Scientific Publications Relating to Insect Vectors from 2005 to 2010





Biology and Ecology Section,
Medical Entomology Group,
National Institute of Health
Department of Medical Sciences,
Ministry of Public Health

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FOREWORD

The National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand, has been conducting comprehensive research programs relevant to the control and management of vector-borne diseases such as Dengue, Dengue Haemorrhagic Fever, Filariasis, Japanese encephalitis, Chikungunya and other diseases. As a part of mission of NIH, medical scientists in the Institute are engaged in a variety of research programs focusing attention on the biology, ecology, and management of insect vectors of important infectious diseases employing chemical control agents, biotic and microbial control agents and possibly genetic manipulations of vectors. The information generated in these applied studies will form the basis for instituting area-wide and local control and management programs against disease vectors. To make this information available to the public at large and the professional cadre of disease control agencies, we here compile published research results obtained by scientists of the Biology and Ecology Section incorporation with overseas scientists, especially Professor Mir S. Mulla of the Department of Entomology, University of California, Riverside, California, USA. Needless to say most of the research information gathered in the research from 2005 to 2010 has been published in internationally renowned peer reviewed journals not readily available in Thailand. We are pleased to offer this information in this booklet.

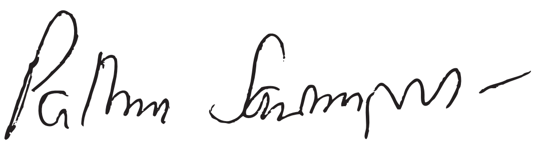
Significant advances were made in this period in identifying and evaluating novel chemical and microbial control agents against the dengue vector *Aedes aegypti* and other mosquitoes. Although these agents showed high level of activity against *Ae. aegypti*, they are equally effective against other mosquito vectors under Thailand environmental conditions. The novel chemical and microbial agents are now becoming available and promoted for vector control in Thailand. Novel synthetic chemicals such as diflubenzuron and novaluron yielded promising results against mosquitoes breeding in clear and polluted water in Thailand. Similar promising results were obtained by applying a naturalized microbial product produced in large fermentation of an actinomycete soil bacterium. The discovery and evaluation of these novel products enhance the arsenal of products available for disease vector control programs.

Other studies on mosquitoes investigated a local predator *Micronecta* and its predatory behavior feeding on mosquito larvae. This predator has a good potential as a biological control agent for mosquito larvae under field conditions. Also NIH scientists investigated host blood meals acquired by *Ae. aegypti*. About 86% of the blood meals were from humans showing this mosquito to be highly anthropophilic, only 1 to 3.6% feeding on pigs, dogs and cattle. Intensity of dengue transmission was elucidated and correlated with some ecological and social factors. NIH researchers along with other experts considered genetic techniques which might render mosquitoes incapable of transmitting pathogens to humans. These genetic strategies have potential for disease control in the future. Much more laboratory and field research is needed before genetically modified mosquitoes can be put to use in halting pathogen transmission.

In 2008, there was an outbreak of chikungunya in the south of Thailand. NIH researchers studied nature and extent of this viral disease in humans in the epidemic region of this outbreak and determined the infection rate in mosquito vectors *Ae. aegypti* and *Ae. albopictus*. The causal agent virus was found in about one half of the mosquitoes (both males and females), including transovarial transmission of the virus, since male mosquitoes do not feed on blood.

Vector and pest species other than mosquitoes were also the subject of detailed studies by NIH and cooperating scientists. The German cockroach was found in some markets, populations high in July and August and the lowest populations recorded in December and January. In cooperative studies, our scientists studied seven plant essential oils for repelling against 3 species of cockroaches in laboratory and found that oils of *Citrus hystrix* was an effective repellent against cockroaches. Field studies with this oil also showed the highest repellency.

The published record of scientific information compiled here, will be of great benefit to professional and vector control experts in formulating and applying effective vector control programs in Southeast Asia and elsewhere. This information will form the basis of area-wide as well as local vector control programs to be instituted against a variety of disease vectors.



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Mosquito Vectors

Mosquito Vectors

Dengue Haemorrhagic Fever in Thailand: Current Incidence and Vector Management

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National Institute of Health, Department of Medical Sciences,

Ministry of Public Health, Thailand

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Abstract: Dengue, dengue haemorrhagic fever and dengue shock syndrome are endemic throughout South East Asia where they present a serious public health concern. The current status of these diseases in Thailand is described along with the challenges that confront those who seek to control the spread of these diseases.

Introduction

Dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) are caused by one or more of four dengue viruses (DEN-1, DEN-2, DEN-3, DEN-4) that transmit to humans through the bites of infective *Aedes* mosquitoes, *Aedes aegypti* (L.) and *Ae. albopictus* Skuse (Service, 1993). The disease is now one of the major public health problems worldwide, especially in the tropical and sub-tropical regions. It is estimated that almost half of the global population are at risk of dengue infection. The disease is currently endemic in more than 100 countries in Africa, the Americas, Eastern Mediterranean, Southeast Asia and Western Pacific (WHO, 2002). However, the Southeast Asia and the Western Pacific have been more seriously affected nowadays. The prevalence of DF/DHF has substantially grown during the past five decades. The annual average number of cases of DF or severe dengue (DHF/DSS) reported to the World Health Organization (WHO) have increased exponentially from 908 cases in 1950s to 925,896 cases in 2000s (Nathan and Dayal-Drager, 2007). The recent global epidemic occurred in 1998, when a total of 1.2 million cases of DF/DHF, including 3,442 deaths were reported to WHO (WHO, 2002).

Incidence of DHF

DF, DHF and DSS are communicable diseases under national surveillance system of Thailand which are required notification to the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health. Among these, DHF is the majority of the reported cases (>90%) each year. DHF was first reported in Bangkok in 1949 (Wangroongsarb, 1997) whereas the first epidemic occurred in 1958 (Ungchusak and Kunasol, 1988). Since then, the annual incidence of DHF (morbidity per 100,000 populations) has fluctuated over time and increased from

8.9/100,000 in 1958 to 74.8/100,000 in 2006, with the highest incidence of 325/100,000 in 1987 (Figure 1). According to classification by Nisalak *et al*. (2003), the severe epidemics (annual morbidity rate >175 per 100,000) occurred in 1987, 1998, 2001 and 2002 whereas moderately severe epidemics (annual morbidity rate 134 – 175 per 100,000) presented in 1984, 1985, 1989, 1990 and 1997. The highest mortality rate of DHF in Thailand (1.88 per 100,000) was observed in 1987. However, it has appeared below 0.8 per 100,000 since 1988 and become less than 0.1 per 100,000 since 2004. Regarding the case fatality rate (CFR) among DHF patients, it was generally greater than 10% during 1958 – 1960 and dropped dramatically thereafter. CFR has been reduced to less than 1% since 1982 and remained below 0.5% during the past 18 years (Figure 2). Recently, in 2004, 2005 and 2006, the CFR was 0.12, 0.15 and 0.13%, respectively. Based on average number of DHF cases between 1981 and 2006, the most prevalent age group of DHF patients was 5 – 9, followed by 10 – 14, 15+ and 0 – 4 years, respectively (Figure 3). DHF occurs in Thailand all year round with high incidence during the rainy season lasting from May to October (Figure 4). Until recently, DHF have been found in all provinces of Thailand; however, most cases were reported from central and southern regions in the present decade.

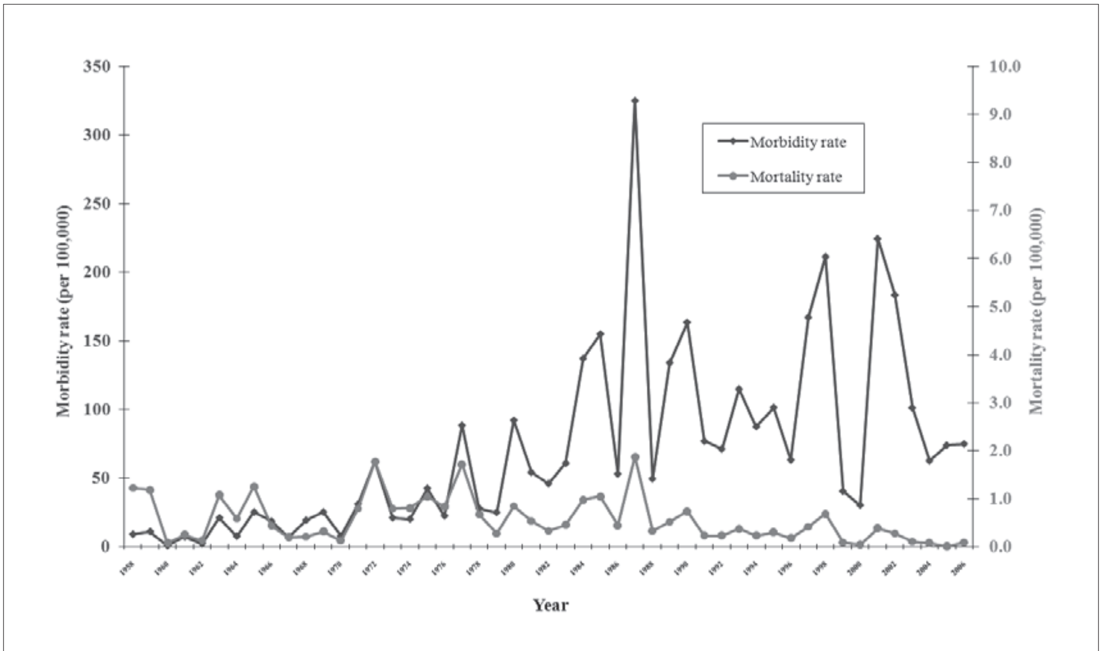

Figure 1 Morbidity and mortality rates of DHF in Thailand, 1958 - 2006.

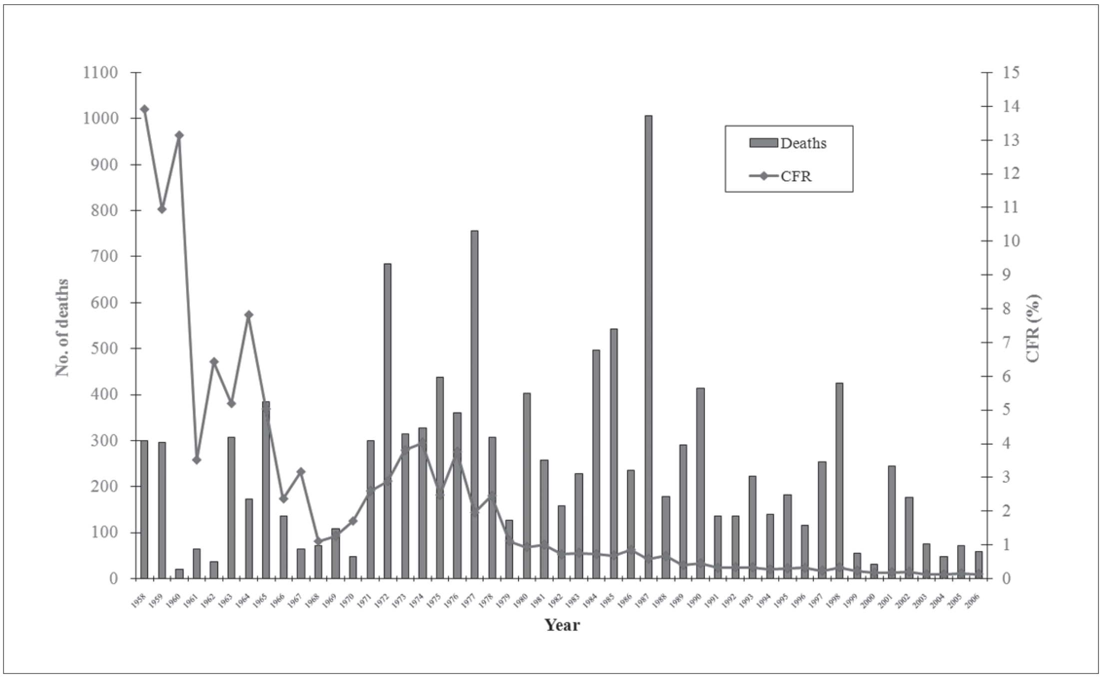

Figure 2 Number of deaths and case fatality rate (CFR) of DHF in Thailand, 1958 - 2006.

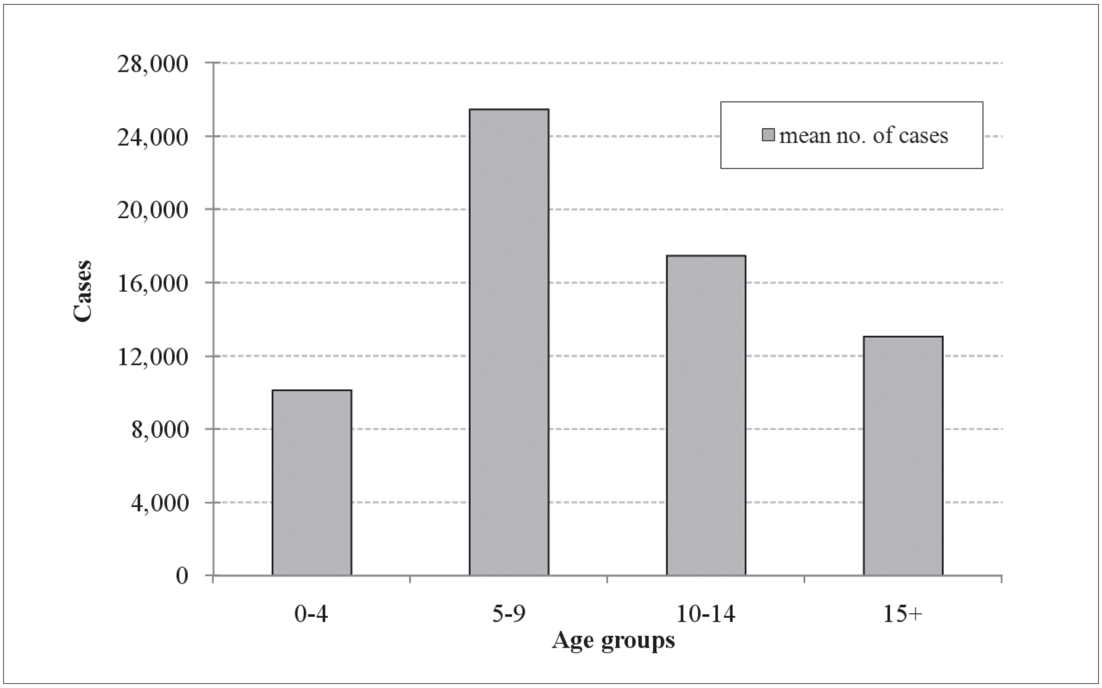

Figure 3 Distribution of DHF in Thailand by age groups, 1981 - 2006

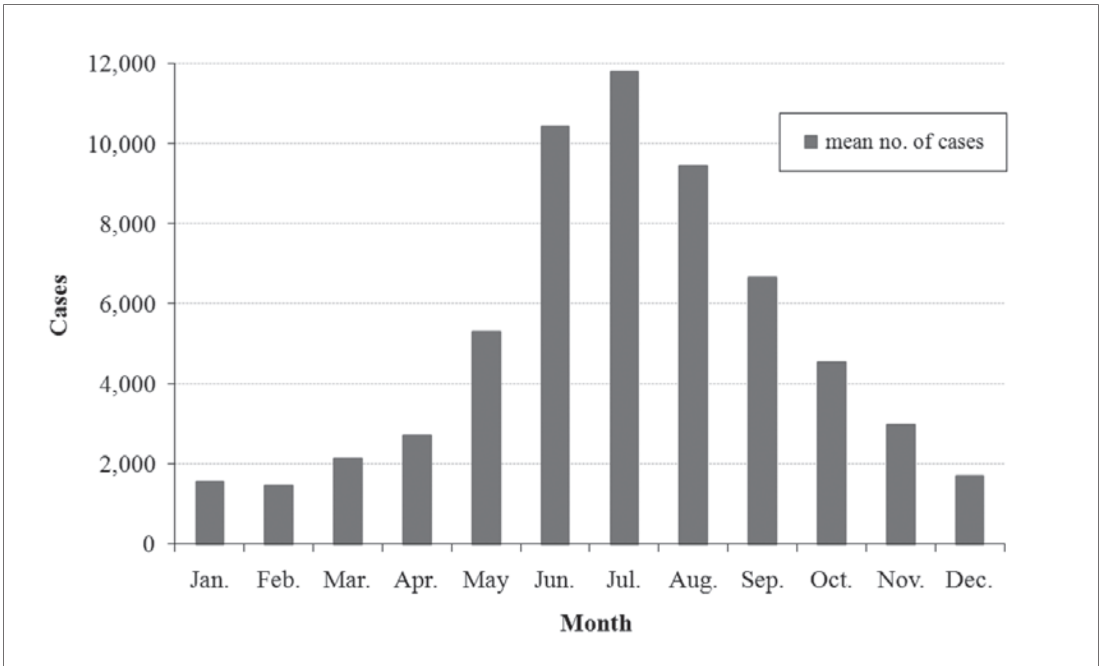

Figure 4 Distribution of DHF in Thailand by months, 1981-2006.

Distribution of Dengue viruses

All four serotypes of dengue viruses (DEN-1, DEN-2, DEN-3, DEN-4) are endemic in Thailand (Nisalak *et al*., 2003). However, the predominant virus serotypes which have been frequently associated with epidemics vary from year to year. According to the data collected between 1973 and 1999 from 15,376 dengue patients admitted in the Queen Sirikit National Institute of Child Health (formerly the Bangkok Children's Hospital) in Bangkok, DEN-2 was predominant from 1973 to 1986 and 1988 to 1989; DEN-3 in 1987, and from 1995 to 1999; DEN-1 from 1990 to 1992; and DEN-4 from 1993 to 1994 (Nisalak *et al*., 2003). Among 50 isolations of dengue viruses obtained from DHF patients admitted at the Rayong Provincial Hospital from 1980 to 1984, DEN-2 was predominant serotype (46%), followed by DEN-1 (32%), DEN-4 (12%) and DEN-3 (10%), respectively (Rojanasuphot *et al*., 1988). Recently, Anantapreecha *et al*. (2005) found that 45% out of 2,715 confirmed specimens collected from six hospitals scattered throughout Thailand between 1999 and 2002 were identified as DEN-1, whereas the rest were DEN-2 (32%), DEN-3 (18%) and DEN-4 (5%), respectively.

DHF vectors

Both species of DHF vectors: *Ae. aegypti* and *Ae. albopictus* are found throughout Thailand. The main breeding places of *Ae. aegypti* in Thailand are mostly man-made water-storage containers, such as earthenware jars, water-storage drums, cement tanks, ant traps, etc (Thavara *et al*., 2001). *Ae. albopictus*, on the other hand, is able to breed in a wide range of natural and artificial types of breeding sources and water holding niches varying from place to place. The main breeding places of *Ae. albopictus* are mainly natural sites, such as leaf axils, tree holes, coconut husks, bamboo stumps, etc., as well as, artificial containers, for example, earthenware jars, water-storage drums, used tyres and a variety of plastic containers found in the domestic environment (Thavara *et al*., 2001). Although the both species normally breed in different habitats, in some certain places they may be found breeding together in the same containers (Thavara *et al*., 2001). The females of both species prefer to oviposit in containers with relatively clean water; however, they may also do so in waters with varying degree of contamination with organic debris.

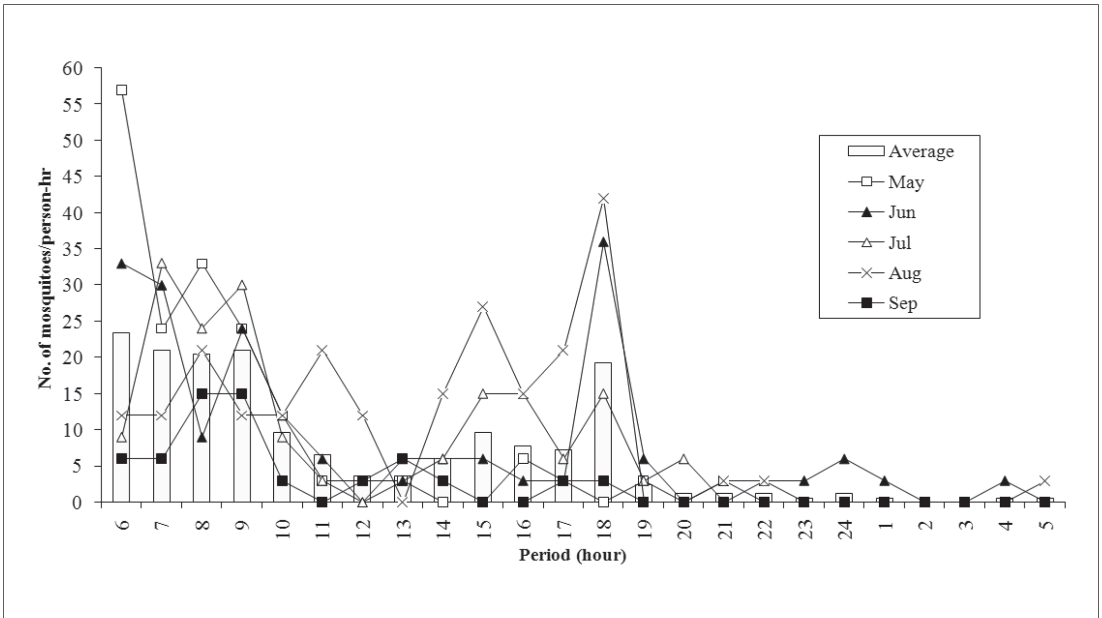

Figure 5 Biting activity of *Ae. aegypti* during 24 hours of the day conducted once a month between May and September 2007, in Nonthaburi, Thailand.

The females of *Ae. aegypti* and *Ae. albopictus* are daytime biters. They may feed on human victims from dawn to dusk, depending on hosts available. The diel biting activity of both species is usually higher in the morning hours than in the afternoon period (Thavara *et al*., 2001). Very recently, we have carried out an intensive study on biting rhythm of *Ae. aegypti* during 24 hours of the day, once a month for five months during raining period in 2007, and we found that the biting patterns change from earlier reports. The average biting remained high (20.4 – 23.4 mosquitoes/person-hr) in the morning period from 6 to 9 am (Figure 5). Then, the average biting activity declined dramatically to less than 10 mosquitoes/person-hr and rose again to about 19.2 mosquitoes/person-hr at dusk (6 pm). It is interesting to note that the biting activity of *Ae. aegypti* also occurred after sunset until early morning of the next day (5 am), except a couple hours between 2 and 3 am (Figure 5). *Ae. aegypti* is primarily an endophagic mosquito, rarely biting outside whereas *Ae. albopictus* is primarily an exophagic mosquito but can be found in significant numbers biting indoors (Thavara *et al*., 2001). Both species are persistent mosquitoes, pursuing the victim until feeding is completed. Actually, feeding continues until the mosquito is fully engorged; however, if this feeding is disturbed, it may return to feed again on the same or other victims. This thus plays an important role in dengue transmission.

In Thailand, dengue viruses have been isolated from or detected in wild caught *Ae. aegypti* (Watts *et al*., 1985; Rojanasuphot *et al*., 1988; Thavara *et al*., 1996; Tuksinvaracharn *et al*., 2004; Thavara *et al*., 2006) as well as from *Ae. albopictus* (Thavara *et al*., 1996; Thavara *et al*., 2006). It was also found that two different serotypes of dengue viruses (DEN-2 and DEN-3) had been detected by polymerase chain reaction (PCR) technique in field-caught individual *Ae. aegypti* males and females and *Ae. albopictus* females (Thavara *et al*., 2006). The evidence of dengue infection in wild-caught individual *Ae. aegypti* males in the previous study obviously reveals phenomenon of transovarial transmission occurring in natural environment in Thailand. This phenomenon could maintain the viruses in the environment during dry period when populations of mosquito vectors are scarce, even though the transovarial transmission of dengue viruses by mosquito vectors could occur naturally at a relative low rate (Rosen *et al*., 1983). These may confirm a high incidence of DHF in Thailand.

Current DHF vector management

At the outset, initial vector control programs, which were implemented in late 1960s, emphasized the application of chemical sprays to control adult mosquitoes, but this intervention had little or no impact on disease transmission. As a result, the national policy on DHF vector control was redirected to community-based strategies with emphasis on source reduction employing village health volunteers since the 1980s. The current strategies used for prevention and control of DHF vectors in Thailand are briefly summarized in Table 1. Satisfactory achievement has been achieved in some areas, depending on the strength of local health authorities and community participation.

Table 1 Current strategies used for prevention and control of DHF vectors in Thailand

Strategies used	Processes / activities
Provisional of health education	➢ School: teaching in classes
	➢ Communities: public announcement, posters, brochures, leaflets
	➢ Mass media: television, radio, newspapers
Massive campaigns	➢ Larval elimination on every Friday
	➢ 3 regular practices for household water-storage containers: covering, changing, fish releasing
Environmental measures	➢ Source reduction of larval breeding
Larval control	➢ Physical approach: covering, draining, filling
	➢ Biological agents:
	-: larvivorous fishes: Guppy (*Poecilia reticulata*), Siamese fighting
fish	(*Betta splendens*)
	-: Bti-based larvicides
	➢ Chemical larvicides:
	-: temephos-based larvicides
Adult control: space spraying*	➢ Thermal fogs
	➢ Cold aerosol sprays
Personal protection	➢ Mosquito nets
	➢ Electrical mosquito-rackets
	➢ Repellents

* Insecticides used: Deltamethrin, Cypermethrin, Tetramethrin, Bioresemethrin, Cyfluthrin, Bifenthrin, Fenitrothion, Fenthion and Malathion.

In 2007, the Department of Disease Control, Ministry of Public Health, established a new scheme for controlling of dengue diseases. This scheme includes five major key performances: rapidness of dengue reporting, complete investigation of index cases at village level, preparedness of vector control team at district level, rapidness of vector control at dengue-reported foci, and coverage of vector control at dengue-reported foci. The details of this new scheme are briefly described in Table 2.

Country obstacles and challenges

The programs for DHF vector control in Thailand have confronted some obstacles. These obstacles are listed as following:

Table 2 The key performance of a new scheme for controlling of dengue diseases established in 2007 by the Department of Disease Control, Ministry of Public Health, Thailand.

Key performance	Targeted action
Rapidness of dengue reporting Objective: To strengthen the ability of dengue reporting network	➢ At least 80% of the dengue-reported cases receiving from hospitals are reported to the local health authorities of the patient dwellings within 24 hours by the provincial health authorities.
Complete investigation of index case at village level Objective: To find out the source of infection in order to stop epidemic	➢ At least 80% of index cases are investigated completely (index case = the first dengue case reported by hospital of each village).
Preparedness of vector control team at district level Objective: To prepare the vector control team ready for emergency control immediately after receiving case report	➢ At least one vector control team is officially appointed in each district. ➢ At least one member of the vector control team is well-trained to operate the space-spraying equipment (ULV and/or thermal fog generator). ➢ At least one set of the space-spraying equipment (ULV and/or thermal fog generator) is ready to use at all time. ➢ Chemical insecticides used for space-spraying and larvicides are sufficiently stocked at all time.
Rapidness of vector control at dengue-reported foci Objective: To eliminate the infected mosquitoes in order to stop dengue transmission	➢ Vector control is carried out by the vector control team within 24 hours after receiving report from the provincial health authorities.
Coverage of vector control at dengue-reported foci Objective: To prevent the second generation of infection at the dengue-reported foci	➢ Thoroughly survey and larval control are conducted at the dengue-infected dwellings and surrounding areas. ➢ Application of space spraying is carried out at the dengue-infected dwellings and 100-m surrounding areas. ➢ The second application of space spraying is carried out 7 days apart from the first one at each dengue-infected dwelling. ➢ Assessment of vector control is conducted at 28 days post reporting of the index case to assure the absence of the second generation of infection.

Difficulty in mobilizing community participation in vector control

The sustainability of an integrated vector control program substantially depends upon community participation and ownership (Gubler, 1989). The previous community-based vector control programs in Thailand were not sustained since they were mainly operated by the public health authorities without partnership from the targeted communities (Gubler and Clark, 1996). Therefore, there is an urgent need to identify the appropriate and effective behavior for vector control to be encouraged in the communities. Recently, Kittayapong *et al*. (2006) demonstrated a successful community-based program employing a combination of horizontal and vertical approaches and some components of this program have been already established and routinely managed by the Local Administrative Authority with financial support from individual households. However, the sustainability of the program remains unclear and the long-term success as well as the community ownership needs to be evaluated over time.

Insufficient supply of materials used for vector control

As the key breeding sites of *Ae*. *aegypti* in Thailand are water-storage jars and cement tanks in bathrooms in each house (Thavara *et al*., 2001; Strickman and Kittayapong, 2002), the application of larvicides and releasing of larvivorous fishes in these containers are then the main strategy used for larval control. However, the larvicides supplied by government agencies are insufficient to apply to all breeding sites in each village. Once a year, each family actually receives a limited amount (approximately 20-60 g) of conventional larvicides (mostly 1% temephos sand granules) that are adequate only for 1 – 3 containers (when applied at the dosage of 20 g/200 L) whereas there are more than 5 water-storage containers in each house. Moreover, the loose granules of these larvicides are washed out and eventually lost during the process of cleaning and washing water-storage containers. There is, therefore, an essential need for sufficient supply of larvicides or other appropriate and effective materials used for larval control. Recently, Tawatsin *et al*. (2007) showed long-lasting efficacy of removable and retrievable formulations of temephos-based zeolite and sand granules in sachets that lasted for at least 6 months against *Ae*. *aegypti* larvae in water-storage jars. These innovative formulations will minimize the waste of scanty and costly larvicides and will expand larval control capacity for treatment in large numbers of water-storage containers that are untreated owing to insufficient amounts of the larvicides currently available.

Lack of good management in vector control

There are frequently changes of the staff responsible for dengue control at various levels, ranging from the policy makers to the operational staff. The staff sometimes were inexperienced to cope well with DHF epidemics. According to the decentralization of health systems in Thailand, the activities with regard to vector-borne disease control, such as pesticide procurement and application have been transferring to and eventually carried out by the Regional and Local Administrative Authorities by the year 2015. This is concern that these agencies have little or no experience in vector control and so will require training, procedures and guidelines for all aspects of vector management.

Lack of systematic monitoring of larval and adult resistance to the insecticides used

Although many chemical insecticides have been used for DHF vector control in Thailand for many decades, there is no systematic monitoring of larval and adult resistance to the insecticides used in the treated areas. Recent studies on insecticide susceptibility of *Ae*. *aegypti* and *Ae*. *albopictus* disclosed the occurrence of insecticide resistance in some particular areas of Thailand (Somboon *et al*., 2003; Paeporn *et al*., 2004; Ponlawat *et al*., 2005; Yaicharoen *et al*., 2005; Jirakanjanakit *et al*., 2007a, b; Pethuan *et al*., 2007). Therefore, there is an essential need to establish the systematic monitoring of larval and adult susceptibility/resistance to insecticides in both periodicity and geographical coverage in Thailand. In 2007, the Department of Disease Control initiated a program for monitoring insecticide resistance in *Ae*. *aegypti* in 19 provinces having high risk to DHF epidemic. It is expected that this program could be expanded to more provinces to cover the whole country in the near future. The information obtained from this program would be beneficial for an effective control against DHF vectors in Thailand as well as to prevent or slow the development of insecticide resistance in the vectors.

More applied research is also needed to develop and implement for effective control programs against DHF vectors in Thailand. These include development of early and predictive warning systems employing epidemiological, entomological and serological data, improvement of entomological indices for vector surveillance, identification of high-risk areas to be subjected to intensive vector control, development of new and safe larvicides and their long-lasting formulations. Recently, we embarked upon the testing and evaluation of microbial agent (*Bacillus thuringiensis israelensis* or Bti), chemicals, novel insect growth regulators (IGRs) and formulations that yielded long-lasting control of *Ae*. *aegypti* in water-storage containers. The test larvicides and their efficacy are briefly summarized in Table 3. These materials are expected increase our arsenal against *Ae*. *aegypti*, the important DHF vector.

Table 3 Newly developed formulations of chemical/microbial larvicides tested against *Ae. aegypti* larvae in 200-L water-storage jars carried out in Thailand recently.

Larvicidal agents	Formulations	Dosages(a.i./L)	Effective days (>90% IE)	References
Bti	DT	19,980 ITU/L	91 - 112	Mulla *et al*., 2004
Temephos	1% SG	1 mg/L	> 180	Mulla *et al*., 2004
	1% ZG	1 mg/L	> 180	
Temephos	1% SG (sachet)	1 mg/L	> 180	Tawatsin *et al*., 2007
	1% ZG (sachet)	1 mg/L	> 180	
Novaluron	10% EC	10 µg/L	68	Mulla *et al*., 2003
		0.05 – 0.1 mg/L	175	
		0.5 – 1 mg/L	190	
Diflubenzuron	2% DT	0.02 mg/L	147	Thavara *et al*., 2007
	2% GR	0.05 - 1 mg/L	161	
		0.02 mg/L	154	
		0.05 - 1 mg/L	161	

Conclusion

Dengue diseases, including DF, DHF and DSS have remained important mosquito-borne diseases of Thailand since late 1950s with high annual incidence but relative low mortality. All of the four dengue serotypes circulate continuously in Thailand with fluctuations in dominant serotypes from year to year and from place to place. Both species of DHF vectors: *Ae. aegypti* and *Ae. albopictus* are found throughout Thailand. The current vector control programs for DHF in Thailand consists of provision of health education to raise public awareness, massive campaigns for larval control, environmental measures for source reduction, larval control, adult control and personal protection measure. Satisfactory control has been achieved in some areas, depending on the strength of local health authorities and community participation. However, the programs have confronted some obstacles, such as difficulties to mobilize community participation in larval control measures, inadequate supply of larvicides, lack of good management in vector control programs, little use of procedures resulting from operational research on vector control and lack of systematically monitoring larval and adult susceptibility to the insecticides used. More applied research is needed to develop and implement sustainable control programs against DHF vectors. These include identification of appropriate and effective behavior for vector control to be encouraged in the communities, development of early and predictive warning systems employing epidemiological, entomological and serological data, improvement of entomological indices for vector surveillance, identification of high-risk areas to be subjected to intensive vector control, development of new and safe larvicides and their long-lasting formulations. Another important thrust toward the control of *Ae. aegypti* is the extensive field evaluations of novel IGRs, assessment of the longevity of the currently used and new temephos formulations as well as controlled released formulations of Bti. Information of these new strategies and tools will be used in developing future control programs against DHF vectors in Thailand.

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Identification of Blood Meal of Field Caught *Aedes aegypti* (L.) by Multiplex PCR

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Abstract: Laboratory bred female *Aedes aegypti* (L.) was used to determine sensitivity of multiplex PCR for detecting human blood meal. Human blood DNA was detected in live fully fed mosquitoes until 3 days after blood feeding, and for 4 weeks when stored at -20°C. Among 890 field caught female mosquito samples examined for vertebrate DNA by multiplex PCR, results were positive for human, pig, dog, cow and mixture of 2 host DNA at 86.1, 3.4, 2.1, 1.0 and 3.6%, respectively, while 3.9% of the samples were negative. Blood feeding pattern must be considered when mosquito control strategies become employed.

Introduction

Aedes aegypti is a major dengue vector in Thailand. Since the first report of dengue outbreak in Thailand in 1958, prevalence of the disease has increased dramatically and has spread throughout the country (Minister of Public Health, 1998). As a specific drug to treat the virus is unavailable and vaccine against dengue is during the development phase, mosquito control strategy is the only effective method to control the disease (Taksinvaracharn *et al*, 2004; Thavara *et al*, 2006).

Blood feeding patterns of the mosquito provide valuable data for disease transmission. The distribution of bites of the mosquito can be used to develop effective mosquito control strategies (Scott *et al*, 1993). Mosquito blood meals have been identified by various immunological techniques, such as capillary precipitin test (Tempelis, 1975), agar gel diffusion (Crans, 1969) and enzyme-linked immunosorbent assay (ELISA) (Burkot *et al*, 1981). Although such immunological methods have been widely used to identify mosquito blood meal

(Burkot *et al*, 1981; Beier *et al*, 1988; Hunter and Bayly, 1991; Chow *et al*, 1993; Ponlawat and Harrington, 2005), these protocols still have some intrinsic problems leading to misidentification of the types of blood due to cross-reactivity of serum proteins from related species (Ngo and Kramer, 2003). Recently, molecular techniques have been developed to identify vertebrate DNA in mosquito blood meals (Ngo and Kramer, 2003; Kent and Norris, 2005). The advantages of these procedures are that they are highly species specific and sensitive. Multiplex PCR has been developed to identify vertebrate host DNA from mosquito using a primer set specific for human, dog, cow, pig and goat (Kent and Norris, 2005). The primers were designed to anneal specifically to cytochrome oxidase b gene of vertebrate hosts (Kent and Norris, 2005). The objectives of this sludy were to determine the time course of the multiplex PCR detection of host blood DNA in *Ae. aegypti* mosquito, to compare different methods for collecting mosquito specimens and to study the prevalence and type of vertebrate blood from field caught *Ae. aegypti*.

Materials and methods

Time course analysis

Five day-old female *Ae. aegypti* mosquitoes were allowed to feed on human blood through a membrane feeding apparatus. Fully fed mosquitoes were then reared in an insectary at 28°C ± 1°C and 80% ± 5% humidity, and supplied with a damp cotton wool pad containing 10% sucrose solution as a carbohydrate source. DNA extraction was performed at 24, 48, 72 and 96 hours after feeding, and extracted DNA samples were kept at -20°C until used.

To determine the best method for preserving host DNA in collected mosquito specimens, blood fed mosquitoes were collected in a microcentrifuge tube containing 70% ethanol and kept at room temperature, or in a micro centrifuge tube without ethanol, and kept at 4°C and -20°C. Host DNA was extracted from the collected mosquitoes every week for 4 weeks.

Mosquito collection

Mosquitoes were collected from various areas of Ratchaburi Province (approximately 100 km from Bangkok, Thailand) using an electronic aspirator during March-July 2008. The total number of female mosquitoes was 890, and after identification mosquitoes were stored in microcentrifuge tubes at -20°C until used.

DNA extraction

DNA from whole blood sample was extracted using an AquaPure Genomic

DNA Isolation Kit (Bio-Rad, CA) followed the manufacturer's instruction. Human (*Homo sapiens*), dog (*Canis familiaris*), cow (*Bos tarsus*) and pig (*Sus scrofa*) blood samples preserved in EDTA were used, and the extracted DNA samples were kept at -20°C.

DNA was extracted from individual mosquito using the method described by Kent and Norris (2005). Mosquito abdomen was ground in 100 µl of extraction buffer [0.1M NaCl, 0.2M sucrose, 0.1M Tris-HCI, 0.05M EDTA, pH 9.1 and 0.5% sodium dodecyl sulfate (SDS)] and incubated at 65°C for one hour. A 15 µl aliquot of cold 8 M potassium acetate was added and the solution was incubated on ice for 45 minutes. The sample then was centrifuged at 15,000g for 10 minutes. To precipitate DNA, 250 µl of 100% ethanol were added to the supernatant, which was incubated at room temperature for 5 minutes and centrifuged at 15,000g for 15 minutes. DNA pellet was dried at room temperature, resuspended in 10 µl 0.1 x SSC (15 mM NaCl, 1.5 mM sodium citrate) and 40 µl of double-distilled water and kept at -20°C until used.

Multiplex PCR

The procedure was modified from that of Kent and Norris (2005). PCR reaction (25µl) contained 2.5 µl of 10x buffer, 2.5 µl of 50 mM $MgCl_2$, 1.5 µl of 100 µM of each primer (universal reverse primer and forward primers of human, pig, dog and cow cytochrome oxidase b gene), 0.5 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA), 2 µl of 10 mM dNTP mixture, 2 µl of extracted DNA and double-distilled water. Thermal cycling was performed in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystem, Foster city, CA) as follows: one cycle of 95°C for 5 minutes; followed by 35 cycles of 95°C for 1 minute, 58°C for 30 seconds and 72°C for 1 minute; a last cycle of 72°C for 7 minutes. A 10 µl aliquot of the PCR products was electrophoresed in 15% agarose gel at 100 volts, stained with ethidium bromide (0.5 µg/ml) and visualized using a Gel Doc EQ system (Bio-Rad, CA). Amplification of human, pig, cow and dog DNA produced the expected PCR amplicon of 334, 453, 561 and 680 bp, respectively.

Results

Time course analysis

Human blood fed mosquitoes were used for time course analysis. DNA from fully blood fed mosquitoes were collected every 24 hours until 96 hours. Extracted DNA was PCR amplified and amplicon (334 bp) was visualized following electrophoresis and staining. Whole blood was used as positive control and sucrose fed mosquito as negative control. Human blood DNA was detected in mosquito until 72 hours after feeding (Figure 1).

