area; *Enterobacter cloacae, Enterobacter cancerogenus*, *Enterobacter hormaechei*, *Enterobacter mori, Klebsiella quasipneumoniae*, *Klebsiella variicola,* and *Microbacterium yannicii.*

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Table: 14 The percentage of bacterial identification in *Aedes albopictus* collected from Chumphon province (Muang district) (n=20)

Bacterial phylum	Closest related bacterial species**	Percent infected mosquitoes*
Actinobacteria	*Microbacterium yannicii*[#]	5.00
Proteobacteria	*Agrobacterium tumefaciens*	5.00
	Enterobacter cancerogenus[#]	5.00
	Enterobacter cloacae[#]	15.00
	Enterobacter hormaechei[#]	5.00
	Enterobacter mori[#]	5.00
	Klebsiella pneumoniae	10.00
	Klebsiella quasipneumoniae[#]	5.00
	Klebsiella variicola[#]	5.00
	Moraxella osloensis	5.00
	Rhizobium pusense	10.00

[#] Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

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4.3.3.3 Bacterial species identification from Aedes albopictus midguts collected from Chumphon province (Thung Tago district) (n=20)

Suan Nai Dum sub-district was the second area that was selected for collecting *Aedes albopictus*. The bacterial species which were identified from these mosquitoes were shown in Table 15. A total of 15 bacterial species were identified and the dominant bacterial species were *Micrococcus luteus* and *Providencia rettgeri.* In addition, there were eight bacterial species were only identified from Thung Tago district and a haft of these species were dominated by *Bacillus kochii, Chryseobacterium taklimakanense, Pantoea dispersa,* and *Pseudomonas*

psychrotolerans. However, the percentage of bacterial species identification was less but there was greater diversity in this area.

Table: 15 The percentage of bacterial identification from *Aedes albopictus* collected from Chumphon province (Thyng Tago district) (n=20)

Bacterial phylum	Closest related bacterial species**	Percent infected mosquitoes*
Actinobacteria	*Actinomyces oris*[#]	5.00
	Microbacterium dextranolyticum	5.00
	Micrococcus luteus	15.00
Firmicutes	*Bacillus kochii*[#]	10.00
	Bacillus pocheonensis[#]	5.00
	Staphylococcus epidermidis	5.00
Proteobacteria	*Acinetobacter lwoffii*[#]	5.00
	Acinetobacter variabilis	10.00
	Agrobacterium tumefaciens	5.00
	Chryseobacterium taklimakanense[#]	10.00
	Erwinia tasmaniensis[#]	5.00
	Novosphingobium panipatense	5.00
	Pantoea dispersa[#]	10.00
	Providencia rettgeri	15.00
	Pseudomonas psychrotolerans[#]	10.00

[#] Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

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4.3.3.4 The percentage of bacterial identification from Aedes albopictus collected from Yala province (Thanto district) (n=30)

The bacterial species that were identified from *Aedes albopictus* midguts collected from Yala province were high diversity and it was summarized in Table 16. A total of 18 bacterial species were identified and the dominating bacterial species from *Aedes albopictus* collected from Muang district were *Rhizobium pusense* and *Agrobacterium tumefaciens.* A total of 12 species were only found in Yala province but only three bacterial species; *Burkholderia seminalis, Candidatus Rhizobium massiliae, and Sphingomonas sanguinis* were dominant. These species were gram-negative bacterial genera. However, this area showed the bacterial species were diversities and difference more than other study areas.

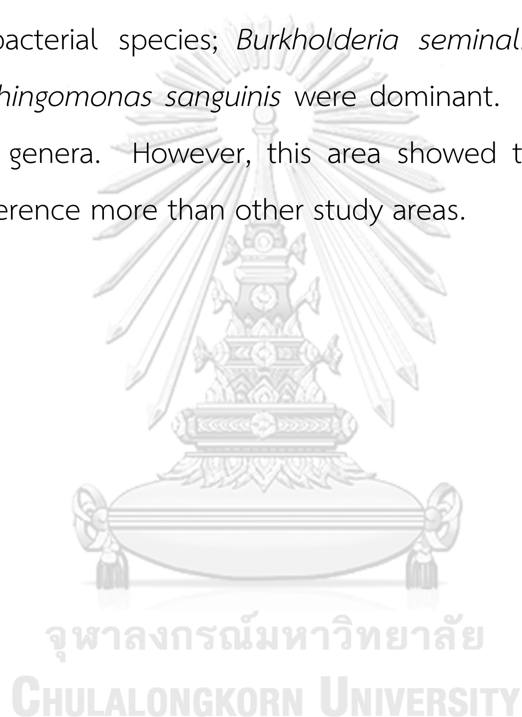

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Table: 16 The percentage of bacterial identification from *Aedes albopictus* collected from Yala province (Thanto district) (n=30)

Bacterial phylum	Closest related bacterial species**	% identification from mosquito
Actinobactira	*Brachybacterium nesterenkovii*	3.33
	Microbacterium aoyamense#	3.33
	Micrococcus luteus	3.33
	Micrococcus yunnanensis	3.33
	Nocardioides zeae#	3.33
Firmicutes	*Bacillus altitudinis*#	3.33
Proteobacteria	*Acinetobacter radioresistens*	3.33
	Agrobacterium tumefaciens	13.33
	Beijerinckia fluminensis#	3.33
	Brevundimonas aurantiaca#	3.33
	Burkholderia seminalis#	6.67
	Candidatus Rhizobium massiliae#	6.67
	Massilia timonae#	3.33
	Pectobacterium carotovorum#	3.33
	Pseudomonas oleovorans#	3.33
	Rahnella aquatilis#	3.33
	Rhizobium pusense	16.67
	Sphingomonas sanguinis#	6.67

#Representing the bacterial species that were found only in this group

* Infected mosquitoes/tested mosquitoes

** All bacterial species were identified on the basis of a % identity higher than 99%

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CHAPTER V
DISCUSSTION AND CONCLUSION

Aedes albopictus can be found throughout Thailand, particularly in rural areas. They are competent vectors for different arboviruses, including Chikungunya (CHIK), Dengue, West Nile (WN), and Zika viruses (59, 64-66). However, the study of mosquito vector competence for CHIKV in Thailand is limited. This study was, therefore, conducted to examine the vector competence of *Ae. albopictus* for CHIKV, and the effects of CHIKV titers in blood meals on virus infection, dissemination, and transmission in *Ae. albopictus*.

The CHIKV used in this study was isolated from a patient during the outbreak of this virus in Thailand in 2010, and it was propagated in the laboratory. It is in the Indian Ocean lineage (IOL) with an alanine-to-valine substitution at the position 226 of the E1 envelope glycoprotein, which is in the same lineage as the 2008 Thailand strain. The genome sequences of CHIKV isolated from the outbreak in 2008 in Thailand are related to the strains isolated from the outbreaks in 2007 in India and in 2008 in Singapore, but different from the virus isolated in 1988 and during 1995-1996 in Thailand (27).

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5.1 The vector competence of *Ae. albopictus* for CHIKV

A study of mosquito vector competence of CHIKV indicated that the mosquito species which were responsible for the current outbreak included *Ae. albopictus*, whereas *Ae. aegypti* was found to have contributed to the previous outbreak in Thailand (67). Tsetsarkin et al. (2007) (6) affirmed that *Ae. albopictus* was more likely to be the potential vector for CHIKV than *Ae. aegypti* due to the mutation of the virus. Mutation has allowed the virus to adapt to different mosquito vectors over time past. Vertical transmission in mosquitoes may contribute to the maintenance of CHIKV in

nature. For example, Chompoosri et al. (2016) (68) demonstrated that *Ae. aegypti* and *Ae. albopictus* mosquitoes from Thailand were capable of transmitting the Indian Ocean lineage of CHIKV vertically in the laboratory. They also showed that *Ae. albopictus* was more susceptible to CHIKV and had a greater ability to transmit the virus vertically than *Ae. aegypti*. However, Wong et al. (2016) (69) investigated the vertical transmission of infectious clones of CHIKV in *Ae. aegypti* from Malaysia in laboratory experiments. Eggs and adult progeny from the second gonotrophic cycles of infected parental mosquitoes were tested by RT-PCR. There was detectable CHIKV RNA in 56.3% of the pooled eggs and 10% of the adult progeny, but there was no detectable infectious virus through the plaque assay. In the present study, the blood-fed mosquitoes were examined for the presence of CHIKV in different parts of mosquitoes on day 14 post blood feeding (PBF). The percent CHIKV infections in *Ae. albopictus* were 83.3, 90, 100, 100, and 100% after being fed on 10^2, 10^3, 10^4, 10^5, and 10^6 CID_{50}/ml of CHIKV, respectively. The percent CHIKV disseminations in *Ae. albopictus* were 70.8, 86.7, 100, 90, and 98% and the percent CHIKV transmissions in *Ae. albopictus* were 41.6, 70, 100, 90, and 82.4% after blood meals with the titers of 10^2, 10^3, 10^4, 10^5, and 10^6 CID_{50}/ml of CHIKV, respectively. Further studies need to be performed of the virus infection, dissemination, and transmission in *Ae. albopictus* after taking blood meals with virus titers less than 10^2 CID50/ml of CHIKV in order to establish the minimum infectious dose of CHIKV in this mosquito. Low CHIKV titers can usually be found in infected animals in nature and laboratory animals.

The percent virus infection, dissemination, and transmission were lowest and highest after being fed on 10^2 and 10^4 CID^{50}/ml of CHIKV, respectively. However, there was no significant difference among the percent infections after being fed on 10^4, 10^5, and 10^6 CID_{50}/ml and there was no significant difference between the percent transmissions after being fed on 10^4 and 10^5 CID_{50}/ml of CHIKV. The lowest percent transmission was 41.6% and the highest percent transmission was 100% after being fed on 10^2 and 10^4 CID_{50}/ml of CHIKV, respectively. The high virus titer in mosquito blood meal might. In fact, cause high mortality in the blood-fed mosquitoes and affect the

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average percent transmission. The difference in mosquito intrinsic factors among each mosquito might also affect the virus infection, dissemination, and transmission. The present study indicated that the CHIKV transmission by infected *Ae. albopictus* occurred after blood meals with a titer of 10^2 CID_{50}/ml, which is the titer that can be found in human and various animals. *Ae. albopictus* is susceptible to CHIKV infection and is an efficient vector for CHIKV transmission. Also, CHIKV titers in blood meals have effects on virus infection, dissemination, and transmission in *Ae. albopictus*. These mosquitoes play important roles in the ecology of CHIKV, therefore mosquito control must be of great concern during the outbreak of this virus. The blood-fed mosquitoes were tested on day 14 PBF because it is the optimal day for examination of virus infection, dissemination, and transmission in mosquito vectors as described in other studies (59, 66, 70, 71). CHIKV susceptibility varies by virus strain, and mosquito species and strain. The Asian strain of CHIKV starts to replicate at 5-6 days post infection (DPI) with the maximum virus yield at 5-10 DPI in both *Ae. aegypti* and *Ae. albopictus*. The variant Central/East/South African (CESA) virus genotype replicates earlier at 1 DPI with the maximum virus yield at 3-6 DPI in *Ae. albopictus* females while the nonvariant virus strain replicates at 1-2 DPI with the maximum virus yield at 6-12 DPI. In *Ae. aegypti*, these viruses replicate at 1-2 DPI, with maximum yields at 4-5 DPI (72). In this study, the lowest virus titer in the blood meal was 10^2 CID_{50}/ml and the percent infection was found to be 83.3%, which is very high when compared with the percent infection of WNV in *Ae. Albopictus* (66). The percent WNV infections in *Ae. albopictus* were 0, 0, 89, 98, 93, 91, and 90% after being fed on $10^{2.5}$, 10^5, 10^7, $10^{7.5}$, 10^8, $10^{8.5}$, and $10^{9.5}$ CID_{50}/ml of WNV, respectively. Even though the percent CHIKV infection was 83.3%, the percent dissemination and transmission were 70.8% and 41.6%, respectively. These findings indicate that there was some degree of virus barrier in the mosquitoes that was acting as an infection, dissemination, and transmission barriers. These barriers were involved in the replication of the virus in the mosquitoes as indicated in other previous studies (13, 58, 73). The present study suggests that *Ae.*

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albopictus is susceptible to CHIKV infection and is an efficient vector for CHIKV transmission. CHIKV titers in blood meals also affect virus infection, dissemination, and transmission in *Ae. albopictus* or vector competence of this mosquito. The information in this study will be useful for the understanding of the ecology of CHIKV in nature in Thailand and also for disease surveillance, vector control, and prevention of CHIKV outbreak in Thailand.

5.2 The presence of midgut microbiota in CHIKV infected mosquitoes

Previous studies have revealed that the colonized microbiota in the gut of mosquitoes influence the mosquito's susceptibility to arboviruses and parasites (17-20). Although, it is the epidemiological importance in diseases transmission, very limited studies are available on *Ae. albopictus* with respect to the identification of gut microbiota and their interaction with CHIKV infection. The study of the correlation of midgut microbiota of *Ae. albopictus* and CHIKV infection are less well known in Thailand.

There are reports suggesting that the microbiota midgut of mosquitoes have an impact on vector control. There is also increasing evidence that interactions occur between resident or introduced microbial taxa in arthropods and invading pathogens (19, 74-76). Previous studies using culturing and denaturing gel electrophoresis methods have found that Proteobacteria and Firmicutes were the dominated bacterial communities associated with *Ae. albopictus* from the Indian Ocean, and the bacterial diversity and composition were influenced by the environment inhabited by the mosquitoes (77, 78). Using a taxonomic microarray that targeted more diverse bacterial taxa, showed the bacterial community in ALPROV strain of *Ae. albopictus* which originating from La Reunion island was more diverse than previously described and the various endosymbionts could interact with each other and with CHIKV within the host (77).

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Nevertheless, influence of midgut microbiota remains poorly investigated from CHIKV infected mosquitoes with varying doses of CHIKV infection. Therefore, in this study, an assessment of the correlation of midgut microbiota with *Ae. albopictus* infected with CHIKV in Thailand. It was found that there are changes in the community of bacterial phylum between CHIKV infected and non-infected *Ae. albopictus*. Herein, we propose that the data for the correlation of the midgut microbiota between CHIKV infected and non-infected *Ae. Albopictus*.

The midgut microbiota was diverse in each mosquito groups, however, there was no correlation between midgut microbiota and CHIKV infection in *Ae. albopictus*. Some different bacterial species was found only one group. For instance, *Agrococcus terreus* and *Bacillus amyloloquefaciens* were isolated only from mosquito group that fed on negative blood meal. These bacterial species have been isolated from various environments including soil samples (79), potato plants, and dried seaweed, as well as from the air (80). These bacterial might be found in the food that were fed by the larval stage or in the sheep blood that were fed by the adult mosquitoes.

For the mosquito group that were fed on 10^6 CID_{50}/ml CHIKV, the bacterial species that were isolated only from this group were *Actinomyces naeslundii, Brachybacterium paraconglomeratum, Micrococcus aloeverae, Streptomyces psuedogriseolus, Bacillus megaterium* and *Paenibacillus timonensis*. These bacterial species have been isolated from a variety of sources including soil, fresh and salt water, food, plants, and insect larvae (81, 82). These bacteria might be found in the food that were fed by the larval stage and might be the effect of high dose of CHIKV infection in mosquito midgut because these bacteria were not isolated from the non-infected mosquitoes except *Streptomyces psuedogriseolus* that was isolated from both infected and non-infected mosquitoes.