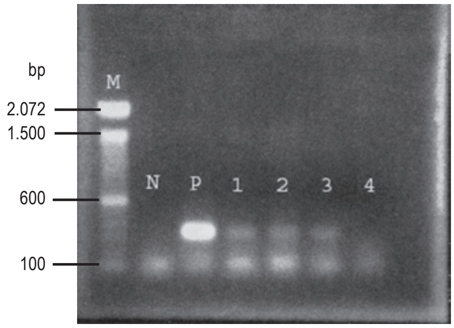

Figure 1 Time course analysis of human DNA from blood fed mosquito. Human DNA was PCR amplified as described in Materials and Methods. M, 100 bp marker; N, negative control; P positive control; 1-4, female mosquitoes collected on day 1 to day 4 after blood feeding.

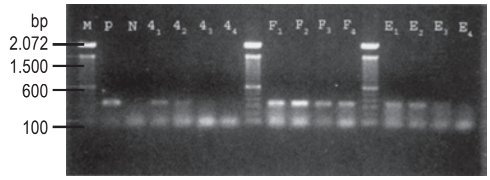

Figure 2 Preservation of mosquito specimen for detecting human host blood DNA. Human DNA was PCR amplified as described in Materials and Methods. M, 100 bp marker; N, negative control; P, positive control; 4_1-4_4, samples stored at 4°C without preservative for 1-4 weeks; F_1-F_4, samples stored at -20°C without preservative for 1-4 weeks; E_1-E_4, samples stored in 70% ethanol at room temperature for 1-4 weeks.

Preservation of mosquito specimens for detecting host blood DNA

Blood fed mosquito specimens were kept for 1-4 weeks in microcentrifuge tube containing 70% ethanol at room temperature, and in microcentrifuge tube without ethanol at 4°C and at -20°C. Human host DNA was detected in mosquito specimens stored at 4°C without preservative for 2 weeks, at room temperature in 70% ethanol for 3 weeks and at -20°C without preservative for 4 weeks (Figure 2).

Identification of blood meal from field caught mosquito

Among the 890 mosquito samples, 766 (86.1%), 30 (3.4%), 18 (2.0%) and 9 (1.0%) were positive for human, pig, dog and cow DNA respectively, and 32 (3.6%) samples were positive for two types of host DNA and 35 (3.9%) were negative. Figure 3 shows a typical result.

Discussion

Time course analysis study in *Ae. aegypti* mosquitoes fed on human blood demonstrated that host DNA was detected 72 hours after feeding. Kent and Norris (2005) showed that this method is able to detect vertebrate host DNA in *Anopheles stephensi* mosquito only up to 48 hours after feeding. Boake et al (1999) studies in black flies (*Simulium damnosum* s.l.) fed on human blood demonstrated that PCR is able to detect human DNA up to 72 hours post-feeding. Ngo

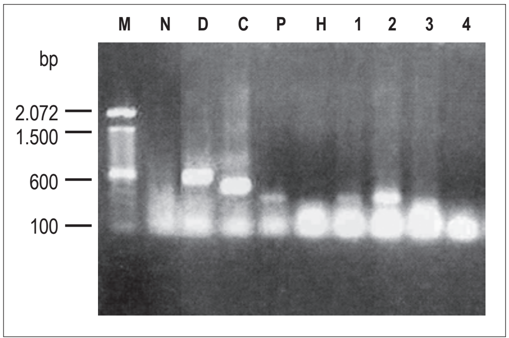

Figure 3 Agarose gel-electrophoresis of multiplex PCR of host DNA from field caught *Ae. aegypti*. Multiplex PCR was performed as described in Materials and Methods. M, 100 bp marker; N, negative control; D, C, P, H, positive control of dog, cow, pig and human DNA; 1-4, field caught mosquito specimens, which were positive for pig, pig, human and negative. respectively.

and Kramer (2003) also demonstrated that avian blood DNA is detected in *Culex p. pipiens* L up to 72 hours after feeding. Lee *et al* (2002) was able to detect Japanese quail DNA from *Cx. tarsalis* Coquillett 7 days post-feeding. Differences in duration of host DNA detection in blood feeding insects depend on several factors, including DNA extraction procedure, different digestive processes in black flies compared with mosquitoes or even differences in mosquito species (*An. stephensi* and *Ae. aegypti*).

Preservation method studies showed host DNA in blood fed *Ae. aegypti* mosquitoes stored at -20°C without any preservative for more than 4 weeks. Less periods were obtained for mosquitoes kept in 70% ethanol at room temperature or at 4°C.

High percentage (86.1%) of human DNA detected in *Ae. aegypti* mosquito is expected as it is considered as highly anthropophilic (Harrington *et al*. 2001). The negative result from this study was higher than the previous studies (Ngo and Kramer, 2003) and may have been caused by the primer set inability to anneal with other vertebrates, such as cat and other avian blood DNA.

In summary, blood feeding pattern of *Ae. aegypti* provides valuable data for dengue vector control. As the mosquito can maintain its life cycle by feeding on other vertebrate hosts, blood feeding pattern must be considered when mosquito control strategies are deployed.

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Intramolecular Integration Assay Validates Integrase Phi C31 and R4 Potential in a Variety of Insect Cells

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Abstract. Phage ϕC31 and R4 integrases are site-specific and unidirectional serine recombinases. We have analyzed the ability of these integrases to mediate intramolecular integration between their *attB* and *attP* sites in 7 important insect cell lines as a means of predicting their relative mobility in the corresponding insect species. Both integrases exhibit significantly higher frequencies in *Drosophila* S2 cells than in the other insect cell lines examined, but do work well in all of the species tested. Our results, coupled with previous results of the activity of ϕC31 integrase in *D. melanogaster* and *Aedes aegypti*, suggest the family of serine catalyzed integrases will be useful site-specific integration tools for functional genome analysis and genetic engineering in a wide range of insect species.

Introduction

Practical genetic engineering of insects for potential utility as moderators of native populations requires new tools for efficient site-specific integration of genes to allow reliable prediction of expression stability and fitness costs to the transgenic insects. The most common method currently employed for engineering eukaryotic chromosomes is random integration facilitated by mobile genetic elements in which foreign or manipulated DNA is introduced into the chromosomes of an organism without control over the ultimate position of the insertion, resulting in unpredictable gene expression and possible insertional mutagenesis leading to fitness costs.

In contrast, phage integration mechanisms can provide high specificity and have been recognized as a powerful genetic tool in a variety of prokaryotic and eukaryotic cells. While site-specific recombinases are structurally and functionally diverse (Smith and Thrope, 2002), most can be classified into either the tyrosine or serine family based on amino acid sequence homologies and catalytic residues.

Recombinases such as Cre and FLP use a catalytic tyrosine to mediate bidirectional recombination between two identical sites (Stark *et al*, 1992). These recombinases recognize DNA sequences that are typically 30-40 bp in length. They require no host-specific co-factors and have successfully functioned in mammalian cells, providing important and widely used tools for genome manipulation (O'Gorman *et al*, 1991; Sauer, 1994; Sorrell and Kolb, 2005). Although Cre and FLP recombinase-mediated integrations can be efficiently performed in mammalian cells, the net integration frequency is low (Sauer and Henderson, 1990) because of the reversibility of the reaction.

In contrast, phage integrases mediate unidirectional recombination between two different DNA sequences, the bacterial attachment site, *attB*, and the phage attachment site, *attP* (Campbell, 1992). These two sequences generally share a short stretch of identical bases where crossing-over occurs. After recombination, an integrated phage genome is flanked by two hybrid sites, *attL* and *attR*, each consisting of half *attP* sequence and half *attB* sequence. Only a subset of phage integrases, including those from phages ϕC31, R4, and TP-901-1, belong to the serine recombinase family (Christiansen *et al*, 1996; Matsuura *et al*, 1996; Thorpe and Smith, 1998; Brondsted and Hammer, 1999; Olivares *et al*, 2001; Hollis *et al*, 2003). Because these integrases cannot carry out the reverse excision reaction without additional co-factors, these enzymes are especially helpful for catalyzing stable integration reactions.

ϕC31 integrase is isolated from a *Streptomyces* phage (Kuhstoss and Rao, 1991; Rausch and Lehmann, 1991) and is reported to mediate intramolecular recombination of plasmids in *Escherichia coli* and *in vitro* with no requirement of host-specific co-factors (Thorpe and Smith, 1998). The 605-amino acid ϕC31 integrase can perform recombination between minimal 34-bp *attB* and 39-bp *attP* sites surrounding a core cleavage and ligation sequence of TTG in human cells (Groth *et al*, 2000). This integrase mediates stable, site-specific integration of plasmids bearing *attB* into *attP* sites randomly integrated into the genomes of cultured human cells (Thyagarajan *et al*, 2001). It also functions in *Rhodococcus equi* (Hong and Hondalus, 2008), *Schizosaccharomyces pombe* (Thomason *et al*, 2001), the silkworm cell line, BmN4 (Nakayama *et al*, 2006), wheat plants (Rubtsova *et al*, 2008), *Methanosarcina* species (Guss *et al*, 2008) and human cells (Thyagarajan *et al*, 2008). ϕC31 integrase has been utilized to efficiently create transgenic *Xenopus laevis* (Allen and Weeks, 2005, 2009), and mice (Belteki *et al*, 2003; Hollis *et al*, 2003).

The ϕC31 integrase may also be used to effectively target naturally occurring chromosomal sequences with partial sequence identity to *attP*, called pseudo *attP* sites (Thyagarajan *et al*, 2001). Site-specific integration of foreign genes at pseudo *attP* sites of *Drosophila* (Groth *et al*, 2004), bovine cells (Ma *et al*, 2006; Ou *et al*, 2009), and mammalian cells (Thyagarajan *et al*, 2001) has been detected. The genomic integrations mediated by ϕC31 were also detected by long-term expression of luciferase in human and mouse cells (Thyagarajan *et al*, 2001; Thyagarajan and Calos, 2005). This ability of ϕC31 integrase to find and utilize pseudo *attP* sites has been successfully used in gene therapy experiments in mouse liver, mouse muscle-derived stem cells, mouse muscle, rat retina, human myoblasts and human keratinocytes (Olivares *et al*, 2002; Ortiz-Urda *et al*, 2002, 2003; Quenneville *et al*, 2004; Chalberg *et al*, 2005; Held *et al*, 2005; Bertoni *et al*, 2006). The ϕC31 integrase system also produces stable transgene expression in adult mouse neural progenitor cells (mNPCs) and their progeny that may be useful in strategies for combating neurodegenerative disorders (Keravala *et al*, 2008).

The ability of ϕC31 integrase to efficiently target transgenes to specific chromosomal locations and the potential to integrate very large transgenes has broad applicability for genetic manipulation of many medically and economically important insect species. ϕC31 integrase has demonstrated effectiveness to promote integration of *attB*-bearing plasmid into *P*-element vectored *attP* sites of two transformed *Drosophila* lines (Groth *et al*, 2004). The ϕC31 integrase system was used in conjunction with recombinase-mediated cassette exchange (RMCE) for precise targeting of transgenic construct to predetermined genomic sites in *Drosophila* (Bateman *et al*, 2006). This integrase system has also been used in the construction and manipulation of transgenic *Aedes aegypti* mosquitoes, increasing integration efficiencies by up to 7.9-fold (Nimmo *et al*, 2006) over alternative transposon-mediated protocols. Most recently, endogenous expression of ϕC31 integrase has been used to generate a collection of *Drosophila* lines having many different predetermined intergenic *attP* sites distributed throughout the fly genome (Bischof *et al*, 2007).

R4 integrase is a similar unidirectional site-specific recombinase derived from the *Streptomyces parvulus* phage R4. This streptomyces phage encodes a 469 amino acid protein termed *sre* for ***s***ite-specific ***re***combinase (Matsuura *et al*, 1996). *sre* mediates recombination between *attP* and *attB* sequences sharing a 12-bp common core region (Shirai *et al*, 1991). This integrase has demonstrated intramolecular integration capability in human cells (Olivares *et al*, 2001), and has been used for recombining two introduced *att* sites on the same chromosome resulting in deletion of the intervening sequences (Hollis *et al*, 2003).

Using extrachromosomal plasmid excision and integration assays, we investigated the ability of both ϕC31 and R4 integrases to mediate intramolecular recombination reactions between compact ϕC31 *attB* and *attP* or R4 *attB* and *attP* sites in a variety of cultured insect cells. We find that both ϕC31 and R4 integrases are capable of serving as effective tools for efficient recombination in a wild range of insects.

Materials and methods

Plasmids

ϕC31 integrase-expressing plasmids were constructed using two promo ters. *Drosophila*-derived heat shock protein 70 (hsp70) promoter (Di Nocera and Dawid, 1983; Lis *et at*, 1983; Steller and Pirrotta, 1984; Spradling, 1986; Linsquist and Craig, 1988) was amplified by the PCR from the plasmid phsp-pBac with the primers 5′-ACTAGTCCCCCAGAGTTCTCTTCTTGTATTCAATAA and 5′-GGTACCATTCCCATCCCCCTAGAATCCCA. The *D. melanogaster* actin 5C promoter was removed from pAct5C-Int by digestion with the restriction enzymes *Acc65*I and *Spe*I, and was replaced by the heat-shock promoter, creating the plasmid phsp-Int. The *sre* gene was amplified by the PCR from the plasmids pCMV-sre (kindly provided by Dr MP Calos) with the primers 5′-GGAT CCTCAAACCTTCCTCTTCTTCTTAGGCTCGGCCACGTCTCGCCACT and 5′-ACTAGTACCATGGGTATGAATCGAGGGGGGCCCACT. ϕC31 integrase was removed from phsp-Int by digestion with the restriction enzymes *BamH*I and *Spe*I, and was replaced by the *sre* gene, creating the plasmid phsp-sre.

Plasmid pBCPB+ (kindly provided by Dr MP Calos) containing ϕC31 *attP* and ϕC31 *attB* and plasmid pBC-P64-B295 (kindly provided by Dr MP Calos) containing R4 *attP* and R4 *attB* were used to detect intramolecular recombination in insect cells.

Cell line cultures

Seven insect cell lines used in this study were presenting 2 orders of insect, Diptera and Lepidoptera. Diptera, *Ae. aegypti* Aag2 cells, *Anopheles gambiae* Sua5B and *D. melanogaster* S2 cells were maintained at 28°C in Schneider's Drosophila medium (Gibco Laboratories) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 10% tryptose phosphate broth (SIGMA-ALDRICH) and 1% antibiotic/ antimycotic solution (SIGMA-ALDRICH). *Ae. aegypti* ATC-10 cells and *Ae. albopictus* C6/36 cells were maintained at 28°C in Leibovitz L-15 medium (Atlanta Biologicals) supplemented with 10% FBS, 10% tryptose phosphate broth and 1% antibiotic/antimycotic solution. Lepidoptera, silkworm *Bombyx mori* BmN4 and Fall armyworm *Spodoptera frugiperda* Sf9 cells were maintained at 28°C in TNMFH insect culture medium supplemented with 5% FBS but no FBS was supplemented in Sf9 cell culture, 10% tryptose phosphate broth and 1% antibiotic/antimycotic solution.

Transfection efficiencies measured by firefly luciferase expression

Insect cells, Aag2, ATC-10, C6/36, S2, Sf9 and Sua5B were transfected with either 1 μg of the firefly luciferase-expressing plasmid, pAct5C-Fluc or 1 μg of enhanced yellow fluorescent protein expressing plasmid, pAct5C-EYFP but BMN4 was transfected with either 1 μg of pAct3C-Fluc or pXLHMASP1- Act3C-EYFP using either TransFectin Lipid Reagent (Bio-Rad Laboratories) or DOTAP Liposomal Transfection Reagent (Roche Applied Science). At 24 hours post-transfection, only S2 cells were harvested for firefly luciferase assay because actin 5C promoter (Act5C) originated from *D. melanogaster* (Fyrberg *et al*, 1981, 1983; Bond and Davidson, 1986), and it is strong promoter in these cells. If these cells were harvested at 96 hours post-transfection, the luciferase protein would be oversaturated and could not be assayed. For other insect cells, they were harvested at 96 hours post-transfection

To assay firefly lueiferase expression, the harvested cells were washed twice with 1x cold phosphate-buffered saline, pH 7.4 (Gibco laboratories). These washed cells were resuspended in 500 μl of 1x cold cell culture lysis reagent (Promega Corporation) and then incubated at room temperature for 5-10 minutes. The lysed cell suspension was transferred to microcentrifuge tubes and stored at -70°C overnight. After overnight storage, this lysed cell suspension was thawed and centrifuged at 10,000 rpm for 30 seconds to remove cell debris. Luciferase activity in crude protein extracts was determined using Luciferase Assay Reagent (Promega Corporation) and the results were analyzed with SoftMax Pro 4.8 software. The luciferase activity in crude protein extracts was normalized by subtracting the measurement of the EYFP activity from that of the firefly luciferase activity.

Intramolecular reaction mediated by either ϕC31 or R4 integrase

Plasmid pBCPB+ or pBC-P64-B295 was used to detect intramolecular recombination, containing a *lacZ* gene flanked by their wild type *attB* and *attP* sequences. Each insect cell line was plated each well of a 6-well plate at $1x10^6$ cells/well. Cells were co-transfected with either 2.5 μg of pBCPB+ and 7.5 μg of phsp-Int or 2.5 μg of pBC-P64-B295 and 7.5 μg of phsp-sre using TransFectin Lipid Reagent but using DOTAP Liposomal Transfection Reagent for C6/36 cells. Control transfection was performed using either pBCPB+-only, phsp-Int-only, pBC-P64-B295- only, phsp-sre-only or no DNA. At 24 hours post-transfection, cells were heat shocked at 37°C for 1.15 hours and changed to complete media. The second heat shock was performed at 48 hours post-transfection and cells were allowed to grow. At 72 hours post-transfection, low molecular weight DNA was recovered as described by Hirt (1967). A half portion of this DNA was *BamH*I digested for reaction mediated by ϕC31 integrase or *Mlu*I digested for reaction mediated by R4 integrase to reduce the background of unreacted DNA. DNA was then electroporated into competent DH10B *E. coli* cells (Invitrogen, Carlsbad, CA) and spread on LB plates containing 25 μg/ml of chloramphenicol and 50 μg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) to select for white recombinant plasmids reacted by ϕC31 integrase and screened by primers 5′-GGCGAACGTGGCGAGAAAGG and 5′-GGAAACCTGTCGTGCCAGCTG and the white recombinant plasmids reacted by R4 integrase are screened by primers 5′-CAGCTGGCACGACAGGTTTCC and 5′- CCTTTCTCGCCACGTTCGCC. PCRs on white bacterial colonies contained 200 nM forward and reverse primers, 2.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 mM dNTPs and 200 pg of either *in vitro*-reacted pBCPB+ or *in vitro*-reacted pBC-P64-B295 plasmid DNA as a positive control. PCR was conducted as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, 60.3°C for 30 seconds, 72°C for 30 seconds. A final extension period of 72°C for 7 minutes then was performed. PCR products were analyzed on 1.5% agarose (SIGMA-ALDRICH). The intramolecular integration frequency was determined as the PCR-screened positive white colonies divided by the total number of colonies X 100.

Results

Transfection efficiencies of each cell line measured by firefly luciferase expression

We determined the relative transfection efficiencies for each insect cell line using either TransFectin or DOTAP and a firefly luciferase reporter plasmid (Table 1). Luciferase counts were 70 to 100-fold greater for S2 cells compared to the other insect cell lines transfected with the same reagent. DOTAP yielded 9-fold higher transfection efficiency for C6/36 cells than TransFectin based on relative luciferase counts, but displayed decreased transfection efficiency in Aag2 relative to TransFectin. The transfection efficiency results demonstrated TransFectin was most efficient for Aag2, ATC-10, BmN4, S2, Sf9 and Sua5B cell lines, while DOTAP was most effective for C6/36 cells.

Table 1 Transfection efficiency measured by firefly luciferase assay.

Cells	TransFectin	DOTAP
	Mean ± SE	Mean ± SE
S2	1.10×10^5 ± 1,605.66	5.7×10^4 ± 1,908.89
Sf9	1.10×10^3 ± 13.22	7.34×10^2 ± 3.61
Sua5B	2.44×10^2 ± 10.85	2.37×10^2 ± 6.62
ATC-10	7.65 ± 0.24	1.12 ± 0.04
Aag2	5.46 ± 0.31	3.2×10^{-3} ± 0.0002
C6/36	4.68 ± 0.06	4.2×10^1 ± 0.50
BmN_4	6.8×10^{-1} ± 0.07	6.6×10^{-1} ± 0.03

Each value is the average of the three independent experiments, with the standard error.

Intramolecular recombination assay for ϕC31 integrase activity

We developed an intramolecular recombination assay to assess the activities of ϕC31 and R4 integrases in a variety of insect cells. For the ϕC31, we used the recombination assay plasmid, pBCPB+, containing ϕC31 *attP* and *attB* sites in direct orientation that flanked a *lacZ* gene driven by the native *lacZ* promoter on a chloramphenicol-resistant ColE1 derivative (Figure 1A). The helper plasmid for the assay expressed the ϕC31 integrase under the control of a minimal heat shock promoter (Lis *et al*, 1983), phsp-Int (Figure 1B). These plasmids were co-transfected into each of the 7 insect cell lines being examined and were subjected to heat shock for 1.15 hours at 37°C at 24 and 48 hours post-transfection to induce integrase expression. Low molecular weight DNA was harvested at 72 hours post-transfection and a half portion of the recovered DNA was *BamH*I digested to reduce the background of unreacted plasmids prior to bacterial transformation.

Site-specific recombination mediated by ϕC31 integrase resulted in deletion of the *lacZ* gene from plasmid pBCPB+ (Figure 2B), yielding plasmids

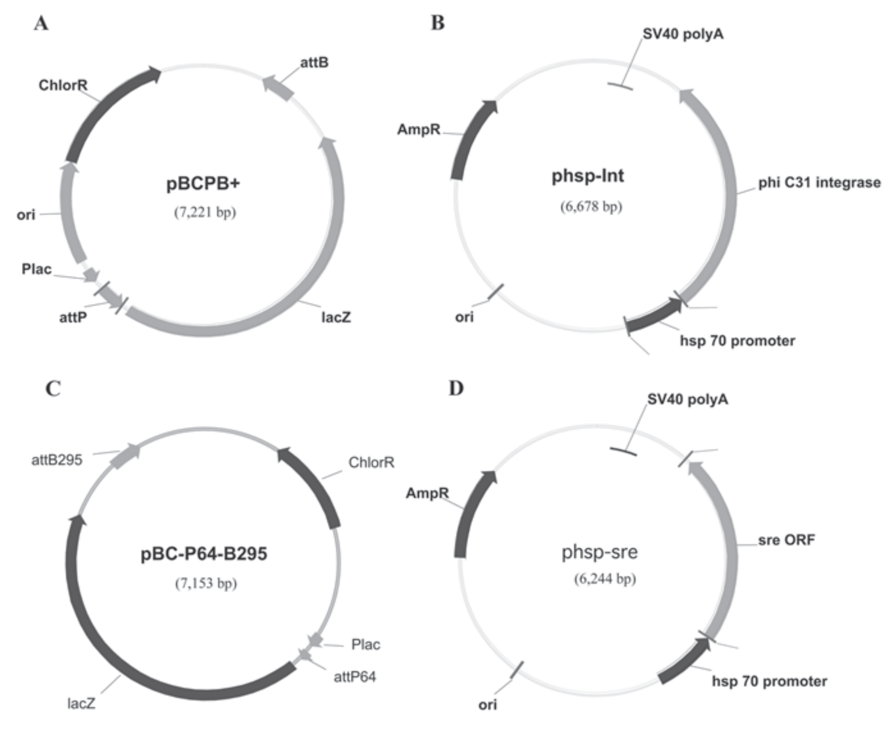


Figure 1 Plasmids used to monitor the ϕC31 and R4-mediated site-specific recombination in 7 insect cells. (A) pBCPB+, intramolecular integration assay vector used in reaction mediated by ϕC31. (B) phsp-Int, a plasmid for expression of ϕC31 integrase. (C) pBC-P64-B295, intramolecular integration assay vector used in reaction mediated by R4. (D) phsp-sre, a plasmid for expression of R4 integrase.

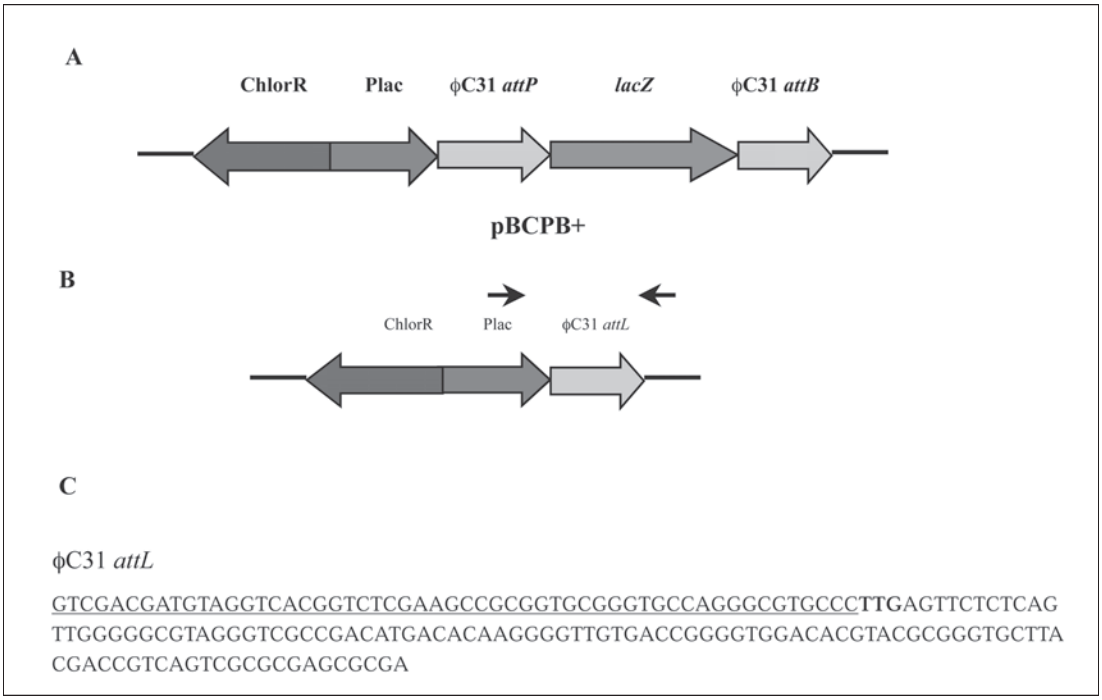


Figure 2 Schematic diagram of φC31-mediated site-specific recombination and DNA sequence. (A) plasmid pBCPB+ was used to assay φC31 integrase-mediated intramolecular integration in a variety of insect cells. DNA fragments of 285-bp φC31 *attB* and 221-bp φC31 *attP* flank *LacZ* gene driven by *lac* Z promoter (Plac). (B) φC31-mediated site-specific recombination resulted in deletion of a *LacZ* gene in pBCPB+. The φC31 *attL* junction is derived from φC31-mediated recombination between φC31 *attB* and φC31 *attP* sites. Arrows show the location of PCR primers that amplify 600 bp fragment specific for φC31 *attL*. (C) φC31 *attB*-derived flanking sequences are underlined and 3-bp common core region between φC31 *attB* and φC31 *attP* in which crossovers occur is shown in bold.

that produced white colonies on chloramphenicol/X-Gal plates upon transformation of DH10B *E. coli* cells. However, the simple digestion and plating assay resulted in a high background of white colonies due to unexpectedly high transfection-associated mutation of the plasmid that was not specific for the *att* site. We therefore verified the site-specific recombination by colony PCR with specific primers that amplified a 600-bp product from the recombinant (Figure 3). DNA sequences of these PCR products were determined to confirm φC31 site-specific integration events (Figure 2C).

φC31 integrase catalyzed intramolecular recombination between φC31 *attB* and *attP* sites in all 7 insect cells (Table 2). The highest recombination frequency was obtained in S2 cells which exhibited a 2-18-fold greater frequency compared to that of other insect cells. The increased frequencies of recombination events recovered in our assays did not necessarily correspond with the relative

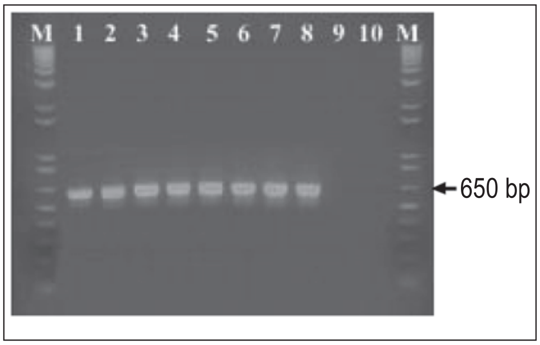


Figure 3 PCR analysis of ϕC31-mediated site-specific recombination in a variety of insect cells. A 600-bp product is shown for the site-specific recombination. Lane 1, positive control, *in vitro*-reacted pBCPB+; lane 2, *in vitro*-reacted pBCPB+ from Aag2 cells; lane 3, *in vitro*-reacted pBCPB+ from ATC- 10 cells; lane 4, *in vitro*-reacted pBCPB+ from BmN4 cells; lane 5, *in vitro*-reacted pBCPB+ from C6/36 cells; lane 6, *in vitro*reacted pBCPB+ from S2 cells; lane 7, *in vitro*-reacted pBCPB+ from Sf9 cells; lane 8, *in vitro*-reacted pBCPB+ from Sua5B cells; lane 9, negative control, *in vitro*-transfected pBCPB+; lane 10, negative control, *in vitro*- transfected phsp-Int; lane M, size markers.

transfection efficiencies for each of the cell lines, suggesting these data do reflect relative activities to some extent recombination.

Intramolecular recombination assay for R4 integrase activity

We used the plasmid pBC-P64-B295, bearing R4 *attP* and *attB* sites flanking a *lacZ* gene (Figure 1C) as the reporter plasmid for R4 integrase activity, with a helper plasmid expressing the R4 integrase under the control of minimal heat shock promoter (Figure 1D). These plasmids were co-transfected into each of the cell lines followed by heat shock at 24 and 48 hours post-transfection to induce integrase expression. Low molecular weight DNA was harvested at 72 hours post-transfection and a half portion of the recovered DNA was *Mlu*I digested to reduce the background of unreacted plasmids, and putative recombination positive white colonies were recovered following transformation of DH10B *E. coli* cells by plating on chloramphenicol/ X-Gal plates. As with the previous recombination assay, R4 integrase-mediated site-specific recombination resulted

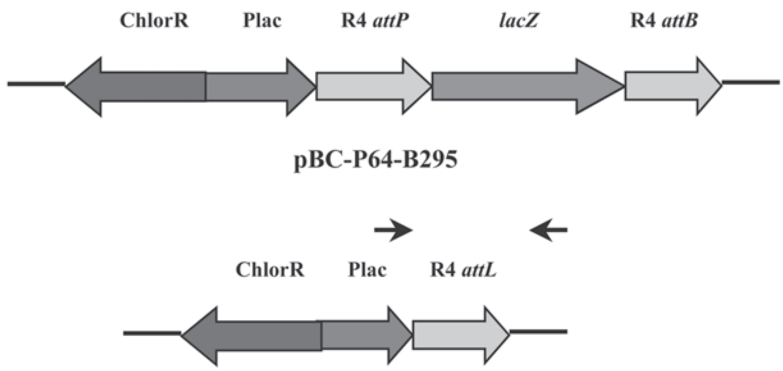
Table 2 Integration frequency in a variety of insect cells.

Cells	% integration frequency mediated by ϕC31 integrase	% integration frequency mediated by R4 integrase
S2	1.65[a] ± 0.2	1.16[a] ± 0.19
C6/36	0.98[b] ± 0.16	0.72[b] ± 0.11
Sua5B	0.24[c] ± 0.06	0.73[b] ± 0.19
ATC-10	0.39[c] ± 0.11	0.64[b] ± 0.12
BmN_4	0.40[c] ± 0.08	0.19[c] ± 0.06
Sf9	0.36[c] ± 0.05	0.13[c] ± 0.04
Aag2	0.09[c] ± 0.03	0.19[c] ± 0.09

Each value is the average of the three independent experiments, with the standard error. Average followed by the different letter is statistically significant ($p < 0.001$).

A

B



C

R4 attL

CGTGGGACGCCGTACAGGGACGTGCACCTCTCCCGCTGCACCGCCTCCAGCGTCGCCGCCGGCTCGAAGGACG
GGGCCGGGATGACGATGCAGGCGGCGTGGAGGTGGCGCCCAAGTTGCCCATGACCATGCC**GAAGCAGTGGTA**
CTGCTTGTGGGTACACTCTGCGGGTG

Figure 4 Schematic diagram of R4-mediated site-specific recombination and DNA sequence. (A) plasmid pBC-P64-B295 was used to assay R4 integrase-mediated intramolecular integration in a variety of insect cells. DNA fragment of 295-bp R4 *attB* and 64-bp R4 *attP* flank *LacZ* gene driven by *lac* Z promoter (Plac). (B) R4-mediated site-specific recombination resulted in deletion of a *LacZ* gene in pBC-P64-B295. The R4 *attL* junction is derived from R4-mediated recombination between R4 *attB* and R4 *attP* sites. Arrows show the location of PCR primers that amplify 678 bp-fragment specific for R4 *attL*. (C) R4 *attB*-derived flanking sequences are underlined and 12-bp common core region between R4 *attB* and R4 *attP* in which crossovers occur is shown in bold.

in deletion of the *lacZ* gene (Figure 4B), but the assay resulted in a high background of white colonies, necessitating PCR screening with specific primers that amplified a 678-bp product from the recombinant (Figure 5) and sequencing confirmation to recover R4 site-specific integration events (Figure 4C).

R4 integrase catalyzed recombination between its *attB* and *attP* sites in all 7 insect cells. The S2 cells displayed recombination frequency 2-9-fold greater than that observed for all other insect cells. Once again, the relative recombination frequencies for this integrase in each of the cell lines did not necessarily correspond with the relative transfection efficiencies, suggesting these frequencies were at least partly related to the activity of R4 integrase within the given cell line.

Control assay for recombination in transformed bacterial cells

While both ϕC31 and R4 integrases are active in bacterial cells, neither should be expressed within bacteria from our heat shock promoter helper

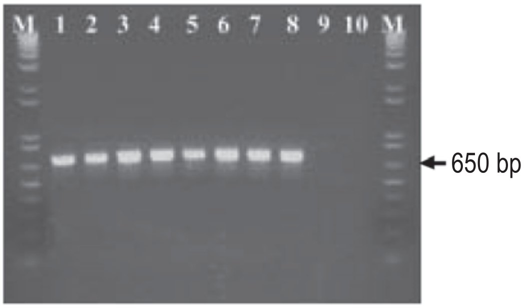


Figure 5 PCR analysis of R4-mediated site-specific recombination in a variety of insect cells. A 678-bp product is shown for the site-specific recombination. Lane 1, positive control, *in vitro*-reacted pBC-P64-B295; lane 2, *in vitro*-reacted pBC-P64-B295 from Aag2 cells; lane 3, *in vitro*-reacted pBC-P64-B295 from ATC-10 cells; lane 4, *in vitro*-reacted pBC-P64-B295 from BmN4 cells; lane 5, *in vitro*- reacted pBC-P64-B295 from C6/36 cells; lane 6, *in vitro*-reacted pBC-P64-B295 from S2 cells; lane 7, *in vitro*-reacted pBCP64-B295 from Sf9 cells; lane 8, *in vitro*-reacted pBC-P64-B295 from Sua5B cells; lane 9, negative control, in *vitro*-transfected pBC-P64-B295; lane 10, negative control, *in vitro*- transfected phsp-sre; lane M, size markers.

plasmids. To assure that the ϕC31 integrase-mediated and R4 integrase-mediated site-specific integration events that we were detecting actually resulted from recombinations within the transfected insect cells and not upon transformation of the bacteria, we coelectroporated the integrase and assay plasmids directly into DH10B *E. coli* and examined the resulting colonies for evidence of recombination events. No white colonies were present on ampicillin/chloramphenicol/X-Gal plates out of total colonies recovered confirming that the site-specific recombination events recovered in our assays could not have arisen from recombination in bacterial cells following harvesting plasmids from the transfected insect cells.

Discussion

Integrase activity assays are greatly influenced by the ability to effectively transfect the plasmid DNA into target cells. A number of diverse methods have been explored for introducing DNA into eukaryotic cells including the use of calcium phosphate or other divalent cations, polycations, liposomes, retroviruses, microinjection and electroporation. An important addition to repertoire of DNA-transfection methodologies is cationic liposome-mediated transfection (lipofection) such as TransFectin Lipid Reagent and DOTAP liposomal Transfection Reagent. For this study, we tested the abilities of these liposome reagents to mediate transfection of firefly luciferase-expressing plasmids into seven of the most widely used insect cell lines.

The promoters chosen for the firefly luciferase (Fluc) reporter plasmids were based upon previous observations of promoter activity in a variety of cell lines (Burn *et al*, 1989; Han *et al*, 1989; Chung and Keller, 1990; Huynh and Zieler, 1999; Zhao and Eggleston, 1999). For most cell lines in this study the *Drosophila* actin 5C promoter proved adequate in reporting firefly luciferase activity. However, this promoter was inadequate for expressing Fluc in *Bombyx mori* cells (data not shown) and we chose instead to utilize the *B. mori* actin 3C promoter previously characterized (Zhang *et al*, 2008).

The transfection frequency obtained with TransFectin was greater for most insect cell lines with the notable exception of the mosquito C6/36 cell line. There are several possibilities that make the difference of both transfection frequencies. It might have to optimize transfection conditions of both transfection methods and cell types including cell density, duration of transfection, volume of medium during transfection and ratio of lipid reagent to DNA that are key factors for transfection efficiency. For TransFectin, it is a mixture of a proprietary cationic lipid and colipid DOPE (1,2-dioleoyl-phosphatidylethanolamine). When cationic lipids are brought into contact with aqueous solutions under special conditions, they form positively charged micelles or liposomes. These micelles associate with the negatively charged phosphates of nucleic acids and form spontaneous complexes with DNA or RNA. The DNA-liposome complexes then fuse with cell membrane via hydrophobic and electrostatic interactions, and the complex is then internalized (Remy *et al*, 1994; Zhou and Huang, 1994). For DOTAP, it is aqueous dispersion (liposomes) in MBS (MES-buffered saline) that there might be some differences from TransFectin in formulation like colipid. In various reported studies, cationic liposomes function most efficiently when cationic lipid is mixed with a helper lipid or colipid like DOPE that is most commonly used in applications (Legendre and Szoka, 1993; Felgner *et al*, 1994; Farhood *et al*, 1995). DOPE is generally believed to rest on its propensity to form nonbilayer structures that are akin to membrane fusion intermediates (Hui *et al*, 1981). It is thought to help facilitate the fusion of cationic liposome in DNA-cationic liposome complexes to cell membrane. Therefore, the fusion between DNA-cationic liposomes and the cell membrane appeared from the use of TransFectin might play better role in gene delivery, expression including in determining transfection efficiency for most insect cells except C6/36 than that occurred from DOTAP. For C6/36 cell transfection, DOTAP works better than TransFectin. It might be because of cell membrane constituents and properties of C6/36 cells to be the critical barriers of cationic liposome-mediated gene delivery. DOTAP formulation might provide the DNA-cationic liposome complexes to better fuse with C6/36 cell type than other cell types resulting in being more taken by cells. Based on the transfection efficiency results, TransFectin was chosen for DNA transfection of Aag2, ATC-10, BmN4, S2, Sf9 and Sua5B cell lines while DOTAP was used only for C6/36 transfection.

Previous work had shown that the serine-catalyzed ϕC31 integrase could occasionally integrate *attB* into endogenous sequences with partial sequence identity to *attP*, pseudo-*attP* sites of *Drosophila* (Groth *et al*, 2004), bovine cells (Ma *et al*, 2006; Ou *et al*, 2009) and mammalian cells (Thyagarajan *et al*, 2001). The genomic integration mediated by ϕC31 integtrase was also detected by long-term expression of luciferase over the 4-week time course (Thyagarajan *et al*, 2001; Thyagarajan and Calos, 2005). This ability of ϕC31 integrase to integrate into pseudo *attP* sites has been used in gene therapy experiments in mouse, human keratinocytes (Olivares *et al*, 2002; Ortiz-Urda *et al*, 2002, 2003; Quenneville *et al*, 2004; Held *et al*, 2005; Bertoni *et al*, 2006), and rat retina (Chalberg *et al*, 2005). The ϕC31 integrase system also produces stable transgene expression in adult mouse neural progenitor cells (mNPCs) and their progeny that may be useful in strategies for combating neurodegenerative disorders (Keravala *et al*, 2008).