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While *Corynebacterium ihumii* was only one bacterial species that were isolated from the mosquito group that were fed on 10^5 CID_{50}/ml CHIKV. This bacterial species has been isolated from human colon and hospital environment (83). Beside that, *Brevibacterium casei, Bacillus aquimaris, Bacillus clausii, Paenibacillus lautus,* and *Brevundiminas diminuta* were the bacterial species that were isolated only from the mosquitoes that were fed on the blood meal with 10^4 CID_{50}/ml CHIKV. These bacterial species might induce the CHIKV infection in mosquito because the percent infection was 100% in this group. In addition, these bacteria can also be found in raw milk, human skin, and animal source (84). In addition, *Bacillus aquimaris* has been isolated from marine environments and recently isolated from sea water of a tidal flat of the Yellow Sea in Korea (85). Normally, these bacteria should not be found in the mosquito midgut in nature. For further studies, the bacterial contamination in larval food and sheep blood for adult mosquitoes should be investigated.

Moreover, *Corynebacterium aurimucosum, Streptomyces griseoauranticus, Bacillus circulans, Bacillus methylotrophicus, Bacillus tiansheni, Staphylococcus saprophyticus,* and *Acinetobacter indicus* are bacterial species which were isolated only from the mosquitoes that were fed on 10^3 CID_{50}/ml CHIKV. Almost bacteria were isolated from infected CHIKV mosquitoes except *Streptomyces griseoauranticus* and *Staphylococcus saprophyticus* which were isolated from non-infected mosquitoes. These bacterial species have also been isolated from water, soil marine environments, air, and dump site (86-90).

In addition, we found that the bacterial species, *Brevibacterium saguinis, Corynebacterium jeikeium, Corynebacterium pilbarense, Kocuria marina, Psychrobacter pulmonis,* and *Enhydrobacter aerosaccus*. were isolated only from the mosquito group that was fed on 10^2 CID_{50}/ml CHIKV. The most of these bacterial species have been isolated from a patient including HIV, sepsis, nosocomial infection, and skin colonization or superficial infections (91-94). Besides, *Kocuria marina* has

been isolated from marine environments (95), and this bacterial species was isolated from non-infected CHIKV mosquito in this group. The results could not be indicated that there were specific bacterial species affecting to CHIKV infection in mosquitoes. Furthermore, the factor that involved in midgut microbiota should be studies such as the bacteria in larval food and blood meal for adult mosquitoes in the laboratory.

However, we were unable to relate the correlation between CHIKV infection and microbiota midgut that were fed on the blood meal with 10^4 CID_{50}/ml CHIKV because there was no non-infected mosquito in this group. In the group which was fed a dose of CHIKV 10^6 CID_{50}/ml, we found that the bacteria diversity in the mosquito midgut was dominated by the bacterial genera *Micrococcus* spp., members of the Actinobacteria phylum. However, we were unable to relate the correlation between CHIKV infection and microbiota midgut because the bacteria were not cultured and isolated non-infected mosquitoes. These results suggest that the high CHIKV titer may have an impact on bacterial isolation because we also cultured and isolated bacteria in the mosquito midgut where the CHIKV titer was low. Overall the Actinobacteria phylum was dominant phyla in this group. The group which was fed CHIKV 10^5 CID_{50}/ml, it was apparent that there was bacterial diversity in both infected and non-infected mosquito. *Micrococcus* spp. was the dominant bacterium in infected mosquitoes and *Staphylococcus* spp. was dominant in non-infected mosquitoes. But the correlation between CHIKV infection and the microbiota in the midgut is not significant in this group. Overall the dominant phyla was Actinobacteria whilst Firmicutes is the dominant phyla in the mosquitoes that were fed on 10^4 CID $_{50}$/ml CHIKV. In group that was fed CHIKV 10^3 CID_{50}/ it was found that *Micrococcus* spp. was dominant in infected mosquito and that *Staphylococcus* spp. was the dominant bacteria genera in non-infected mosquitoes. However, Actinobacteria was also the dominant phyla in both infected and non-infected mosquitoes.

In addition, the low CHIKV titer group of mosquitoes, 10^2 CID $_{50}$/ml, the bacteria isolated were dominated by *Micrococcus* spp. It was observed that the bacterial genera, *Micrococcus* were significantly different between infected and non-infected mosquitoes. This result indicated that *Micrococcus* may correlate with CHIKV infection and which may induce susceptibility of *Ae. albopictus* to CHIKV infection. Although there is no research that shows the effect of *Micrococcus* on inducing virus and parasite infection, a recent study shown that *Micrococcus* can produce the protein that contributes to antibiotic tolerance, reemergence from latent infections, and even quorum sensing and biofilm formation (96). However, others bacterial genera may also induce susceptibility of *Ae. albopictus*. The previous studies found that a core bacterial community in *Ae. albopictus* was not either by infection or by the bloodmeal and these was mostly represented by Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Acidobacteria. The presence of core taxa has been noted previously in other environments, and it may function to stabilize the community. Zouache and colleague (2012) (52)) indicated that the midgut microbiota found in CHIKV infected *Ae. aegypti* were Beta- and Gammaproteobacteria. Here, we classified the bacterial genera that were identified to the three phyla Actinobacteria, Firmicutes, and Proteobacteria. It is shown in figure 11 that Actinobacteria dominates communities in the midgut microbiota of *Ae. albopictus* when infected with CHIKV. The Actinobacteria phyla may correlate to CHIKV infection when they dominate in the midgut of infected mosquitoes. The CHIKV infection induced a change in the composition of the bacterial community, but not in its structure, however, the density of the bacteria changed slightly with ageing of mosquitoes, and this was probably results of modified nutritional conditions (97).

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In this study, the bacterial community in *Ae. albopictus* can be modified after being infected with CHIKV and the bacterial phyla found in the present study were Actinobacteria, Firmicutes, and Proteobacteria. The bacterial identification was limited by the TSA agar-based aerobic culturing method used in this study. The TSA agar culturing method has some limitations in providing the complete composition of the mosquito midgut microbiota since a large fraction of bacteria are likely to be unculturable, similar to the human intestinal microbiota (49). We require easily propagated dominant bacterial species for further investigation. Although no function can be assigned to the bacterial communities identified through conventional the RT-PCR methodology, it is apparent that these bacterial genera inhabit diverse environments and some are even known to establish facultative or mutualistic symbioses with insects (98).

It is not clear whether CHIKV infection affects distinct bacterial genera or whether there is a parallel effect resulting from the *Ae. albopictus* innate immune response to fight viral infection (99-103). Previous research shown that virus infection affects the composition and dynamics of the total bacterial community in mosquitoes. Nowadays, there is no available treatment or vaccine is for most arboviruses including CHIKV. Previous studies have shown that *Wolbachia* is a candidate that limit the transmission and spread of arboviruses using symbiosis-based control (reviewed in (50)). However, *Wolbachia* are found to be present in some *Ae. albopictus* reproductive organs and not in the gut tissue (reviewed in (104)). Although many studies have reported that the native *Wolbachia* from *Ae. albopictus* was associated with a decrease of DENV transmission in the mosquitoes from La Reunion island, no significant impact of *Wolbachia* was observed in CHIKV transmission (35, 105, 106). Moreover, there are many reports that have suggested that other bacteria could also be candidates. For instance, the *Enterobacter* Esp_Z isolate was shown to produce reactive oxygen species (ROS) that inhibited the malaria parasite (75). A specific strain

of *Serratia* that has enhanced motility suppresses *Plasmodium* compared to a non-motile strain. These instances provide an insight into the mechanisms behind the interference of the phenotype and highlights the importance of bacterial inter-strain variation on vector competence (107). In other studies, *Enterobacter*, *Proteus* and *Paenibacillus* have been shown to inhibit La Crosse virus (LACV) and DENV (48, 108). Ramirez and colleague (2014) (109) also found that *Chromobacterium* isolates had both anti-*Plasmodium* and anti-viral properties and reduced the survival of larvae and adult mosquitoes. The mechanism for these effects was possibly linked to the secretion of metabolites such as cyanide.

In addition to studies on arboviruses and malaria, bacterial microbes can alter pathogens in other vector species. *Serratia*, which is a dominant component of the gut microbiome of Triatomine bugs, appears to be an important determinant of *Trypanosome* infection (110, 111). The trypanocidal activity of *Serratia* could be related to prodigiosin production, which affects the mitochondrial activity of the parasite, and the ability of this bacterium to attach to the parasite (112, 113). Studies in sandflies also imply that microbes reduce the *Leishmania* parasite load (114) whilst tsetse flies that were cured of their symbionts were more susceptible to *Trypanosome* infection (115).