Previous studies have demonstrated the potential for ϕC31 integrase to mediate site-specific recombination in some insect species. This integrase mediated site-specific integration of two ϕC31 *attP* flanking DsRed gene into the cultured silkworm cell line, BmN4, in which ϕC31 *attB* sites inserted between a baculovirus IE2 promoter and a polyadenylation signal are present in one chromosome (Nakayama *et al*, 2006). ϕC31 integrase was employed to target the *P* element- predetermined *attP* sites in transgenic *Drosophila* by injecting integrase mRNA together with ϕC31 *attB*, resulting in up to 55% of fertile adults producing transgenic offspring (Groth *et al*, 2004). The ϕC31 integrase system was used in conjunction with recombinase-mediated cassette exchange (RMCE) for precise targeting of transgenic construct to predetermined genomic sites in *Drosophila* that the efficient RMCE events were observed at all four genomic target sites tested and the transgene lacking a visible marker can be integrated with high efficiency by selecting only for a phenotypic marker in genomic target (Bateman *et al*, 2006, Bateman and Wu, 2008). It has also been shown as improved current method to reproducibly produce transgenic *Ae. aegypti* in range of 17-32% (Nimmo *et al*, 2006).

Actually, transgenesis has been performed through many techniques. In *Drosophila*, transgenesis mostly relies on *P* element transposon. This transposon was introduced and has been one of the most important breakthroughs in germ line transgenesis in *Drosophila*. *P* element belongs to a group of transposable elements (designated class II, Finnegan, 1992). It contains two terminal repeats, including inverted repeat sequences and other internally located sequences motifs absolutely required for their mobilization and transposition (Beall and Rio, 1997).

Attempts to transform *Aedes* mosquitoes began in the late 1980s using the *P* element from *D. melanogaster* (Morris *et al*, 1989). This element would mobilize and integrate into the mosquito genome. The result of transformed lines containing exogenous constructs was obtained after microinjecting embryos with plasmid DNA. However, transformed lines were obtained at frequencies of less than 0.1% of the embryos injected and transformation appeared not be *P*-element mediated, but to result from illegitimate recombination. The low frequency of integration precludes the use of this approach for the routine transformation of mosquitoes.

Because of the failure of *P* element, many other methods of transformation have been developed. Transformation of *Ae. aegypti* and other mosquitoes was made possible by the isolation and characterization of additional class II transposable elements such as *Hermes*, *Mariner*, *Minos* and *piggyBac* (Franz and Savakis, 1991; Medhora *et al*, 1991; Atkinson *et al*, 1993; Fraser *et al*, 1996). These transposons were first discovered in insects, all except *piggyBac* belong to large families of elements that appear widespread throughout eukaryotes. Having learned a lesson from the many failed experiments with *P* element, researchers first tested these new elements for mobility with *in vivo* transposition assays. These experiments involve microinjecting embryos with three different plasmids, a donor that contains a marker gene flanked by the ITRs (inverted terminal repeats) of the transposable element, a target that carries a number of marker genes distinct from the donor, and a helper that contains the appropriate transposase under the control of an inducible promoter (O'Brochta and Handler, 1988). Induction of the transposase mobilizes the marker gene construct from the donor to the target. Successful transposition assays in *Ae. aegypti* embryos were reported for *Hermes* (Sarkar *et al*, 1997) and *Mariner* (Coates *et al*, 1998).

The *piggyBac* element is the only identified mobile member of a larger family of what are now called TTAA-specific elements (Fraser, 2000). This short inverted terminal repeat element is 2.5 kb long, having a paired inverted terminal repeat configuration, a 2.1 kb ORF and specificity for the target sequence TTAA which it duplicates upon insertion and precisely regenerates upon excision (Cary *et al*, 1989; Elick *et al*, 1996; Fraser *et al*, 1996). Both excision and interplasmid transposition assays indicate that this element is mobile in a wide variety of insects (Lobo *et al*, 1999; Lobo N, unpublished data) suggesting a ubiquitous mechanism of mobilization.

piggyBac has an experimentally demonstrated capability to mediate germ-line transformation in a myriad of insects including the Mediterranean fruit fly, *Ceratitis capitata* (Handler *et al*, 1998), the pink boll-worm, *Pectinophora gossypiella* (Peloquin *et al*, 2000), *D. melanogaster* (Handler and Harrell, 1999), the silkworm, *B. mori* (Tamura *et al*, 2000), the Caribbean fruit fly, *Anastrepha suspense* (Handler and Harrell, 2001), the Oriental fruit fly, *Bactrocera dorsalis* (Handler and McCombs, 2000), the housefly, *Musca domestica* (Hediger *et al*,

2001) and the mosquitoes, *Ae. aegypti* (Lobo *et al*, 1999, 2001, 2002; Kokoza *et al*, 2001; Nimmo *et al*, 2006; Sethuraman *et al*, 2007), *Ae. albopictus* (Lobo *et al*, 2001), *Ae. triseriatus* (Lobo *et al*, 2001), *An. gambiae*, (Grossman *et al*, 2001), *An. albimanus* (Perera *et al*, 2002) and *An. stephensi* (Nolan *et al*, 2002).

In this study, we demonstrated the ability of ϕC31 integrase to mediate unidirectional site specific recombination in 5 Dipteran cell lines *Ae. aegypti* Aag2, *Ae. aegypti* ATC-10, *Ae. albopictus* C6/36 , *An. gambiae* Sua5B, as well as *D. melanogaster* S2. We also demonstrated the effectiveness of this integrase in two Lepidopteran cell lines *B. mori* BMN4, and *S. frugiperda* Sf9. Based upon these and previous analyses, there is ample evidence that this integrase can provide for effective integration in virtually any insect species.

This study also establishes that the R4 integrase derived from *S. parvulus* phage R4, like the ϕC31 integrase, mediates site-specific recombination in the 7 assayed insect cell lines. As with ϕC31, the R4 integrase has wide effective host range (Olivares *et al*, 2001). The ability of these integrases to function in such a wide range of species is directly related to their lack of cofactor requirement (Thrope and Smith, 1998).

Because the transfection abilities varied greatly among the cells examined, it is impossible to directly compare the relative effectiveness of ϕC31 integrase among these cells. However, there may be a correlation between the relative recombination frequencies and expression of the integrase from the hsp70 promoter. According to our results these integrases seem to perform best in *Drosophila* S2 cells, exhibiting a statistically significant higher integration frequency. This may be due to the relative hsp70 promoter activity in each of these cell lines. The hsp70 promoter was first discovered as a heat-induced puff on *Drosophila* polytene chromosomes (Ritossa, 1962). Since hsp70 promoter originated from *Drosophila* as a strong promoter, it is most commonly used promoter for transgenic work in *D. melanogaster* and with *Drosophila* cell lines (Cobreros *et al*, 2008; Siddique *et al*, 2008; Singh *et al*, 2008). It is heat-inducible and functions at all stages of development and in all *Drosophila* tissues (Di Nocera and Dawid, 1983; Steller and Pirrotta, 1984; Spradling, 1986; Lindquist and Craig, 1988). The hsp70 promoter has been widely used in mosquito cells (Lycett and Crampton, 1993; Shotkoski *et al*, 1996; Zhao and Eggleston, 1999), transgenic *Ae. aegypti* (Pinkerton *et al*, 2000; Nimmo *et al*, 2006; Sethuraman *et al*, 2007), *An. gambiae* cells and embryos (Miller *et al*, 1987; Windbichler *et al*, 2007) and lepidopteran cell lines and larvae (Helgen and Fallon, 1990; Morris and Miller, 1992; Plymale *et al*, 2008). Also, it was employed in driving the gene expression in *S. frugiperda* Sf9 cells (Sah *et al*, 1999) and transgenic silkworm, *B. mori*, (Suzuki *et al*, 2005; Dai *et al*, 2007). Because of its applicability in many insects, we chose hsp70 promoter to drive the expression of integrase genes for mediating the site-specific integration in a variety of insect cell lines. Phage ϕC31 and R4 integrases may come to represent a powerful site-specific integration tools for genetic manipulation of medically and economically important organisms.

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Outbreak of Chikungunya Fever in Thailand and Virus Detection in Field Population of Vector Mosquitoes, *Aedes aegypti* (L.) and *Aedes albopictus* Skuse (Diptera: Culicidae)

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Abstract. We investigated chikungunya fever outbreak in the southern part of Thailand. Human plasma specimens obtained from suspected patients and adult wild-caught mosquitoes were detected for chikungunya virus employing reverse transcriptase polymerase chain reaction technique. Chikungunya virus was detected in about half of the blood specimens whereas a range of 5.5 to 100% relative infection rate was found in both sexes of the vector mosquitoes, *Aedes aegypti* (L.) and *Ae. albopictus* Skuse. The infection rate in *Ae. albopictus* was higher than in *Ae. aegypti*, with relative infection rate in male of both species being higher than in female. The appearance of chikungunya virus in adult male mosquitoes of both species reveals a role of transovarial transmission of the virus in field population of the mosquito vectors. These findings have provided further understanding of the relationship among mosquito vectors, chikungunya virus and epidemiology of chikungunya fever in Thailand.

Introduction

Chikungunya fever is an arthropod-borne disease caused by chikungunya virus (Family: Togaviridae, Genus: *Alphavirus*) and is transmitted to humans by the bite of infected mosquitoes. Chikungunya virus was first isolated from man and mosquito during an epidemic of fever in Newala, Tanzania between 1952 and 1953 (Ross, 1956). Two species of *Aedes* mosquitoes, *Ae. aegypti* (L.) and *Ae. albopictus* Skuse, are well recognized as vectors of this disease (McIntosh and Gear 1981; Gratz, 2004; Vazeille *et al*, 2007; Reiskind *et al*, 2008). However, many studies have revealed that *Ae. albopictus* shows a higher susceptibility to chikungunya virus and more efficiency to transmit the virus than *Ae. aegypti* (Mangiafico, 1971; Turell *et al*, 1992; Yamanishi, 1999; Schufferenecker *et al*, 2006; Vazeille *et al*, 2007, 2008). Chikungunya fever is rarely life-threatening and milder than dengue infection as it has no severe hemorrhage manifestations or shock (Nimmannitya and Mansuwan, 1966). When compared with dengue infection, chikungunya fever seems to be more acute (short onset of illness) and predominant in high fever (with short duration), erythematous maculopapular eruption, headache and muscle pain (Nimmannitya and Mansuwan, 1966). The disease also frequently causes rash and severe arthralgia (joint pain without inflammation) or arthritis (joint pain with inflammation), which sometimes persisted for weeks to months (Thaikruea *et al*, 1997). Until now, no vaccine is available against chikungunya viral infection.

Sporadic outbreaks of chikungunya fever were reported from many countries in Asia since late 1950s. Since then, chikungunya virus has been isolated or detected in Thailand in 1958 (Hammon *et al*, 1960), and subsequently in Cambodia (Chastel, 1963) and India (Sarkar *et al*, 1964) in 1963, Vietnam (Dai and Kim-Thoas, 1967) and Sri Lanka (Hermon, 1967) in 1967, the Philippines in 1969 (Campos *et al*, 1969), Myanmar in 1970 (Khai *et al*, 1974), Malaysia in 1978 (Marchette *et al*, 1978) and Indonesia in 1982 (Slemons *et al*, 1984). Since 2000, indigenous and imported cases of chikungunya fever have been reported from several countries in various continents, including Africa (Congo, Gabon and Kenya), Asia (India, Sri Lanka, Indonesia, Malaysia, Singapore and Thailand), Europe (Italy, France, Germany, Norway and Spain) and some islands in the Indian Ocean (Comoros, Madagascar, Mauritius, Mayotte, La Reunion and Seychelles) (WHO, 2007). In 2004, the disease affected almost 500,000 people in Africa (Epstein, 2007). Recently, chikungunya fever affected 266,000 cases (approximately one third of total population) in La Reunion Island during the period from February 2005 to June 2006, and about 1.42 million cases in India during the epidemic between January 2006 and August 2007 (WHO, 2007).

The first record of chikungunya fever in Thailand as well as in Southeast Asia was found in Bangkok in 1958 by virus isolation from blood specimens collected from patients during the epidemic of dengue fever and dengue hemorrhagic fever (Hammon *et al*, 1960). In 1962, 160 blood specimens out of 815 patients with hemorrhagic fever admitted to the Children's Hospital, Bangkok, were randomly selected for virus isolation and serological studies and 135 cases were confirmed as dengue (98 cases), chikungunya (29) and possible double infection (8) (Nimmannitya and Mansuwan, 1966). Chikungunya fever disappeared from Thailand for about 14 years until some cases were reported from Prachin Buri in 1976. The disease reemerged in the country with reported cases of chikungunya fever in 1988 from Surin, in 1991 from Khon Kaen, in 1993 from

Loei and Phayao, and in 1995 from Nong Khai and Nakhon Si Thammarat. Since August 2008, chikungunya fever has re-emerged again in Thailand with several thousands of reported cases from at least 47 provinces of Thailand. This paper provided information of a recent incidence of chikungunya fever in Thailand together with data of viral infection in vector mosquitoes conducted in Songkhla Province, a particular area with high inci-dence of the disease in southern Thailand.

Materials and methods

Incidence of chikungunya fever in Thailand

The data of reported cases of chikungunya fever in Thailand between January 1, 2008 and June 30, 2009 were obtained from the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand.

Collection of blood specimens

Blood specimens were taken from suspected patients who were admitted to hospitals in various provinces of Thailand, including Songkhla Province. Blood samples were drawn into test tubes containing EDTA as anticoagulant, and centrifuged to obtain plasma, which were kept in liquid nitrogen and then transported to the Arbovirus Section, National Institute of Health, Department of Medical Sciences, Nonthaburi, Thailand for determination of chikungunya infection.

Virus detection in blood specimens

Viral RNA was extracted from 100 µl of patient plasma using the QIAamp viral RNA mini kit (QIAGEN, Germany) following the manufacturer's protocol. The procedures for chikungunya virus (CHIKV) detection in plasma followed the methods described by Parida *et al* (2007) with minor modifications. In brief, one-step reverse transcriptase polymerase chain reaction (RT-PCR) was performed using a primer pair of CHIKV E1 gene [CHIK-F3 (ACGCAATTGAGCGAAGCAC) (genome position 10294 to 10312) and CHIK-B3 (CTGAAGACATTGGCCCCAC) (10498 to 10480)]. Amplification was carried out in a 25 µl total reaction volume using Superscript III one-step RT-PCR kit (Invitrogen, USA) with 50 pmol of each primer and 2 µl of RNA. Thermal cycling of RT-PCR was 48°C for 30 minutes and 94°C for 2 minutes, followed by 35 cycle of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute and a final extension cycle at 72°C for 10 minutes. RT-PCR products were detected by electrophoresis in 2% agarose gel.

Study site for mosquito collection

Songkhla, a province of southern Thailand was selected as the study site. This province has been recognized as an area with high incidence of chikungunya since 2008 and it also has two species of mosquito vectors, *Ae. aegypti* and *Ae. albopictus*. Eight villages from two districts, Muang and Hat Yai (four villages from each district), were randomly selected. At least 20 houses from each village were randomly chosen for collection of adult mosquitoes.

Mosquito collection

Mosquito collection was carried out at the study sites during late January 2009. The dwellings for mosquito collection were randomly selected from the eight villages of the study sites. Eight volunteers collected mosquitoes indoors for 20 minutes in each dwelling. The collectors usually situated themselves in dark areas of the room where most mosquito landing and biting activities occur. The collectors bared their legs between knee and ankle and collected all landing mosquitoes individually in vials capped. Using a similar procedure, mosquito collecting was also conducted outdoors (approximately 5-10 m away from dwellings) to catch *Ae. albopictus* in the same environment. Mosquito collection was usually carried out from 9:00 AM to 5:00 PM. The collected mosquitoes were visually identified, as there were only two species, *Ae. aegypti* and *Ae. albopictus*, present. These live mosquitoes were inactivated by placing in a refrigerator, and then separated by species, sex and locality. Pools were stored in liquid nitrogen for subsequent chikungunya viral detection. A maximum of 5 mosquitoes were placed in each pool.

Virus detection in mosquitoes

In each pool, mosquito wings and legs were removed and the remaining bodies were ground in the lysis solution provided with the test kit and centrifuged. The supernatant was then processed for RNA extraction as described above.

Results

In 2008, a total of 2,233 cases of chikungunya fever were reported from 4 provinces of southern Thailand, Narathiwat, Pattani, Yala and Songkhla. The first official report of chikungunya fever in Thailand in 2008 was at week 33 (August 10-16, 2008) from Narathiwat. The reported cases of chikungunya fever increased gradually week by week until the end of 2008 with the highest incidence of about 400 cases per week (Fig 1). However, chikungunya fever has increased dramatically from the first week of 2009 with an incidence of about 1,040 cases. The incidence of chikungunya fever in 2009 has fluctuated between 463 and 2,068 cases per week with 3 peaks appearing at week 4 (1,791 cases),

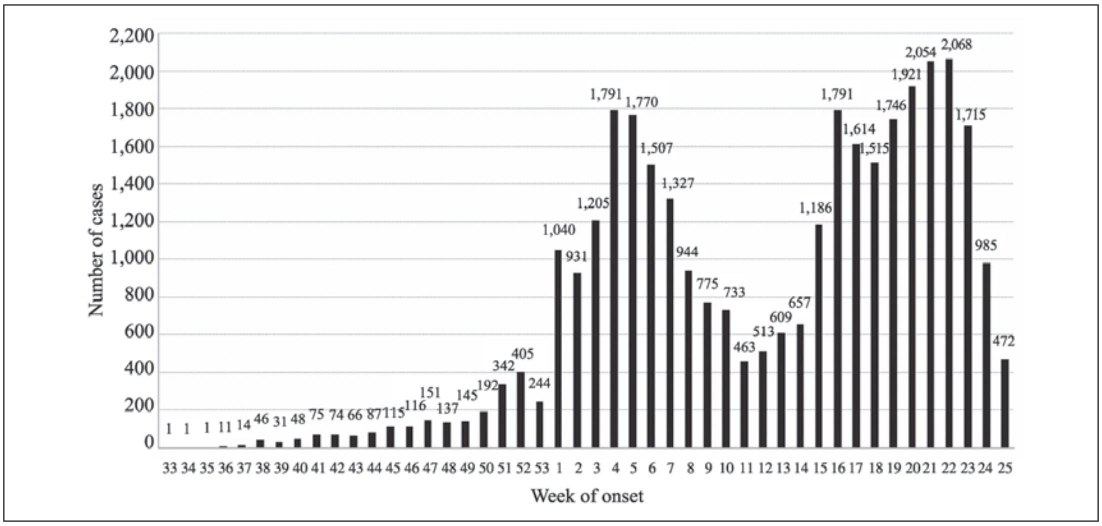

Figure 1 Number of reported cases of chikungunya fever in Thailand by week of onset between August 2008 and June 2009.

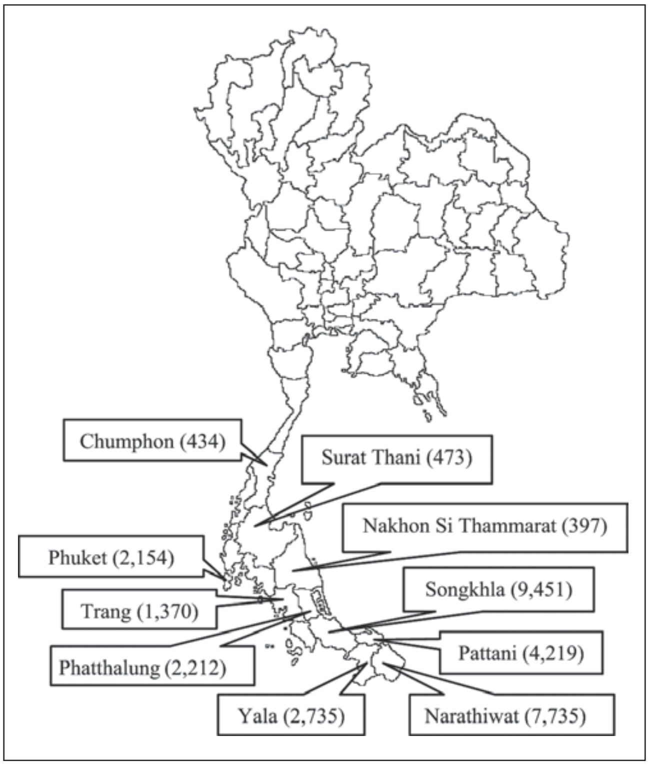

Figure 2 Map of Thailand showing 10 provinces with high reported cases of chikungunya fever from January 1 to June 30, 2009.

week 16 (1,791 cases) and week 22 (2,068 cases) (Fig 1). A total of 32,102 cases of chikungunya fever were reported from 47 out of 76 provinces of Thailand during January 1 and June 30, 2009. Among these, 31,768 cases (98.96%) were from the southern region (14 provinces) whereas those from central (14 provinces), north (9 provinces) and northeastern region (10 provinces) were 137 cases (0.43%), 129 cases (0.40%) and 68 cases (0.21%), respectively. The top-ten highest incidence were reported from Songkhla (9,451 cases), followed by those from Narathiwat (7,735 cases), Pattani (4,219 cases), Yala (2,735 cases), Phatthalung (2,212 cases), Phuket (2,154 cases), Trang (1,370 cases), Surat Thani (473 cases), Chumphon (434 cases) and Nakhon Si Thammarat (397 cases), all located in southern Thailand (Fig 2). The reported cases of chikungunya fever from the other 37 provinces ranged from 1 to 262 cases. Interestingly, only 72 cases of chikungunya fever were reported from Bangkok during this period.

Table 1 Age distribution of totally 32,102 reported cases of chikungunya fever in Thailand from January 1 to June 30, 2009.

Age (Year)	<1	1 - 6	7 - 9	10 - 14	15 - 24	25 - 34	35 - 44	45 - 54	55 - 64	>65
Proportion (%)	0.4	4.5	3.5	8.7	15.9	18.0	19.1	15.2	8.4	6.3

Table 2 Prevalence of chikungunya infection detected in blood specimens collected from patients admitted to hospitals from various provinces of Thailand and Songkhla, between October 2008 and June 2009.

Area	Year	Total test specimens	PCR result		Relative infection rate (%)
			Positive	Negative	
Thailand	2008	1,011	550	461	54.4
	2009	745	414	331	55.6
	Total	1,756	964	792	54.9
Songkhla	2008	100	36	64	36.0
	2009	69	40	29	58.0
	Total	169	76	93	45.0

Regarding age distribution, most reported cases were frequently found in patients aged 35-44 (19.1%), 25-34 (18.0%), 15-24 (15.9%) and 45-54 years (15.2%), and less than 10% were found in other age groups (Table 1). The lowest percentage of 0.4% was found in children aged less than 1 year. There was no mortality among all reported cases of chikungunya fever during the current outbreak.

From 1,756 blood specimens collected from patients admitted in hospitals from various provinces throughout Thailand during the period from October 2008 to June 2009 and determined for presence of chikungunya virus, 964 specimens (54.9%) were positive for chikungunya infection (Table 2). The relative infection rate obtained in 2008 (54.4%) was almost equal to that detected in 2009 (55.6%). Some 76 out of 169 specimens obtained from Songkhla were positive for chikungunya virus, but the infection rate (36%) in 2008 was substantially lower than that (58%) in 2009.

Mosquito collections were carried out randomly in eight villages of two districts of Songkhla Province, Mueang and Hat Yai (four villages from each one) during late January. A total of 169 houses from the eight villages were surveyed in this study. Of 192 adult mosquitoes collected from the study sites 165 were *Ae. aegypti* (41 males and 124 females) and 27 *Ae. albopictus* (2 males and 25 females), and subsequently they were pooled according to species, sex and locality of collection. There were 101 pools of *Ae. aegypti* (28 pools of males and 73 pools of females) and 17 pools of *Ae. albopictus* (2 pools of males and 15 pools of females), and all 118 pools were identified for chikungunya virus by RT-PCR assay (Fig 3 and Table 3). Nineteen out of 118 pools (16%) were positive for

Table 3 Prevalence of relative infection of chikungunya virus in *Ae. aegypti* and *Ae. albopictus* mosquitoes collected from Songkhla Province, Thailand, during late January 2009.

Mosquito species		Total test mosquitoes	Total test pools	Total positive pools	Relative infection rate (%)
Ae. aegypti	Male	41	28	6	21.4
	Female	124	73	4	5.5
	Total	165	101	10	9.9
Ae. albopictus	Male	2	2	2	100
	Female	25	15	7	46.7
	Total	27	17	9	52.9
Grand total		192	118	19	16.1

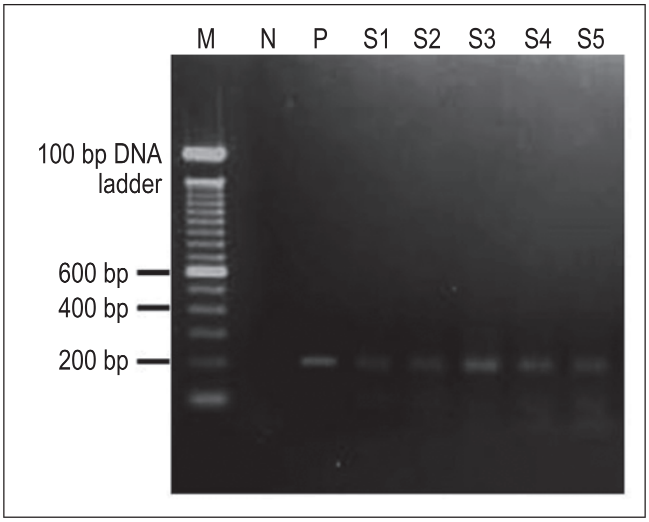

Figure 3 Gel electrophoresis of RT-PCR amplicon of chikungunya virus. RT-PCR was carried out as described in Materials and Methods. M, molecular weight marker; N, negative control; P, positive control; S1 – S5, samples positive for chikungunya virus.

chikungunya virus. Of these positive pools, 10 pools were *Ae. aegypti* (6 males and 4 females) and 9 pools were *Ae. albopictus* (2 males and 7 females). Overall, the relative infection rate of chikungunya virus in *Ae. albopictus* (53%) was higher than in *Ae. aegypti* (10%). In addition, the relative infection rate of the former species in both males (100%) and females (47%) was higher than those of the latter species, 21% and 5%, respectively.

Discussion

After the first appearance in Thailand in 1958, epidemics of chikungunya fever have re-occurred many times (in 1962, 1976, 1988, 1991, 1993, 1995 and 2008). Apparently, chikungunya fever has shown remarkable epidemiological appearance, *ie* epidemics occur and disappear periodically, with interepidemic periods of a few years and sometimes as long as more than 10 years. A long silence of 10 years or more was also observed in other countries, such as India and Malaysia. The reason for a long period of disappearance of chikungunya fever in these places is still unknown. It may be due to a broken transmission cycle of the disease between infected humans and vector mosquitoes. The chikungunya virus appears in blood circulation of the infected person only for a few days or so, and if no vector mosquitoe takes a blood meal during this viremic period, the transmission cycle then will be broken. Chikungunya fever could re-emerge when a carrier person travels and stays in a place which already has the vector mosquitoes, namely, *Ae. aegypti* and *Ae. albopictus*.

The recent outbreaks of chikungunya fever in India (2006-2007) and Thailand (2008-2009) similarly affected a larger number of people than previous epidemics. These could be due to variations in genotypic and antigenic charac teristics of chikungunya virus in the regions. Formerly, the Asian genotype of chikungunya virus was responsible for the epidemics in the whole continent of Asia, whereas the other two genotypes of the virus, West African (WA) and East Central South African (ECSA) strains, were responsible for the African countries (Arankalle *et al*, 2007). However, the ECSA genotype of chikungunya virus has been introduced into the Asian continent and was responsible for the explosive outbreak in India in 2006 (Yergolkar *et al*, 2006) and Singapore in 2008 (Leo *et al*, 2009). During the current epidemic in Thailand, similar strains of chikungunya virus, as isolated from the outbreaks in India in 2007 and in Singapore in 2008, were also found in clinical specimens collected from patients in Narathiwat (Theamboonlers *et al*, 2009), and both species of mosquito vectors were collected from Prachuab Khiri Khan (unpublished data). Regarding the variation in antigenic characteristics of chikungunya virus, a mutation at position 226 of the E1 gene with the substitution of alanine by valine was observed from virus isolated in La Reunion in 2006 (Schuffenecker *et al*, 2006) and in India in 2007 (Arankalle *et al*, 2007). This mutant strain of chikungunya virus, is also suspected to be in Thailand during the current outbreak, and studies on genomic sequencing of chikungunya virus are required.

Regarding the prevalence of chikungunya infection in blood specimens, the positive rates found in this study were relatively low, ranging from 36% to 58%. This could be due to at least three main factors affecting the results, namely, quality of blood specimens, sensitivity of detection method and misdiagnosis. The most appropriate blood specimen for viral detection should be collected from suspected patient during the acute phase of onset. Beyond this period, the possibility to detect virus from clinical specimens appears to be low, although the patient is infected with chikungunya virus. In this study, we used a conventional RT-PCR method, which has a detection limit of about 200 copy numbers, to determine chikungunya virus in blood specimens. The sensitivity of this method, however, may be insufficient to detect chikungunya virus in some specimens having low concentration of virus. Therefore, a more sensitive method to detect chikungunya virus, such as reverse transcription loop-mediated isothermal amplification (RT-LAMP) that has a detection limit of about 20 copy numbers

(Parida *et al*, 2007) should be used in future studies of chikungunya infection in clinical specimens. Among the specimens that were negative for chikungunya infection, there were also some samples positive for dengue infection. This could be due to a misdiagnosis between chikungunya and dengue infection, which has similarly clinical symptoms as it was found that there were some specimens positive for dengue infection among the specimens negative for chikungunya. However, co-infection of both viruses was also observed in this study as some specimens were positive for both dengue and chikungunya infections (data not shown). A recent report from India also reveals the co-infections with chikungunya virus and dengue virus occurred in Delhi areas in 2006 during a dengue outbreak, and these concurrent infections might result in overlapping clinical symptoms, making diagnosis and treatment difficult for physicians (Chahar *et al*, 2009).

The first reported case of chikungunya fever in Thailand in the current outbreak was found in mid-August 2008 in Narathiwat, the southernmost province of Thailand-Malaysia border. Prior to this report, there was no evidence of chikungunya fever within any area of the southern provinces or elsewhere in Thailand. During that period, a total of 1,703 cases of chikungunya fever were already present in 5 states of Malaysia and 117 cases also occurred in Singapore (Bureau of Epidemiology, 2008a,b). It is possible that the chikungunya virus was introduced into Thailand from people who traveled between the epidemic areas and Thailand during that period. Afterwards, the incidence of chikungunya fever had increased and spread to 4 southern provinces along Thailand-Malaysia border (Narathiwat, Yala, Pattani and Songkhla) in 2008 and 47 provinces throughout Thailand in 2009. This indicates the Thai people are highly susceptible to the current strain of chikungunya virus, and that the disease could spread rapidly within a short period. As seen in Fig 2, high incidences of this disease were in the southern provinces of Thailand. This could be due to the prevalent of vector mosquitoes, *Ae. aegypti* and *Ae. albopictus*, especially the latter. We have found that *Ae. albopictus* is abundant in all 14 provinces of southern Thailand, especially in such habitats as rubber plantations, palm plantations, orchards, waterfalls and public parks. Besides the southern region, *Ae. albopictus* is also found in other provinces throughout Thailand, possibly with lower abundance than in the southern provinces (Huang, 1972; Benjaphong and Chansang, 1998; Thavara, 2001).

In this study, we could collect only small numbers of *Ae. aegypti* and *Ae. albopictus*, especially the latter, because it was during the dry season with no rain when the mosquito collection was carried out. It was documented previously that *Ae. albopictus* populations are markedly suppressed during the dry season when their natural breeding sites are mostly dry, whereas *Ae. aegypti* could be present all year round since their breeding sites are human-made water-storage containers that are usually filled with water, even in the dry season (Thavara *et al*, 2001). Although the collected numbers of both mosquito species were low, the relative infection rates of chikungunya virus of the two species were quite high, especially in *Ae. albopictus*. This may imply that *Ae. albopictus* in Thailand has a higher potential to transmit chikungunya virus than *Ae. aegypti*. This is supported by a number of studies (Mangiafico, 1971; Turell *et al*, 1992; Reiskind *et al*, 2008). Genetics is likely to be one factor that controls the susceptibility of *Ae. albopictus* to chikungunya virus infection (Tesh *et al*, 1976). Recently, it was reported that the mutant strain of chikungunya virus (E1: A226V) shows a shorter incubation period in *Ae. albopictus*, which enables the mosquito to transmit the virus as early as two days after an infected blood-meal (Vazeille *et al*, 2007). However, more subsequent studies would be needed to demonstrate this capability of *Ae. albopictus* in Thailand.

It is interesting to note that chikungunya virus was also detected in males of *Ae. aegypti* and *Ae. albopictus* collected in our study from different locations. It is, therefore, an obvious evidence for the phenomenon of transovarial transmission occurring in the natural environment in the study sites in Thailand. This phenomenon is similar to that of dengue virus as described previously by Thavara *et al* (2006). Based on laboratory studies, the positive rates of infection in larvae and adult females of their progeny in *Ae. albopictus* are higher than those of *Ae. aegypti* and the infected females of *Ae. aegypti* and *Ae. albopictus* are capable of vertically transmitting chikungunya virus to their offsprings to at least the third generation (Zhang *et al*, 1993).

To control the epidemic of chikungunya fever, many measures have to be carried out by public health officers with active community participation. These measures include active case management, space spraying of insecticides, larval source reduction of the vector mosquitoes, public health education and personal protection. The viremic patients, especially during the high fever period, should be treated in screened ward at hospital or under mosquito net at home in order to prevent biting by vector mosquitoes (Townson and Nathan, 2008). Space spraying of insecticides, employing thermal fogging or ultra low volume (ULV) spraying, has to be carried out thoroughly in the village as soon as possible after a case of chikungunya fever is detected, and at least one more spraying should also be repeated 7 days after the first application. Larval source reduction of *Aedes* mosquitoes in the village also have to be implemented concurrently with space spraying of insecticides. Effective larvicides against *Ae. aegypti* larvae containing various active ingredients, such as temephos (Mulla *et al*, 2004; Thavara *et al*, 2004; Tawatsin *et al*, 2007), novaluron (Mulla *et al*, 2003; Arredondo-Jimenez and Valdez-Delgado, 2006), diflubenzuron (Thavara *et al*, 2007; Chen *et al*, 2008), and *Bacillus thuringiensis israelensis* (Bti) (Mulla *et al*, 2004; Lee *et al*, 2008) should be applied in water-storage containers in and around houses, whereas the natural habitats of *Ae. albopictus* could be applied with Bti (Lee *et al*, 1996) employing ULV applicator. In addition, the integration of larvicides and adulticides could provide the possibility of achieving both larvicidal and adulticidal effects against targeted mosquitoes, such as *Ae. aegypti* and *Ae. albopictus* when applied as ULV spraying (Yap *et al*, 1997).

Public health education is an important measure that could prevent epidemic of chikungunya fever. The relevant information about chikungunya fever, such as etiology of the disease, disease prevention, vector mosquitoes and their control should be supplied to the public in an understandable format, especially in the high risk areas. Personal protection from biting mosquito is also one of the critical measures that could minimize the expansion of chikungunya fever in the epidemic areas. This measure requires mosquito net for infants or children who always sleep during daytime and mosquito repellents, for instance, mosquito coils, vaporizers and topical repellents. Effective topical repellents containing such active ingredients as deet (N, N-di-ethyl -3- methylbenzamide), IR3535 (Ethyl butylacetylaminopropionate), and essential oils extracted from plants, have demonstrated a high degree of repellency against *Ae. aegypti* and *Ae. albopictus* (Thavara *et al*, 2001; Tawatsin *et al*, 2001, 2006a,b).

In summary, chikungunya fever has re-emerged in Thailand after a disappearance of about 13-14 years. The current outbreak of this disease in 2009 has infected some 32,000 people, mostly in the southern provinces of Thailand. Chikungunya virus was detected in blood specimens obtained from suspected patients and in both sexes of wild-caught vector mosquitoes, *Ae. aegypti* and *Ae. albopictus*. The presence of chikungunya virus in adult male mosquitoes of both species revealed a role of transovarial transmission of the virus in field population of the mosquito vectors. As no vaccine is currently available for chikungunya infection, vector control employing various measures and personal protection from biting mosquito still remain the main effective strategies to control the disease.

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Field Evaluation in Thailand of Spinosad, a Larvicide Derived from *Saccharopolyspora spinosa* (Actinomycetales) against *Aedes aegypti* (L.) Larvae

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Abstract: Two formulations of spinosad, direct application tablet (DT) and 0.5% granules (GR), at 3 dosages (0.25, 0.5 and 1.0 mg/l) in 200-liter earthen jars were evaluated against the larvae of *Aedes aegypti*. Two water regimens were used in the jars: jar full all the time and a full jar in which half the volume of the water was removed and replaced at each assessment interval. All treatments and controls were replicated 4 times and challenged with cohorts of 25 third-instar larvae of *Ae. aegypti* at weekly intervals during the study. The number of pupal skins (indicating successful emergence of adults) in the treated and control regimens were counted 7 days post-addition and they were used to calculate inhibition of emergence (% IE) based on the original number of larvae used. The DT formulation at the highest concentration (1.0 mg/l) yielded 79-100% IE for 34 days in the full jars, efficacy declining beyond this period. However, the longevity of this dosage was much longer with 90-100% IE for 62 days post-treatment in the water exchange regimen. The target and manufacturer-recommended concentration of 0.5 mg/l of DT gave good control (92-100% IE) for 20 days, declining below 92% IE thereafter in full jars. This dose also yielded good control with IE of 97-100% for 27 days in the water exchange regimen. The 0.5% GR formulation at all 3 dosages showed higher efficacy and greater longevity in the jars than the DT. In the full jars, all 3 dosages produced IE of 76-100% for 55 days post-treatment. In the water exchange regimen, the efficacy and longevity were increased by about one week, up to 62 days post-treatment. It is clear that the DT formulation can be used effectively against *Ae. aegypti* larvae at a target dose of 0.5 mg/l in 200-liter jars. This dose can be increased to 1.0 mg/l if slightly longer residual activity is desired. In containers where water is consumed and more water added, the longevity of efficacy will be longer for the DT than in jars which remain full all the time. GR (0.5%) gave longer control than DT. GR (0.5%) floated

on the surface and produced scum and an oily film, features not desirable in stored water.

Introduction

Spinosad is a natural product derived from the bacterium *Saccharopolyspora spinosa*. This bacterium is normally responsible for the decomposition of organic material in soil. This organism was first isolated in certain soil samples in 1988 (Thompson *et al*, 1997). The most active metabolites from spinosad fermentation were identified as Spinosyn A and D (Sparks *et al*, 1997). Spinosad exhibits stomach and contact poisoning properties and affects specifically the function of γ-aminobutyric acid (GABA) receptors and nicotinic acetylcholine receptors of the target insects (Salgado 1997, 1998). This product has been widely tested against injurious insects in a variety of crops, such as cotton (Banerjee *et al*, 2000), wheat (Fang *et al*, 2002) and tobacco (Blanc *et al*, 2004). In Thailand, spinosad has also been evaluated against tomato pests, such as thrips, *Ceratothripoides claratris* (Shumsher) (Premachandra *et al*, 2005) and the sweetpotato whitefly, *Bemisia tabaci* (Genadius) (Kumar and Poehling, 2007). This bioactive agent has also been shown to have a high level of activity against larvae of various mosquito species, such as *Aedes aegypti* (L.), *Ae. albopictus* (Skuse), *Culex quinquefasciatus* Say, *Cx. pipiens* L., *Anopheles albimanus* Weidemann, *An. stephensi* Liston and *An. quadrimaculatus* Say in recent studies (Bond *et al*, 2004; Cetin *et al*, 2005; Dariet *et al*, 2005; Dariet and Corbel, 2006; Paul *et al*, 2006; Romi *et al*, 2006; Pridgeon *et al*, 2008).

The objective of this study was to evaluate a novel formulation (DT) of spinosad and compare it with GR (0.5%) in earthen jars (200 liter water) according to WHO guidelines (WHO, 2005). Multiple dosages of each formulation were employed in two water regiments of either full jars or full jars with half the volume of water removed and replaced weekly. This regimen simulated water-use patterns in rural Thailand and elsewhere.

Materials and methods

Field study site

During the course of this research, the experimental field facilities in Bang Bua Thong District, Nonthaburi Province, Thailand, were employed. We have used these facilities successfully in the past evaluating larvicides (Mulla *et al*, 2004; Fansiri *et al*, 2006; Tawatsin *et al*, 2007; Thavara *et al*, 2007). Earthen water-storage jars in the tests were placed on a concrete slab covered with a roof, but open on the sides. The containers were in shade at all times. The facility is located in a semi-rural area about 50 km from Bangkok. The jars were fitted with aluminum fabricated lids covering the jars at all times except during assessment for about 34 hours per week.