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Pathogen enhancement mediated by microbes has also been documented in mosquitoes. The midgut microbiota were suppressed by antibiotic treatment in *Anopheles* mosquitoes decreased O'nyong nyong virus (ONNV) infections (116), indicating that the constituents of the microbiota are required for pathogenic infection. Re-infection of live, but not heat-killed bacteria, into antibiotic treated mosquitoes degenerated viral titers to levels comparable to untreated controls (116). These effects are in contrast to what is observed with *Plasmodium* where there is an increase

in titer after mosquitoes were treated antibiotic (117-119). A similar pathogen enhancement effect was also seen in *Ae. aegypti* re-infected with *Serratia odorifera,* which increases both DENV and CHIKV infections (49, 120). The ability of bacterial taxa to both enhance and suppress pathogens in insects suggests complex interplay between the host, the microbiome and the pathogen, that dictates vector competence. Furthermore, specific vector-pathogen-microbe combinations may have unique outcomes, which means intervention strategies need to be understood thoroughly before implementation.

This was the first study to show that varying the dose of virus infection affects the bacterial isolation and identification in mosquito midgut. Our results suggested that other bacteria could also be candidates. While there was increasing evidence for both positive and negative effects of natural or introduced bacteria on virus infection and transmission (19, 108, 117). However, this study has shown the importance of considering the whole microbial community and their mutual interactions, in order to better appreciate and understand the phenomenon of interference in determining ultimate vector competence (52).

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5.3 The presence of midgut microbiota in filed-collected mosquitoes

Previous studies reported that the midgut bacteria of mosquitoes play a significant role in modulating overall vector competence (117, 120). This work was carried out to study the diversity of midgut bacteria of laboratory-reared *Ae. albopictus* and field-collected *Ae. albopictus* from the Sigha Buri, Chumphon and Yala provinces in Thailand. The mosquitoes were cultured and the microbiota in mosquito midgut were identified in the laboratory-reared and also in the field-collected *Ae. albopictus*. In addition, this study was based on the 16S rRNA gene for identification using the two

*Ae. albopictu*s line (Laboratory-reared and field-collected), so that variation was influenced only by the host food source since control was lacking in field-collected *Ae. albopictus*. In this study, the focus was on the characterization of culture-dependent aerobic bacteria from the midgut of both strains of *Ae. albopictus*, because only culturable bacteria can be used for further applications in the management of disease transmission such as paratrangenesis.

Consideration of phyla in field-collected *Ae. albopictus* revealed the Proteobacteria is the dominant phyla, while the Actinobacteria is the dominant phyla in laboratory-reared *Ae. albopictus*. The Proteobacteria in field-collected mosquitoes may be result of the source of food or environment in which they inhibit and this may be a determinant in the differences found in dominant phyla. Although, the field-collected *Ae. albopictus* have shown high variation of midgut microbiota, in the laboratory *Ae. Albopictus* shown it was found that *Staphylococcuc* was the dominant genera and were significantly different when compared with field-collected *Ae. albopictus*. Although the effect of bacterial *Staphylococcus* genera on inducing viral and parasitic infection was not identified in this study these organisms have a propensity to form biofilms, which aid in surface colonization and provide enhanced tolerance to antibiotics (90). This limitation is potentially significant because the source food may affect the interactions within the bacterial community that is undergoing change. This line of reasoning is directly applicable to the variation of the bacterial genera that were identified in the wild or field-collected *Ae. albopictus*.

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A total of 31 different bacterial genera were identified by a 16S rRNA gene sequence analysis for both strains of *Aedes* mosquitoes. Most of the bacterial genera from the midgut of *Aedes* as well as other mosquito species had already been reported. The bacterial genera of *Enterobacter, Bacillus, Pseudomonas, Staphylococcus, Klebsiella, Pantoea, Acinetobacter,* and *Aeromonas* from the midguts of mosquitoes have been reported by others and the results of the present study corroborate these (121-124). It was apparent from the results, of this study, that in

both the mosquito strains the main bacterial species belonged to the phylum Proteobacteria. It has been reported that, bacteria in the mosquito' s midguts are primarily acquired either through vertical inheritance or acquisition from the environment (125). The bacterial genera such as *Acinotobacter, Agrobacterium, Klebsiella, Microbacterium, Micrococcus, Pseudomonas,* and *Staphylococcus* have also been isolated from the midgut of both laboratory-reared and field-collected *Ae. albopictus*. Other species present in the midgut, but of very low prevalence, were also isolated and identified from both the laboratory-reared or field-collected *Ae. albopictus*. For instance, *Enhydrobacter, Leucobacter,* and *Pandoraea* were only present in the laboratory-reared *Ae. albopictus* whereas, *Actinomyces, Bacillus, Beijerinckia, Brachybacterium, Brevundimonas, Burkholderia, Candidatus Rhizobium, Chryseobacterium, Enterobacter, Erwinia, Massillia, Moraxella, Nocardioides, Novosphingobium, Pantoea, Pectobacterium, Providencia, Rahnella, Rhizobium, Serratia,* and *Sphingomonas* were exclusively isolated from the field-collected *Ae. albopictus*.

In addition, the presence of *Microbacterium yannicii* was observed in the midgut of the field-collected *Ae. albopictus* for the first time. Earlier, this bacterial species was isolated and identified from arabidopsis thaliana root (126). The presence of *Bacillus kochii* was also not isolated from *Aedes* mosquitoes but it had been reported earlier in the gut of *Drosophila melanogaster* (reviewed in (127)). *Brachybacterium nesterenkovii*, is proposed for a group of coryneform bacteria that were have been isolated from various milk products. Also, *Bacillus kochii*, *Bacillus pocheonensis, Acinetobacter lwoffii*, *Nocardioides zeae, Beijerinckia fluminensis*, *Brevundimonas aurantiaca*, and *Burkholderia seminalis* were previously isolated from the soil, plant, and normal flora, but none of these have been observed in the midgut of mosquitoes up to the present time (reviewed in (128-132)).

Besides the bacterial species, *Serratia marcescens* was isolated from filed-collected mosquitoes (Sighha Buri province), this bacterial species has been found in

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food, particularly in starchy variants which provide an excellent growth environment. It is an important cause of nosocomial infection (133). For the bacterial species that isolated only in Muang district, Chumphon province, were *Microbacterium yannicii, Enterobacter cancerogenus, Enterobacter cloacae, Enterobacter hormaechei, Enterobacter mori, Klebsiella quasipnuemoniae,* and *Klebsiella variicola*. Which the most bacterial species are part of the normal flora of the gastrointestinal tract of 40 to 80% of people and are widely distributed in the environments (134-139). In the Thung Tago district, Chumphon province, the bacterial species that isolated only in this group were *Actinomyces oris, Bacillus kochii, Bacillus pocheonensis, Acinetobacter lwoffii, Chryseobacterium taklimakanense, Erwinia tasmaniensis, Pantoea dispersa,* and *Psuedomonas psychrotolerans*. These bacterial species have been isolated from soil, plants, flowers, water, clinical environments, and normal flora (128, 132, 140-144).

Moreover, the bacterial species that isolated only from Yala province mosquitoes were *Microbacterium aoyamense, Nocardioides zeae, Bacillus altitudinis, Beijerinckia fluminensis, Brevundimonas aurantiaca, Burkholderia seminalis, Candidatus Rhizobium massiliae, Massilia timonae, Pectobacterium carotovorum, Psuedomonas oleovorans, Rahnella aquatilis,* and *Sphingomonas sanguinis.* These bacterial species have been isolated from forest soil, plants, rice seed, plant roots, fresh water, environments and the upper atmosphere (130, 145-152). Interestingly, *Massilia timonae* is an environmental organism, which it could be coinfected with malaria affects to patient have been high fever (153). These results might be the effects of food which mosquitoes fed including to the host blood that mosquitoes bitten.