Test units

Earthen water-storage jars, the most commonly used containers were placed in rows on the concrete slab, on each side of a gutter. The jars have a capacity of 200 liters, when filled have a depth of 62 cm, when half full (100 liter water), the depth was 32 cm. Fitted aluminum lids covered the mouths of the jars at all times except during filling, emptying, adding larvae and counting larvae and pupal skins. The covers precluded light entry and prevented deposit of debris and oviposition by wild mosquitoes, as well as invasion by predacious macro-invertebrates. The jars were filled with tap water, 0.5 g of grow1d up larval food was added initially and then 25 third-instar larvae from a laboratory colony of *Ae. aegypti* were added at weekly intervals. Water loss due to evaporation was replenished on a monthly basis and larval food (0.25 g) was added weekly. One month after the start of the experiment, a food suspension (25 g ground up mouse food/l00 ml water) was prepared, then 1 ml of this food suspension (equal to 0.25 g dry food/container) was added weekly.

Formulations and treatments

The spinosad product in this study was a mixture of Spinosyn A and D with ratio of 85% to 15%, respectively. Two formulations of spinosad: a direct application tablet (DT: GF-1855, Lot NB 115-44-29) and granules (0.5% GR: GF-1578, Lot NB104-3532) were employed in this study and were provided by Dow Agro Sciences, Indianapolis, USA. Both the DT (7.5% active ingredient) and 0.5% GR (0.5% active ingredient) formulations were administered at 3 dosages: 0.25, 0.5 and 1.0 mg/l. Both formulations were applied directly to water in the jars without stirring. Controls were also run. All treatments and controls were replicated 4 times and challenged with cohorts of 25 third-instar larvae (lab reared) of *Ae. aegypti* at weekly intervals after treatment. Two water regimens were used in the jars: water jars full all the time and full jars from which half the water volume (100 liters) was removed and refilled weekly at each assessment interval. For water removal, a submersible water pump was lowered to the mid-depth of the jar and the water was then pumped out. Water exchange began one week after the granules sank or 13 days after treatment.

Assessment of efficacy

After treatment, 25 third-instar larvae/ jar were added weekly to challenge the efficacy of the treatments. By the 7th day post-addition, all surviving larvae had pupated and emerged as adults. The exact number of pupal skins (indicating successful emergence of adults) was counted 7 days post-addition by removing pupal skins which float on the water surface along the margins of the water using a syringe. The pupal skins in each container were removed and placed in a white pan containing water. The efficacy was reported as the level of inhibition of emergence (IE%) calculated on the basis of successful surviving larvae, pupae or emergence (based on pupal skins) in the treated and control regimens divided by the original number of larvae used (25 larvae/jar). The assessment of efficacy was made at weekly intervals until the level of control dropped below 80%.

Results

Data regarding DT efficacy is found in Fig 1 (without water exchange) and Fig 2 (water exchanged weekly). This formulation at all 3 dosages (1.0, 0.5 and 0.25 mg/l) resulted in 100% inhibition of emergence (IE) in the full jars for 13 days (Fig 1). The DT formulation at the highest concentration (1.0 mg/l) yielded 79-100% IE for 48 days in the full jars, with the efficacy declining beyond this period. However, in the water exchange regimen, the longevity of efficacy was much longer with 90-100% IE for 62 days post-treatment (Fig 2). There was one reading on Day 27 when the IE was 69%, an obvious anomaly since the 5 sub-sequent readings gave 90-100% IE (Fig 2). The manufacturer recom-mended dose of 0.5 mg/l of DT gave good control (IE 92-100%) for 20 days, declining below 92% IE thereafter reaching 79% IE 27 days post-treatment in the full jars (Fig 1). This dose also yielded good control with an IE of 97-100% for 27 days in the water exchange regimen and thereafter the efficacy fluctuated between 74% and 96% IE from 34 to 55 days post-treatment (Fig 2). The DT formulation at the lowest dosage of 0.25 mg/l

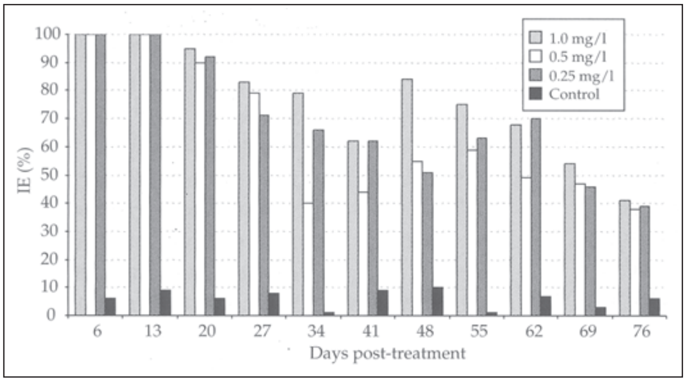

Figure 1 Field evaluation of spinosad (DT tablets) against larvae of *Ae. aegypti* in 200 liter water in earthen jars (water full jars) at Bang Bua Thong, Nonthaburi, Thailand (treated on April 18, 2007).

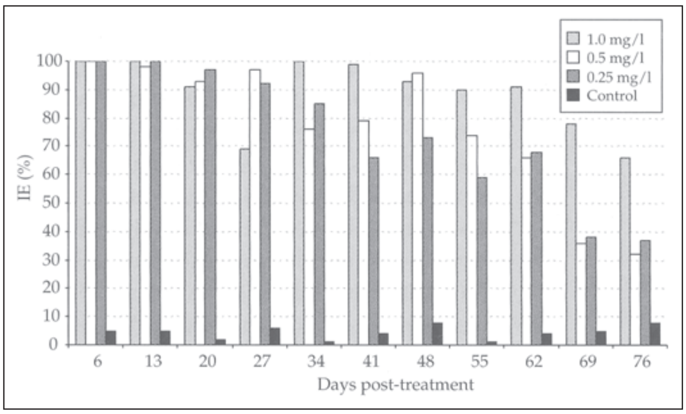

Figure 2 Field evaluation of spinosad (DT tablets) against larvae of *Ae. aegypti* in 200 liter water in earthen jars (water full jars, 1/2 emptied - refilled weekly) at Bang Bua Thong, Nonthaburi, Thailand (treated on April 18, 2007).

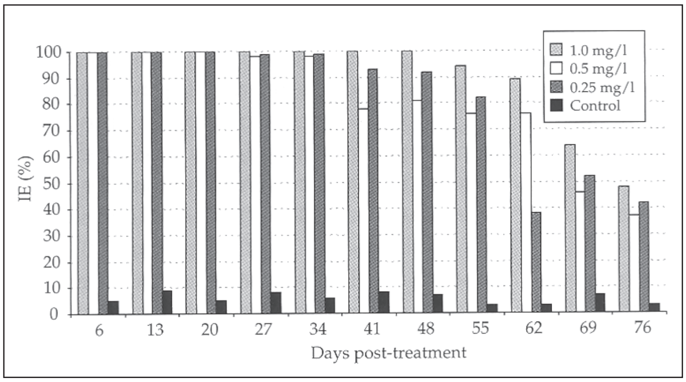

Figure 3 Field evaluation of spinosad (GR 0.5) against larvae of *Ae. aegypti* in 200 liter water in earthen jars (water full jars) at Bang Bua Thong, Nonthaburi, Thailand (treated on April 18, 2007).

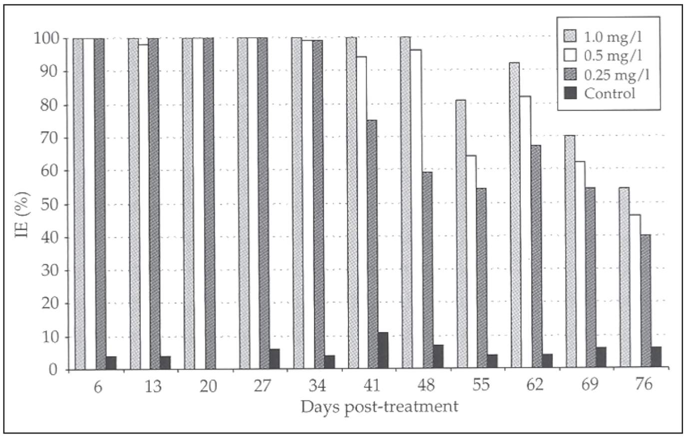

Figure 4 Field evaluation of spinosad (GR 0.5) against larvae of *Ae. aegypti* in 200 liter water in earthen jars (water full jars, 1/2 emptied - refilled weekly) at Bang Bua Thong, Nonthaburi, Thailand (treated on April 18, 2007).

provided satisfactory control (about 92-100% IE) in the jars with water exchange for 27 days post-treatment, one week longer than those in the jars without water exchange (20 days post-treatment). Beyond these periods, this dosage showed a continuing decline in efficacy in both water regimens.

Regarding the 0.5% GR formulation, the dosages of 1.0, 0.5 and 0.25 mg/l produced almost complete efficacy (IE 98-100%) in both the full and exchanged water jars for 34 days post-treatment (Figs 3 and 4). Beyond this period, the highest dosage (1.0 mg/l) still showed more than 80% IE for up to 62 days post-treatment in both water regimens. At 69 and 76 days post-treatment, this dosage (1.0 mg/l) exhibited a lower efficiency with an IE of 70% or lower in both water regimens. The GR formulation at 0.5 mg/l provided 76-81 % IE in full jars 62 days post-treatment (Fig 3) and yielded at least 82% IE for up to 62 days post-treatment in the water-exchanged jars (Fig 4). In contrast, the 0.5% GR formulation at the lowest dosage (0.25 mg/l) in the jars without water exchange demonstrated somewhat better efficacy of least 82% IE (from 41 to 55 days post-treatment) better than the jars with water exchange regimen (Figs 3 and 4).

Discussion

The efficacy and longevity of both formulations of spinosad in our study were dose dependent. Overall, the DT and 0.5% GR formulations at dosages of 0.25, 0.5 and 1.0 mg/l provided at least 90% IE for 20-62 and 41-62 days, respectively. However, the duration of the residual efficacy of the 0.5% GR formulation in this study was shorter than that obtained in our previous study in 2006 (83-111 days) under the same dosages and simulated field conditions (WHO, 2007). In comparison, a similar study conducted in Malaysia with the same testing protocol showed that the DT formulation of spinosad yielded excellent control (97-100% mortality) against *Ae. aegypti* larvae in earthen jars at all three concentrations tested (0.25, 0.5 and 1.0 mg/l) for up to 16 weeks in both water regimens while the granular formulation (0.5% GR) also gave the same results for up to 16 weeks in the jars without water exchange, but shorter longevity (12 weeks) in the jars in which water was removed and refilled weekly (Jaal, 2007). These differences in efficacy and longevity could be due to various factors, such as the test material, biological response of the test larvae, differences in earthen jars used or in the environment. According to the results obtained from WHOPES supervised trials, the WHO (2008) has recommended the use of the spinosad DT formulation at dosages of 0.25-0.5 mg/l to control *Aedes* larvae in breeding containers with an expected duration of residual efficacy for 4-6 weeks under field conditions. Prior to this study, spinosad 12% sc and 0.5% GR have been also recommended by the WHO for the control of container-bred mosquitoes at dosages of 0.1-0.5 mg/l, with an expected duration of efficacy of 10-12 weeks (WHO, 2007).

The status and behavior of the two formulations after application were also observed in this study. These observations are important in terms of the physical characteristics, ease of application and acceptability of the formulations in water storage containers. The granules prepared on corn grit were of uniform size (15/20 mesh estimated size) with good flow ability. After application a considerable amount of the granule formulation sank to the bottom of the earthen jars, but a substantial amount (about 25%) remained floating on the water surface. At the two higher dosages of 1.0 and 0.5 mg/l, the amount of the granules formulation floating in the containers was quite large which will be objectionable for use in water-storage containers. The floating granules made counting the larvae, pupae and pupal skins difficult. Six days post-treatment the granules were mixed with the surface water using a spoon whereupon all the granules sank to the bottom. The granules at the two higher dosages produced a film of scum on the water surface. A film, scum or floating granules are objectionable in water storage containers. The film disappeared after one month and was not visible until the date of the termination of the experiment. No such scum or film was seen with the DT treatments.

The speed of action of spinosad was also observed in order to provide a critical insight into the activity and efficacy of the two formulations. During the first month after treatment, mosquito larvae with the granular treatment died within one to two hours of being placed in the treated water. The larvae were noted to undulate and curve attempting to bite their siphons. A quick effect was not noted with the DT treatment. With the DT treatment mortality occurred more slowly and later. This phenomenon is reflected by the activity and efficacy of DT, which were shorter compared to the granules (Figs 1, 2, 3 and 4). On termination of the experiment (83 days post-treatment), none of the larvae in any of the treatments (DT and 0.5% GR) succumbed quickly. They were noted to be active and alive, even at high dosages of DT and 0.5% GR.

The activity and longevity of many mosquito larvicides depends on the characteristics of the formulation. This also applies to spinosad. Spinosad has been formulated as SC (11.6%), GR (0.5%) and recently as controlled release tailor-made direct treatment tablets (DT), weighing 1.35 g, containing 7.5% spinosad. DT is a unique formulation, consisting of two homogeneous layers. Each layer has a different concentration of spinosad and a different release profile. The first layer is effervescent, facilitating quick release of spinosad for initial concentration and action. The second layer containing a different concentration of spinosad will ensure slow release of the active ingredient for long-lasting control of mosquito larvae. The tablets were easy to apply. They sank to the bottom instantly, started to fizz and kept moving on the bottom surface and resettled soon. After fizzing, the tablets remained stationary. They remained mostly intact and visible at the bottom of the containers until the termination of the experiment 83 days post-treatment. There was no scum or visible film with the DT treatments. DT, therefore, should have greater acceptability in treating artificial water-storage containers. The DT application at all dosages provided greater and longer efficacy in jars with the water exchange regimen as compared to the full jars. Water removal and replenishment either increased the release of spinosad from DT or facilitated dissolution of spinosad from the active ingredients absorbed and adsorbed on surfaces. Our prior studies with other chemicals also showed this trend in jars (Mulla *et al*, 2004; Thavara *et al*, 2007). In general, the IE (%) was higher in water exchange regimens than in full jars, except in one case, the dosage of 1.0 mg/l at 27 days post-treatment. This anomaly could be due to the interruption releasing of spinosad from the second layer of the DT formulation. In all assessments up to 62 days, the IE (%) was consistently higher in the water-exchange jars than in the full ones.

In conclusion, it is clear that the DT formulation can be used effectively against *Ae. aegypti* larvae at the dose of 0.5 mg/l in 200 liter jars. This dose can be increased to 1.0 mg/l if slightly longer residual activity is desired. In containers where water is consumed and added, the longevity of efficacy is longer for DT than in the jars which remained full all the time. 0.5% GR gave longer control than the DT formulation. With the GR formulation, there was not much difference between full and water exchanged jars. The spinosad DT formulation is a satisfactory choice for controlling *Ae. aegypti* larvae in water-storage containers.

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Feeding Ability of *Micronecta grisea* Nymphal Instars and Adults on Third Instar *Aedes aegypti* Larvae

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Abstract: Pygmy waterboatmen, *Micronecta grisea*, were collected and used to establish laboratory cultures in order to study the predation rates and feeding behavior of nymphal instars (N) and adults upon third instar larvae (L3) of *Aedes aegypti* to assess their potential for biological control. The body length, head capsule size and head length of 330 nymphs and 71 adults of *M. grisea*, collected from Nonthaburi Province, Thailand, were measured using a stereo microscope. In contrast to head capsule size and head length, which yielded overlapping size distributions, five discrete nymphal instars (N1 – N5) plus adults could be classified by body length; the 1st (N1; 0.54 – 0.65 mm), 2nd (N2; 0.69 – 0.84 mm), 3rd (N3; 0.9 – 1.11 mm), 4th (N4; 1.29 – 1.56 mm) and 5th (N5; 1.74 – 1.98 mm) nymphal instars plus adults (2.07 – 2.43 mm). Using body length to define developmental stadia, nymphs were classified as three discrete size categories, small (N1 & N2), medium (N3 & N4) and large (N5), and together with adults these four classes were examined for predation rates and prey handling times when fed L3 *Ae. aegypti* at different predator to prey ratios. Prey searching and handling times decreased with increasing *M. grisea* size (developmental stadia), and were consistent with a Type II functional predator-prey response.

Introduction

Aedes aegypti L. (Diptera: Culicidae), the yellow fever or dengue mosquito, is the principal vector for transmission of the dengue flavivirus in humans across the tropics and subtropical regions, including within Thailand, as well as for yellow virus in endemic regions. Infection with one of the dengue virus strains can cause Dengue or the more serious Dengue Hemorrhagic Fever (DHF), and has been estimated to cause an average morbidity in some 40 million and 2-300,000 people annually worldwide, Thailand, although regional epidemics can be large. Urban *Ae. aegypti* populations are well adapted to human (their principal blood host) environments and breed in both indoor and outdoor water-storage containers, as well as a diverse array of other suitable temporal sources of relatively stagnant water, including empty coconut shells, flag pole holders, gutters, plant pot trays, refuse bins, etc. This makes total larval elimination or control unrealistic and restricts these measures to the control of the main breeding sites (human water storage containers) and attempted reduction of the minor sites so as to reduce adult mosquito numbers in the proximity of human habitations. To this end, larval control programs in Thailand are traditionally performed using a mixture of three imperfect approaches, that is physical (water container covers, upturning to drain dustbins, prevention of blockage of gutters and similar drainage channels to reduce pooled rainwater), chemical (e.g. use of the insect growth regulator pyriproxyfen) and biological (e.g. use of bacterial toxins such as *Bacillus thuringensis israelensis* or predators such as *Mesocyclops* copepods) control methods (Vu et al., 1998). However, the choice(s) available are somewhat restricted by whether the water is intended for human consumption or not and the ability to locate, and so treat, all such temporally dynamic potential breeding sites for *Ae. aegypti*. Thus, for example household water jars may be easily covered (physical) but external natural and man made water sources cannot all be located let alone be covered, whilst chemical control suffers from the same problem of the inability to locate and treat all breeding sites, increasing insecticide and larvicide resistance, and the fact that the larval rearing sites are in close proximity to humans, such as in the house or even drinking water reservoirs, and so is restricted by the risks of long term human exposure in addition to environmental and economic concerns. There is thus an increasing demand for alternative treatments, including environmentally acceptable biocontrol based methods, for the treatment of non-household water container based breeding grounds. Many biological control methods are, however, not mobile and so again are limited by the problem of locating and treating all the temporally dynamic breeding grounds, with loss of the control agent as a given site dries up or the mosquito moves away as a pharate adult. Thus mobile, prey seeking (or imago phoretic, such as, perhaps, microsporidia) biological agents would potentially offer an alternative weapon, being able to also control neighboring unlocated breeding sites and also new larval rearing sites as they become available.

Micronecta grisea Fieber (Heteroptera: Micronectidae), the pygmy waterboatmen, are true aquatic hemipteran insects with paddle-like legs that have all their developmental stages in water. They are excellent and active swimmers both on the surface and under water, feeding on insect larvae, including mosquito larvae, by capturing the prey in the water and then sucking out the haemolymph from the prey body. Combined with their widespread distribution from India and Sri Lanka to Taiwan and Indonesia and Java (Nieser & Chen, 1999), their relatively small size and water size requirements, mobility between water sites, their natural habitat being stagnant water or parts of streams with little current flow, broadly the same as *Aedes aegypti* larvae, and that they are potential predators of *Ae. aegypti* larvae (Lekprayoon, 2006), makes them potential alternative biocontrol agents in mosquito control programs.

A survey on the distribution of *Micronecta* spp. in all regions of Thailand, including inspections of water-storage jars which were breeding sites of *Ae. aegypti*, reported that the presence of *Micronecta* spp. was found to be 100, 89, 62 and 25 % of the outdoor jars in the central and eastern, north-eastern, northern and southern parts of Thailand, respectively (Suphapathom et al., 2002).

As *Micronecta* spp., including *M. grisea*, are common species found throughout the country, this study was carried out to examine the body length, head capsule size and head length of *M. grisea* nymphal instars and adults, together with their predation efficiency and feeding ability upon L3 *Ae. aegypti* larvae as a potential biocontrol agent.

Materials and methods

Micronecta grisea

M. grisea were collected from water-storage clay jars at the Research Station for Mosquito Biology and Control, Department of Medical Sciences, Ministry of Public Health, Bang Bua Thong District, Nonthaburi Province, Thailand. All samples were brought to, and used to establish laboratory cultures at, the Department of Biology, Faculty of Science, Chulalongkorn University. Laboratory cultures and experiments were performed at 25 ± 1 °C and an L: D period of 12:12. Five adult female and male *M. grisea* were kept in a 1.2 l clay jar for stock cultures and fed on *Ae. aegypti* larvae *ad libitum*. All clay jars used were covered with nylon meshes (1 mm mesh size).

Aedes aegypti larvae

Freshly laid *Ae. aegypti* eggs, attached on filter paper and dry, were obtained from the laboratory cultures of the Department of Medical Sciences, Ministry of Public Health, and were reared in 2 l plastic rectangular containers in the laboratory at Chulalongkorn University to obtain late L3 *Ae. aegypti* for use in the experiments. Excess larvae were not reared further but killed and in-house *Ae. aegypti* cultures were not established.

Tap water, for rearing all stadia of *M. grisea* and *Ae. aegypti* larvae, was dechlorinated by leaving to air in containers for 24 h. For *Ae. aegypti*, the dried eggs on filter paper were added to this water whereupon they hatched within 30 minutes and the larvae were fed daily with crushed "Pedigree" dog food at 0.1 g of food per 2 l of water containing 200 larvae, and maintained at 25 ± 1 °C and an L: D period of 12:12. After 3 – 4 days under these conditions, larvae molted to the L3 stadia, and when observed under a stereo microscope were within the size range of 6 – 8 mm long, which were then selected and used in the experiments.

M. grisea nymphal instars and adult

Measurements of the body length, head capsule size and head length were made on individual *M. grisea* nymphs and adults using a stereo microscope (32 x). *M. grisea* were put individually on the petri dish by using a dropper. After that, tissue paper was used to dry up the water surrounding the insect body. The three characters were measured once for each individual by a stage micrometer attached under the microscope. To determine the size range of each of the *M. grisea* nymphal instars, as well as adults, the data for the three measured characters for the nymphal instars and adults were analyzed by scatter graph plots. The individuals were then ascribed to a given nymphal instar or adult category based upon the size distribution scatter plot analysis (see results) and then each category was reanalyzed for size range and variation within and between each defined nymphal instar and adults. The nymphal instars were grouped into three sizes; small (N1 & N2), medium (N3 & N4) and large (N5) for evaluation of their predation and feeding efficiencies and handling time of the different developmental stages of *M. grisea* at different predator (*M. grisea*) and prey (L3 *Ae. aegypti*) densities.

Mosquito larvae consumption

Feeding tests were conducted to determine the ability of the three nymphal size categories, plus adults, of *M. grisea* to feed on L3 *Ae. aegypti* as determined by predation efficiency and handling times.

To broadly standardize the hunger level of *M. grisea* (predator), and thus the potential hunting desire and, when prey is not limiting, the total consumption rate, the three size categories of *M. grisea* nymphs (small, medium and large) and adults were selected randomly from stock cultures and kept separately without food for the same period of time, that is for 24 h prior to experimentation. *M. grisea* were housed at three different densities, *viz.* 5, 10 and 20 nymphs or adults in 1.2 l clay jars filled with 1 l of dechlorinated tap water. After that, third instar *Ae. aegypti* larvae at one of three different densities, *viz.* 10, 20 and 40 larvae, were put into each clay jar containing the *M. grisea* at different densities to start the tests. After 24 h the number of living larvae and the cadaver remains in the jars was recorded. In all experiments three replicates were performed for each combination.

The mortality numbers were adjusted by Abbott's formula (Abbott, 1925), and then used to calculate the percentage mortality of mosquito larvae in all experiments, and as a measure of predation levels. Plots of prey density against prey attacked were plotted to determine if the predator-prey relationship best fitted a type I or type II functional response.

Statistical analyses

Data for the mean sizes of the three measured parameters for nymphal instars and adults were subject to ANOVA and Duncan's multiple means tests with significant differences accepted at the $P \leq 0.05$ level. For evaluation of the predation levels, the data obtained from all experiments were calculated as the mean percentage mortality from all replicates and then the percentage means of *Ae. aegypti* L3 mortality induced by each *M. grisea* category (nymphal instar and adults), as a measure of predation efficiency, was analyzed by Mann –Whitney U-test to compare predator efficiency within 24 h.

Feeding behavior of *M. grisea*

During the feeding tests with L3 *Ae. aegypti* mosquito larvae as the prey, the searching and handling times of individual *M. grisea* waterboatmen nymphs and adults were recorded separately using a timer for each of the four size categories. Searching time started from the time when the prey (L3 *Ae. aegypti*) were put into containers until the time that the first prey was captured by a predator. The handling time started from the time the prey were captured until the time that predators released their prey or prey remnants, and included capturing, killing, eating and digesting (Holling, 1959).

The feeding time of *M. grisea* nymphs and adults were calculated by summation of both the searching and handling times.

Table 1 The range and mean (± S.E.) body length, head capsule size and head length of each developmental stage of *M. grisea*.

	N	Body Length (mm)			Head Capsule (mm)			Head Length (mm)		
		MIN	MAX	MEAN±SE	MIN	MAX	MEAN±SE	MIN	MAX	MEAN±SE
1st nymphal instar	73	0.54	0.65	0.60±0.002a	0.21	0.36	0.31±0.003a	0.06	0.15	0.11±0.002a
2nd nymphal instar	34	0.69	0.84	0.76±0.005b	0.21	0.40	0.35±0.008b	0.09	0.18	0.13±0.003b
3rd nymphal instar	63	0.90	1.11	1.03±0.005c	0.42	0.54	0.48±0.003c	0.12	0.23	0.17±0.003c
4th nymphal instar	70	1.29	1.56	1.40±0.006d	0.48	0.69	0.61±0.005d	0.12	0.27	0.22±0.004d
5th nymphal instar	90	1.74	1.98	1.86±0.007e	0.63	0.84	0.75±0.004e	0.18	0.38	0.28±0.003e
Adult	71	2.07	2.43	2.23±0.010f	0.72	0.90	0.81±0.004f	0.21	0.39	0.28±0.005e

* Means in the same column with different letters are significantly different ($P \leq 0.05$; One-way ANOVA)

Table 2 Mean (± 1 S.E.) mortality (%) of third instar *Ae. aegypti* mosquito larvae observed within 24 hours using different instars and densities of *M.grisea*(M*; predator) with different densities of *Ae. aegypti* larvae (A*; prey).

M*	S			M			L			ADULT		
A*	5	10	20	5	10	20	5	10	20	5	10	20
10	0a^{1*}	0a^{1}	0a^{1}	33.3 ± 8.8a^{1}	40 ± 5.8a^{1}	46.7 ± 3.3a^{1}	50 ± 5.8a^{1}	80 ± 5.78a^{2}	80 ± 5.8a^{2}	96.7 ± 3.3a^{1}	100 ± 0.0a^{1}	100 ± 0.0a^{1}
20	0a^{1}	0a^{1}	0a^{1}	31.7 ± 7.3a^{1}	30 ± 8.ab^{1}	43.3 ± 4.4a^{1}	45 ± 2.9a^{1}	71.7 ± 4.4a^{2}	80 ± 5.8a^{2}	95 ± 5.0a^{1}	100 ± 0.0a^{1}	100 ± 0.0a^{1}
40	0a^{1}	0a^{1}	0a^{1}	19.2 ± 4.2a^{1}	25.8 ± 1.7b^{1}	35.8 ± 5.1a^{1}	28.3 ± 0.8b^{1}	50.8 ± 0.8b^{2}	69.2 ± 6.0a^{3}	95.8 ± 3.0a^{1}	95.8 ± 2.2a^{1}	100 ± 0.0a^{1}

* Means in the same column with different letters, or * across rows (within the same size of predator) with different superscript numbers are significantly different($P \leq 0.05$; *Mann-Whitney U*-test).

Results

Measurements of the body length, head capsule size and head length of 401 *M. grisea* specimens of mixed developmental stadia revealed six discrete developmental stages of *M. grisea*, comprised of five nymphal instars (N1–N5) and the adults, were recognized from the three measured characters when the data was analyzed by scatter graphs, as shown in Figs. 1A and 1B with $r^2 = 0.95$ and 0.81, respectively. No overlap in the body length distribution was found between each nymphal instar of the examined pygmy waterboatmen, whereas some overlap in the head capsule size and head length size distributions was noted between nymphal instars and also adults.

The normality test and homogeneity test showed that the data were normally distributed and the variances were significantly homogenous at $P \leq 0.05$. A one-way analysis of variance (ANOVA) with Duncan's multiple comparison tests on the data for the three different measurements for each instar of the pygmy waterboatmen showed a significant difference in the mean body length between each instar (df = 5, 395, F = 9383.19, $P = 0.000$), but no significant difference was found within each instar of pygmy waterboatmen. With discrete non-overlapping

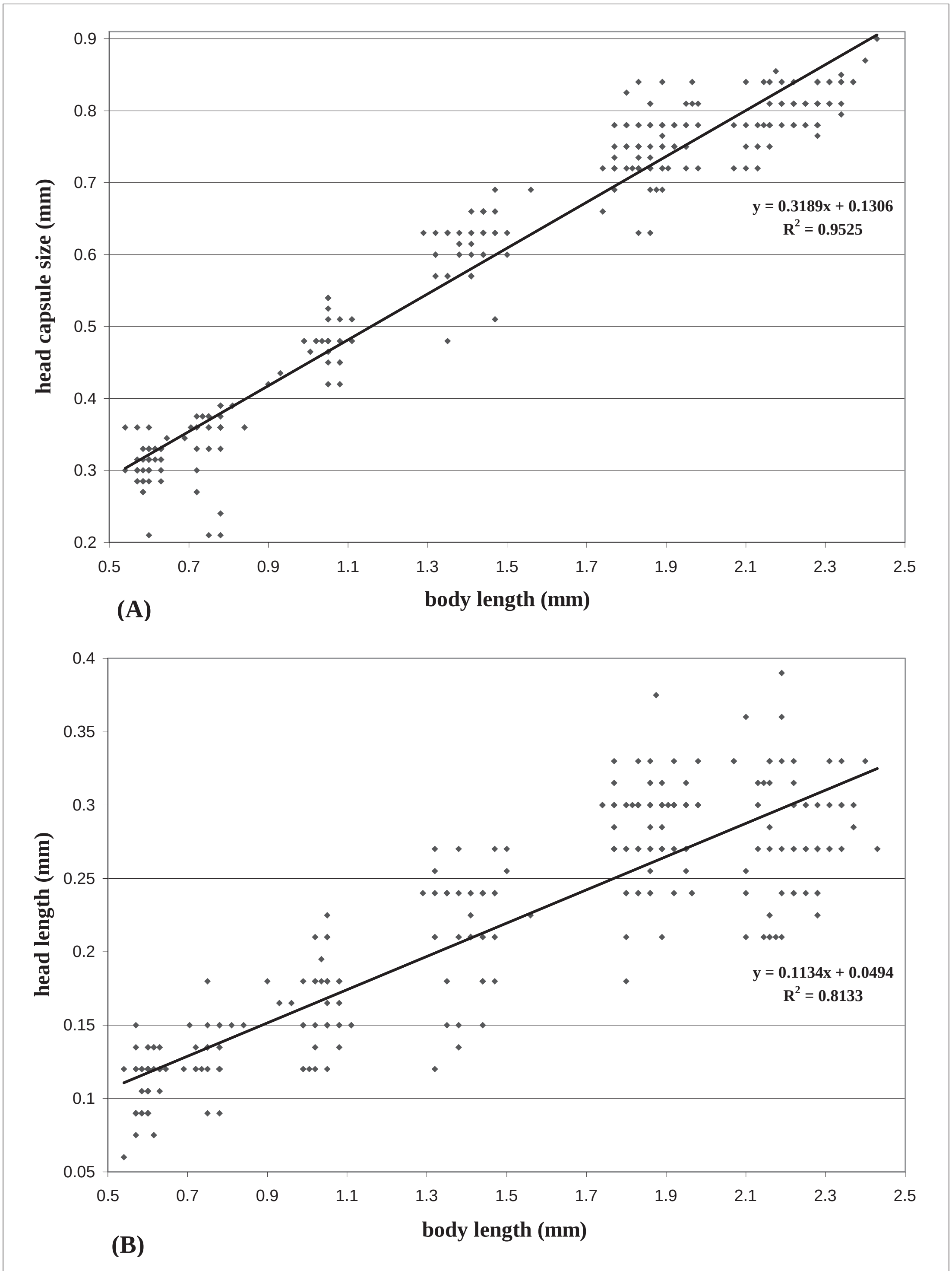

Figure 1 The (A) body length and head capsule size (mm) and (B) body and head lengths (mm) of *M. grisea*. Data are shown for 401 individuals of mixed developmental stadia.

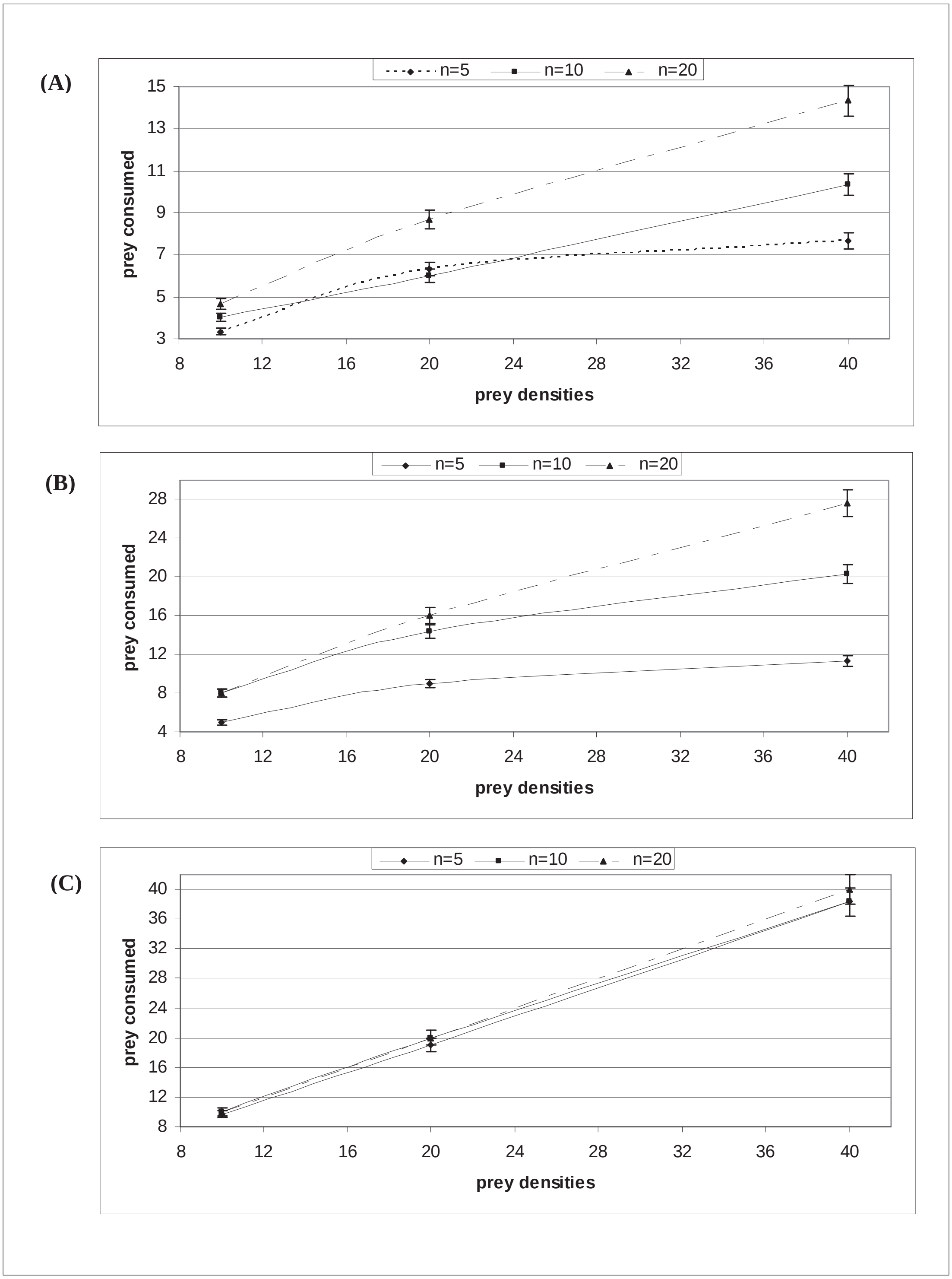

Figure 2 Type II functional response of the predator (*M. grisea*) – prey (L3 *Ae. aegypti*) relationship at different predator densities (n). Data, shown as the mean ± S.E. and derived from 3 repeat trials, are shown for (A) medium nymphs, (B) large nymphs and (C) adults of M. grisea

distributions between the five apparent nymphal instars plus the adults, the body lengths could be and were subsequently used for classifying these six discrete developmental stages of *M. grisea*, and as such split the 401 specimens into 73, 34, 63, 70, 90 and 71 individual N1, N2, N3, N4, N5 and adults of *M. grisea*, respectively. Analysis of the body length, head capsule size and head length within these six categories (developmental stadia) revealed that each nymphal instar and the adults were significantly different ($P \leq 0.05$), except for head lengths between the N5 and adults (Table 1).

Using the discrete body size distributions as markers for the different developmental stages of *M. grisea*, the nymphal instars were grouped into small (N1 & N2), medium (N3 & N4) and large (N5) size groups for the predatory feeding tests with L3 *Ae. aegypti*. The feeding tests were performed with four size categories of *M. grisea* at three different densities (5, 10 and 20 per l container), each of which was supplied with live L3 *Ae. aegypti* as prey at three different densities (10, 20 and 40 larvae per l). The mean *Ae. aegypti* larval mortality within 24 h, used as the marker for predation rate, was evaluated and the data is summarized in Table 2. Analyses of the predator-prey relationship by plots of prey density against prey attacked were consistent with a Type II functional response (Fig. 2), and were tested using the Holling's disc equation. Predator satiation occurred to a slight extent in the M and L nymphs' functional response curves, but not in the adult's curve. The proportion of prey consumed by predators declined with increasing prey densities. Functional response curves revealed that the decreasing rate, as for five medium nymphs, five large nymphs and ten large nymphs, was due to prey saturation of the pygmy waterboatmen, while the constant consuming rate showed unsaturated conditions, higher prey densities were still available for predators to consume. The adult, medium and large nymphal categories predated the L3 *Ae. aegypti* in all experiments, whilst in contrast the small nymphs showed no evidence of predation upon L3 *Ae. aegypti* in all tests.

The normality test showed that the data were not normally distributed and the homogeneity test also showed the variances to differ significantly at $P \leq 0.05$. Using Mann-Whitney U-test, the results revealed that as the prey numbers (and thus the prey: predator ratio) were increased no significant difference in the predation level (larval mortality) was observed for *M. grisea* of the same developmental stage at the same density ($P \leq 0.05$). Comparisons of larval mortality, as a marker for predation levels, amongst the different nymphal stages of *M. grisea* at different predator densities showed that only the large nymphal size category consumption caused a significantly different mortality amongst the different predator densities (Table 2).

For a density of five *M. grisea* with 10, 20 and 40 prey (L3 *Ae. aegypti*), the consumption by medium and large nymphs were numerically, but not statistically significantly, different, whereas adults, caused a significantly higher predation level than both medium and large nymphs at all prey densities (Fig. 3).

When the *M. grisea* density increased to 10 there were significant differences amongst the predation levels (mortality of L3 *Ae. aegypti*) between each size category of nymphal instars and adults of pygmy waterboatmen, with the predation levels increasing with increasing predator size (developmental stage).

Table 3 Mean (± S.E.) searching, handling and feeding times of medium (M) and large (L) size category nymphs and adults of M. grisea upon third instar *Ae. aegypti* larvae (One-way ANOVA) (P < 0.05)

M. grisea stage	No. of M. grisea: No. of mosquito larvae	Searching time (s)	Handling time (s)	Feeding time (s)
M-size	10 : 20	92.8 ± 13.1a*	1680 ± 312a	1773 ± 314a
L-size	10 : 20	57 ± 10.1b	867 ± 182b	924 ± 185b
Adult	10 : 20	13.8 ± 1.0c	480 ± 42b	494 ± 43b

* Means in the same column with the different letters are significantly different (P ≤ 0.05; One-way ANOVA).

At predator densities of 20, the prey consumption of medium sized nymphs was significantly lower than that of large nymphs, with that of adults being significantly higher still.

The laboratory no-choice feeding tests with *M. grisea* (predator) feeding on L3 *Ae. aegypti* (prey) revealed that at a predator: prey ratio of 10:20 the searching and handling times of predators were significantly different between the medium and large nymphal stages and the adults (Table 3), with adult and medium sized nymphal *M. grisea* having the shortest and longest, respectively, searching and feeding times. Therefore, with feeding time being simply the summation of searching and handling times, then the same trend was noted with adults having the shortest feeding time and medium stage nymphs the longest. Note, however, that no data on searching, handling and feeding times is presented for the small *M. grisea* nymphs since no prey were ever caught or killed.