In the present study, it was found that *Acinetobacter*, *Agrobacterium Klebsiella*, *Microbacterium*, *Micrococcus, Pseudomonas*, and *Staphylococcus* were the dominant genera in both laboratory-reared and field-collected *Ae. albopictus*. While, *Enhydrobacter*, *Leucobacter,* and *Pantoea* were the dominating genera in laboratory-

reared *Ae. albopictus*. In the bacterial species that was isolated from the field-collected *Ae. albopictus* that were collected from the Chumphon provinc, *Enterobacter cloacae* was the most common bacterial species. This finding is significant in that previous studies have shown that this species of bacteria has been found to block the development of *Plasmodium falciparum* in *Anopheles gambiae* and sporogonic development of *Plasmodium vivax* in *An. Albimanus* (75), such as to induce the expression of mosquito immune components in the midgut of *An. stephensi* (154). Moreover, *E. cloacae* has also been found to inhabit the midgut of the sand fly *Phlebotomus papatasi* and has potential application in the paratransgenic approach to reduce the transmission of *Leishmania* has been suggested recently (155). Apart from these potential applications, *E. cloacae* have also been successfully used to deliver, express, and spread foreign genes in termite colonies (156). *E. cloacae* transformed with an ice nucleation (IN) gene have also been shown to be useful for the reduction of the mulberry pyralid moth, *Glyphodes pyloalis* (156). Considering these findings, direct application of *E. cloacae* for pathogen reduction, through the paratransgenic approach, appears to have potential as a powerful strategy towards the effective management of vector-borne diseases (157).

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The bacterial genera *Serratia* and *Enterobacter* produce hemolytic enzymes that might take part in the digestion of blood in hematophagous Diptera (46, 158). Another important bacterium was *Acinetobacter* which obtained from the *Ae. albopictus* in this study are also known to be involved in blood digestion. Minard and colleagues reported that *Acinetobacter baumannii* and *A. johnsonii* isolated from *Ae. albopictus* may play a role in the absorption of nectar and in blood digestion (159).

In the recent years, it has reported that some midgut inhabiting bacteria play an important role in disease transmission, host-parasites interaction, and also affects the vector competence of mosquitoes. The midgut serves as the first contact point between parasites and the epithelial surfaces, where significant parasite numbers are reduced (19). The microbiota involved in the blocking of the *Plasmodium*

development may be used in the modulation of vector competence of mosquitoes (117). Midgut microbiota are known to augment the immune response of the mosquito (17, 117, 160). Whereas immunocompetent mosquitoes are less likely to transmit other parasites such as malaria (161), it might be that a related strategy might also be helpful in dengue control through the use of bacterial species that augment the mosquito immune system.

The midgut microbiota composition had an important role on the susceptibility of chikungunya and dengue viruses. It has been shown that the susceptibility of *Ae. aegypti* to chikungunya and dengue virus increases in the presence of *Serratia odorifera* due to the suppression of the immune response of *Ae. Aegypti* (49, 120). It has also been reported that *Ae. aegypti* were more susceptible to DENV-2 when fed with the *Aeromonas* spp. and *Escherichia coli* (120).

From the above studies, it was clear that the midgut bacteria can be significantly involved in host-parasite interactions and may decrease or increase the vector competence through various mechanisms including enhancement of the immune response or by impeding the development of parasites. Midgut microbiota may be genetically manipulated to express molecules against the infecting parasites, which could be used as a novel strategy for vector control. The understanding of midgut microbiota in mosquitoes could be used for the development of novel, cost effective, eco-friendly and a highly effective defense mechanism in order to reduce the vector competence of mosquitoes and therefore on disease transmission control.

Conclusion

To the best of our knowledge, this is the first study in which an attempt has been made towards a comprehensive study and understanding of the correlates of varying doses of CHIKV infection and how these effects the bacterial communities found in the midgut of *Ae. albopictus*, and what differences might arise between

laboratory-reared and field-collected *Ae. Albopictus*. The involvement of the midgut bacteria in the defense mechanism of the vector has been reported previuosly, but the information is very limited. *Enterobacter* was found to be the common culturable midgut bacteria in the field-collected *Ae. albopictus* and previously reported data supports its involvement in *P. falciparum* development blockage and blood digestion. While the *Micrococcus* was found to be the dominant culturable midgut bacteria genera in the infected CHIKV *Ae. albopictus*. However, this study did not no report on its involvement in the insect but detailed their properties in particular in producing proteins for antibiotic tolerance, re-emergence from latent infections, and even quorum sensing and biofilm formation, that may induce susceptibility to CHIKV in *Ae. albopictus*. While the dominant bacterial *Staphylococcus* genera in the laboratory-reared *Ae. albopictus,* have shown a propensity to form biofilms, which aid in surface colonization and provide enhanced tolerance to antibiotics, which may also act differently when compared with the filed-collected *Ae. albopictus*. Other important bacterial genera such as *Acinetobacter* were also identified from *Ae. albopictus* and these are known to take part in the blood digestion of mosquitoes. A comprehensive understanding of the role of the midgut bacteria may leads towards a better understanding of the direct or indirect involvement of microbiota in the immune response, and the nutrition, and reproduction of mosquitoes, which may. In the end, be of significant help in improving upon current vector control strategies.

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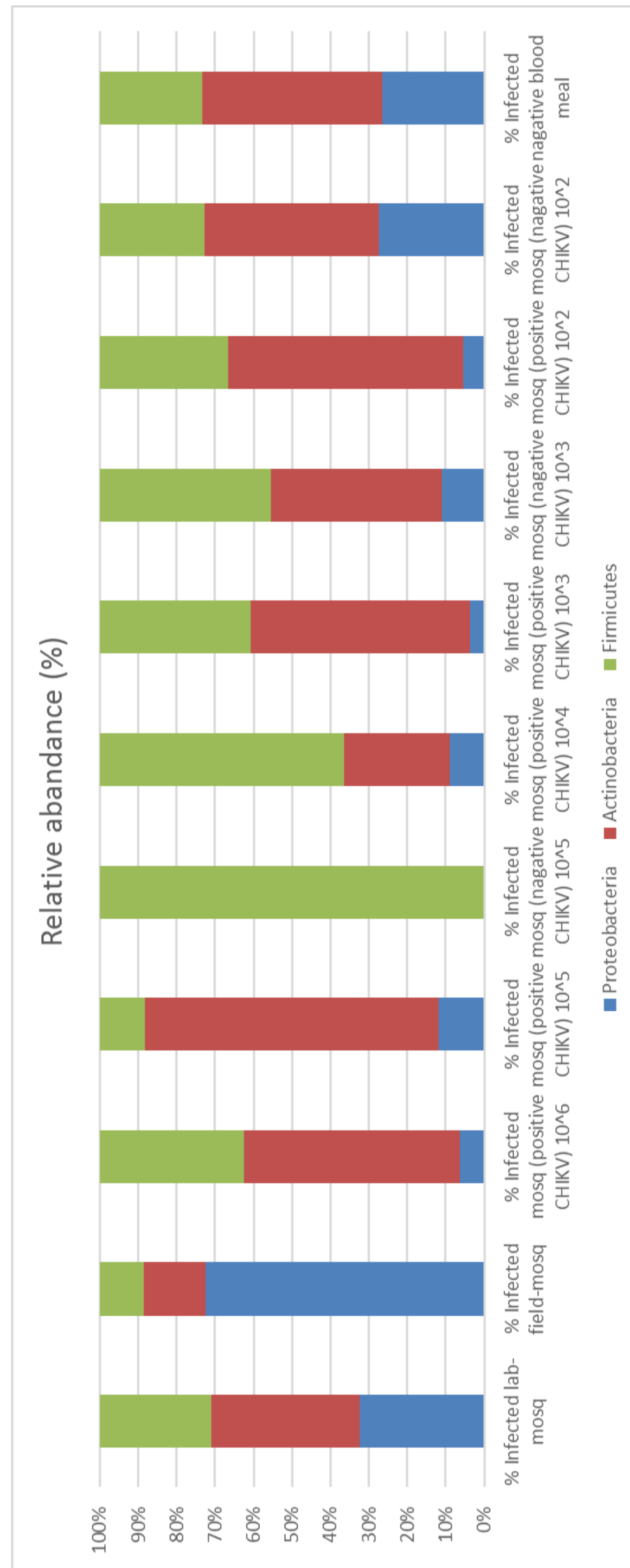