Discussion

Measurement of the body and head lengths and the head capsule size of different nymphal instars and adults of the pygmy waterboatman, *M. grisea*, revealed that five discrete nymphal instars, plus the adult stage, could be distinguished. Although no allometry was found between the three characters measured (analysis not shown), the body length was found to have a discrete and well separated distribution pattern between these six developmental stages, whereas both the head capsule size and head length showed some overlap in their size distribution profiles between related developmental stadia. This notion of five

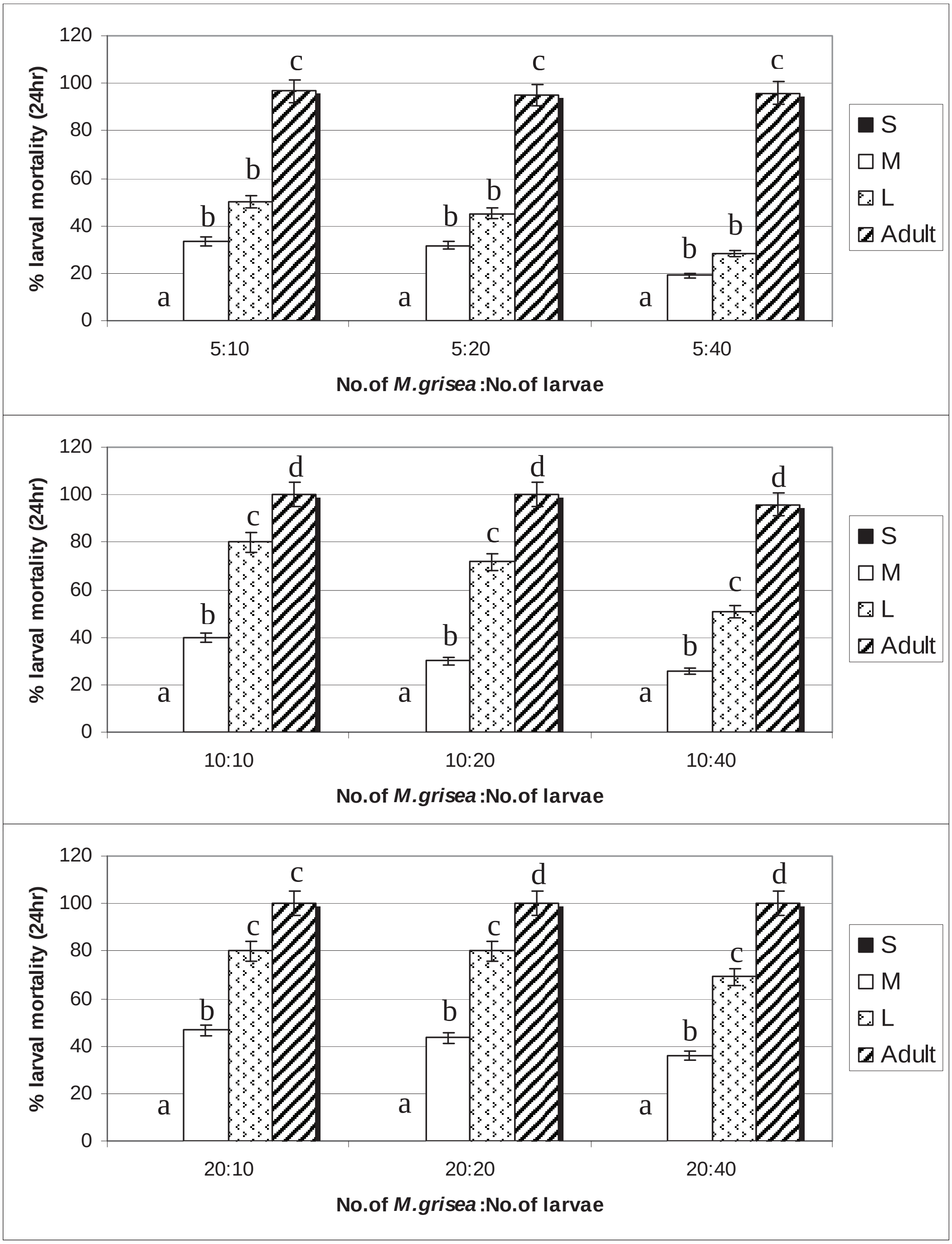

Figure 3 Mean mortality (predation) of third instar *Ae. aegypti* larvae by different instars of *M. grisea* at different densities (5, 10 and 20) with different densities of mosquito larvae (10, 20 and 40). Data are shown as the mean ± S.E. and derived from 3 repeats. Means with a different letter are significantly different (P<0.05; Mann-Whitney U tests)

nymphal instars is consistent with studies in the biology of the related waterboatmen (Gerridae) and the backswimmers, *Enithares* sp., which have also been reported to have five preadult nymphal instars in their development (Chittihunsa, 1980).

The body lengths of each instar, showing no overlap, were used for classifying each *M. grisea* instar. The non-overlapping discrete body length ranges were 0.54 – 0.65, 0.69 – 0.84, 0.9 – 1.11, 1.29 – 1.56 and 1.74 – 1.98 mm for the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} (N1 – N5) nymphal instars, respectively, and 2.07 – 2.43 mm for adults. Although the adult size range is lower than that previously reported from the study of *M. grisea* in Singapore and Peninsular Malaysia, such as 2.6 – 3.2 mm (Nieser, 2002), it is in agreement with the results of a recent survey on the distribution of *Micronecta* spp. across all the regions of Thailand which found that adult *Micronecta* spp. were approximately 2 – 3.5 mm long (Suphapathom et al, 2002). This may be due to environmental conditions and diet sufficiency at different distribution regions.

In this study differences in the body length, head capsule size and head length (with no evidence of allometry between these three parameters) of each nymphal and adult stage were noted, except for the head lengths between the N5 and adult stages which were approximately the same. This may be due to the fact that the anterior part of the head segment in adults was hidden underneath during measurement leading to variable underestimates in their size.

Within the 24 h feeding window, the predation and feeding ability of the pygmy waterboatmen (*M. grisea*) on L3 *Ae. aegypti* differed amongst each size category. Adult waterboatmen showed the highest predation rates (as *Ae. aegypti* L3 mortality) followed by large, medium and small nymphs, in that order, with small nymphs revealing no detectable predation of L3 *Ae. aegypti* in all tests. Consistent with this observation is that under laboratory conditions larger sized backswimmers (*Enithares* sp.) caused a higher larval mortality (predation rate) than those of a smaller size (Chittihunsa, 1980). For the small sized nymphs of *M. grisea* in this study it seems likely that they were too small, compared with the prey size available (L3 *Ae. aegypti*) in these no-choice experiments, to capture their prey, whilst the newly hatched instar nymphs would also still have nutrients from the yolk available to them.

M. grisea of the same size categories (small, medium and large nymphal and adults) and at the same densities caused no significant difference in the larval mortality percentages (predation levels). Rather, within each size category *M. grisea* (predators) when at the same predator number (density), they consumed about the same amount of L3 *Ae. aegypti* prey items even when at higher prey densities, and so higher prey: predator ratios, suggesting that prey saturation may influence predator consumption. Of course, as the prey densities are increased for a given number of predators, and the total numbers of prey consumed remain the same (satiation), the evaluated prey mortality percentages would appear to decrease.

Although in general, predators may spend time on three types of activities, searching, handling their prey and then satiation related activities, we observed the former two in these assays. At the same prey density, the searching times of adults, large and medium sized nymphs of *M. grisea* were significantly different. Adults showed the shortest searching time followed by large and finally medium sized nymphs, respectively. The handling time showed a similar relationship, except that the handling time for large nymphs and adults were not significantly different. Consequentially, feeding time paralleled the predator handling time.

Overall, adult *M. grisea* provided the highest L3 *Ae. aegypti* mortality in all tests with the shortest feeding times. This may be due to adults being more active and larger than nymphs, but it may also reflect the relatively large prey size used. Thus, whilst it remains important to evaluate the predation efficiency and feeding times of all developmental stages of *M. grisea* upon all larval developmental stages of *Ae. aegypti*, it also remains of interest to evaluate if different developmental stages of *M. grisea* preferentially feed upon different larval developmental stages of *Ae. aegypti*, as well as other prey items, since *Ae. aegypti* development in urban water resources is frequently derived from multiple females and is asynchronous.

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Geographical Structure of Dengue Transmission and Its Determinants in Thailand

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Abstract: Expansion of dengue has been attributed to urbanization. To test this concept, we examined dengue transmission intensities in Thailand. We used the inverse of mean age of dengue haemorrhagic fever (DHF) cases as a surrogate of dengue transmission intensity (or force of infection). The transmission intensity in Bangkok decreased rapidly since the mid-1990s, to levels that are currently lower than in other regions. Regression analysis revealed that transmission intensity is highest in the Northeastern rural region, mainly due to scarcity of private water wells. Private wells reduce the need for household water containers, the major breeding sites for vectors. Cumulatively, these results show that urbanization is not necessarily associated with intense dengue transmission in Thailand. Paradoxically, the DHF incidence in Bangkok has surpassed other regions despite declines in transmission intensity. This finding implies the existence of endemic stability (i.e. low incidence of a clinical illness in spite of high transmission intensity).

Introduction

Dengue haemorrhagic fever (DHF), a life-threatening manifestation of dengue infection, emerged in the 1950s [1] and has rapidly increased [2]. In the years following its emergence, DHF was most often reported in urban areas [3]. For this reason, the expansion of dengue virus and DHF has been attributed to urbanization [4]. Bangkok, which is inhabited by 9% of the Thai population, was proposed to be the epicentre from which annual waves of DHF have been emitted to the other parts of Thailand [5].

The idea that urbanization constitutes a risk for dengue infection has not been thoroughly tested. To begin to test this idea, transmission intensities (or force of infection) of dengue virus among different regions of Thailand were compared. Our analysis centred on DHF, but not on the more benign dengue fever, since dengue fever is not regularly reported in endemic countries [6]. The incidence of DHF fluctuates dramatically with a cycle of 3-4 years [7], possibly due to the oscillation in population immunity [8]. Hence, the incidence may not accurately represent the transmission intensity. Instead, we assumed that the inverse of the mean age of DHF cases (IMADHF) could be used as a surrogate for the transmission intensity of dengue virus. The inverse of mean age of infected individuals is generally regarded as an approximation of the force of infection (i.e. '*per capita* rate at which susceptibles acquire infection') for infectious diseases which confer life-long immunity [8]. This relationship has not been confirmed for DHF, since DHF occurs mainly in secondary infections [9, 10]. However, a secondary infection, as well as a primary infection, would be expected to occur at an earlier age under more intense transmission. Consistent with this, recent simulations have predicted that the mean age of DHF is negatively correlated with the force of infection of dengue virus (Nagao & Sakamoto, unpublished observations). In addition, an epidemiological study conducted in Thailand revealed a strong negative correlation between mean age of DHF cases and proportion of houses infested with larvae/pupae of vector mosquitoes (Thammapalo *et al*, unpublished observations). Therefore, we based our analysis upon the assumption that the IMA-DHF reflects the force of infection of dengue virus, albeit not necessarily linearly.

The present study illustrates the spatial patterns of transmission intensities of dengue across Thailand, as determined by IMA-DHF. In addition, the observed spatial patterns are explained by using climatic and socioeconomic conditions as independent variables. The results highlight the rapidly shifting epidemiological state of dengue transmission, which may also be present in other tropical countries.

Methods

Epidemiological data

In Thailand, there are currently 926 districts in 76 provinces. Among these districts, 50 are considered to be part of the Bangkok metropolitan province. Detailed epidemiological data were obtained from the Bureau of Epidemiology, Ministry of Public Health of Thailand. Data regarding the annual number of DHF cases were stratified by age group (0, 1-4, 5-9, 10-14, 15-24, 25-34, 35-44, 45-54, 55-64, ≥65 years). The mean age of DHF cases was obtained by averaging the mid-point age of each group (i.e. 0·5, 3, 7·5, 12·5, 20, 30, 40, 50, 60, 75 years) weighted by the number of DHF cases in each group. Then, by adjusting the number of DHF cases in each group to the national demographic structure in 2000, the adjusted mean age of DHF was obtained. The DHF case data were also used to derive the annual DHF incidence, based upon population data obtained from the National Statistics Office of Thailand. Between 1981 and 1994, the spatial resolution of these data was province level. Between 1995 and 2004, it was district level. We used the district-level data for our analysis.

Map data

The geographic information system software employed was MapInfo 7.0 (MapInfo, Troy, NY, USA). A digital map that delineates the boundaries of the 926 districts was obtained from MapInfo Thailand.

Detection of spatial clustering

To examine the spatial clustering of transmission intensity, we employed a methodology proposed by Getis & Ord [11-13]. In this methodology, the G^*i spacial statistic describes clustering of the variable of interest, Y, around location i. Large G^*i values indicate local clustering of large Y values; whereas, a small G^*i indicates clustering of low Y values. We derived G^*i and associated one-tailed P values for the variable of interest, IMA-DHF, at each district i. The binary distance matrix required by this methodology was generated based upon a cut-off distance of 200 km between paired district centres. Spatial clusterings (at the level of $P<0.001$) were noted on the map.

Climatic data

Climatic data beginning from 1987 were obtained from the University Corporation of Atmospheric Research [14]. For each year between 1995 and 2004, we extracted the following data: the annual mean of daily mean temperatures (T_{mean} in the official data definition, °C), the daily minimum temperature (T_{min}, °C), the daily maximum temperature (T_{max}, °C), the total reported precipitation per month (RPCP, mm/month), the average vapour pressure deficit (A VPD, hPa) which is the saturation vapour pressure minus the observed vapour pressure, and the average pan evapo-transpiration (APET, mm/day) which is the amount of water transformed into vapour from a pan of a standard size. This study enrolled 89 weather stations on the Indochina peninsula, which consistently reported all these variables between 1995 and 2004. These climatic variables were interpolated to the geographic centre (centroid) of each district, as reported previously [15].

Socioeconomic data

Over 250 socioeconomic variables have been surveyed from each of the 61 000 Thai villages on a bi-annual basis, as described previously [15]. Data were collected in even-numbered years between 1994 and 1998 and in odd-numbered years since 2001. This was performed under the initiative of the National Rural Development Committee of Thailand. This rural database, officially coded 'NRD2C database', does not include Bangkok. We selected the variables that (i) were consistently recorded in all six surveys conducted between 1994 and 2005, (ii)

Table 1 Socioeconomic variables as explanatory variables for inverse of mean age of DHF cases

Variable	Definition
Surface water	Proportion of villages with surface water
Piped water	Proportion of villages with supply of piped water
Health station	Proportion of villages with health station
Kindergarten	Proportion of villages with kindergarten
Primary school	Proportion of villages with primary school
High school	Proportion of villages with high school
Electricity	Proportion of villages with electricity
Road to district centre	Proportion of villages connected to the district centre by roads
Public bus	Proportion of villages where public bus service is available
Firewood	Proportion of villages where firewood is used for cooking
Working remotely	Proportion of villages where residents work outside of the subdistrict
Public large wells	*Per capita* number of public large water wells
Private large wells	*Per capita* number of private large water wells
Public small wells	*Per capita* number of public small water wells
Private small wells	*Per capita* number of private small water wells
High-school graduates	Proportion of population who graduated from senior high school
Pickup trucks	Proportion of households which possess pickup trucks
Motorcycles	Proportion of households which possess motorcycles
Land ownership	Proportion of households which own land
Rent houses	Proportion of households which do not own any land
Area for agriculture	Proportion of area which is used for agriculture
Population density	Population per km^2
Household density	Number of households per km^2
Population per house	Average population per household
Birth rate	Number of births in the previous year per 1000 population

covered more than 90% of all villages in any survey, and (iii) were not related to technical detail for a specific industry. Twenty-five variables were selected (Table 1). Among these, six variables were related to local water resources, four to education, two to public health, four to transportation, three to demographic characteristics, and six to other aspects. These variables were averaged for each district and linearly interpolated to the years intervening the surveyed years. This database can be accessed at: http://www.vector-borne-diseases.org/socioeconomics_thailand.

Regression analyses

To explain the spatial pattern of transmission intensity of dengue (represented by IMA-DHF) based upon climate and socioeconomics, regression analyses were performed. Two regression methodologies were used in tandem in order to select the most robust explanatory variables. The first methodology, the random-effect linear regression model, adjusted for the possible biases caused by repeated measurement or temporal autocorrelation. In the present study, this bias would have arisen from the fact that each district reported a maximum of ten annual values of IMA-DHF. The second methodology was employed to adjust for spatial autocorrelation [16, 17]. For this spatial regression analysis, both dependent and independent variables were averaged from the entire study period for each district. For the random-effect linear regression analysis, we first selected an 'optimal time lag' at which transmission intensity is optimally predicted by each climatic variable as follows. For each climatic variable, the mean value of the previous j years (j = 0-8) was incorporated as a single independent variable in the random-effect univariate linear regression. The time lag, j, which resulted in the maximum R^2, was selected as the optimal time lag for each climatic variable. Climatic variables averaged from their corresponding optimal time lags were incorporated into subsequent multivariate random-effect regression. Only the socio-economic variables that were recorded in the same year as the dependent variable were used as independent variables. To consider the possible confounding by ecological differences among the regions, dummy variables were included to stratify the analysis by region. The year value of each record was also incorporated as an independent variable to represent the temporal trend. The independent variables (i.e. climate, socioeconomics, region-specific dummy variables and year) that significantly contributed to the multivariate random-effect model were selected following a stepwise process of elimination.

Finally, multivariate spatial regression analysis, which has been frequently used in socioeconomic and health studies [16, 17], was performed using the selected variables. As a result, the variables that remained after stepwise elimination in both random-effect and spatial regressions should be the most robust. In both random-effect and spatial regressions, the final models were retested after replacing the crude IMA-DHF with the IMA-DHF calculated from the adjusted mean age, to exclude confounding by heterogeneity in the demographic structure. Statistical significance was based upon a two-sided a-level of 0.05. In all regression analyses, the records with no DHF cases were omitted from the analysis. In preliminary regression analyses, we normalized IMA-DHF [18], and obtained largely similar results to the results from non-normalized IMA-DHF. Considering the difficulty in the interpretation of results, we presented the results from non-normalized IMA-DHF. Stata 9.0 (Stata Corp., College Station, TX, USA) was used for statistical analysis. Software for the spatial analysis was provided by Maurizio Pisati (University of Milano, Bicocca).

Results

DHF epidemiological data

We divided Thailand into four regions, with Bangkok located in the central region (Fig. 1). Changes in the mean age of DHF cases were gradual, while DHF incidence fluctuated dramatically across all regions (Fig. 2*a*, *b*). The mean age of DHF cases was consistently the lowest in Northeastern region (Fig. 3). The mean age of DHF cases in the entire country increased gradually beginning in the 1980s, and this increase accelerated beginning in the mid-1990s (Fig. 2*a*). The increase in mean age of DHF, which is equivalent to a decrease in transmission intensity, was most prominent in Bangkok. In Bangkok, the mean age of DHF increased from 11 to 22 years between 1995 and 2004, while the average age in the population increased only from 31 to 34 years. DHF incidence in Bangkok was generally lower than other regions before the mid-1990s, as has been reported [19]; however; Bangkok frequently surpassed other regions afterwards (Fig. 2*b*).

Figure 1 Regions in Thailand. Thailand is classified into four regions: North (N), Northeastern (NE), Central (C), and Southern (S). Bangkok is shown in black.

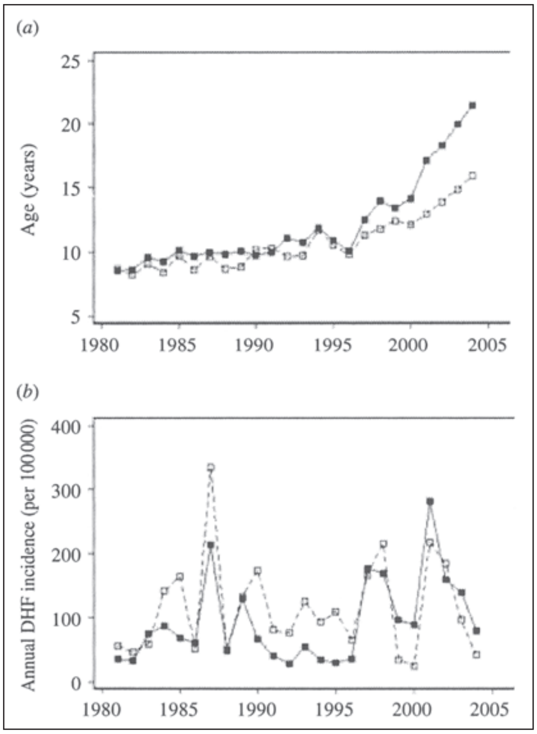

Figure 2 Temporal shift in the mean age and annual incidence of dengue haemorrhagic fever (DHF) cases across Thailand from 1981 to 2004. (a) Mean age and (b) annual incidence (per 100000 individuals) of DHF are presented for Bangkok (–■–) and for other regions (- -□- -).

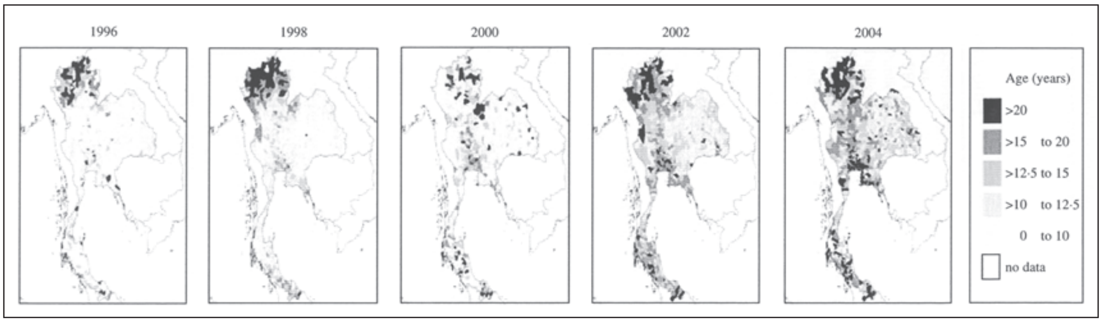

Figure 3 The spatial pattern of mean age of dengue haemorrhagic fever (DHF) cases in Thailand from 1995 to 2004. Mean age of DHF cases was calculated for each district in each year between 1995 and 2004. Data is presented only for even-numbered years.

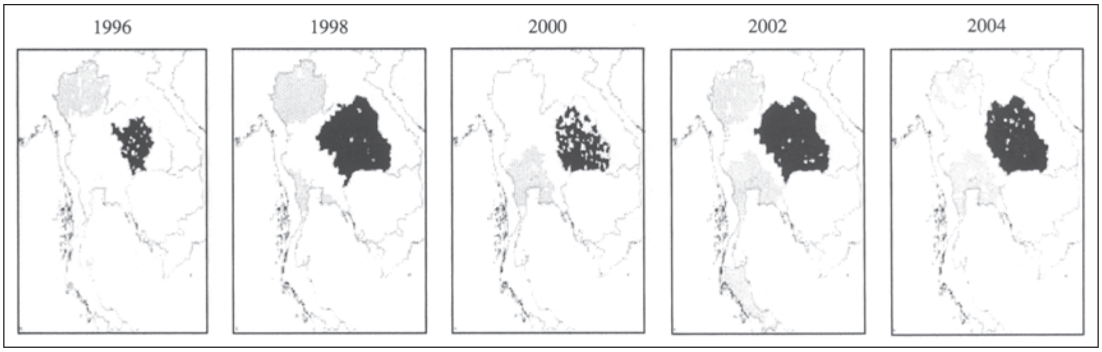

Figure 4 Spatial clustering of dengue transmission intensity, represented by inverse of mean age of DHF cases (IMA-DHF), in Thailand from 1995 to 2004. Local clusterings of both high (.) and low (.) values of IMA-DHF are indicated. Data is presented only for even-numbered years.

Cluster of transmission intensities

A clustering of high transmission intensities (represented by high IMA-DHF values) was consistently present in the Northeastern region (Fig. 4). In contrast, a clustering of low transmission intensities initially existed in the Northern region. In 2000, the mean age in this region was comparable to the Central region, hence this cluster of low transmission intensities disappeared. Another clustering of low transmission intensities subsequently emerged in the late 1990s in the area surrounding Bangkok and expanded thereafter.

Regression analyses

The optimal time lag was determined by univariate random-effect regression analysis for each climatic variable as follows: 5 years for T_{mean}, T_{min}, T_{max} and RPCP; 8 years for APET and AVPD. These climatic variables with corresponding optimal time lags, along with socioeconomic variables, were incorporated into multivariate random-effect linear regression to select variables with significant contributions to IMADHF [Table 2, column (a)]. The mean age was then predicted based upon this multivariate random-effect model. The trend

Table 2 Variables which explain transmission intensity, represented by inverse of mean age of dengue haemorrhagicfever cases (IMA-DHF)

	Random-effect regression (n= 7902)		Spatial regression (n = 873)[¶]	
IMA-DHF[#]	(a) Crude	(b) Adjusted	(c) Crude	(d) Adjusted
Independent variables[§]		Regression coefficients		
Year	- 0.0029***	- 0.0030***	Not used	Not used
APET (mm/day)	0.054***	0.056***	0.036***	0.030***
Public large wells	0.21*	0.21**	0.56**	0.82***
Public small wells	0.65***	0.38**	0.57**	n.s.
Private small wells	- 0.081***	- 0.081***	- 0.053**	- 0.059***
Birth rate	0.00063***	0.00046***	0.0014***	0.0010***
Land ownership	0.0086**	0.0087**	0.025***	0.027***
High school	0.017**	0.015**	0.032***	0.032***
Northeastern region	0.018***	0.019***	Not used	Not used
Southern region	0.011***	0.0083***	Not used	Not used
Constant	5.7***	5.9***	- 0.038	0.0058
R^2	0.14	0.21	0.33	0.39

APET, Average pan evapo-transpiration.
¶ Independent and dependent variables were averaged in each district outside Bangkok.
# IMA-DHF was obtained from either crude or adjusted mean age of DHF cases.
§ Each socioeconomic variable is defined in Table 1.
*** P<0.001, ** P<0.01, * P<0.05, n.s., not significant.

towards a low mean age in the Northeastern region was well reproduced (Fig. 5*a* vs. Fig. 3, 2004). This was mainly because public ‘large water wells’ (*bobadan* in Thai), a positive risk factor, were most prevalent in this region (Fig. 5*b*). Conversely, private ‘small water wells’ (*bonamten*), a negative risk factor, were scarce (Fig. 5*c*).

The variables selected by random-effect linear regression were further tested by spatial regression analysis [Table 2, column (c)]. The results suggested that private small water wells are potential risk reducers of the transmission intensity. APET, public large/small water wells, high school, land ownership, and birth rate remained positive risk factors. The spatial patterns of some of these variables are presented in Fig. 5. In both random-effect and spatial regressions, the adjustment of mean age of DHF affected the results only slightly [Table 2, columns (b), (d)], ensuring that the interference by the demographic structure was minimal.

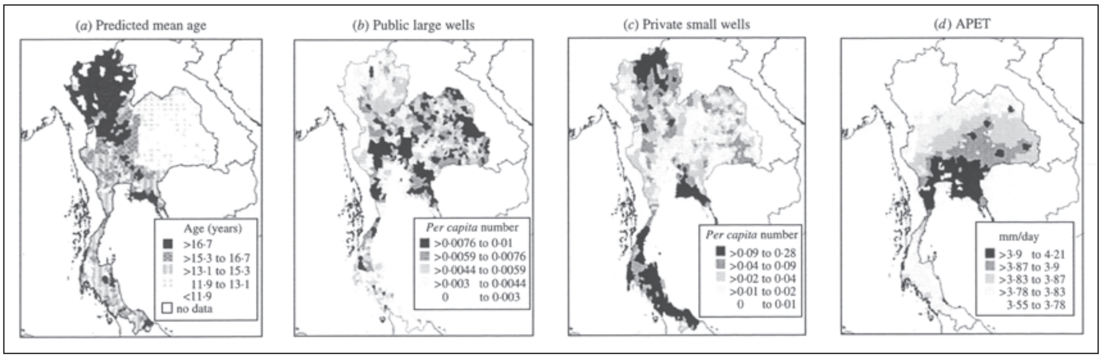

Figure 5 Mean age of DHF cases predicted by random-effect regression model and predictor variables used in the model. (a) inverse of mean age of DHF cases in 2004 was predicted using the final random-effect regression model (see Table 2) and was transformed into mean age. Some socio-economic and climatic variables are illustrated: (b) per capita number of public large water wells, (c) per capita number of private small water wells, and (d) average pan evapo-transpiration (APET, mm/day). Data for all of variables presented are from 2004, with the exception of APET. APET data are the average of the 1996-2003 period since the optimal time lag for APET was 8 years.

Discussion

It has often been held that dengue transmission is more intense in urban areas, based on the observation that cities are often associated with a high incidence of DHF. However, DHF incidence was highly volatile, suggesting that incidence may not accurately reflect the transmission intensity. In addition, incidence is subject to biases caused by over-(underreporting and local modification of diagnostic criteria for DHF [6,10,20,21]. In contrast, mean age of DHF showed higher continuity and is less likely to be affected by over-/under-reporting or modified diagnostic criteria. Based on this rationale, the inverse of mean age of DHF was employed as a surrogate of transmission intensity. Use of this methodology revealed clustering of high transmission intensity in the North-eastern rural region and clustering of low transmission intensity in Bangkok and the Northern region.

Regression analysis was employed to explain the transmission intensity in light of climatic and socioeconomic conditions. As an initial step, optimal time lag was selected for each climatic variable. For all the climatic variables, the optimal time lags were longer than 4 years. This may suggest that the transmission intensity at a time-point is determined by the accumulated effect of climate during the preceding years.

The highest transmission intensity in the Northeastern region could be explained by a combination of the highest prevalence of public large water wells and the lowest availability of private small water wells, as shown by multivariate regression analysis. The residents who obtain water from public wells store the water in household containers, which provide breeding sites of vector mosquitoes, *Aedes*. On the other hand private wells reduce the necessity of storing water in houses. These results are consistent with our previous report that public wells and private wells were positive and negative risk factors, respectively, for infestation by *Aedes* [15].

The birth rate remained a positive risk factor perhaps because it represents the supply of susceptible population. The finding that 'land ownership' was a positive risk factor may be explained by the fact that the large water containers frequently observed in rural Thailand require a yard. In addition, garbage, used tyres, and tree hollows that may be found in a yard often provide breeding sites [22, 23]. The proportion of villages in which high schools are present remained a positive risk. This might suggest that high schools represent a centre of contact between hosts and vectors, persisting after decades-long vector control efforts targeted at primary schools. An alternative explanation might be that pupils in districts with fewer high schools may lodge near their school, in another district, affecting the age profile of DHF in these districts. APET remained a positive risk factor possibly because people in the arid area have to store water in household containers. The high APET in the Northeast (Fig. 5*d*) explains the high transmission intensity observed in this region. Taken together, biological causality can be explained by most of the independent variables selected in the final model. Moreover, these findings provide reciprocal support for the use of IMA-DHF as a surrogate for dengue transmission intensity.

The variable 'year' showed strong negative contribution in the random-effect regression suggesting that there has been a downward trend in dengue transmission intensity that cannot be explained solely by the climatic and socio-economic factors examined. This trend may reflect, to some extent, vector control efforts. Regional vector control offices have been distributing larvicide freely and conducting educational campaigns within communities and schools. It is probable that such vector control efforts were more intense in urban areas than in remote areas due to the fact that regional offices are located in cities. If so, the rapid increase of mean age of DHF cases around Bangkok might reflect these intense vector control activities. Air-conditioned or window-screened houses, which have been increasingly prevalent in Bangkok, may also have contributed to the rapid decrease of transmission intensity there.

Dengue transmission intensity, represented by IMA-DHF, has been lower in Bangkok than in other regions since the mid-1990s. In contrast, DHF incidence in Bangkok surpassed other regions during the same period. These paradoxical findings are consistent with a state of 'endemic stability' [24, 25]. Endemic stability is defined as a state in which the incidence of a clinical illness is low, while the transmission intensity is high. In support of the existence of endemic stability, we recently found that the correlation between DHF incidence and vector abundance were partially 'negative' (Thammapalo *et al*, unpublished observations; Nagao *et al*, unpublished observations). We hypothesize that the unique aetiology of DHF (i.e. enhancement in secondary infections) may give rise to this endemic stability (Nagao & Sakamoto, unpublished observations). Regardless of its underlying mechanism, the possibility of such a partially negative correlation between DHF incidence and transmission intensity has an important implication. It implies that insufficient vector reduction might transiently increase DHF incidence in areas at high transmission intensity; Bangkok may provide such an example. Hence, a greater awareness of the relationship between DHF incidence and transmission intensity is warranted.

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Larvicidal Efficacy of New Formulations of Temephos in Non-woven Sachets against Larvae of *Aedes aegypti* (L.) (Diptera: Culicidae) in Water-Storage Containers

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Abstract: Three new formulations of temephos (LAVIFOS SG 1 %, MOSQ SG 1 % and AZAI-SS ZG 1 %) were evaluated for larvicidal efficacy against larvae of *Aedes aegypti* (L.) in water-storage jars under field-simulated conditions. LAVIFOS SG 1 % and MOSQ SG 1 % are sand granule formulations, whereas AZAI-SS ZG 1 % is zeolite granule formulation. Each formulation contained 1 % temephos as an active ingredient. Each formulation was packed in a non-woven sachet at quantity of 20 g per sachet and placed in a 200-liter glazed clay jar to obtain a dosage of 1 mg/l (one sachet per jar). Each treatment and control (jar without larvicide) was replicated four times. A concurrent set of treatments and controls were carried out in parallel, but the water in each treated and control jars was removed and refilled weekly. All jars (treatment and control) were challenged weekly by adding 25 third-instar larvae per jar and assessment was made of larval mortality by counting pupal skins one week after the addition of larvae. The three formulations provided complete larvicidal efficacy (100%) for at least 24 weeks post-treatment (the length of this study). In the jars where all the water was removed and refilled weekly, LAVIFOS SG 1 %, and MOSQ SG 1 % provided complete larvicidal efficacy for at least 24 weeks post-treatment, whereas AZAI-SS ZG 1 % showed complete larvicidal efficacy for 16 weeks post-treatment. AZAI-SS ZG 1 % still demonstrated a high degree of larvicidal activity (93-99%) from 17 to 24 weeks post-treatment. The present study reveals an excellent residual efficacy of the three new formulations of temephos against larvae of *Aedes aegypti* in water-storage jars lasting for at least 16 to 24 weeks post-treatment. These new formulations will make the control of DHF vectors in Thailand more cost effective as they are removable and retrievable sachets that can be reused after cleaning the water-storage containers.

Introduction

Aedes aegypti (L.) is generally accepted as a primary vector of dengue viruses in tropical and subtropical regions (Halstead, 1966; Gubler and Casta-Valez, 1991; Thavara *et al,* 1996). One of the main strategies to control this vector is application of larvicides to control larval population of mosquitoes. Various larvicidal formulations that possess long-lasting residual activity have been developed, tested and used to control this mosquito vector. In Thailand, temephos sand granule formulation (1 % active ingredient) has been used since the early 1970s in operational control programs at a dosage of 1 mg/l a.i. in water-storage containers (Bang and Tonn, 1969a,b; Bang *et al,* 1972). Until now, the temephos sand granule formulation (1 %) is still an effective larvicide against *Ae. aegypti* larvae. In practice, temephos sand granules possess two negative features: they cause an objectionable odor and water turbidity (Thavara *et al,* 2001). These factors cause objection by household members regarding the use of temephos sand granules in their water-storage containers. As a result, a new formulation of temephos zeolite granules was recently developed to overcome these disadvantages. Mulla *et al* (2004) revealed the newly developed formulation of temephos zeolite granules lacked these undesirable characteristics and possessed high residual efficacy against *Ae. aegypti* larvae for more than six months under field-simulated conditions. This formulation also provided high larvicidal activity for at least three months in village-scale trials under normal water-use practices with high acceptance by villagers (Thavara *et al,* 2004). However, loss of granules during the process of cleaning water-storage containers is another problem with granular formulations of temephos larvicide found in the field (Thavara *et al,* 2004). As a result, Thavara *et al* (2005) initiated an experiment to show that temephos 1 % sand granules enclosed and tied in a muslin cloth and placed in 200-liter water storage jars then transferred sequentially to new sets of jars four time successively provided high larvicidal efficacy (92-100 % emergence inhibition) for about five months or longer. It was therefore necessary to develop a new effective formulation of temephos larvicide that is retrievable during the cleaning process, which is more practical and desirable. This study was carried out to evaluate the larvicidal efficacy of three new formulations of temephos larvicides packed in non-woven sachets against the larvae of *Ae. aegypti* in water-storage jars under field-simulated conditions. The results obtained from this study could provide information regarding a cost effective strategy for the control of *Ae. aegypti* larvae in Thailand and elsewhere.

Materials and methods

Study site

The experiment was carried out at a research station for the evaluation of larvicidal products and other experimental agents for vector control, Nonthaburi Province, Thailand. Description of the research facilities are given in Mulla *et al* (2004).

Materials and treatments

Three new formulations of temephos larvicide, namely: LAVIFOS SG 1 %, MOSQ SG 1 % and AZAI-SS ZG 1 %, were evaluated against larvae of *Ae. aegypti* (L.) in water-storage jars under field-simulated conditions. LAVIFOS SG 1 % and MOSQ SG 1 % are sand granule formulations, whereas AZAI-SS ZG 1 % is a zeolite granule formulation, each one contained 1 % temephos as an active ingredient. These formulations were provided by Ikari Trading (Thailand), Bangkok, Thailand. Each formulation was placed in a non-woven sachet at a quantity of 20 g per sachet and placed in a 200-liter glazed clay jar to obtain a final dosage of 1 mg/l a.i. (one sachet per jar). Each treatment and control jar without larvicide) was replicated four times. A concurrent set of treatments and controls was also carried out in parallel, but all of the water in each treated and control jar was removed and refilled weekly. The treatment and control jars were arranged in a block design and set in a row from east to west. All the jars were positioned in the shade under a roof and were covered with celocrete sheets to prevent wind-borne debris and oviposition by wild mosquitoes. The treatments and controls were challenged weekly with a fresh cohort of laboratory-reared third-instar larvae of *Ae. aegypti,* where a total of 25 larvae were added to each jar. About 0.5 g of ground mouse food was added weekly per jar for larval food. The experiment was carried out for 24 weeks post-treatment.

Assessment of efficacy

Mortality occurred almost completely in the larval stages, not many surviving to lead to the adult stage. Pupal skins (indicating adult emergence) floating on the water surface, mostly at the meniscus level can be picked up easily with a dropper without disturbing the water in the jars. Adult emergence based on pupal skins was then used as an assessment parameter of the overall effectiveness of treatment. The rates of failure to emerge are shown in percentages, which were calculated on the basis of the total number of pupal skins compared to the initial number of larvae added.

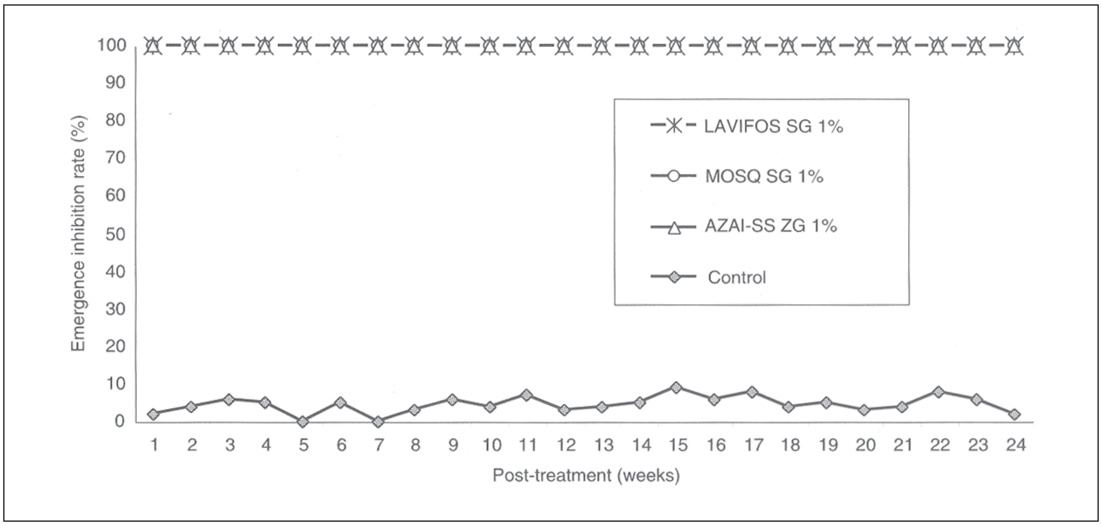

Figure 1 Larvicidal efficacy of three new formulations of temephos larvicides against *Ae. aegypti* larvae in 200-liter water-storage jars.

Data analysis

Comparison of larvicidal efficacy among test larvicides was carried out employing the one-way analysis of variance (ANOVA) with Duncant's multiple range test. All differences were considered significant at $p \leq 0.05$.

Results

The larvicidal efficacy of LAVIFOS SG 1 %, MOSQ SG 1 % and AZAI-SS ZG 1 % applied at 1 sachet per jar (1 mg/l a.i.) against *Ae. aegypti* larvae in constantly full jars is presented in Fig 1. The three formulations provided complete larvicidal efficacy against *Ae. aegypti* for at least 24 weeks post-treatment. In comparison, the mortality rates of larvae in control jars (without larvicides) remained low at about 0-9% (Fig 1).

In jars where the water was removed and refilled weekly, LAVIFOS SG 1 % and MOSQ SG 1 % provided complete larvicidal efficacy for at least 24 weeks post-treatment, whereas AZAI-SS ZG 1 % showed complete larvicidal efficacy for 16 weeks post-treatment (Fig 2). However, the AZAI-SS ZG 1 % still demonstrated a high degree of larvicidal activity (93-99%) during the period from 17 to 24 weeks post-treatment. There was no significant difference in larvicidal efficacy among the three formulations during the course of this study ($p > 0.05$). The mortality rate of larvae in control jars in this experiment was less than 10% (Fig 2).

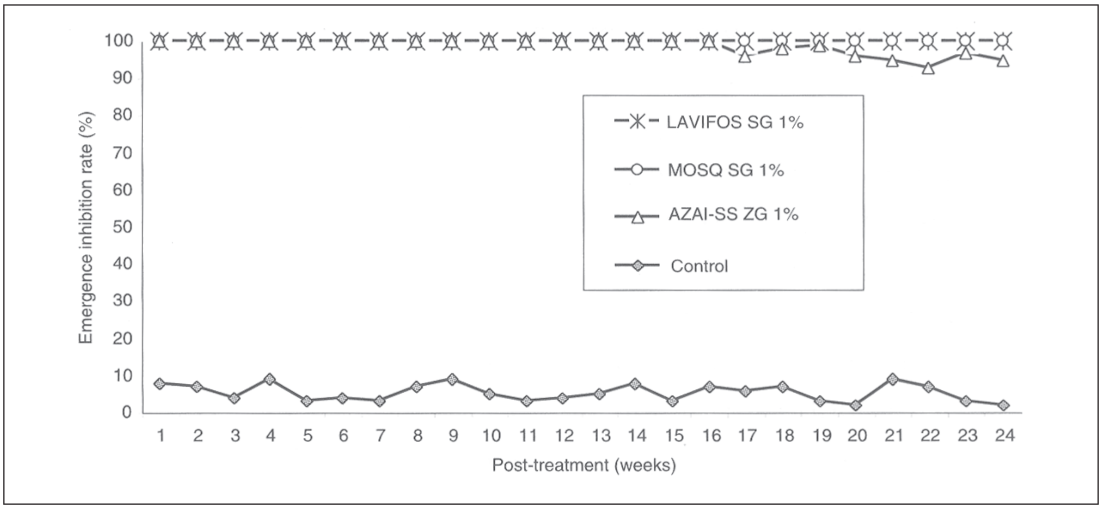

Figure 2 Larvicidal efficacy of three new formulations of temephos larvicides against *Ae. aegypti* larvae in 200-liter water-storage jars with the condition of all water removed ahd refilled weekly.

Discussion

The residual efficacy of temephos (1 %) sand granules in early studies (Bang and Tonn, 1969a,b) and temephos (1 %) zeolite granules in recent trials (Thavara *et al*, 2004) was reported to be about three months against *Ae. aegypti* larvae. Based on these field studies, effectiveness over two to three months in water-storage containers can be expected under normal water-use practices. This residual activity is desirable for larval control of DHF vectors. However, many factors, especially water-use practices, such as adding, removing, draining, and cleaning, will affect the residual efficacy of the larvicides used, even long-lasting formulations may lose efficacy due to dilution or removal of water. Thavara *et al* (2004) found small sizes of water-storage containers (50-200 liters) are usually used for daily consumption of water and are frequently cleaned by homeowners, whereas larger containers (200-2,500 liters) used for long-term storage are only cleaned occasionally. During cleaning, the loose sand or zeolite granules of temephos larvicide are washed out and eventually lost. Thus, newly developed formulations of temephos larvicides packed in sachets, as used in this study, provide an advantage over loose temephos granules in the containers. These formulations will certainly minimize the waste of larvicide and extend the capacity of the control program against *Ae. aegypti* larvae as the larvicides are retrievable and reuseable in the same water-storage containers or in other untreated ones.

As pointed out by Thavara *et al* (2005), temephos was released slowly from sand granules over a long period of time and once it was released in adequate quantities, it remained in the treated containers and yielded excellent control of *Ae. aegypti* larvae for a period of several weeks. Temephos sand granules enclosed and tied in muslin cloth and placed in 200-liter water-storage jars and then transferred sequentially to new sets of jars four time successively still provided high larvicidal efficacy (92-100% larval mortality) even at the low dosage of 0.05 mg/l a.i. for five months or longer (Thavara *et al,* 2005). This observation provided the idea to develop new formulations of larvicide that are retrievable, and more practical. Three new formulations of temephos larvicides in this study, LAVIFOS SG 1 %, MOSQ SG 1 % and AZAI-SS ZG 1 %, were then formulated and packed in non-woven sachets that were very thin and allowed release of active ingredient. The three new formulations demonstrated excellent control of *Ae. aegypti* larvae for at least six months without water replacement. However, villagers usually wash their water-storage containers used for daily consumption of water. It was assumed that people use water at about 200 liters per week and then cleaned their water-storage containers. On this basis, the experiment involved removing all the water in the treated jars and refilling weekly. It is clear that all three larvicidal formulations tested provide excellent results (92 -100% larval mortality) for the control of *Ae. aegypti* larvae in water-storage jars for at least six months when all water was removed and refilled weekly. This implies that temephos was released slowly and continuously from the sachets during the course of this study. It is possible the three new formulations of temephos larvicide could last longer than six months if the experiment were extended.

In conclusion, the present study reveals an excellent residual efficacy of the three new formulations of temephos placed in non-woven sachets against larvae of *Ae. aegypti* in water-storage jars lasting for 16-24 weeks post-treatment depending upon water use practices. These new formulations could make the larval control program of DHF vectors in Thailand more cost effective as they are removable and retrievable sachets that can be reused after cleaning the water-storage containers.

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The authors would like to thank Ikari Trading (Thailand) Co, Ltd, Bangkok, Thailand, for supplying the samples of the new formulations of temephos larvicides for experimentation. We appreciate the encouragement and constant support of Dr. Pathom Sawanpanyalert, Director of the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand. We also acknowledge the able assistance of Patcharawan Sirisopa, Deanpen Deepolngam, Laddawan Wansopa, Thanittha Chumphonrak and Chumphon Chumphonrak of NIH, Thailand, in larval counting, and timely assessment and maintenance of the experimental site. We thank Yutthana Phusap for his supply of mosquito larvae used in the experiment.

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Field Evaluation of Novaluron, a Chitin Synthesis Inhibitor Larvicide, against Mosquito Larvae in Polluted Water in Urban Areas of Bangkok, Thailand

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Abstract: Novaluron, an insect growth regulator, a benzoylphenyl urea insecticide, was evaluated in the field against the larvae of polluted-water mosquitoes. The study was carried out in highly polluted sites infested with populations of mosquito larvae, mostly *Culex quinquefasciatus* Say, in low-income communities in urban areas of Bangkok, Thailand. An EC10 formulation was premixed in water and applied by pressurized spray tank to plots ranging from 180 to 1,000 m^2 at the rate of 0.1 ml EC1 0/m^2 (equal to 10 mg a.i./m^2) of the breeding sites. Assessments were made by sampling mosquito larvae and pupae to determine the trends of immature populations before treatment and weekly after treatment. Reduction of the populations in percents were then computed by comparing counts of immature mosquitoes (larvae and pupae) to the pretreatment counts at each particular site. It was found that the immature populations of mosquitoes in the treated areas were dramatically suppressed and remained at extremely low levels for 3-7 weeks after the treatment depending on the prevailing conditions of each experimental site. No negative impact on fishes or aquatic plants in the treated areas were detected during the study period and three months after the experiment was discontinued. Novaluron is an effective agent to control immature populations of polluted-water mosquitoes, especially *Cx. quinquefasciatus* in habitats in urban areas. This IGR larvicide may play an important role in vector control programs in terms of effectiveness, environmental friendliness and strategies for insecticide-resistance management in vector mosquitoes.

Introduction

The control of polluted-water mosquitoes, especially *Culex quinquefasciatus* Say in urban and suburban areas in Thailand is usually carried out by the municipality, district health office or local administrative unit of each area. These agencies always use thermal fogging with various chemical insecticides, mainly pyrethroids as the main strategy to control mosquitoes. This operation, however, mainly affects adult mosquitoes for a short period, Evidence of resistance to various groups of chemical insecticides by *Cx. quinquefasciatus* has been reported in several provinces of Thailand (Wattanachai *et al*, 1996; Somboon *et al*, 2003; Sathahtriphop *et al*, 2006) as well as in various parts of the world, such as France (Yebakima *et al*, 1995), Colombia (Bisset *et al*, 1998), West Africa (Chandre *et al*, 1998), USA (Liu *et al*, 2005) and Malaysia (Nazni *et al*, 2005). The resistance to chemical insecticides by *Cx. quinquefasciatus* mosquitoes may have been induced by repeated applications of the same kind or group of insecticides by mosquito control programs. The common use of household products containing mainly pyrethroids, carbamates and organophosphates to control insect pests in dwellings may cause insecticide resistance in mosquitoes (Charoenviriyaphap *et al*, 1999). Attempts have been made to control mosquitoes by using the microbial agent *Bacillus sphaericus* Neide. Many formulations of *B. sphaericus* larvicide have demonstrated high degrees of efficacy and long residual control of the larvae of *Cx. quinquefasciatus* in polluted water in artificial pools (Pantuwatana *et al*, 1989) and in urban and suburban communities in Thailand (Mulla *et al*, 1997, 1999, 2001). However, the emergence of resistance in field populations of *Cx. quinquefasciatus* to *B. sphaericus* has been described in Thailand (Mulla *et al*, 2003) and India in the past decade (Rao *et al*, 1995). This is an urgent need to develop strategies to control polluted-water mosquitoes, especially *Cx. quinquefasciatus* that have already developed resistance to chemical insecticides as well as the microbial larvicide *B. sphaericus*. Insect growth regulators (IGRs) are then considered as a novel group of insecticides for controlling these insecticide-resistant mosquitoes.

IGRs, in general, exhibit a good margin of safety to most non-target biota, thus offering some advantages in mosquito control programs (Mulla, 1995). Many IGR compounds and products have been evaluated for larvicidal activity against various mosquito species, such as *Culex peus* Speiser and *Cx. quinquefasciatus* (Mulla and Darwazeh, 1988), *Aedes aegypti* (L.) (Mulla *et al*, 2003), *Culex mosquitoes* (Su *et al*, 2003), *Anopheles* and *Culex mosquitoes* (Batra *et al*, 2005). Most IGRs provide good larvicidal efficacy for the control of targeted mosquitoes, depending on the active ingredients, formulations, dosages, and the habitats treated.

Novaluron is an IGR of the benzoyl urea group, which inhibits chitin synthesis, affecting the moulting process of insects. It has low acute, sub-acute and chronic toxicity to humans, birds, earthworms, fish and aquatic plants, but is highly toxic to some crustaceans (WHO, 2005). Recently, a few reports documented the larvicidal efficacy of novaluron under laboratory and field conditions against the larvae of *Ae. aegypti* (Mulla *et al*, 2003) and *Culex mosquitoes* (Su *et al*, 2003). The present study was carried out to evaluate the field efficacy of a formulation of novaluron against immature mosquitoes in highly polluted developmental sites infested with heavy populations of mosquito larvae and pupae in low-income communities in urban areas of Bangkok, Thailand.

Materials and methods

Experimental material An EC10 (emulsifiable concentrate containing 10% active ingredient) formulation of novaluron was provided by SCK (269) Co, Ltd, Thailand. This material was produced by Makhteshim Chemical Works Ltd, Israel. This formulation is a new product that has not been previously evaluated against polluted-water mosquitoes in the field in Thailand.

Study sites

The four polluted-water mosquito breeding sites used in this study (three sites were waste-water accumulations but another one was a stagnant canal) are located in communities in the suburbs of Bangkok, Thailand. These communities have clusters of low-income housing without adequate waste-water disposal systems. All houses are elevated on posts; the domestic waste-water accumulates under and between the houses. The study sites are discribed below.

Krunai site 1. This site has waste-water accumulation, having the largest area of about 1,000 m^2, with a water depth ranging from 10 to 30 cm. Most of the area beneath the houses is covered with stagnant polluted water. The water level at this site is influenced by domestic water usage and rains; there is no gate for flood control.

Krunai site 2. This site has waste-water accumulation, having an area of about 180 m^2, with a water depth ranging between 10 and 30 cm. The water level fluctuates with domestic water usage and rains. It is located near Krunai site 1 (~ 400 m apart).

Janpradittharam site 1. This site is located about 30 km from Krunai site 1 and Krunai site 2. It also has waste-water accumulation, covering an area of about 220 m^2, with a water depth ranging from 15 to 40 cm. The water level is influenced by domestic water usage and rains. The water is more polluted than the above 2 sites, there was some open space in the site exposed to the sun.

Janpradittharam site 2. This site is located near Janpradittharam site 1 (~ 600 m apart). It is a stagnant canal, having an area of about 500 m^2, with a water depth ranging from 50 to 100 cm. The water was highly polluted and the water level was influenced by opening and closing the flood-control gate, domestic water usage and rains. The flow rate of the water in the canal was relatively low, except some days when the gate was opened for water draining.

Application

The required amount of the EC10 formulation of novaluron for the given site and dosage was placed in a compression spray tank (8 liters), then the required amount of tap water was added and stirred with a wooden paddle. The tank was sealed, shaken well, and pumped to pressurize the mixture for spraying. The mixture was sprayed through a cone nozzle, which produced a stream that reached up to 5 meters away. In this study novaluron was applied at a concentration of 0.1 ml of EC10 per m^2 (equal to 10 mg a.i./m^2) to the breeding sites.

Sampling

Sampling for immature mosquitoes (larvae and pupae) was conducted by dipping with a standard 400 ml dipper at each study site before and after treatment. Samples were taken from locations where heavy aggregations of larvae and pupae occurred. The contents of each dipping were transferred into white plastic trays (30 x 15 x 4 cm deep) for counting. The study sites having large areas, such as Krunai site 1 and Janpradittharam site 2, were sampled by taking 30 dips for each assessment, whereas 15 dips were taken from the smaller sites (Krunai site 2 and Janpradittharam site 1). Some larvae and pupae from each study site were taken and brought to laboratory for species identification.

Efficacy assessment

Assessments were made by sampling mosquito larvae and pupae before treatment (two weeks) and weekly after treatment. Mean and standard error (SE) values for the immature populations (larvae and pupae) for each sampling date and treatment were determined. Reduction rates (%) for the immature mosquitoes post-treatment (weeks 3-12) were computed by comparing counts of immature mosquitoes to the average number obtained from the pre-treatment counts (weeks 1 and 2) for each particular site. These values (mean number, SE and % reduction) were inserted into the figures.

Results

Krunai site 1, a treated area in this study, received a dose of EC10 of novaluron at 10 mg a.i./m^2. The population sizes of immature mosquitoes during preliminary surveys (weeks 1 and 2) were relatively high (average 310-434 larvae

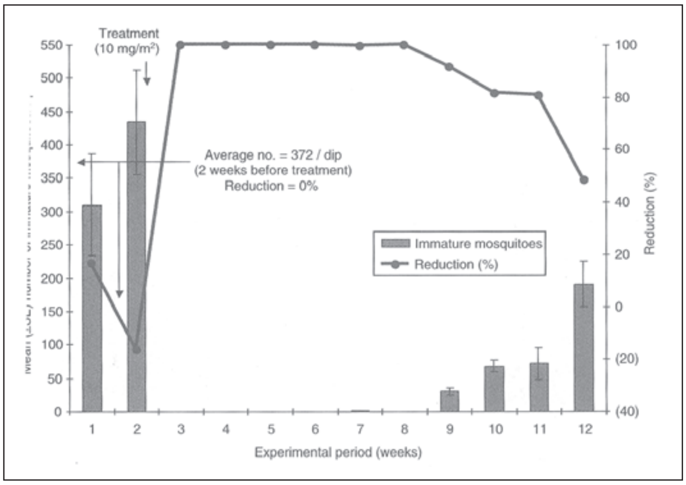

Figure 1 Field efficacy of novaluron treated at the dosage of 10 mg/m^2 at Krunai site 1.

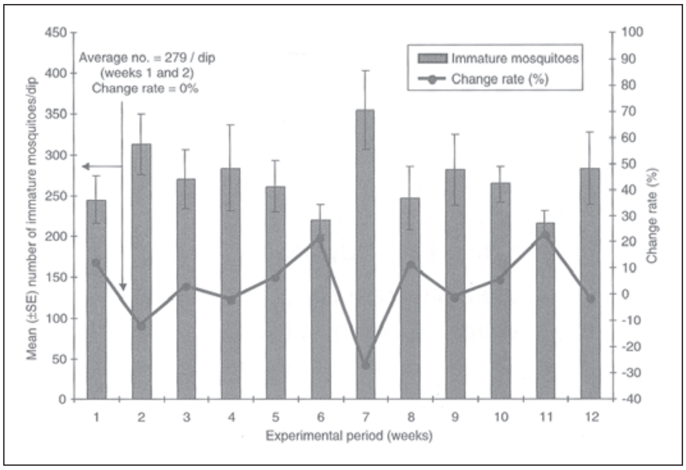

Figure 2 Field populations of immature mosquitoes at Krunai site 2 (check area).

and pupae per dip) with an average number of 372 per dip. The treatment provided excellent control (92-100% reduction) in immature mosquitoes for seven weeks post-treatment (Fig 1). After this period, a high degree of control (81-82% reduction) was achieved for two more weeks, with a moderate resurgence of immature mosquitoes. Ten weeks after treatment, the efficacy had dramatically declined to about 49% compared to the average number of the immature mosquitoes before treatment. There were some light rains at the Krunai site 1 during the course of this study.

Fig 2 demonstrates the natural fluctuation in field populations of immature mosquitoes at Krunai site 2, the control area of this study. No treatments were made at this site in order to observe concurrent immature populations in comparison with those of the treated sites. The population of immature mosquitoes remained moderate to high throughout the experimental period (12 weeks). The average number of immature mosquitoes fluctuated between 216 and 355 per dip, whereas the average number obtained during weeks 1 and 2 (before treatment) was 279 per dip (Fig 2). The immature mosquito population in the control area did not change much compared to the average number for weeks 1 and 2 (279 per dip), but did during week 6 (21 %), week 7 (-27%) and week 11 (23%). Some light rains were recorded in this area.

Janpradittharam site 1, another treated area, also received a single treatment with novaluron at the same dosage on the same day as the Krunai site 1. The population sizes of immature mosquitoes during preliminary surveys were heavy (average 226-272 larvae and pupae per dip). The average number of immature mosquitoes before treatment (weeks 1 and 2) was 249 per dip.

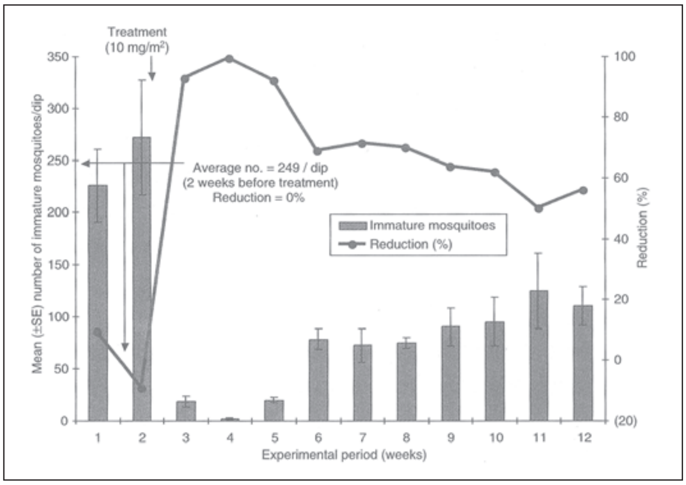

Figure 3 Field efficacy of novaluron treated at the dosage of 10 mg/m^2 at Janpradittharam site 1.

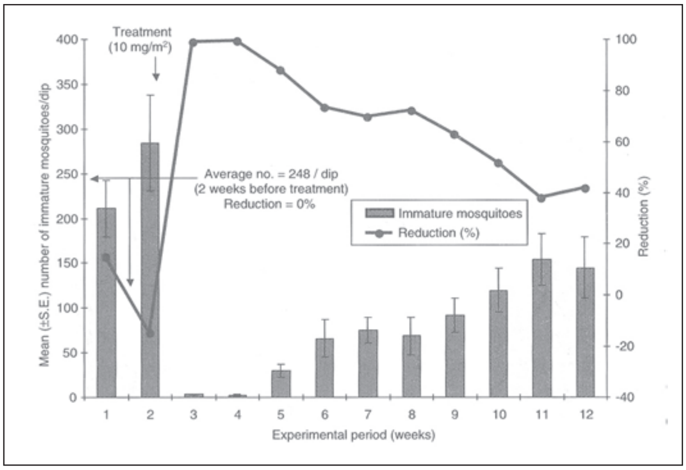

Figure 4 Field efficacy of novaluron treated at the dosage of 10 mg/m^2 at Janpradittharam site 2

Treatment with novaluron yielded excellent control (92-99% reduction) in all immature mosquitoes for three weeks post-treatment (Fig 3). After that, a moderate degree of control (69-71 % reduction) was achieved for three more weeks. The residual efficacy then dropped over the time and remained at about 56% by the end of the experiment (10 weeks post-treatment). Light to heavy rains occurred in the Janpradittharam site 1 during the study period.

Janpradittharam site 2, the stagnant canal, was treated with novaluron at a dosage of 10 mg a.i./m^2 on the same day, as the other two treated sites (Krunai site 1 and Janpradittharam site 1). The populations of immature mosquitoes before treatment were high (211-284 larvae and pupae per dip) with an average number of 247 per dip. Similar to the Janpradittharam site 1 , the treatment provided excellent control (88-99% reduction) of all immature mosquitoes for three weeks post-treatment (Fig 4). Afterward, a moderate degree of control (70-73% reduction) was obtained for three more weeks. The field efficacy of novaluron then declined over the experimental period. The reduction rate remained about 41 % by the time the study was discontinued (10 weeks post-treatment). Janpradittharam site 2 also received some light to heavy rains during the course of this study; as a result, some water was drained out several times.

In this study, *Cx. quinquefasciatus* was the dominant species (>98%) found in all experimental sites, whereas the other species, *Cx. gelidus* Theobald and *Armigeres subalbatus* Coquillett were present in small proportions (<1 %). According to our direct observations, no negative impact on mosquito fish (*Gambusia affinis* Baird & Girard) or aquatic plants, such as water morning glory (*Ipomoea aquatica* Foisk) and water hyacinth [*Eichhornia crassipes* (Mart.) Solms] in the treated areas was detected during the study and for three months after the experiment was discontinued.

Discussion

In our experiment, we used the dosage (10 mg a.i./m^2) for novaluron as a single treatment and we attempted to obtain residual effects against target mosquitoes in the treated sites, containing highly polluted waters. It is interesting to note that a single treatment of an EC10 formulation of novaluron at a dosage of 10 mg a.i./m^2 provided excellent control (90-100% reduction) of the immature populations of polluted-water mosquitoes in the treated areas for three to seven weeks. The differences in residual efficacy depended on the prevailing conditions for each site, such as the degree of water pollution, sun exposure and extent of rains and water drainage. These conditions were encountered in two treated sites (Janpradittharam site 1 and Janpradittharam site 2), which yielded excellent residual control for only three weeks. In contrast, Krunai site 1, another treated site where less rain and drainage was noted provided longer residual control for seven weeks. Similarly, a study conducted in India also showed that novaluron 10% EC when applied at the same dosage (10 mg a.i./m^2) exhibited high degrees of residual effects against *Cx. quinquefasciatus* larvae for 13 days in cesspits, 17 days in drains and 69 days in unused wells (Jambulingam *et al*, unpublished report to the WHO Pesticide Evaluation Scheme, 2004). In smaller breeding sites, such as buckets (18 liter capacity), novaluron 10% EC provided excellent control (>90% inhibition of the emergence) of wild populations of *Cx. quinquefasciatus* for as long as 10 weeks (Arredondo-Jimenez and Valdez-Delgado, unpublished report to the WHO Pesticide Evaluation Scheme, 2004).

Su *et al* (2003) revealed the duration of a high level of efficacy (>90% inhibition of emergence) of novaluron against the larvae of *Cx. quinquefasciatus* tested in mesocosms (27 m^2) were 7 and 13 days at dosages of 1-5 and 10 mg/m^2, respectively; whereas a duration of 14 days was achieved at a dosage of 1.25-5 mg/m^2 tested in a microcosm (240 liters) where the water was enriched with rabbit pellets. Field evaluation of novaluron at dosages of 110 mg/m^2 against *Cx. quinquefasciatus* conducted in India also demonstrated that the effective duration (>80% inhibition of emergence) obtained in cesspits (1-15 m^2), street drains (4-6 m^2) and wells (1.3-4 m^2) were 1113, 8-17 and 33-69 days, respectively (Jambulingam *et al*, unpublished report to the WHO Pesticide Evaluation Scheme, 2004). As can be seen, the extent residual activity of novaluron against *Cx. quinquefasciatus* depends markedly on two major factors: dosages used and types of larval habitats. The activity of novaluron is also degraded by environmental factors, such as ultraviolet light and organic pollution (Su *et al*, 2003).

As mentioned earlier, *Cx. quinquefasciatus* has already developed resistance to some chemical insecticides as well as the microbial larvicide *B. sphaericus* in Thailand and other places around the world; therefore, IGRs including novaluron may be effective larvicides for the control of *Cx. quinquefasciatus* mosquitoes. There have been no reports of resistance to novaluron in this mosquito species. However, monitoring of resistant status in target mosquitoes should be carried out when novaluron or other IGRs are applied in the field.

As tested against other mosquito species, novaluron demonstrated a high level of residual activity against *Anopheles* larvae (*An. culicifacies* Giles and *An. subpictus* Grassi) in riverine pools (at a dosage of 0.01 mg a.i./l) for 63 days and in gem pits (at dosages of 0.010.1 mg a.i./l) for 124 days (Yapabandra, unpublished report to the WHO Pesticide Evaluation Scheme, 2004). Novaluron provided complete coverage (at dosages of 0.0166-0.0498 mg a.i./l) against the larvae of *An. albimanus* Wiedemann and *An. pseudopunctipennis* Theobald confined in sentinel cages placed in artificial pools was also achieved for 16 weeks (Arredondo-Jimenez and Valdez-Delgado, unpublished report to the WHO Pesticide Evaluation Scheme, 2004). Novaluron provided excellent control of *Ae. aegypti* larvae in 200 liter water-storage jars at dosages of 10-20 µg a.i./l for at least two months (Mulla *et al*, 2003); applications at dosages of 0.055-0.165 mg a.i./l yielded high mortality (>85%) for *Ae. aegypti* and *Ae. albopictus* (Skuse) larvae in sentinel cages placed in 18 liter buckets for 14 weeks (Arredondo-Jimenez and ValdezDelgado, unpublished report to the WHO Pesticide Evaluation Scheme, 2004).

The WHO (2005) recommends the use of novaluron as larvicide applied in non-drinking water-storage containers, temporary mosquito habitats and polluted waters at the dosage of 10-50 µg a.i./l or 10-100 g a.i./l ha; however, the higher dosages are required for polluted and vegetated habitats and for obtaining longer residual efficacy. It was stated by the WHO (2005) the level of control by novaluron at various dosages is comparable to pyriproxyfen and better than methoprene (Mulla *et al*, 1986, 1989).

Regarding environmental concerns, novaluron at a dosage of 10 mg a.i./m^2 had no impact on mosquito fishes and aquatic plants in treated areas during and after the experiment. A similar safety profile for novaluron with nontarget fauna in riverine pools was also found for guppies (Poecilia reticulata), a native fish species of Sri Lanka (Rosbora daniconis) and aquatic beetles, when applied at concentrations of 0.01-2.5 mg a.i./l (Yapabandra, unpublished report to the WHO Pesticide Evaluation Scheme, 2004).

In conclusion, our findings show novaluron (10% EC) may be used as an effective larvicide to control immature polluted-water mosquitoes, such as *Cx. quinquefasciatus* larvae, with residual effects for three to seven weeks after a single treatment at a dosage of 10 mg a.i./m^2. Re-treatment with this IGR, may be justified, depending on the prevailing conditions of the treated sites. This IGR larvicide may play an important role in operational vector control programs in terms of effectiveness, environmental friendliness and as a strategy for managing insecticide-resistant vector mosquitoes. Further field studies of novaluron against a range of mosquito species in various habitats in Thailand are warranted.

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Simulated Field Evaluation of Two Formulations of Diflubenzuron, a Chitin Synthesis Inhibitor against Larvae of *Aedes aegypti* (L.) (Diptera: Culicidae) in Water-Storage Containers

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Abstract: Tablet (40 mg a.i./tablet) and granular (2% a.i.) formulations of diflubenzuron, a chitin synthesis inhibitor, insect growth regulator, were evaluated for larvicidal efficacy against the larvae of *Aedes aegypti* (L.) in water-storage containers under field conditions in Thailand. Each formulation was applied to 200-1 clay jars at 5 different dosages (0.02, 0.05, 0.1, 0.5 and 1 mg/l a.i.). The jars were covered with solid celocrete sheets and placed in the shade under a roof. Another experiment was also carried out using 3 different dosages (0.1, 0.5 and 1 mg/l) where half the water in each treated jar and the control was removed and refilled weekly. Each treatment was replicated four times. The treatments were challenged by adding 25 3rd instar larvae/jar weekly. Assessments were made of each treatment through emergence inhibition (%EI) by removing and counting pupal skins one week after larval addition. Using these assessment techniques, a high degree of larvicidal efficacy (96-100%EI) was achieved with 4 dosages (0.05, 0.1, 0.5 and 1 mg/l) of both (tablet and granular) formulations for a period of 23 weeks post-treatment. The efficacy of the lowest dosage (0.02 mg/l) of tablet and granular formulations lasted for 21 and 22 weeks post-treatment, respectively. Under the conditions of water removal and weekly refilling, a high degree of larvicidal efficacy (96-100%EI) at the 3 dosages was obtained with the tablet formulation 18 to 21 weeks post-treatment, whereas the efficacy of the granular formulation persisted 15 to 23 weeks post-treatment depending on the dosage. This study clearly demonstrates a high level of residual activity with both formulations of diflubenzuron against larvae of *Ae. aegypti* in water-storage containers. Considering environmental factors and water-use conditions, it is likely that dosages of 0.05 to 0.1 mg a.i./l are effective dosages providing long-lasting control for 3 to 4 months in the field.

Introduction

Aedes aegypti (L.) is generally recognized as the most important vector responsible for transmission of dengue viruses causing dengue fever, a major mosquito-borne disease. This mosquito species is widely spread throughout the world, including in tropical, subtropical and temperate regions. Chemical and microbial larvicides containing a variety of active ingredients, such as temephos, Bti and insect growth regulators (IGRs), have been developed and recommended for the control of *Ae. aegypti* larvae (Mulla *et al,* 2004; Thavara *et al,* 2004). The IGRs are diverse groups of synthetic chemical compounds which are highly effective against immature stages of mosquitoes and also other insects, but they possess a good margin of safety for most non-target organisms (Mulla, 1995). Their safety and environmental friendliness offer a potential advantage for their use as larvicides in vector control programs. The IGRs have a specific ability to interrupt the life cycle of insects by inhibiting the maturity of insects and keeping them from reaching the critical adult stage. The compounds having insect growth regulating properties belonging to the classes of benzamides, benzoylureas, carbamates, terpenoids, triazines, and other classes of chemicals (Mulla, 1995). IGRs are now available for testing against *Ae. aegypti.* Mulla and Darwazeh (1988), Mulla (1995), Mulla *et al* (2003), Su *et al* (2003), Martins and da Silva (2004) and Batra *et al* (2005) have reported studies on laboratory evaluation and field efficacy of a number of IGRs against mosquito larvae.

This study was initiated to evaluate residual efficacy of two formulations of diflubenzuron (tablets and granules) against larvae of *Ae. aegypti* in water-storage containers under field conditions in Thailand. Multiple dosages of each formulation were used and the treated jars and controls were challenged weekly with larvae for about 27 weeks.

Materials and methods

Study site

This study was carried out at a field research station for the evaluation of mosquitocidal products and other experimental agents for vector control, in Bang Bua Thong District, Nonthaburi Province, Thailand. A detailed description of the research facilities is given in Mulla *et al* (2003, 2004).

Materials and treatments

Diflubenzuron [1-4(chlorophenyl)-3-(2,6 difluorobenzoyl) urea] is a chitin synthesis inhibitor applied to water in order to control breeding of disease vectors. Two formulations of diflubenzuron: tablets (Bi-Larv T, 2 g in weight/ tablet with 40 mg a.i./tablet) and granules (Bi-Larv G, 2% a.i.) were evaluated in this study. These formulations were provided by Crompton Corporation. Each formulation was applied to 200-l glazed-clay jars using 5 dosages (0.02, 0.05, 0.1, 0.5 and 1 mg/l a.i.) and each dosage consisted of 4 jars. To achieve these dosages, each jar of each particular dosage was treated with 1/10, 1/4, 1/2, $2^1/_2$, and 5 tablets of Bi-Larv T, respectively, whereas those treatments with Bi-Larv G were 0.2, 0.5, 1, 5, and 10 g, respectively. The 200-l glazed-clay jars used in this study are commonly used water-storage containers in Thailand. The jars were covered with solid celocrete sheets to prevent wind-borne debris from entering the jars and oviposition by wild mosquitoes and were placed in the shade under a roof. Another concurrent experiment was also carried out using 3 dosages (0.1, 0.5 and 1 mg/l) of both formulations, where half of the water in each treated jar and controls was removed and refilled weekly. The treatments, including controls were arranged in a block design and set in a row from east to west. The jars were treated after addition of the first cohort of larvae and the water loss was replaced weekly.

Assessment of efficacy

The treatments were challenged weekly with a fresh cohort of laboratory-reared larvae, where 25 larvae (third instars) of *Ae. aegypti* transferred in water in cups, were added per jar. About 1 g of ground mouse food was added per jar for the larvae. Larval mortality at the start and later adult emergence, evaluated by counting pupal skins, were assessed. It was noted that almost complete mortality occurred in the larval stage, not surviving to adult emergence. Assessment of pupal skins provide a good measure of the yield of emerging adults. Pupal skins were always found in expected numbers in control jars, but very few if any in the treated jars, except toward the end of the experiment. Pupal skins float on surface, mostly at the meniscus level and can be picked up with a syringe without disturbing the water. The syringed pupal skins were placed in water in white plastic trays and counted. The magnitude of inhibition of emergence was presented in percentage (%EI), which was calculated on the basis of the number of pupal skins (indicating adult emergence) compared to the initial number of larvae added.

Results

The residual efficacy of diflubenzuron (tablet formulation) at five different dosages (0.02, 0.05, 0.1, 0.5 and 1 mg/l active ingredient) against larvae of *Ae. aegypti* in constantly full jars is presented in Fig 1. As can be seen, the lowest dosage (0.02 mg/l) of the tablet formulation provided excellent efficacy with a high emergence inhibition rate (96-100%EI) for 21 weeks post-treatment, after which its efficacy declined continuously over the remaining period, reaching about 50%EI at 26 weeks post-treatment, and dropped to about 19%EI by the end of the experiment (27 weeks post-treatment). In contrast, the higher dosages (0.05, 0.1,

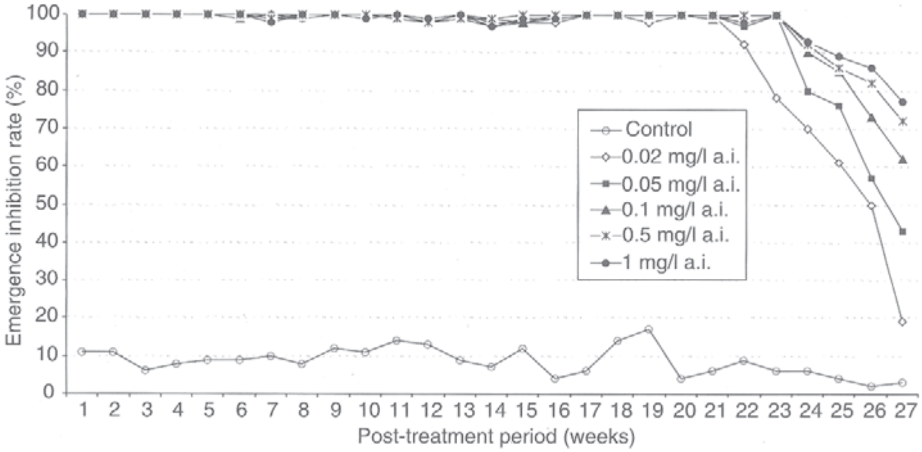

Figure 1 Residual efficacy (% emergence inhibition) of diflubenzuron tablet (40 mg a.i./tablet) at various dosages (mg/l a.i.) in water-storage jars (200 l), kept full without exchange of water.

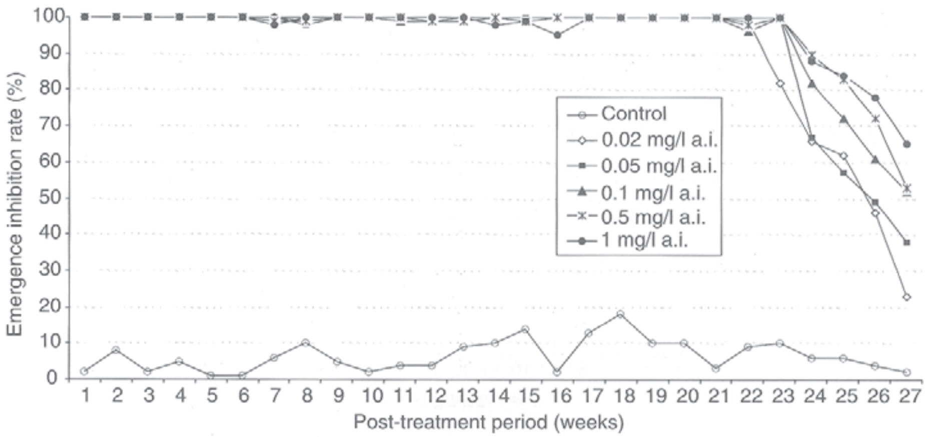

Figure 2 Residual efficacy (% emergence inhibition) of diflubenzuron granules (2% a.i.) at various dosages (mg/l a.i.) in water-storage jars (200 l), kept full without exchange of water.

0.5 and 1 mg/l) of the tablet formulation demonstrated somewhat longer efficacy for 23-25 weeks post-treatment. After that, the efficacy of these dosages also declined, but the efficacy remained higher than the dosage of 0.02 mg/l during the same period. By the end of this experiment (27 weeks post-treatment), the efficacy of the diflubenzuron tablet at dosages of 0.05, 0.1, 0.5 and 1 mg/l were 43, 62, 72 and 77% EI, respectively. The emergence inhibition rate of the control group in this study was usually low, except in some weeks when it was more than 10%.

Fig 2 shows the residual efficacy of diflubenzuron (granular formulation) at five dosages: 0.02, 0.05, 0.1, 0.5 and 1 mg/l a.i. against the larvae of *Ae. aegypti*

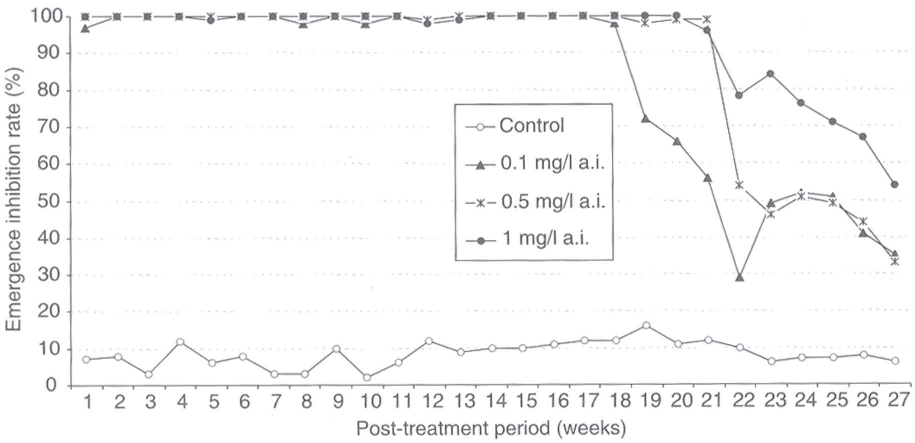

Figure 3 Residual efficacy (% emergence inhibition) of diflubenzuron tablet (40 mg a.i./ tablet) at various dosages (mg/l a.i.) in water-storage jars (200 l), 1/2 water volume removed and refilled weekly.

in constantly full jars. A high degree of larvicidal efficacy at four dosages (0.05, 0.1, 0.5 and 1 mg/l) of this formulation lasted for 23 weeks post-treatment, similar to that of the tablet formulation. The longevity of efficacy at the lowest dosage (0.02 mg/l) of diflubenzuron granular formulation (22 weeks) was slightly longer than that of the tablet formulation (21 weeks). It is evident that the residual patterns of efficacy of diflubenzuron granular formulation were almost the same as those of the tablet formulation. However, when both formulations reached their maximum period of excellent efficacy, the efficacy of the granular formulation group declined somewhat more rapidly than that of the tablet formulation group. At the end of this study (27 weeks post-treatment), the efficacy of diflubenzuron granules at the dosages of 0.02, 0.05, 0.1, 0.5 and 1 mg/l a.i. were 23, 38, 52, 53 and 65%EI, respectively.