Figure: 11 Percent Relative abandance of Bacterial Phylum

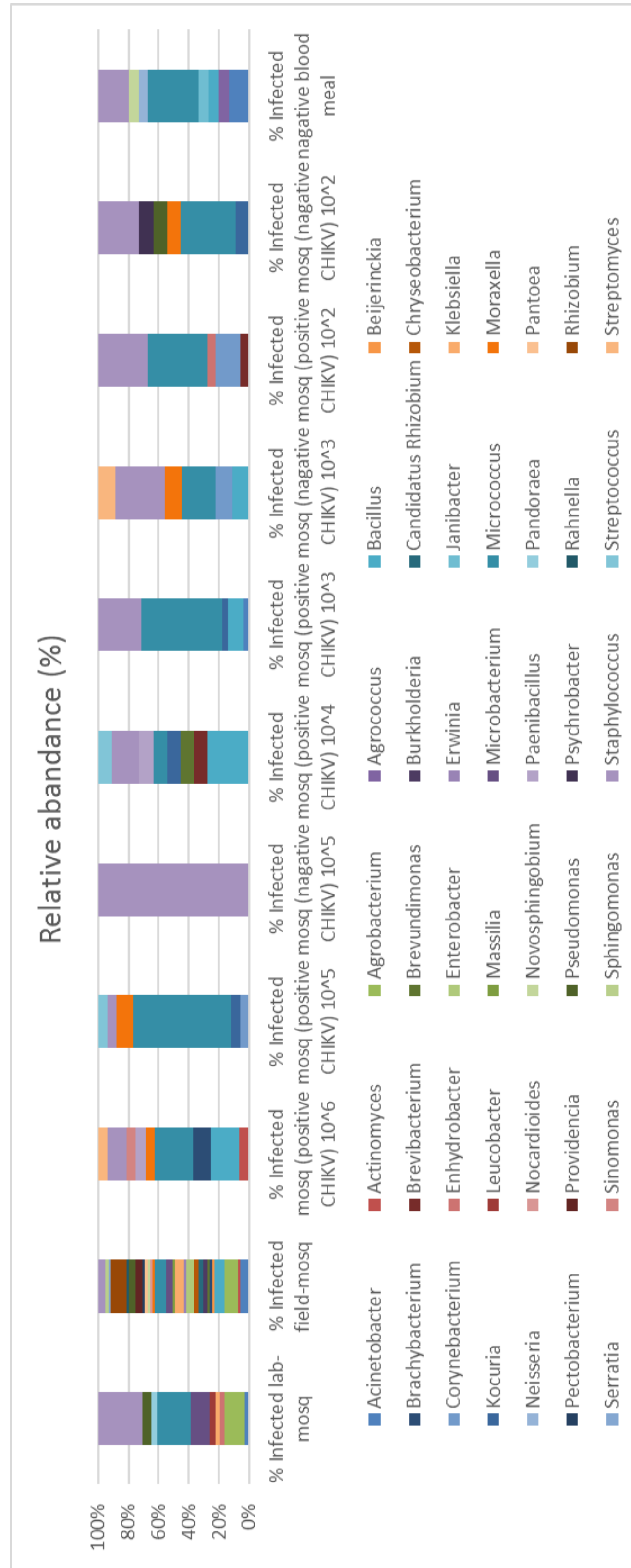

Figure : 12 Percent relative abandance of Bacterial Genus

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จุฬาลงกรณ์มหาวิทยาลัย
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APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
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The details of isolated midgut microbiota from laboratory-reared mosquitoes

Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria sequence
No.1	92	-	+	*Pseudomonas luteola*
	130	+	+	*Staphylococcus epidermidis*
	12	+	+	*Leucobacter chironomi*
No.2	86	+	+	*Micrococcus luteus*
	32	+	+	*Micrococcus yunnanensis*
No.3	292	(+/-)	+	*Pandoraea sputorum*
	14	-	+	*Klebsiella pneumoniae*
No.4	38	+	+	*Micrococcus luteus*
	12	+	+	*Micrococcus yunnanensis*
No.5	16	+	+	*Micrococcus luteus*
	10	-	+	*Enhydrobacter aerosaccus*
No.6	24	+	+	*Micrococcus luteus*
No.7	16	-	+	*Pseudomonas aeruginosa*
	10	+	+	*Microbacterium dextranolyticum*
No.8	14	(+/-)	+	*Acinetobacter variabilis*
	2	+	+	*Microbacterium laevaniformans*
No.9	12	+	+	*Staphylococcus pasteuri*
	4	+	+	*Microbacterium dextranolyticum*
No.10	-		-	-
No.3 (25-9-14)	18	(+/-)	+	*Staphylococcus arlettae*
No.5 (25-9-14)	33	+	+	*Staphylococcus epidermidis*
No.6 (25-9-14)	6	+	+	*Staphylococcus epidermidis*
No.11	-		-	-
No.12	36	-	+	*Agrobacterium tumefaciens*
	57	+	+	*Micrococcus luteus*
	3	+	+	*Staphylococcus warneri*
No.13	57		-	-
	717		-	-
No.14	21		-	-
No.15	-		-	-

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria sequence
No.16	9	+	+	*Staphylococcus epidermidis*
	3	+	+	*Staphylococcus arlettae*
No.17	-		-	-
No.18	-		-	-
No.19	-		-	-
No.20	-		-	-
No.21	-		-	-
No.22	621	+	+	*Microbacterium dextranolyticum*
No.23	6	-	+	*Agrobacterium tumefaciens*
No.24	333		-	-
	60		-	-
No.25	-		-	-
No.26	234		-	-
	66		-	-
No.27	189		-	-
	93		-	-
	63		-	-
No.28	180	+	+	*Staphylococcus warneri*
	3		-	
No.29	12	-	+	*Agrobacterium tumefaciens*
	66		-	
No.30	258	-	+	*Agrobacterium tumefaciens*
	513		-	
	3		-	
				31 sample strains

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The details of isolated midgut microbiota from Sigha Buri Province

Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria Sequence
No.1	3	+	+	*Bacillus subtilis*
	3		-	-
	3	-	+	*Serratia marcescens*
	6		-	-
No.2	-		-	-
No.3	-		-	-
No.4	66	+	-	*Staphylococcus hominis*
No.5				
No.6	294		-	-
	249		-	-
	3		-	-
No.7	-		-	-
No.8	-		-	-
No.9	-		-	-
No.10	3	+	+	*Staphylococcus haemolyticus*
				4 sample strains

The details of isolated midgut microbiota from Meang district Chumphon Province

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria Sequence
No.1	6	-	+	*Klebsiella pneumoniae*
	15		-	-
	120		-	-
	114		-	-
	12		-	-
No.2	3	-	+	*Enterobacter cloacae*
	3	-	+	*Enterobacter hormaechei*
	87		-	-
No.3	>>>+		-	-
	>>>+		-	-

Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria Sequence
No.4	15		-	-
	12		-	-
	438		-	-
	756		-	-
	12		-	-
No.5	246		-	-
	18		-	-
	3		-	-
No.6	3	-	+	*Enterobacter mori*
	15	+	+	*Microbacterium yannicii*
No.7	156		-	
	18	-	+	*Klebsiella variicola*
	3	-	+	*Klebsiella pneumoniae*
No.8	3	-	+	*Agrobacterium tumefaciens*
No.9	-			
No.10	3			
	12	-	+	*Rhizobium pusense*
No.11	279	-	+	*Enterobacter cancerogenus*
	273		-	- *Enterobacter cloacae*
	3,336		-	-
No.12	51		-	-
	9		-	-
No.13	3		-	-
	96		-	-
	81	-	+	*Rhizobium pusense*
No.14	-		-	-
No.15	-		-	-
No.16	-		-	-
No.17	3		-	-
	2,391		-	-
No.18	30	(+/-)	+	*Klebsiella quasipneumoniae*
No.19	18		-	- *Enterobacter cloacae*