Under the condition of exchange of half the volume of water (100 l) weekly, the residual efficacy of diflubenzuron (tablet formulation) at three dosages (0.1 ,0.5 and 1 mg/l a.i.) against the larvae of *Ae. aegypti* is presented in Fig 3. The high degree of emergence inhibition rate of diflubenzuron tablet at the dosage of 0.1 mg/l a.i. lasted for about 18 weeks after treatment, after which its efficacy decreased rapidly and fluctuated between 29 and 52%EI during the last six weeks of this experiment. Under the same conditions of water removal and refilling weekly, the higher dosages (0.5 and 1 mg/l a.i.) of the diflubenzuron tablet exhibited a longer period of excellent efficacy for 21 weeks post-treatment. After reaching the maximum period of excellent efficacy at 21 weeks post-treatment, the efficacy of the diflubenzuron tablet at a dosage of 0.5 mg/l a.i. dropped rapidly to about 54%EI by the 22^{nd} week, and then fluctuated between 51 and

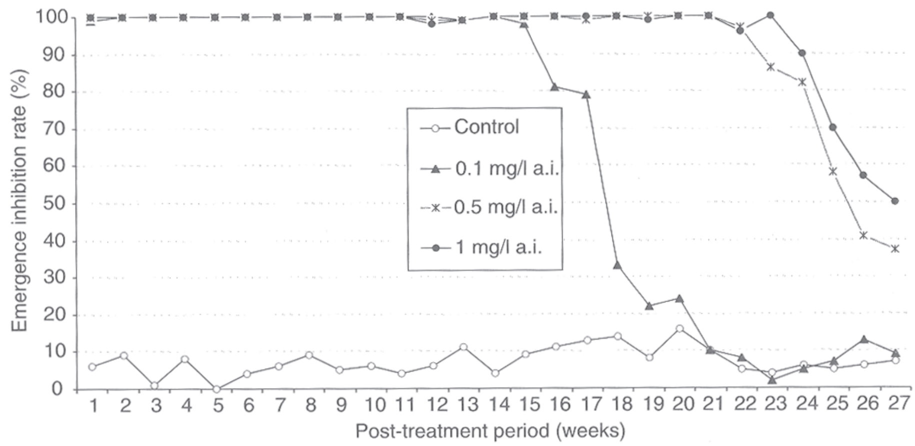

Figure 4 Residual efficacy (%emergence inhibition) of dif/ubenzuron (granules, 2% a.i.) at various dosages (mg/l a.i.) in water-storage jars (200 l), 1/2 water volume removed and refilled weekly.

33%EI during the last five weeks of this experiment. In contrast, the efficacy of the diflubenzuron tablet at the highest dosage (1 mg/l a.i.) declined gradually over the test period from 96%EI at the 21st week post-treatment to about 54%EI at the 27th week post-treatment.

Fig 4 reveals the residual efficacy of diflubenzuron (granular formulation) at three dosages (0.1, 0.5 and 1 mg/l a.i.) against larvae of *Ae. aegypti* under the conditions of half the volume of water (100 l) removal and refilling weekly. The lowest dosage (0.1 mg/l a.i.) showed a high degree of emergence inhibition rate for a period of 15 weeks post-treatment. Its efficacy decreased to about 80%EI during the period between the 16th and 17th week, then dropped sharply to about 33%EI by the 18th week post-treatment, remaining lower than 24%EI until the end of experiment. On the contrary, a longer efficacy was noted for dosages of 0.5 and 1 mg/l for a period of 22 and 23 weeks post-treatment, respectively. After these periods, the efficacy of both dosages declined gradually over the test period. At the end of this study (27 weeks post-treatment), the emergence inhibition rate of the diflubenzuron granules at the dosages of 0.5 and 1 mg/l were 37 and 50%EI, respectively.

Discussion

Diflubenzuron is an IGR which inhibits chitin synthesis of mosquito larvae during ecdysis, affecting larval development at all larval instars and other stages. However, there are significant differences in diflubenzuron inhibiting activity among the instars, and the 3rd instar larvae were found to be the most resistant to the diflubenzuron inhibiting effect (Martins and da Silva, 2004) and diflubenzuron does not cause any reduction in the reproduction potential of *Ae. aegypti* (Fournet *et al,* 1993). The larvae on ingestion of diflubenzuron were unable to complete their molt and subsequently died. This is a completely different mode of action than the other synthetic chemical larvicides and thus provides a novel strategy for resistance management where resistance to conventional larvicides occurs. As temephos has been used for the control of *Ae. aegypti* larvae in Thailand for over 3 decades (Bang *et al*, 1972), it is now suspected that temephos resistance may already have developed or could soon occur in some areas subjected to temephos treatments for long periods. Development of some degrees of resistance to temephos in *Ae. aegypti* in the field has been reported in various places, such as the Caribbean (Georghiou *et al*, 1987), Santa Domingo (Mekuria *et al*, 1991), British Virgin Islands (Wirth and Georghiou, 1999), Brazil (Campos and Andrade, 2001), Thailand (Paeporn *et al*, 2004), and Malaysia (Chen *et al*, 2005).

The present study documents excellent larvicidal efficacy of two formulations of diflubenzuron at various dosages against 3rd instar larvae of *Ae. aegypti in* water-storage jars (200-1 capacity) under field conditions. We used the 3rd instar larvae of *Ae. aegypti* for evaluation in order to determine the larvicidal efficacy against the most resistant stage of the tested species. Regarding the water-storage containers used in this study, we employed glazedclay jars (200-1 capacity) as they are the most commonly used containers by dwellers throughout the country, which constitutes a major habitat for *Ae. aegypti* in Thailand (Chansang *et al*, 1993; Kittiyapong and Strickman, 1993; Thavara *et al*, 2001). To obtain actual larvicidal efficacy, we also simulated water-use and exchange practices by removal and refilling the water weekly. However, the longevity of diflubenzuron in water-storage jars against *Ae. aegypti* larvae was somewhat decreased by water exchange practices. In the jars without water removal and refill, the two higher dosages (0.5-1 mg/l a.i.) of both formulations provided almost complete efficacy (100 %EI) for 23-24 weeks post-treatment, whereas a similar residual efficacy of both dosages in the jars with water removal and refill lasted for 20-21 weeks (tablets) and 22-24 weeks (granules) post-treatment. The data in our study, support a high level of residual efficacy for both formulations of diflubenzuron against *Ae. aegypti* larvae in water-storage containers under field conditions. Recently, the World Health Organization (WHO) recommended the use of two formulations of diflubenzuron (2% DT and 2% GR) for the control of container-breeding mosquitoes, such as *Ae. aegypti*, at a dosage of 0.02-0.25 mg/l a.i., with an expected residual efficacy of 2 to 4 months (WHO, 2006). In addition, higher rates of application were also recommended for containers with exposure to sunlight or with high organic content. On the other hand, Ansari *et al* (2005) suggested using another two formulations of diflubenzuron (25WP and 22SL) at a dosage of 8 mg/m^2 to control *Ae. aegypti* larvae in unused coolers with complete inhibition

(100%EI) for 7 weeks.

In Thailand, the most commonly used larvicide for the control of *Ae. aegypti* larvae is temephos which has been used since the early 1970s (Bang *et al,* 1972). Various formulations of temephos have shown excellent residual effectiveness against *Ae. a egypti* larvae in water-storage containers for several months (Mulla *et al*, 2004; Thavara *et al*, 2004). However, some formulations of temephos sand granules are objected to by dwellers for use in their water-storage containers because of unpleasant smell and water turbidity after application (Phanthumachinda *et al*, 1985; Thavara *et al*, 2001). Hence, it is likely that at least two formulations of diflubenzuron tested in this study could provide a substitute or an alternative larvicide for the control of *Ae. aegypti* larvae in Thailand as these formulations possess no unpleasant characteristics.

In addition to good effectiveness against *Ae. aegypti* larvae, diflubenzuron also has a wide spectrum of larvicidal activity against various mosquito species, such as *Anopheles quadrimaculatus* Say and *Culex tarsalis* Coquillett (Estrada and Mulla, 1986), *An. culicifacies* Giles, *An. stephensi* Liston and *Cx. quinquefasciatus* Say (Ansari *et al*, 2005). WHO (2006) has also recommended the use of diflubenzuron (2% GR and 25% WP formulations) for mosquito control in open bodies of water at the dosage of 25-100 g/ha a.i. However, higher dosages are required in polluted and vegetated habitats, whereas lower dosages are probably adequate for the control of flood-water mosquitoes (WHO, 2006).

Regarding safety, WHO classifies diflubenzuron as unlikely to present an acute hazard in normal use since it has low-acute and chronic toxicity to mammals, with no indication of carcinogenicity, mutagenicity or teratogenicity (WHO, 2006). Diflubenzuron is also environmentally friendly with a low toxicity to birds, fish and aquatic plants. Although diflubenzuron is highly toxic to non-target biota, such as some crustaceans and macro-invertebrates, the resurgence of the affected target and non-target organisms takes place fairly quickly (WHO, 2006).

In conclusion, a high degree of larvicidal efficacy (96-100% EI) was achieved with 4 dosages (0.05, 0.1, 0.5 and 1 mg/l a.i.) of both formulations of diflubenzuron for a period of 23 weeks post-treatment, whereas the efficacy of the lowest dosage (0.02 mg/l a,i.) of the tablet and granular formulations lasted for 21 and 22 weeks post-treatment, respectively. Under conditions of water removal and refilling weekly, high degrees of larvicidal efficacy (96-100%EI) at the 3 higher dosages was obtained with the tablet formulation for 18 to 21 weeks post-treatment, whereas the efficacy of the granular formulation persisted 15 to 23 weeks post-treatment depending on the dosage used. This study clearly demonstrated a high level of residual activity for both formulations of diflubenzuron (tablets and granules) against the larvae of *Ae. aegypti* in water-storage containers. Considering environmental factors and water-use conditions, it is likely that dosages 0.05 to 0.1 mg/l a.i. will be effective dosages to provide long-lasting control for at least 3 to 4 months in the field.

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Repellency of Essential Oils Extracted from Plants in Thailand against Four Mosquito Vectors (Diptera: Culicidae) and Oviposition Deterrent Effects against *Aedes aegypti* (Diptera: Culicidae)

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Abstract: In this study we evaluated and reported repellent effects of essential oils from Thai plants against 4 mosquito vectors: *Aedes aegypti*, *Ae. albopictus*, *Anopheles. dirus* and *Culex quinquefasciatus* under laboratory conditions using human volunteers. The essential oils were extracted from 18 plant species, belonging to 11 families, and the oils were then prepared as 10% solution in absolute ethanol with additives. Two chemical repellents, deet and IR3535, were also prepared in the same formulation as the essential oil repellents and tested for repellency as controls. The essential oils were also evaluated for oviposition deterrent effects against *Ae. aegypti* under laboratory conditions. The results show night-biting mosquitoes (*An. dirus* and *Cx. quinquefasciatus*) and *Ae. albopictus* were more sensitive to all the essential oils (repellency 4.5-8 hours) than was *Ae. aegypti* (repellency 0.3-2.8 hours), whereas deet and IR3535 provided excellent repellency against all four mosquito species (repellency 6.7-8 hours). All essential oils exhibited oviposition deterrent activity against *Ae. aegypti* with various degrees of repellency ranging from 16.6 to 94.7%, whereas deet and IR3535 had no repellency. The present study demonstrates the potential for using essential oils as mosquito repellents and oviposition deterrents. These findings may lead to new and more effective strategies for protection from and control of mosquitoes.

Introduction

Many mosquito-borne diseases, such as malaria, dengue fever (DF), dengue hemorrhagic fever (DHF) and filariasis, are serious public health problems in tropical regions, especially in Africa and Asia. These diseases are transmitted to human beings through mosquito bite only. Since there is no effective vaccine available for the control of these diseases, prevention of mosquito bites is one of the main strategies to control or minimize incidence of these diseases. The use of insect repellents can provide a practical and economical means of preventing mosquito-borne diseases. It is important not only for local people in disease risk areas, especially in tropical countries, but also for travelers who are vulnerable to diseases spread by mosquito vectors when they visit and seek leisure away from their home country.

Although the most common mosquito repellents currently available on the market containing deet (N, N-diethyl-3-methylbenzamide) have shown excellent protection from mosquito bites (Yap, 1986; Walker *et al*, 1996; Thavara *et al*, 2001) and other biting insects (Coleman *et al*, 1993), there were reports of toxicity problems after application of deet range from mild effects, such as contact urticaria (Maibach and Johnson, 1975) and skin eruption (Reuveni and Yagupsky, 1982), to severe reactions, such as toxic encephalopathy (Zadikoff, 1979; Roland *et al*, 1985; Edwards and Johnson, 1987). To overcome these adverse effects, attempts to find and develop repellents derived from plant extracts have been made by many researchers. In Thailand, some plant extracts, such as basil (Chokechaijaroenporn *et al*, 1994), galanga (Choochote *et al*, 1999), turmeric (Tawatsin *et al*, 2001), aromatic turmeric (Pitasawat *et al*, 2003), celery (Choochote *et al*, 2004; Tuetun *et al*, 2004) and clove (Trongtokit *et al*, 2004) have been investigated for repellent activity against various mosquito species under laboratory and field conditions. The development and use of locally available plants showing repellent activity avails an alternative strategy for the control or minimization of mosquito-borne diseases, especially in developing countries. In the present study, we evaluated and report on the repellent effects of essential oils extracted from 18 species of Thai plants against four mosquito vectors: *Aedes aegypti* (L.), *Ae. albopictus* (Skuse), *Anopheles dirus* Peyton & Harrison, and *Culex quinquefasciatus* Say under laboratory conditions. Comparison of repellency over different exposure periods was also carried out to standardize repellent testing methods. In addition, we evaluated the oviposition deterrent activity of each repellent composition against *Ae. aegypti* under laboratory conditions.

Materials and methods

Plant species

Eighteen plant species belonging to 11 families were selected for this study because most of them are known or used traditionally as mosquito repellents by Thai people. They were *Eleutherococcus trifoliatus* (L.) (Phak paem), *Schefflera leucantha* R. Vig. (Hanuman prasankai), *Ocimum sanctum* L. (Holy basil), *Vitex trifolia* L. (Khon thi so), *Litsea cubeba* (Lour.) Pers. (Ta khrai ton), *Manglietia garrettii Craib* (Montha doi), *Aglaia odorata Lour*. (Prayong), *Myristica fragans Houtt*. (Nutmeg tree), *Melaleuca cajuputi Powell* (Cajuput tree), *Psidium guajava* L. (Guava), *Piper betle* L. (Betel pepper), *Piper nigrum* L. (Black pepper), *Murraya paniculata* (L.) Jack (Orange jasmine), *Houttuynia cordata Thunb*. (Fishwort), *Zingiber officinale Roscoe* (Ginger), *Alpinia galanga* (L.) Wild (Galanga), *Curcuma Ionga* L. (Turmeric), and *Hedychium coronarium* J. Konig (White ginger).

Extraction of essential oils

Essential oils were extracted from each plant by steam distillation. One to two kilograms of fresh plant material (by particular part of each plant, see Table 1) were cut into small pieces and placed in a distillation flask with approximately five times as much water, and 10 glass beads. The distillation chamber was heated in a liquid paraffin bath at about 120°C until the distillation was completed. The distillate was collected in a separate funnel in which the aqueous portion was separated from the essential oil (oily phase). The aqueous phase (lower layer) was slowly drawn off until only the oil layer remained. This procedure was repeated until at least 5 ml of essential oil was collected. Each essential oil was kept in a screwed-cap glass vial at 4°C until it was tested for mosquito repellency and ovi-positional deterrent activity.

Analysis of chemical constituents

All essential oils were analyzed for chemical constituents employing the Gas Chromatography / Mass Spectroscopy (GC/MS) assay. Briefly, the essential oil (50 µl) was diluted with 1.5 ml of hexane and CH2Cl2 (1:1) to a final concentration of 3.33% v/v. The diluted sample (0.1 µl) was then injected into the column (DB^{TM}-1 ms, 30 m x 0.25 mm x 0.25 µm, 100% dimethylpolysiloxane) for analysis with a GC-MS instrument (QP2010, Shimatzu). The operation conditions were as follows: the injection temperature was 200°C. Helium was used as a carrier gas and the purge flow rate was 3 ml/minute. The pressure was 69.4 kPa and the split ratio was 1:100. The chemical constituents of each essential oil were obtained by searching each peak and comparing with data from the National Institute of Science and Technology (NIST) library spectra. The relative amounts of the individual chemical components of each essential oil were computed from the GC peak areas (%).

Preparation of repellents for testing

The essential oils were formulated as 10% lotion in absolute ethanol and additives (vanillin, propylene glycol and polyethylene glycol). For comparison with standard repellents, two chemical repellents, N, N-diethyl-3-methylbenzamide (deet) and ethyl butylactylamino-propionate (IR3535), were formulated as 10% lotion similar to the essential oil repellents. All formulated repellents were placed in screw-cap vials and kept at room temperature before testing.

Test mosquitoes

The mosquitoes used in this study were laboratory-reared female mosquitoes (age 4-5 days) *Ae. aegypti*, *Ae. albopictus*, *An. dirus* and *Cx. quinquefasciatus*. These were reared according to the standard protocol of the National Institute of Health, Thailand, and maintained at the insectary of the institute.

Repellent test

The repellency of essential oils and standard repellents was assessed in the laboratory using a human-bait technique (Tawatsin *et al*, 2001). Ethical clearance was approved by the Ethics Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (TM-IRB004/2005). Six volunteers (age 25-61 years) participated in the laboratory tests. The testing period lasted up to eight hours, depending on the efficacy of repellent. The timing of the tests depended on whether the target mosquitoes were day or night-biters. *Ae. aegypti* and *Ae. albopictus* were tested during the daytime from 0900 to 1700, while *An. dirus* and *Cx. quinquefasciatus* were tested during the night from 1900 to 0300. Evaluations were carried out in a 6x6x3 m room, at 25-29°C with relative humidity of 60-80%. An area of 3x10 cm on each forearm of the six human volunteers was marked out with a permanent marker. Each test repellent formulation (0.1 ml) was applied to the marked area of one forearm of each volunteer while the other forearm was treated with 0.1 ml of solution base (without active ingredient) as a control. Before the start of each exposure period, the bare hand of the test person, used as control area for each volunteer, was exposed for up to 10 seconds in a mosquito cage (30x30x30 cm), containing 250 host-seeking female mosquitoes (4-5 days old). If at least two mosquitoes landed on or bit the hand, the repellency test was then continued. This was done to ensure that the mosquitoes were host seeking. Then each volunteer put the test forearm and hand covered by a paper sleeve with a hole corresponding to the marked area into the mosquito cage for the first three minutes of each half-hour interval. The number of mosquitoes biting the treated area of each volunteer was recorded each minute (at 1, 2 and 3 minutes) of each 3 minute exposure. To determine the duration of protection for each repellent, the exposures continued until at least two bites occurred in a given exposure period, or until a bite in the previous exposure period was followed by a confirmatory second bite in the following exposure period. The time between application of the test repellent and the second successive bite was recorded as the protection time.

Ovipositional deterrent test

Ovipositional deterrent activity of essential oils and standard repellents were studied for gravid *Ae. aegypti* under laboratory conditions at room temperature. Two black plastic cups (300 ml in capacity) were filled with 200 ml deionized water. One cup was a control and the other cup was treated with essential oil (undiluted) or standard chemical repellent (deet or IR3535) at dosage of 20 µl/cup. The final concentration of the treated material (essential oil or chemical repellent)in each treated cup was 0.01%. Each cup was fitted inside with a white filter-paper sheet (7 x 28 cm) for deposition of mosquito eggs. The paper was located in each cup so as the lower half of the paper was submerged in water. The cups were placed in a mosquito cage (30 x 30 x 30 cm) containing 50 gravid female mosquitoes for 48 hours then, the eggs laid in each cup were counted after removal of the oviposition paper. Each test repellent was tested in six cages. The percentage of repellency for each essential oil and standard repellent was calculated by Xue *et al* (2001) as follows:

$$\text{Repellency (\%)} = \frac{C - T}{C} \times 100$$

where *C* stands for the number of mosquito eggs collected from the control cup and *T* denotes the number of mosquito eggs collected from the treated cup.

Data analysis

The mean protection time was used as a standard measure of repellency for the essential oils, deet and IR3535 against the four mosquito species. Comparison of repellency for each test repellent derived from the different exposure periods and oviposition deterrency against gravid *Ae. aegypti* were carried out employing the one-way analysis of variance (ANOVA) with Duncan's multiple range test. All differences were considered significant at $p \leq 0.05$.

RESULTS

Yields of the 18 essential oils distillated from different parts of each plant species and the chemical constituents identified by GC/MS are shown in Table 1. Of these, 11 oils were extracted from leaves, four oils from rhizomes (Zingiberaceae family), and the remaining oils were from seeds (*Litsea cubeba*), fruits (*Piper nigrum*) and flowers (*Houttuynia cordata*). Most of the plants in this study yielded less than 1% essential oil, except *Litsea cubeba* (3.16%). Moderate yields were obtained from *Alpinia galanga* (0.83%), *Myristica fragrans* (0.66%) *Melaleuca cajuputi* (0.43%) and *Piper betle* (0.37%), whereas the other species provided low yields of 0.20% or less. The lowest yields (less than 0.10%) were obtained from *Piper nigrum* (0.08%), *Manglietia garrettii* (0.07%), *Eleutherococcus trifoliatus* (0.05%), *Murraya paniculata* (0.05%), *Schefflera leucantha* (0.04%) and *Aglaia oclbrata* (0.04%).

Numerous chemical constituents, ranging from 12 to 30 peaks of different chemicals were detected in the essential oils (see Table 1). These included both common and commonly known chemicals. The commonly known chemicals were α-pinene, β-pinene, borneol, linalool, d-limonene, cymene, eucalyptol, citronellal, caryophyllene. However, a few chemical peaks found in an essential oil (*Melaleuca cajuputi*) could not be identified, since they were less than 80% similar to other compounds in the database spectra library.

Repellency (as shown in hours of protection time) obtained for the three different exposure times (1, 2 and 3 minutes) for each essential oil repellent and the two chemical repellents (deet and IR3535) assessed by mean protection time (+ SE) against the four mosquito species under laboratory conditions is shown in Table 2. The blank controls (solution base without any active ingredients) showed no repellency against the four mosquito species. The average numbers of mosquitoes landing or biting the bare hand of the volunteers (within 30 seconds before the start of each exposure) were 17, 12, 5 and 10 against *Ae. aegypti*, *Ae. albopictus*, *An. dirus* and *Cx. quinquefasciatus*, respectively (data not shown). This confirms the test mosquitoes were host seeking during the test periods.

There were significant differences in repellency obtained during the three different exposure times (1, 2 and 3 minutes) for each repellent against the test mosquitoes, especially *Ae. aegypti* and *Cx. quinquefasciatus* (Table 2). The results show the repellency was inversely proportional to the exposure time. In other words, repellency of most of the essential oils declined with time of exposure. Of the 18 essential oils tested, 17, 3, 1, and 8 oils provided significantly different repellencies during the three exposure periods against *Ae. aegypti*, *Ae. albopictus*, *An. dirus* and *Cx. quinquefasciatus*, respectively. In contrast, there was no significant difference in repellency obtained for deet and IR3535 against the four mosquito species at the three exposure periods.

Table 1 Yields and chemical constituents of essential oils obtained from the study plants.

Plant names	Part used	Yield (%)	Chemical constituents (%)[a]
Eleutherococcus trifoliatus (L.) S.Y.Hu (ARALIACEAE)	leaves	0.05	(1 S,3R,5S)-2(1 0)-pinen-3-ol (23.81), 2-pinen-10-ol (10.71), 1,2-epoxy-p-menth-8-ene (10.59%), 2-pinen-10-ol (10.37), (Z)-verbenol (20.49), (±)-p-menth-6-ene-2,8-diol (5.01), 2,2,3-trimethyl-3-cyclopentene-1-acetaldehyde (4.28), [1S-(1α, 2β, 4β)-1-ethenyl-1-methyl-2,4-bis(1-methylethenyl) -cyclohexane (4.22), (1 R)-(-)-myrtenal (3~59), (-)-spathulenol (2.83), (±)- 2(10)-pinen-3-one (2.20), eucalyptol (1.89)
Schefflera leucantha R.Vig. (ARALIACEAE)	leaves	0.04	[1S-1α, 4α, 7α]-1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7 -(1-methylethenyl)-azulene (44.68), (-)-3,7,7-trimethyl-11-methylene-spiro[5.5]undec-2-ene (19.49), 2,3,4,-4a,5,6-hexahydro-1,4a-dimethyl-7-(1-methylethyl)-naphthafene (10.12), [1S-1α, 7α, 8aβ]-1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-azulene (3.95), (-)-spathulenol (3.77), [1S-1α, 7α,8aβ]- 1,2,3,5',6,7,8,8a-octahydro-1 ,4-dimethyl-7-(1-methylethenyl)-azulene (3.49), [2R-(2α,4aα,8aβ)]- 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-naphthalene (2.50%), caryophyllene (2.26), α-caryophyllene (1.91), 8-isopropenyl-1 ,5-dimethyl-cyclodeca-1, 5-diene (1.83), [1S-(1α,2β,4β)]-1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-cyclohexane (1.59), aristolene (1.32), copaene (1.20), (1α,4aβ,8aα)-1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-naphthalene (0.95), (-)-α-panasinsen (0.95)
Ocimum sanctum L ... (LABIATAE)	leaves	0.16	eugenol methyl ether (66.89), caryophyllene (1.8.95), [3aS-(3aα, 3bβ)]-odahydro-7-methyl-3-methylene-4-(1-methylethyl)-1H-cyclopenta[1,3]cyclopropa[1,2]benzene (4.01), 8-isopropenyl-1,5-dimethyl-1,5-cyclodecadiene (3.09), [1S-(1α, 2β, 4β)]-1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)-cyclohexane (1.65), α-caryophyllene (1.21), (-)-borneol (1.10), (+)-epibicyclo-sesquiphellandrene (0.45), caryophyllene oxide (0.45), β-pinene (0.29), limonene (0.27), linalool (0.27), eucalyptol (0.24%), α-pinene (0.23), camphene (0.20), eugenol (0.20), β-terpinene (0.16), cadina-1 (10),4-diene (0.15), 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-cycloheptane (0.11), cycloheptane (0.08)

Table 1 Yields and chemical constituents of essential oils obtained from the study plants. (continued)

Plant names	Part used	Yield (%)	Chemical constituents (%)[a]
Vitex trifolia L. (LABIATAE)	leaves	0.16	eucalyptol (31.26), p-menth-1-en-8-ol acetate (13.48), β-phellandrene (9.99), caryophyllene (7.58), α-pinene (6.93), p-menth-1-en-8-ol (7.33), p-menth-1-en-4-ol (4.57), (-)-spathulenol (3.31), caryophyllene oxide (2.97), β-isomethyl ionone (2.80), [3R-(3α,4aβ,6aβ,10aβ)]-3-ethenyldodecahydro-3,4a,7, 7,10a-pentamethyl-1H-naphtho[2,1-b]pyran (2.43), β-pinene (2.28), [1 R-[1α,2β]]-α,2,5,5,8a-pentamethyl-α-ethenyldecahydro-2-hydroxy-1-naphthalenepropanol (1.02)
Litsea cubeba (Lour.) Pers. (LAURACEAE)	seeds	3.16	*(E)*-3,7-dimethyl, 2,6-octadienal (75.56), 6-methyl- 5-hepten-2-ohe (7.92), *(R)*-(+)-citronellal (3.54), linalool (2.21), limonene (2.03), eucalyptol (1.23), 4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol (1.18), *(E)*- 2-[2' -(2''- methyl-1''-propenyl) cyclopropyl] propan- 2-ol (1.01), neric acid (0;87), 2,7-dimethyl-2,7-octanediol (0.85), *(E)*-4,5-epoxy-carene (0~39), (E)-geraniol (0.37), 2,3-dimethyl-1,3-heptadiene (0.36). β-pinene (0.34), isopulegol (0.30), α-pinene (0.27), p-menth-1-en-8-ol (0.26), (Z)-p-mentha-6,8-dien-2-ol (0.22), β-myrcene (0.21), (Z)-verbenol (0.20), 1-methyl-4-(1-methylethenyl)-7-oxabicyclo[4.1.0]heptane (0.18), 2,3-dimethyl-3-buten-2-ol (0.18), 3-methyl-6-(1-methylethyl)-2-cyclohexen-1-one (0.17), caryophyllene oxide (0.17)
Manglietia garrettiCraib (MAGNOLIACEAE)	leaves	0.07	±-*(E)*-nerolidol (27.04), caryophyllene (17.57), α-caryophyllene (11.14), (-)-globulol (6.38), (τ)-cadinol (9.44), α-cadinol (4.94), (1α, 4aα,8aα)-1,2,3,4-,4a,5.6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-naphthalene (4.13), cadina-1 (10),4-diene (3.71), 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-cyclohexane (3.29), caryophyllene oxide (2.23), cedr-9-ene (2.04), β-pinene (1.58), 2-isopropenyl-4a,8-dimethyl-1,2.3,4,4a.5,6,7-octahydronaphthalene(1.38), selina-6-en-4-ol (1.33), (1 S-Z)-1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-naphthalene (1.03), [1aR-(1aα, 4aα,7α,7aβ,7bα)]-decahydro-1,1,7-trimethyl-4-methylene-1H-cycloprop[e]azulene (0.97), [1aR-(1aα, 4α,4aβ,7bα)]-1a,2.3,4,4a.5,6,7b-octahydro-1,1,4,7-tetramethyl-1H-cycloprop[e]azulene (0.96), [2R-(2α,4aβ,8β)]-α,α,4a,8-tetramethyl-2,3,4,4a,5,6,7,8-octahydro-2-naphthalenemethanol (0.83)

Table 1 Yields and chemical constituents of essential oils obtained from the study plants. (continued)

Plant names	Part used	Yield (%)	Chemical constituents (%)[a]
Aglaia odorata Lour. (MELIACEAE)	leaves	0.04	[1aR-(1aα,4aβ,7α,7aβ,7bα)]-decahydro-1,1,7-trimethyl-4-methylene-1 H-cycloprop[e]azulene (17.31), (1α,3aα,7α,8aβ)-2,3,6,7,8,8a-hexahydro-1,4,9,9-tetramethyl-1 H-3a,7-methanoazulene (15.22%). [3R-(3α,3aβ,7β,8aα)]-octahydro-3,8,8-trimethyl-6-methylene-1H-3a,7-methanoazulene (12.74%), [1S-(1α,3aβ,4α,7α)]-octahydro-4-methyl-8-methylene-7-(1-methylethylht)-1,4-methano-1H-indenne (12.18), humulane-1,6-dien-3-ol (11.38), α-cadinol (6.10), (-)-spathulenol (4.58), cedr-8-ene (3.50), (Z)-β-farnesene (3. i 5), caryophyllene (2.96), (1α,4aα,8aα)-1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-naphthalene (2.85), ylangene (2.72), thunbergol (2.47), (τ)-cadinol (2.03), eudesma-4(14),11-diene (0.80)
Myristica fragrans Houtt. (MYRISTICACEAE)	leaves	0.66	myristicin (27.09), p-menth-1-en-4-ol (18.60), β-pinene (11.96),α-pinene (10.15), linalool (7.23), (S)-(-)-p-menth-1-en-8-ol (6.23), 1,2,3-trimethoxy-5-(2-propenyl)-benzene (4.78), β-phellandrene (2.26), isosafrole (1.46), kaur-16-ene (1.07), p-menth-1-en-8-ol acetate (1.02), bornyl acetate (0.78), 1-methyl-4-(1-methylethyl)-2-cyclohexen-1-ol (0.75), eucalyptol (0.71), 1-methyl-4-(1-methylethyl)-2-Cyclohexen-1-ol (0.63), 1,2-dimethoxy-4-(2-propenyl)-benzene (0.62), d-limonene (0.54), 3-carene (0.47), terpinolene (0.45), *(E)*-p-menth-1-en-3-ol (0.64), cadina-1(10),4-diene (0.43), γ-terpinene (0.35), [S-*(E,E)*-1-methyl-5-methylene-8-(1-methylethyl)-1,6-cyclodecadiene (0.32), pergamol (0.27), caryophyllene (0.26), β-myrcene (0.18), camphene (0.17), neryl acetate (0.17)
Melaleuca cajuputi Powell (MYRTACEAE)	leaves	0.43	unidentified (28.37), unidentified (9.86), 1-methyl-4-(1-methylethylldene)-cyclohexene (6.93), 1-methyl-2-(1-methylethyl)-benzene (6.68), p-menth-1-en-4-ol (6.18), γ-terplnene (5.07), (-)-spathulenol (4.14), caryophyllene (4.09), α-pinene (3.77), p-menth-1-en-8-ol (2.60), selina-6-en-4-ol (2.37), caryophyllene oxide (2.28), α-caryophyllene (2.07), 2-methyl-5-(1-methylethyl)-bicyclo [3.1.0] hex-2-ene (1.64), [1S-(1α,2β,4β)]-1-ethenyl-1-methyl-2,4-bis (1-methylethehyl)-cyclohexane (1.75), 1-methyl-7-(1-methylethyl)-phenanthrene (1.25), eucalyptol (1.14), α-caplnol (1.05), 8-isopropenyl-1,5-dimethyl-1,5-cyclodecadiene (1.01), τ-cadinol (0.89), linalool (0.85),

Table 1 Yields and chemical constituents of essential oils obtained from the study plants. (continued)

Plant names	Part used	Yield (%)	Chemical constituents (%)[a]
			3,4-dimethyl-3-cyclohexen-1-carboxaldehyde (0.85), α-terpinene (0.83), d-limonene (0.83), (-)-globulol(0.83), α-phellandrene (0.64), 3-cyclohexerie-1-carooxaldehyde (0.62), (-)-spathulenol (0.50), β-pinene (0.47), 3,7-dimethyl-2,6-octadienal (0.45)
Psidium guajava L. (MYRTACEAE)	leaves	0.16	Caryophyllene oxide (21.97), 4,4-dimethyl-tetracyclo [6.3.2.0(2,5).0(1,8)] tridecan-9-ol (14.49), caryophyHene (11.76), ±-*(E)*-nerolidol (9.39), [1ar-(1aα,4β,4aβ,7α,7aβ,7bα)]-decahydro-1, 1,4,7-tetraniethyl-1H-Cycloprop[e]azulene-4-ol (8.26), (-)-globulol (5.96), ledol (5.53), eucalyptol (5.13), (1S-(1α,4α,4aβ,8aβ)]-1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl) -1-naphthatenol (4.28), α-caryophyllene (1.60), copaene (1.39), cadina, 1,3,5-triene (1.36)
Piper betle L. (PIPERACEAE)	leaves	0.37	4-allyl-2-methoxy-phenol acetate (31.47), 3-allyl-6-methoxyphenol (25.96), 4-allylphenyl acetate (5.21), [3aS-(3aα,3bβ)-octahydro-7-methyl-3-methylene-4(1-methylethyl)-1H- Cyclopenta [1,3]cyclopropa[1,2]benzene (2.48%), caryophyllene (2.16), p-allylphenol (1.47), (1α,4aα,8aα)-1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-naphthalene (1.43), eucalyptol (1.03), α-caryophyllene (0.51), 3,7-dimethyl-1,6-octadien-3-ol (0.49), 1,2-dimethoxy-4-(2-propenyl)-benzene (0.44), [1S-(1α,2β,4β)]-1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-cyclohexane (0.24), (1α,4aα,8aα)-1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-naphthalene (0.19), 3,7-dimethyl-(Z)-1,3,6-octatriene (0.11), camphene (0.09)
Piper nigrum L. (PIPERACEAE)	fruits	0.08	caryophyllene (54.92), caryophyllene oxide (13.26), α-caryophyllene (3.97), copaene (2.90), cadina-1 (10),4-diene (2.61), ar-tumerone (2.38), (3R,*E*)-4-etheny)-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-cyclohexene (1.93), caryophyllene oxide (1.80), 1-(1,5-dimethyl-4-hexenyl)-4-methyl-benzene (1.74), eudesma-4(14), 11-diene (1.40), 4,4-dimethyl-tetracyclo [6.3.2.0(2,5). 0(1,8)]tridecan-9-ol (1.21), isocaryophyllene (1.16), linalool (1.13), [2R-(2α,4aα,8aβ]-1,2,3,4,4a, 5,6,8a-octahydro-4a,8-dimethyl-2 -(1-methylethenyl)-naphthalene (0.94), limonene (0.85), 3-carene (0.75), β-pinene (0.56), (1S-(1α,2β,4β)]-1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-cyclohexane(0.62). (R)-(-)-p-menth-1-en-4-01 (0.60)

Table 1 Yields and chemical constituents of essential oils obtained from the study plants. (continued)

Plant names	Part used	Yield (%)	Chemical constituents (%)[a]
Murraya paniculata (L.) Jack (RUTACEAE)	leaves	0.05	caryophyllene (32.44), [3aS-(3aα,3bβ)]-octahydro-7-methyl-3-methylene-4-(1-methylethyl)-1H-cyclopenta [1,3] cyclopropa [1,2] benzene (20.94), 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-cyclohexane (10.76), α-caryophyllene (5.03), germacrene B (4.89), [3aS-(3aα, 3bβ)]-octahydro-7-methyl-3-methylene-4-(1-methylethyl)-1 H-cyclopenta [1,3] cyclopropa [1,2] benzene (4.16), (1α,4aβ,8aα)-1,2,3,4,4a,5,6,8a-octahydro-7 -methyl-4-methylene-1-(1-methylethyl)-naphthalene (3.75), [1ar-(1aα,4aα,7β,7aβ,7bα)]-decahydro-1,1,7-trimethyl-4-methylene-1H-cycloprop[e]azulen-7-ol (2.88), α-cadinol (2.31), caryophyllene oxide (2.08), cadina-1 (10),4-diene (1.95), (1α,4aα,8aα)-1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-naphthalene (1.53), [1R-(1α,3α,4β)]-4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)-cyclohexanemethanol (1.41), copaene (1.03), (τ)-cadinol (0.94), phytol (0.90), ±-*(E)*-nerolidol (0.80), germacrene 0-4-01 (0.79), [3R-(3α,3aβ,7β, 8aα)]-2,3,4, 7,8,8a-hexahydro-3,6,8,8-tetramethyl-1H-3a,7-methanoazurene (0.77), [1ar-(1aα,4aα,7β,7aβ. 7bα)]-decahydro-1,1,7-trimethyl-4-methylene-1H-cycloprop[e]azulen-7-ol (0.64)
Houttuynia cordata Thunb. (SAURURACEAE)	flowers	0.2	α-pinene (1.28), Gamphene (0,92), β-pinene (1.25), β-myrcene (1.17), d-limonene (0.85), (5Z)-2,6,10-trimethyl-1,5,9-undecatriene (0.49), l-nonanol (0,72), bornyl acetate (8.66), n-decanoic acid (69.31), 2-dodecanone (8.42), 2-tridecanone (0,89), caryophyllene oxide (0.54), docanoic acid, ethyl ester (1.69), 2-propenoic acid, 2-ethylhexyl ester (1.28)
Zingiber officinale Roscoe (ZINGIBERACEAE)	rhizomes	0.12	*(E)-3,*7-dimethyl-2,6-octadienal (24.72), eucalyptol (20.77), *(Z)*-3,7-dimethyl-2,6-octadienal (18.27), camphene (8.71), (-)-borneol (5.46), (S)- (-)-p-menth-1-en-8-ol (3.90), linalool (2.88), 2-heptanol (2.72), 2-[2-(2-methyl-1-propenyl) cyclopropyl]-2-propanol (2.06). 1-(1,5-dimethyl-4-hexenyl)-4-methyl-benzene (2.70), α-pinene (1.74), 6-methyl-5-hepten-2-one (1.89), 2-undecanone (1.44), 2-nonanol (1.37), 13-heptadecyn-1-ol (1.36)

Table 1 Yields and chemical constituents of essential oils obtained from the study plants. (continued)

Plant names	Part used	Yield (%)	Chemical constituents (%)[a]
Alpinia galangal (L.) Willd. (ZINGIBERACEAE)	rhizomes	0.83	eucalyptol (39.50), 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-ol acetate (28.61), 4- allylphenyl acetate (5.73), caryophyllene (3.35), 4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol (3.15), 2-isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8a-octahydronaphthalene (2.22), eudesma-4(14), 11-diene (2.09), (S)-1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-cyclohexene (2.05), (Z)-β-farnesene (1.72), α-caryophyllene (1.67), germacrene B (1.67), (S)-(-)-p-menth-1-en-8-ol (1.60), d-limonene(1.00), (E)-3,7-dimethyl-2,6-octadien-1-ol acetate (0.99), p-allylphenol (0.98), α-pinene (0.85), p-menth-1-en-8-ol (0.69), 1,2,-dimethoxy-4-(2-propenyl)-benzene (0.68), selina-6-en-4-ol (0.66)
Curcuma longa L. (ZINGIBERACEAE)	rhizomes	0.18	tumerone (41.11), ar-tumerone (23.12), curlone(19.14), α-phellandrene (5.04), eucalyptol (3.92), 1-methyl-2-(1-methylethyl)-benzene (1.66), 2,2-dicyclohexylmalononitrile(1.28), (Z)-α-*(E)*-bergamotol (0.98), 1-zingiberene (0.75), 5-fluoro-2-nitrophenyl 4-methylbenzoate (0.72), 1-(1,5-dimethyl)-4-hexenyl)-4-methyl-benzene (0.63), β-sesquiphellandrene (0.62), 2-cyclohexyl-2-isobutylmalononitrile (0.52)
Hedychium coronarium J. Konigv (ZINGtBERACEAE)	rhizomes	0.2	eucalyptol (56.91), β-pinene (17.21), α-terpineol (7.45), α-pinene (6.02), p-menth-1-en-4-ol (3.00), *(E)*-pinocarveol (2.58), 2-pinen-10-ol (1.96), borneol (1.28), d-limonene (0.82), 1-methyl-2-(1-methylethyl)-benzene (0.71), linalool (0.54), 2(10)pinen-3-one (0.50), 2(10) pinen-3-ol (0.34), thujol (0.34), β-thujene (0.33)

[a] Values in parentheses represent relative amounts (% area) of each chemical constituent.