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria Sequence
No.20	9	(+/-)	+	*Moraxella osloensis*
				13 sample strains

The details of isolated midgut microbiota from Suan Nai Dum, Thung Tago District, Chumphon Province

Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria Sequence
No.1	3	(+/-)	+	*Acinetobacter variabilis*
	3	-	+	*Chryseobacterium taklimakanense*
	9	-	+	*Providencia rettgeri*
No.2	12	-	+	*Pantoea dispersa*
No.3	3	-	+	*Providencia rettgeri*
	3	(+/-)	+	*Acinetobacter variabilis*
	6	+	+	*Micrococcus luteus*
No.4	3	-	+	*Pantoea dispersa*
	3	-	+	*Agrobacterium tumefaciens*
No.5	3	+	+	*Bacillus kochii*
No.6	12	-	+	*Chryseobacterium taklimakanense*
	135		-	-
	228		-	-
No.7	-		-	-
No.8	-		-	-
No.9	3	+	+	*Microbacterium dextranolyticum*
No.10	-		-	-
No.11	12		-	-
No.12	3	-	+	*Acinetobacter lwoffii*
No.13	3	-	+	*Erwinia tasmaniensis*
	9	(+/-)	+	*Pseudomonas psychrotolerans*
No.14	3	+	+	*Staphylococcus epidermidis*
	6	+	+	*Bacillus kochii*
No.15	3	+	+	*Micrococcus luteus*
	9	+	+	*Bacillus pocheonensis*

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria Sequence
No.16	6	(+/-)	+	*Pseudomonas psychrotolerans*
No.17	-		-	-
No.18	3		-	-
	24	+	+	*Actinomyces oris*
	24		-	-
No.19	15	-	+	*Providencia rettgeri*
	15	+	+	*Micrococcus luteus*
No.20	3	(+/-)	+	*Novosphingobium panipatense*
				24 sample strains

The details of isolated midgut microbiota from Tarn To Dictrict and Meang Dictrict, Yala Province

Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria Sequence
No.1	15	-	+	*Rahnella aquatilis*
	546	(+/-)	+	*Pectobacterium carotovorum*
No.2			-	
No.3	3	-	+	*Agrobacterium tumefaciens*
	3	(+/-)	+	*Sphingomonas sanguinis*
No.4	6	-	+	*Agrobacterium tumefaciens*
	9	+	+	*Brachybacterium nesterenkovii*
No.5	3	-	+	*Rhizobium pusense*
No.6	-		-	
No.7	3	-	+	*Rhizobium pusense*
	6	-	+	*Pseudomonas oleovorans*
	9	+	+	*Microbacterium aoyamense*
No.8	-		-	
No.9	3	(+/-)	+	*Burkholderia seminalis*
No.10	3	(+/-)	+	*Brevundimonas aurantiaca*
No.11	3	+	+	*Bacillus altitudinis*
No.12	-		-	
No.13	9	-	+	*Candidatus Rhizobium massiliae*

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria Sequence
No.14	15	-	+	*Rhizobium pusense*
No.15	3	-	+	*Agrobacterium tumefaciens*
No.16	-		-	
No.17	3	-	+	*Rhizobium pusense*
No.18	3	+	+	*Micrococcus yunnanensis*
	3	(+/-)	+	*Sphingomonas sanguinis*
No.19	18	(+/-)	+	*Massilia timonae*
	3	(+/-)	+	*Beijerinckia fluminensis*
No.20	6	-	+	*Agrobacterium tumefaciens*
	3	-	+	*Acinetobacter radioresistens*
No.21	3	-	+	*Rhizobium pusense*
	9	+	+	*Nocardioides zeae*
	3	+	+	*Micrococcus luteus*
No.22	3	(+/-)	+	*Burkholderia seminalis*
	3		-	
No.23	-		-	
No.24	9	-	+	*Candidatus Rhizobium massiliae*
No.25	-		-	-
No.26	-		-	-
No.27	-		-	-
No.28	-		-	-
No.29	-		-	-
No.30	-		-	-
				28 sample strains

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The details of isolated midgut microbiota in mosquitoes after fed on 10^{6} CID_{50}/ml chikunkunya virus

Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.1	-		-	-	-
No.2	3	+	+	+	*Streptomyces pseudogriseolus*
	3	+	+		*Micrococcus luteus*
No.3	-		-	-	-
No.4	-		-	+	-
No.5	3	+	+	+	*Bacillus megaterium*
	6	+	+		*Sinomonas halotolerans*
No.6	-		-	+	-
No.7	3	+	+	+	*Micrococcus luteus*
No.8	3	+	+	+	*Bacillus megaterium*
	3	(-)	+		*Moraxella osloensis*
No.9	-		-	+	-
No.10	-		-	+	-
No.11	-		-	-	-
No.12	3	+	+	+	*Staphylococcus hominis*
	3	(-)	+		*Brachybacterium paraconglomeratum*
	3	+	+		*Actinomyces naeslundii*
No.13	9	+	+	+	*Bacillus subtilis*
	3	+	+		*Micrococcus luteus*
	3	+	+		*Staphylococcus epidermidis*
No.14	-		-	-	-
No.15	-		-	+	-
No.16	3	+	+	+	*Micrococcus aloeverae*
No.17	-		-	+	
No.18	-		-	+	-
No.19	-		-	+	-
No.20	-		-	+	-
No.21	-		-	+	-

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.22	-		-	+	-
No.23	-		-	+	-
No.24	9	(-)	+	+	*Paenibacillus timonensis*
No.25	-		-	+	-
No.26	-		-	+	-
No.27	-		-	+	-
No.28	-		-	-	-
No.29	-		-	+	-
No.30(93)	15	(-)	+	+	*Brachybacterium nesterenkovii*
					16 sample strains

The details of isolated midgut microbiota in mosquitoes after fed on CHIKV titer 10^5 CID_{50}/ml

Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.1	3	+	+	+	*Micrococcus luteus*
No.2	12	+	+	-	*Staphylococcus haemolyticus*
	3	+	+		*Staphylococcus warneri*
No.3	-		-	+	-
No.4	-		-	+	-
No.5	-		-	+	-
No.6	3	+	+	+	*Micrococcus yunnanensis*
	9	+	+		*Micrococcus luteus*
No.7	6	+	+	+	*Micrococcus luteus*
	3	(-)	+		*Kocuria palustris*
No.8	6	+	+	+	*Staphylococcus cohnii*
No.9	6	(-)	+	+	*Moraxella osloensis*
No.10	-		-	+	-
No.11	9	+	+	+	*Micrococcus luteus*
No.12	-		-	-	-
No.13	6	+	+	+	*Micrococcus luteus*
No.14	-		-	-	-

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.15	6	+	+	+	*Corynebacterium ihumii*
No.16	-		-	+	-
No.17	36	+	+	+	*Micrococcus luteus*
	105	+	+		*Micrococcus yunnanensis*
	6	+	+	+	*Streptococcus mitis*
No.18	-		-	+	-
No.19	-		-	+	-
No.20	-		-	+	-
No.21	30	+	+	+	*Micrococcus luteus*
No.22	18	+	+	+	*Micrococcus luteus*
No.23	-		-	+	-
No.24	3	(-)	+	+	*Moraxella osloensis*
No.25	3	+	+	+	*Micrococcus yunnanensis*
No.26	-		-	+	-
No.27	-		-	+	-
No.28	-		-	+	-
No.29	-		-	+	-
No.30					-
					19 sample strains

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The details of isolated midgut microbiota in mosquitoes after fed on CHIKV titer 10^4 CID_{50}/ml

Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.1	3	+	+	+	*Bacillus cereus*
No.2	-		-	+	-
No.3	21	+	+	+	*Bacillus aquimaris*
No.4	-		-	+	-
No.5	3	(-)	+	+	*Brevundimonas diminuta*
No.6	-		-	+	-
No.7	30	+	+	+	*Staphylococcus haemolyticus*
	3	+	+		*Kocuria palustris*
	6	(+/-)	-		-
No.8	-		-	+	-
No.9	3	+	+	+	*Brevibacterium casei*
No.10	3	+	+	+	*Micrococcus luteus*
No.11			-	+	-
No.12			-	+	-
No.13			-	+	-
No.14			-	+	-
No.15	9	+	+	+	*Streptococcus mitis*
No.16	-		-	+	-
No.17	-		-	+	-
No.18	3	+	+	+	*Bacillus clausii*
No.19				+	
No.20				+	
No.21				+	
No.22				+	
No.23	3	+	+	+	*Paenibacillus lautus*
No.24	-		-	+	-
No.25	-		-	+	-
No.26	-		-	+	-
No.27	-		-	+	-
No.28	-		-	+	-

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.29	-		-	+	-
No.30	9	+	+	+	*Staphylococcus epidermidis*
					11 sample strains

The details of isolated midgut microbiota in mosquitoes after fed on CHIKV titer 10^3 CID_{50}/ml

Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.1	3	(-)	+	-	*Streptomyces griseoaurantiacus*
No.2	6	+	+	-	*Micrococcus yunnanensis*
	3	(-)	+		*Moraxella osloensis*
No.3	-		-	-	-
No.4	69	+	+	+	*Micrococcus luteus*
No.5	18	+	+	+	*Staphylococcus epidermidis*
	30	+	+		*Micrococcus yunnanensis*
	126	+	+		*Micrococcus luteus*
No.6	3	+	+	-	*Micrococcus yunnanensis*
No.7	6	(-)	+	-	*Staphylococcus pasteuri*
	6	+	+		*Bacillus tianshenii*
No.8	18	+	+	-	*Staphylococcus hominis*
	9	+	+		*Corynebacterium aurimucosum*
No.9	-		-	-	-
No.10	24	+	+	-	*Staphylococcus saprophyticus*
No.11	-		-	+	-
No.12	-		-	-	-
No.13	57	+	+	+	*Bacillus circulans*
	3	+	+		*Staphylococcus hominis*
	24	+	+		*Micrococcus yunnanensis*
No.14	-		-	+	-
No.15	9	+	+	+	*Micrococcus luteus*
No.16	3		+	+	*Kocuria palustris*
No.17	6	+	+	+	*Staphylococcus haemolyticus*

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
	33	+	+		*Micrococcus luteus*
No.18	6	+	+	+	*Bacillus cereus*
No.19	93	+	+	+	*Staphylococcus haemolyticus*
No.20	231	+	+	+	*Staphylococcus haemolyticus*
	3	(-)	+		*Acinetobacter indicus*
No.21	84	+	+	+	*Staphylococcus haemolyticus*
No.22	15	+	+	+	*Staphylococcus pasteuri*
No.23	63	+	+	+	*Micrococcus luteus*
	39	+	+		*Micrococcus yunnanensis*
No.24	6	+	+	+	*Micrococcus luteus*
	30	+	+		*Micrococcus yunnanensis*
No.25	15	+	+	+	*Micrococcus luteus*
No.26	450	+	+	+	*Micrococcus luteus*
No.27	168	+	+	+	*Micrococcus yunnanensis*
No.28	6	+	+	+	*Staphylococcus pasteuri*
	75	+	+		*Micrococcus luteus*
	33	+	+		*Micrococcus yunnanensis*
No.29	3	+	+	+	*Bacillus methylotrophicus*
No.30	-			+	-
					37 sample strains

The details of isolated midgut microbiota in mosquitoes after fed on CHIKV titer 10^2 CID_{50}/ml

Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.1	42	+	+	-	*Micrococcus luteus*
No.2	-		-	-	-
No.3	12	+	+	+	*Micrococcus luteus*
	3	+	+		*Staphylococcus hominis*

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.4	27	+	+	+	*Micrococcus luteus*
	12	(-)	+		*Enhydrobacter aerosaccus*
	3	+	+		*Staphylococcus hominis*
No.5	30	+	+	+	*Micrococcus luteus*
No.6	3	+	+	+	*Micrococcus luteus*
	42	(-)	+		*Corynebacterium pilbarense*
	33	+	+		*Staphylococcus haemolyticus*
	27	(-)	+		*Corynebacterium jeikeium*
No.7	-		-	+	-
No.8	3	+	+	+	*Brevibacterium sanguinis*
	6	+	+		*Micrococcus yunnanensis*
No.9	6	+	+	-	*Micrococcus luteus*
No.10	-		-	-	-
No.11	42	+	+	+	*Staphylococcus epidermidis*
	24	(-)	+		*Corynebacterium pilbarense*
No.12	42	+	+	-	*Micrococcus luteus*
	18	(-)	+		*Psychrobacter pulmonis*
No.13	3	+	+	-	*Staphylococcus epidermidis*
No.14	3	+	+	-	*Kocuria marina*
No.15	3	+	+	-	*Micrococcus yunnanensis*
	1149	+	+		*Staphylococcus epidermidis*
No.16	6	+	+	-	*Staphylococcus hominis*
No.17			-	-	-
No.18			-	-	-
No.19			-	+	-
No.20	6	+	+	+	*Micrococcus luteus*
No.21	3	(-)	+	-	*Pseudomonas luteola*
No.22	-		-	-	-

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	CHIKV in saliva	Bacteria Sequence
No.23	24	+	+	+	*Staphylococcus hominis*
	111	+	+		*Staphylococcus epidermidis*
	3	+	+		*Micrococcus luteus*
No.24	69	(-)	+	-	*Moraxella osloensis*
					29 sample strains

The details of isolated midgut microbiota in mosquitoes after fed on non-infected blood meal

Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria sequence
No.1	6	(-)	+	*Acinetobacter radioresistens*
No.2	6	+	+	*Micrococcus luteus*
No.3	3	+	+	*Micrococcus yunnanensis*
No.4	3	+	+	*Micrococcus yunnanensis*
No.5	-		-	-
No.6	-		-	-
No.7	3	(-)	+	*Agrococcus terreus*
No.8	3	+	+	*Micrococcus luteus*
No.9	24	+	+	*Staphylococcus pasteuri*
	6	+	+	*Micrococcus luteus*
	3	(-)	+	*Novosphingobium panipatense*
No.10	-		-	-
No.11	-		-	-
No.12	-		-	-
No.13	-		-	-
No.14	-		-	-
No.15	-		-	-
No.16	-		-	-
No.17	-		-	-
No.18	-		-	-

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Mosquito No.	Total colony (per mosquito)	Gram	PCR	Bacteria sequence
No.19	-		-	-
No.20	-		-	-
No.21	3		-	-
	3	(+)	+	*Janibacter indicus*
No.22	3	+	+	*Staphylococcus hominis*
No.23	3	(+/-)	+	*Neisseria perflava*
	3	+	+	*Staphylococcus cohnii*
No.24	3	+	+	*Bacillus amyloliquefaciens*
	3	(+/-)	+	*Acinetobacter radioresistens*
				15 sample strain

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VITA

Miss Ranida Tuanudom was born on October 30, 1979 in Pattani, Thailand. She completed with the Bachelor Degree of Science (B.Sc.) from the Faculty of Science, Kasetsart University, Bangkok, Thailand in 2002. And then, she completed with the Master Degree of Science (M.Sc.), Graduate School (Physiology), Chulalongkorn University, Bangkok Thailand in 2008. After that, she worked at i-tissue laboratory, King Chulalongkorn Memorial Hospital until 2009. Then she has been a scientist at Virology Unit and Emerging and re-Emerging Disease in Animals (CU-EIDAs), Faculty of Veterinary Science, Chulalongkorn University until now. After that, she enrolled in Doctor of Philosophy Program at Inter-Department of Biomedical Science, Graduate School, Chulalongkorn University since academic year 2012. She received the scholarship from the 60/40 Scholarship (co-funded by Chulalongkorn University and Graduated School) during study in Ph.D. program.

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