Table 2 Repellency obtained from three different exposure times for each repellent against four mosquito species.

Repellents	Exposure time (min)	Mean* repellency in hours (± S.E.) against each mosquito species			
		Ae. aegypti	***Ae. albopictus***	***An. dirus***	***Cx. quinquefasciatus***
Eleutherococcus trifoliatus	1	2.3 (± 0.3)a	8.0 (± 0.0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	2	1.4 (± 0.4)b	8.0 (± 0.0)a	8.0 (± 0.0)a	7.8 (± 0.2)ab
	3	1.0 (± 0.2)b	8.0 (± 0.0)a	8.0 (± 0.0)a	7.4 (± 0.2)b
Schefflera leucantha	1	3.6 (± 0.5)a	8.0 (± 0.0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	2	2.8 (± 0.6)ab	8.0 (± 0.0)a	8.0 (± 0.0)a	7.7 (± 0.3)a
	3	1.9 (± 0.4)b	8.0 (± 0.0)a	8.0 (± 0.0)a	7.5 (± 0.5)a
Ocimum sanctum	1	2.0 (± 0.3)a	7.8 (± 0.1)a	8.0 (± 0.0)a	5.8 (± 0.9)a
	2	1.9 (± 0.3)a	7.6 (± 0.2)a	8.0 (± 0.0)a	5.8 (± 0.9)a
	3	1.3 (± 0.2)b	7.6 (± 0.2)a	8.0 (± 0.0)a	5.4 (± 0.9)a
Vitex trifolia	1	3.7 (± 0.5)a	8.0 (± 0.0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	2	2.3 (± 0.7)b	8.0 (± 0.0)a	8.0 (± 0.0)a	7.8 (± 0.2)ab
	3	1.8 (± 0.3)b	8.0 (± 0.0)a	8.0 (± 0.0)a	7.5 (± 0.3)b
Litsea cubeba	1	2.5 (± 0.4)a	7.7 (± 0.2)a	8.0 (± 0.0)a	7.5 (± 0.5)a
	2	1.8 (± 0.2)b	6.3 (± 0.8)b	8.0 (± 0.0)a	7.2 (± 0.8)a
	3	1.7 (± 0.3)b	6.2 (± 0.8)b	8.0 (± 0.0)a	7.0 (± 1.0)a
Manglietia garrettii	1	2.6 (± 0.5)a	7.5 (± 0.5)a	8.0 (± 0.0)a	7.7 (± 0.3)a
	2	1.5 (± 0.3)b	6.6 (± 0.7)ab	8.0 (± 0.0)a	7.4 (± 0.4)a
	3	1.4 (± 0.3)b	6.0 (± 0.9)b	8.0 (± 0.0)a	6.9 (± 0.5)a
Aglaia odorata	1	2.2 (± 0.6)a	6.8 (± 1.0)a	8.0 (± 0.0)a	8 0 (± 0.0)a
	2	1.7 (± 0.4)a	6.8 (± 1.0)a	8.0 (± 0.0)a	8 0 (± 0.0)a
	3	1.2 (± 0.3)b	5.3 (± 1.2)a	8.0 (± 0.0)a	7 2 (± 0.6)b
Myristica fragrans	1	3.5 (± 1.0)a	6.2 (± 1.1)a	8.0 (± 0.0)a	8 0 (± 0.0)a
	2	1.2 (± 0.5)b	5.8 (± 1.1)a	8.0 (± 0.0)a	7 5 (± 0.5)ab
	3	0.8 (± 0.3)b	4.5 (± 1.2)a	8.0 (± 0.0)a	6 9 (± 0.6)b
Melaleuca cajuputi	1	2.1 (± 0.6)a	8.0 (± 0.0)a	8.0 (± 0.0)a	7 9 (± 0.1)a
	2	1.3 (± 0.7)ab	8.0 (± 0.0)a	8.0 (± 0.0)a	7 6 (± 0.3)a
	3	0.7 (± 0.3)b	7.9 (± 0.1)a	8.0 (± 0.0)a	6.9 (± 0.4)b
Psidum guajava	1	4.4 (± 0.8)a	7.4 (± 0.4)a	8.0 (± 0.0)a	7.7 (± 0.3)a
	2	3.3 (± 0.8)a	6.8 (± 0.5)ab	8.0 (± 0.0)a	7.3 (± 0.4)a
	3	2.8 (± 0.9)a	5.6 (± 1.0)b	8.0 (± 0.0)a	6.9 (± 0.5)a
Piper betle	1	2.3 (± 0.4)a	7.6 (± 0.4)a	8.0 (± 0.0)a	7.8 (± 0.2)a
	2	1.8 (± 0.4)ab	7.6 (± 0.4)a	7.8 (± 0.2)a	7.3 (± 0.5)a
	3	1.3 (± 0.3)b	7.1 (± 0.6)a	7.6 (± 0.3)a	6.7 (± 1.0)a
Piper nigrum	1	4.1 (± 0.6)a	8.0 (± 0.0)a	8.0 (± 0.0)a	7.8 (± 0.2)a
	2	2.8 (± 0.5)b	8.0 (± 0.0)a	8.0 (± 0.0)a	7.8 (± 0.2)a
	3	2.3 (± 0.4)b	8.0 (± 0.0)a	8.0 (± 0.0)a	7.8 (± 0.2)a

Table 2 Repellency obtained from three different exposure times for each repellent against four mosquito species. (continued)

Repellents	Exposure time (min)	Mean* repellency in hours (± S.E.) against each mosquito species			
		Ae. aegypti	*Ae. albopictus*	*An. dirus*	*Cx. quinquefasciatus*
Murraya paniculata	1	3.4 (± 0.6)a	6.8 (± 0.7)a	8.0 (± 0.0)a	6.5 (± 0.8)a
	2	2.6 (± 0.5)a	6.4 (± 0.7)a	8.0 (± 0.0)a	6.3 (± 0.8)a
	3	1.5 (± 0.3)b	5.7 (± 1.1)a	8.0 (± 0.0)a	5.0 (± 0.8)a
Houttuynia cordata	1	1.8 (± 0.6)a	7.5 (± 0.3)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	2	0.8 (± 0.2)b	7.5 (± 0.3)a	8.0 (± 0.0)a	7.9 (± 0.1)ab
	3	0.6 (± 0.2)b	7.5 (± 0.3)a	8.0 (± 0.0)a	7.5 (± 0.4)b
Zingiber officinale	1	3.4 (± 0.4)a	7.2 (± 0.5)a	8.0 (± 0.0)a	7.7 (± 0.2)a
	2	2.3 (± 0.4)b	7.2 (± 0.5)a	8.0 (± 0.0)a	7.2 (± 0.3)a
	3	1.7 (± 0.3)b	5.9 (± 1.0)a	8.0 (± 0.0)a	5.9 (± 0.6)b
Alpinia galanga	1	2.2 (± 0.8)a	7.8 (± 0.2)a	8.0 (± 0.0)a	7.2 (± 0.6)a
	2	0.8 (± 0.3)b	7.8 (± 0.2)a	8.0 (± 0.0)a	6.9 (± 0.8)a
	3	0.6 (± 0.2)b	7.8 (± 0.2)a	8.0 (± 0.0)a	6.1 (± 0.9)a
Curcuma longa	1	3.6 (± 0.6)a	8.0 (± 0 0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	2	2.7 (± 0.5)ab	8.0 (± 0 0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	3	2.3 (± 0.4)b	7.7 (± 0 3)a	8.0 (± 0.0)a	8.0 (± 0.0)a
Hedychium coronarium	1	0.9 (± 0.3)a	8.0 (± 0 0)a	8.0 (± 0.0)a	7.5 (± 0.3)a
	2	0.4 (± 0.2)ab	7.5 (± 0 5)a	7.5 (± 0.3)b	6.8 (± 0.7)ab
	3	0.3 (± 0.1)b	7.5 (± 0 5)a	7.1 (± 0.6)b	5.8 (± 1.1)b
Deet	1	7.7 (± 0.3)a	8.0 (± 0.0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	2	7.6 (± 0.3)a	8.0 (± 0.0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	3	7.5 (± 0.2)a	8.0 (± 0.0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
IR3535	1	7.5 (± 0.3)a	8.0 (± 0.0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	2	7.1 (± 0.4)a	8.0 (± 0.0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
	3	6.7 (± 0.8)a	7.8 (± 0.2)a	8.0 (± 0.0)a	8.0 (± 0.0)a
Control (solution base)	1	0.0	0.0	0.0	0.0

* Means of each repellent in each column against each mosquito species followed by the same letter are not significantly different ($p > 0.05$, by one-way ANOVA with Duncant's multiple range test). Comparisons are made only among the repellencies obtained from different exposure times for each repellent against each mosquito species.

Table 3 shows the mean repellency (in hours) for 3 minutes of exposure with the 18 essential oils and 2 chemical repellents against the four mosquito species. This data was retrieved from Table 2 to compare repellencies of the essential oils and chemical repellents. The repellencies of the 18 essential oils against *Ae. aegypti* were between 0.3 and 2.8 hours, whereas those of deet and IR3535 were 7.5 and 6.7 hours, respectively. All the essential oils provided significantly lower repellency than deet and IR3535 ($p < 0.01$). Of the essential oils tested, a high degree of repellency was obtained from *Psidium guajava* (2.8 hours), *Curcuma longa* (2.3 hours), *Piper nigrum* (2.3 hours), *Schefflera leucantha* (1.9 hours), *Vitex trifofia* (1.8 hours), *Litsea cubeba* (1.7 hours), and *Zingiber officinale* (1.7 hours). However, there were no significant differences in repellency of *Ae. aegypti* among these essential oils ($p > 0.05$). When tested against *Ae. albopictus*, the repellency of the 18 essential oils ranged from 4.5 to 8.0 hours, while deet and IR3535 were 8.0 and 7.8 hours, respectively (Table 3). Repellency of the eight essential oils: *Eleutherococcus trifoliatus, Schefflera leucantha, Vitex trifolia, Melaleuca cajuputi, Piper nigrum, Alpinia galanga, Curcuma longa* and *Hedychium coronarium* were statistically equal to the chemical repellents, deet and IR3535 ($p > 0.01$).

Regarding the repellency against *An. dirus*, it is interesting to note that 16 out of 18 essential oils provided excellent repellency of 8 hours, equally to deet and IR3535 (Table 3). High degrees of repellency against *An. dirus* were also detected in the other two essential oils, *Piper betle* (7.6 hours) and *Hedychium coronarium* (7.1 hours). As for the repellency results against *Cx. quinquefasciatus*, the 18 essential oils demonstrated a relatively high degree of repellency, ranging from 5.0 to 8.0 hours, while those of deet and IR3535 were 8.0 hours (Table 3). Unlike the effect against *An. dirus*, excellent repellency against *Cx. quinquefasciatus* was found in only three essential oils, *Curcuma longa* (8.0 hours), *Piper nigrum* (7.8 hours), and *Schefflera leucantha* (7.5 hours), which were statistically equal to those of deet and IR3535 ($p > 0.05$).

The oviposition deterrent effects of essential oils and the two chemical repellents, deet and IR3535 (at 0.01% concentration) against *Ae. aegypti* are shown in Table 4. The average number of mosquito eggs in the control group ranged from 2,171 to 4,805, while those of the treated groups were between 232 and 2,903. As can be seen, all essential oils exhibited oviposition deterrent activity against the mosquitoes with various degrees of repellency, ranging from 16.6 to 94.7%, whereas deet and IR3535 provided no repellency. Of the essential oils tested, 12 out of 18 provided repellency of at least 80%. Relatively high oviposition deterrencies were obtained from *Curcuma longa* (94.7%), *Schefflera leucantha* (91.6%) and *Zingiber officinale* (90.1%), *Vitex trifolia* (89.1%), *Melaleuca cajuputi* (87.9%), *Hedychium coronarium* (87.5%), *Psidium guajava* (87.1%), *Manglietia* *garrettii* (86.1%) and *Houttuynia cordata* (85%). There were no significant differences of repellency among these essential oils. Moderate degrees of deterrency were obtained from three plant species: *Piper nigrum* (82%), *Litsea cubeba* (80.6%) and *Eleutherococcus trifoliatus* (80.2%). The remaining plants showed deterrency below 80%.

Table 3 Repellency obtained from a 3-minute exposure time for each repellent against four mosquito species.

Repellents	Mean* repellency in hours (± S.E.) against each mosquito species			
	Ae. aegypti	*Ae. albopictus*	*An. dirus*	*Cx. quinquefasciatus*
Eleutherococcus trifoliatus	1.0 (± 0.2)de	8.0 (± 0.0)a	8.0 (± 0.0)a	7.4 (± 0.2)b
Schefflera leucantha	1.9 (± 0.4)bc	8.0 (± 0.0)a	8.0 (± 0.0)a	7.5 (± 0.5)ab
Ocimum sanctum	1.3 (± 0.2)cd	7.6 (± 0.2)b	8.0 (± 0.0)a	5.4 (± 0.9)cd
Vitex trifolia	1.8 (± 0.3)bc	8.0 (± 0.0)a	8.0 (± 0.0)a	7.5 (± 0.3)b
Litsea cubeba	1.7 (± 0.3)bc	6.2 (± 0.8)cd	8.0 (± 0.0)a	7.0 (± 1.0)bc
Manglietia garrettii	1.4 (± 0.3)cd	6.0 (± 0.9)cd	8.0 (± 0.0)a	6.9 (± 0 5)bc
Aglaia odorata	1.2 (± 0.3)cd	5.3 (± 1.2)de	8.0 (± 0.0)a	7.2 (± 0 6)bc
Myristica fragrans	0.8 (± 0.3)de	4.5 (± .2)de	8.0 (± 0.0)a	6.9 (± 0 6)bc
Melaleuca cajuputi	0.7 (± 0.3)de	7.9 (± 0.1)ab	8.0 (± 0.0)a	6.9 (± 0 4)bc
Psidium guajava	2.8 (± 0.9)b	5.6 (± 1.0)d	8.0 (± 0.0)a	6.9 (± 0 5)bc
Piper betle	1.3 (± 0.3)cd	7.1 (± 0.6)bc	7.6 (± 0.3)b	6.7 (± 0)bc
Piper nigrum	2.3 (± 0.4)bc	8.0 (± 0.0)a	8.0 (± 0.0)a	7.8 (± 0 2)ab
Murraya paniculata	1.5 (± 0.3)cd	5.7 (± 1.1)cd	8.0 (± 0.0)a	5.0 (± 0 8)d
Houttuynia cordata	0.6 (± 0 2)e	7.5 (± 0.3)b	8.0 (± 0.0)a	7.5 (± 0 4)b
Zingiber officinale	1.7 (± 0.3)bc	5.9 (± 1.0)cd	8.0 (± 0.0)a	5.9 (± 0.6)c
Alpinia galanga	0.6 (± 0.2)e	7.8 (± 0.2)ab	8.0 (± 0.0)a	6.1 (± 0.9)c
Curcuma longa	2.3 (± 0.4)bc	7.7 (± 0.3)ab	8.0 (± 0.0)a	8.0 (± 0.0)a
Hedychium coronarium	0.3 (± 0.1)f	7.5 (± 0.5)ab	7.1 (± 0.6)b	5.8 (± 1.1)c
Deet	7.5 (± 0.2)a	8.0 (± 0.0)a	8.0 (± 0.0)a	8.0 (± 0.0)a
IR3535	6.7 (± 0.8)a	7.8 (± 0.2)ab	8.0 (± 0.0)a	8.0 (± 0.0)a
Control (solution base)	0.0	0.0	0.0	0.0

* Means in each column against each mosquito species followed by the same letter are not significantly different (p >0.05, by one-way ANOVA with Duncant's multiple range test).

Table 4 Oviposition deterrent effect of each repellent against *Ae. aegypti*.

Repellents	Mean no. of eggs (± S.E.)		Repellency* (%)
	Control	Treated	
Eleutherococcus trifoliatus	3,629 ± 529	717 ± 88	80.2b
Schefflera leucantha	3,950 ± 384	331 ± 73	91.6ab
Ocimum sanctum	3,992 ± 689	1,194 ± 142	70.1c
Vitex trifolia	4,805 ± 553	524 ± 167	89.1ab
Litsea cubeba	3,986 ± 338	774 ± 138	80.6b
Manglietia garrettii	3,815 ± 510	532 ± 150	86.1ab
Aglaia odorata	3,141 ± 334	1,190 ± 460	62.1c
Myristica fragrans	2,423 ± 276	2,021 ± 433	16.6d
Melaleuca cajuputi	3,797 ± 684	461 ± 151	87.9ab
Psidium guajava	3,518 ± 570	455 ± 104	87.1ab
Piper betle	3,076 ± 127	649 ± 128	78.9b
Piper nigrum	3,998 ± 660	719 ± 174	82.0b
Murraya paniculata	2,575 ± 381	1,092 ± 254	57.6c
Houttuynia cordata	4,575 ± 314	685 ± 40	85.0ab
Zingiber officinale	4,476 ± 498	443 ± 47	90.1ab
Alpinia galanga	3,569 ± 326	1,073 ± 139	69.9c
Curcuma longa	4,386 ± 438	232 ± 72	94.7a
Hedychium coronarium	3,557 ± 524	445 ± 127	87.5ab
Deet	2,171 ± 191	2,202 ± 336	0.0**e
IR3535	2,504 ± 453	2,903 ± 314	0.0**e

* Repellency followed by the same letter is not significantly different ($p > 0.05$, by one-way ANOVA with Duncant's multiple range test)

** Repellency is considered as zero when the mean number of mosquito eggs in the treated group was greater than the control group.

Discussion

The quality of essential oils, such as yield, chemical constituents and physical properties depends on many factors. Factors affecting the quality of essential oils include plant species (variety), cultivating conditions, maturation of harvested plants, plant storage, plant preparation and methods of extraction (Tawatsin *et al*, 2001). Unfortunately, we could not describe all the factors of the plants used in this study. Data of the chemical constituents of essential oils in our study is valuable for further research regarding plant-based insect repellents. It is difficult to point out which chemicals are responsible for the repellent effects against mosquitoes in this study, since several were uncommon or unidentified chemicals found in th essential oils. Even though there are some known chemicals found in the essential oils, they are not presented in all the essential oils that possess the same repellency against the same mosquito species. Repellent activity against particular mosquito species may be due to the synergistic effects of a combination of phytochemicals in each essential oil. Further studies would reveal more information about the relationship of phytochemicals and the repellent effects against mosquitoes.

There was inconsistency in the differentexposure periods in the mosquito cage when determining the repellency of mosquito under laboratory conditions. Some earlier studies used short exposure times of one minute only. Our study clearly shows a substantial difference in repellency obtained during different exposure periods. Two hours was the minimum protection time needed against *Ae. aegypti* and *Cx. quinquefasciatus* (with a 3 minute exposure period) specified for mosquito repellents to be registered and sold in Thailand. On the basis of this regulation, there are only three repellents (ie, *Psidium guajava, Curcuma longa* and *Piper nigrum*) that meet the established criteria for registration. If the exposure time was one minute, the qualified repellents against *Ae. aegypti* (repellency >2 hours) would then be 16 out of 18 essential oils (except only *Houttuynia cordata* and *Hedychium coronarium*). As can be seen, a shorter exposure time, such as one minute, may indicate a higher repellency than a longer exposure time of two or three minutes. Similar differences in repellency among the three different exposure times were also detected in almost half of tests against *Cx. quinquefasciatus* (8 out of 18 tested essential oils). It is therefore recommended that the exposure time in mosquito cage testing should be at least three minutes in order to better reflect repellency.

The repellency of essential oils against various mosquito species obtained in our study was affected by synergism of some additives used in our formulation. However, all the essential oils and chemical repellents (deet and IR3535) were formulated in the same way for repellency comparison. We believe the essential oils without formulation would provide lower repellency than our results. Tawatsin *et al* (2001) confirmed that the repellency of volatile oils was improved dramatically when they were formulated with vanillin. Formulation technology, therefore, plays an important role for long lasting repellents.

Regarding the repellency obtained in the 3 minute exposure period, the night-biting mosquitoes (*An. dirus* and *Cx. quinquefasciatus*) and *Ae. albopictus* were more sensitive to all the essential oils (repellency 4.5-8 hours) than was *Ae. aegypti* (repellency 0.3-2.8 hours). These results indicate more aggressive biting behavior of *Ae. aegypti* over other mosquito species in this study. Different species of mosquitoes react differently to the same repellents (Rutledge *et al*, 1983). Based on the repellent results against *Ae. aegypti*, we recommend three essential oils, *Psidium guajava*, *Curcuma longa* and *Piper nigrum,* for further development as commercial repellents. These three essential oils also provided high repellency against other mosquito species. Recently, the same formulation of two essential oil repellents in this study, *Psidium guajava* and *Curcuma longa,* were evaluated for repellency in the field against mosquitoes, black flies and land leeches (Tawatsin *et al*, 2006). The results show that both *Psidium guajava* and *Curcuma longa* provided complete protection from mosquito landing and biting for up to 9 hours, and 100% protection against black flies and land leeches for 9 and at least 8 hours, respectively. These results, therefore, clearly confirm promising repellent effects against blood-sucking organisms by both *Psidium guajava* and *Curcuma longa* in the present study.

The studies on oviposition deterrent activity of chemical compounds and insect repellents have been carried out continuously against mosquito vectors, whereas those of plant extracts are scarce. Xue *et al* (2001, 2003) reported the ovipositional deterrent effects of deet and several repellent compounds, such as AI3-37220, AI3-35765, AI3-54995, AI3-55051 against *Ae. albopictus* under laboratory and field conditions. Until recently, Xue *et al* (2006) also pointed out the oviposition deterrent effectiveness (76-100% repellency) against *Ae. albopictus* of 21 commercial insect repellent products (at 0.1% concentration), including 12 botanical, 6 deet-based and 3 synthetic organics. As for the plant extracts, Mehra and Hiradhar (2002) revealed that the crude acetone extract of *Cuscuta hyalina* Roth. was an effective oviposition deterrent against *Cx. quinquefasciatus* at a concentration of 80 ppm. With reference to the relatively high repellency and yields, our study reveals the large potential of essential oils, such as *Curcuma longa*, *Zingiber officinale*, *Vitex trifolia*, *Melaleuca cajuputi*, *Hedychium coronarium*, *Psidium guajava* and *Houttuynia cordata*, to be used as oviposition deterrent agents to disrupt oviposition by *Ae. aegypti* at breeding sites. These oils (at 0.01% concentration) provided 85- 94.7% repellency, with 0.12-0.43% yields. In most prior oviposition deterrent studies, high levels of deterrent activity against *Ae. aegypti* have been rare. Although *Schefflera leucantha* and *Manglietia garrettfi* have shown high oviposition deterrent activity (91.6 and 86.1%), both plants provided substantially low yields of essential oils (0.04 and 0.07%). These two oils, therefore, may not be appropriate for development as antioviposition agents. Further studies are needed to formulate active essential oils needed for treatment of water-storage containers, the most common breeding sites of *Ae. aegypti* in Thailand. Oviposition avoidance of insecticide-treated water-storage containers by gravid female mosquitoes can reduce levels of larval populations (Moore, 1977). Active essential oils that possess oviposition deterrent activity and include larvicidal effects against *Ae. aegypti* would be of interest as plant-based products for the control of mosquitoes.

The present study demonstrates a high potential for using essential oils as mosquito repellents against four species and ovipositional deterrent activity against

Ae. aegypti. This may lead to new and more effective strategies to prevent and control mosquitoes.

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Double Infection of Heteroserotypes of Dengue Viruses in Field Populations of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) and Serological Features of Dengue Viruses Found in Patients in Southern Thailand

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Abstract: In order to understand more about the epidemiology of DHF, a study of the type of dengue viruses and vectors under natural conditions was carried out. Mosquito vectors in the field and the serum of DHF patients in southern Thailand were examined. The two mosquito species are abundant and DHF incidence remains high in this region. Dengue viruses were examined in field-caught mosquitoes by RT-PCR technique. The mosquitoes were caught in 4 provinces: Krabi, Phuket, Phang-Nga and Surat Thani during the late dry season until the early rainy season in 2005. Three dengue serotypes (DEN-2, DEN-3, DEN-4) were detected in *Ae. aegypti* males and females, and 2 (DEN-2, DEN-3) were detected in *Ae. albopictus* females. Double infection with 2 serotypes of dengue viruses (DEN-2 and DEN-3) were detected in *Ae. aegypti* males and females and *Ae. albopictus* females. DEN-2 and DEN-1 were the most prevalent serotypes found in the serum of the patients in this area, followed by DEN-4 and DEN-3. The prevalence of the predominant dengue serotype varied from province to province. Detection of viruses in adult male mosquitoes reveals the role of transovarial transmission of dengue viruses in field populations of DHF vectors and elucidates circulation of dengue viruses in vectors in the natural environment of endemic areas. The incidence of multiple serotypes of dengue virus in Ae. *aegypti* and *Ae. albopictus* in the same area points toward a high risk for an epidemic of DHF. These findings provide greater understanding of the relationship among mosquito vectors, virus transmission and DHF epidemiology in endemic areas.

Introduction

Dengue fever (DF) and its more severe form, dengue hemorrhagic fever (DHF), are important mosquito-borne diseases, caused by four serotypes of dengue virus and are transmitted by *Aedes* mosquitoes: *Aedes aegypti* (L.) and *Ae. albopictus* Skuse (Service, 1993). The disease has worldwide distribution in some 100 countries, but is more prevalent in Africa, the Americas, the Eastern Mediterranean, Southeast Asia, and the Western Pacific (WHO, 2002). It is estimated that 2,500 million people are at risk for DF/DHF, and about 50 million cases of DF/DHF infection are reported annually (WHO, 2002). In Thailand, five decades after the first report of the disease in the late 1950s, DHF has spread across the country and has become a major vector-borne disease, with increasing incidence, especially since the late 1980s. Major efforts using a variety of approaches have been directed toward prevention and control of DF/DHF. *Ae. aegypti* is the major vector of DF/DHF in almost all countries, including Thailand, whereas *Ae. albopictus, Ae. scutellaris* Colless and *Ae. polynesiensis* Marks are vectors in some areas (Japan, islands of the Pacific) (Kettle, 1995). There is currently no effective vaccine available to prevent this disease (Service, 1993). A tetravalent vaccine for DHF is being investigated but it may take years before an effective vaccine can be widely used in the main disease areas (Gratz and Knudsen, 1997). Therefore, prevention and control of the disease should be conducted through understanding of DHF epidemiology and vector control strategies. To understand more about the epidemiology of the disease, a study of the association between viruses and vectors under natural conditions was carried out evaluating viruses in field populations of mosquito vectors and in the serum of DHF patients in southern Thailand, where the two mosquito species are abundant and DHF incidence remains high.

Materials and methods

Study areas

Four southern provinces of Thailand (Krabi, Phuket, Phang-Nga and Surat Thani) were selected for the study sites (Figure 1). These provinces have been recognized as areas with a high incidence of DHF and the places having two species of DHF vectors: *Ae. aegypti* and *Ae. albopictus.*

Mosquito collection

Mosquito collection using human bait (WHO, 1997) was carried out at the study sites from the late dry season (March) to the early rainy season (May) 2005. The chosen dwellings for mosquito collection were those villages which had experienced recent DHF cases. Five volunteers captured mosquitoes indoors for

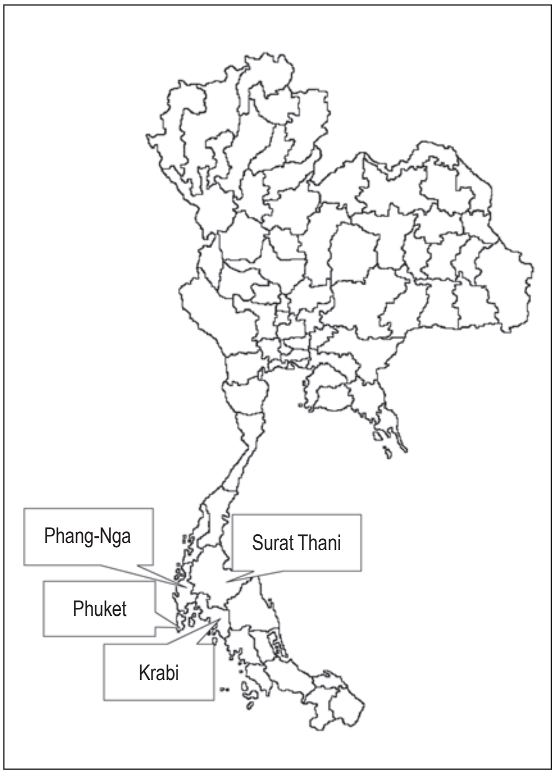

Figure 1 Map of Thailand showing the location of the four provinces in this study.

20 minutes in each dwelling. The collectors usually situated themselves in dark areas of the room where most biting activity occurs. The collectors bared their legs between knee and ankle and collected all landing and biting mosquitoes individually in vials, which were capped. Using a similar procedure, the collecting was also conducted outdoors (approximately 10m away from dwellings) to catch *Ae. albopictus* in the same environment. The collecting was carried out from 0900 to 1700 hours. The collected mosquitoes were visually identified, as there were only two species *(Ae. aegypti* and *Ae. albopictus)* present. These live mosquitoes were inactivated by putting them in a refrigerator, and separated by species, sexes and localities and then kept individually in cryogenic vials to store in liquid nitrogen for subsequent dengue viral detection.

Viral detection in mosquitoes

The procedure for dengue virus detection in mosquitoes followed the methods described by Tuksinvaracharn *et al* (2004). Viral RNA was extracted from individual mosquitoes. The wings and legs were removed then the mosquitoes were ground in a lysis solution (provided with the kit), centrifuged, then the supernatant was processed for RNA extraction using RNeasy mini kit (QIAGEN, Germany).

The six oligonucleotide primers within the core and pre-membrane protein gene (C-prM) of dengue viruses used in this study were designed by Lanciotti *et al* (1992). Two consensus primers (01 and 02) were designed to be homologous to the genomic RNA for all four dengue serotypes, whereas the type-specific nucleotide primers (TS1, TS2, TS3 and TS4) were designed to anneal specifically to each of their respective genomes. These primers were positioned such that a differently sized product was generated from each type.

The procedure of semi-nested RT-PCR performed in this study was modified from that of Lanciotti *et al* (1992). The first step was performed using Superscript III one-step RT-PCR with Platinum III® *Taq* (Invitrogen, USA) which followed the manufacturer's protocol. The second step was to identify type-specific DNA products. The RT-PCR products from the second step were examined by agarose gel electrophoresis and visualized by ethidium bromide staining.

Mosquitoes inoculated with dengue viruses were used as positive controls. These viruses were DEN-1 Hawaii, DEN-2 strain TR 1751, DEN-3 strain H87, and DEN-4 strain H241. The method for mosquito inoculation was modified from Pervin *et al* (2003). Briefly, 3-5 day old female *Ae. aegypti* reared in the laboratory were collected and inactivated on ice for 10 minutes. Each mosquito was then inoculated intra-thoracically with 0.3 µl of 2.5×10^3 pfu/ml of dengue virus antigen diluted with PBS diluents (PBS pH 7.4, 0.5% gelatin and 5% fetal calf serum). After inoculation, the mosquitoes were kept in a double door insectary at 28°C with 80% humidity and supplied with 10% sucrose solution for 7-10 days. Uninfected laboratory-reared *Ae. aegypti* mosquitoes were used as negative controls. The sensitivity of the RT-PCR technique used in the present study was approximately 25 viral particles/µl.

Collection of blood specimens

Blood specimens were taken from DHF patients admitted to hospitals in the study areas. The blood samples were drawn into tubes with EDTA anticoagulant, and centrifuged to obtain plasma. The plasma specimens were kept in liquid nitrogen tanks and then transported to the Arbovirus Laboratory, National Institute of Health, Department of Medical Sciences, Nonthaburi, Thailand for determination of serotypes against dengue viruses.

Viral determination in blood samples

Reverse transcriptase - polymerase chain reaction (RT-PCR) was carried out to identify dengue virus serotypes as described in previous reports (Yenchitsomanus *et al, 1996;* Chanama *et al*, 2004). Viral RNA was extracted from 100 µl of patient plasma with a QIAamp viral RNA mini kit (Qiagen Gmbh, Hilden, Germany). Then, RT-PCR was processed using the one-step RT-PCR kit (Qiagen) and dengue-specific oligonucleotide primers. Positive and negative controls were always included in each batch of tests. Finally, the second PCR products were electrophoresed through agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

Incidence of DHF in Thailand

The data of DHF incidence in Thailand and those of provinces in the study areas between 2000 and 2005 was obtained from the Department of Disease Control, Ministry of Public Health, Thailand. The annual incidence of dengue per 100,000 populations was ranked as percentiles: 71 was 50^{th}, 134 was 75^{th}, and

175 was 90th (Office of the Permanent Secretary for Public Health, 1999). The epidemics with annual dengue incidence ranging between the 75th and 90th percentile were classified as moderately severe epidemics, whereas those greater than the 90th percentile as severe epidemics.

RESULTS

A total of 469 *Ae. aegypti* mosquitoes (145 males and 324 females) were collected from the study sites and subsequently identified individually for dengue virus by RT-PCR assays. The distinct patterns of some products from the RT-PCR assays for dengue viruses are illustrated in Figure 2. Out of those, 75 *Ae. aegypti* (22 males and 53 females) were positive for dengue viruses (Table 1). The relative infection rate varied from place to place, ranging from 5.3% to 25.9%. The three serotypes of dengue viruses: DEN-2, DEN3 and DEN-4 were found in *Ae. aegypti* individual males and females, whereas no DEN-1 was detected in any mosquito (Table 1). It is interesting to note that the three serotypes (DEN-2, DEN-3 and DEN-4) were detected in both male and female *Ae. aegypti* mosquitoes collected from Krabi but only DEN-4 virus was found in one male. *Ae. aegypti* mosquitoes caught from Phuket and Phang-Nga were positive for two serotypes: DEN-2 and DEN-3, whereas of those collected from Surat Thani, only DEN-3 was found in two *Ae. aegypti* females. Double infections with two serotypes of dengue viruses (DEN2 and DEN-3) were also detected in *Ae. aegypti* males and females captured from Krabi and Phuket, while this double infection was found in only one *Ae. aegypti* male collected from Phang-Nga.

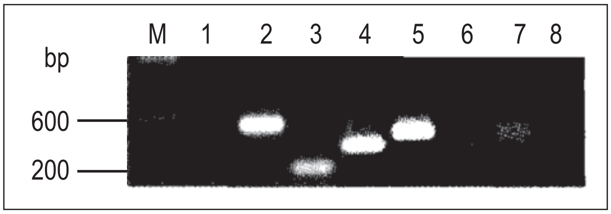

Figure 2 The distinct patterns of products from RT-PCR assays for dengue viruses. Lanes: M = molecular weight marker; 1 = negative control; 2 = positive control (DEN-1); 3 = positive control (DEN-2); 4 = positive control (DEN-3); 5 = positive control (DEN-4); 6 = sample 1 positive for DEN-2 and DEN-3; 7 = sample 2 positive for dengue 3; 8 = sample 3 negative.

Since outdoor mosquito collection for *Ae. albopictus* was carried out in the dry period when natural developmental sites were scarce, the number of mosquitoes collected was extremely low. As shown in Table 2, only 58 *Ae. albopictus* females were caught: Krabi (28), Phuket (5) and Phang-Nga (25). whereas no *Ae. a/bopictus* mosquitoes were collected from Surat Thani in the present study. The relative infection rates were 21.4% in Krabi, 60% in Phuket and 48% in Phang-Nga. Of the field-caught mosquitoes, a total of 21 were positive for DEN-2 (10), DEN-3 (7) and double serotypes: DEN-2 and DEN-3 (4), as shown in Table 2. The mosquitoes collected from Krabi were infected with

Table 1 Prevalence of relative infection of dengue virus in *Ae. aegypti* mosquitoes collected from four southern provinces in Thailand.

Province	Total tested mosquitoes[a]	No. of mosquitoes positive for virus	Relative infection rate (%)	Frequency of dengue serotypes found				
				DEN-1	DEN-2	DEN-3	DEN-4	DEN-2&3
Krabi	82 (M)	9	11	0	4	2	1	2
	149 (F)	18	12.1	0	6	9	0	3
	231 (M+F)	27	11.7	0	10	11	1	5
Phuket	61 (M)	10	16.4	0	2	6	0	2
	114 (F)	29	25.4	0	3	17	0	9
	175 (M+F)	39	22.3	0	5	23	0	11
Phang-Nga	17 (M)	3	17.7	0	2	0	0	1
	10 (F)	4	40	0	0	4	0	0
	27 (M+F)	7	25.9	0	2	4	0	1
Surat Thani	2 (M)	0	0	0	0	0	0	0
	36 (F)	2	5.6	0	0	2	0	0
	38 (M+F)	2	5.3	0	0	2	0	0
Total	145 (M)	22	15.2	0	8	8	1	5
	324 (F)	53	16.4	0	9	32	0	12
	469 (M+F)	75	16	0	17	40	1	17

[a]M = Male mosquitoes, F = Female mosquitoes

Table 2 Prevalence of relative infection of dengue virus in *Ae. albopictus* mosquitoes collected from three southern provinces in Thailand.

Province	Total tested mosquitoes[a]	No. of mosquitoes positive for virus	Relative infection rate (%)	Frequency of dengue serotypes found				
				DEN-1	DEN-2	DEN-3	DEN-4	DEN-2&3
Krabi	28 (F)	6	21.4	0	6	0	0	0
Phuket	5 (F)	3	60	0	2	0	0	1
Phang-Nga	25 (F)	12	48	0	2	7	0	3
Total	58 (F)	21	36.2	0	10	7	0	4

[a]F= Female mosquitoes

Table 3 Prevalence of dengue infection detected in blood specimens collected from some DHF patients admitted to hospitals in the study provinces in 2005.

Provinces	Dengue serotypes, no. and (%) positive				Total
	DEN-1	DEN-2	DEN-3	DEN-4	
Krabi	25 (32.5)	20 (26)	7 (9)	25 (32.5)	77
Phuket	0	10 (100)	0	0	10
Phang-Nga	1 (16.7)	5 (83.3)	0	0	6
Surat Thani	17 (54.8)	10 (32.3)	4 (12.9)	0	31
Total	43 (34.7)	45 (36.3)	11 (8.9)	25 (20.1)	124

Percentage in each parenthesis is derived from comparison with the total number of each line in the last column.

Table 4 Annual incidence of DHF in Thailand (whole country) and four provinces in the study areas from 2000 to 2005.

Place	Population (Year 2004)	Total DHF reported cases/deaths and incidence (per 100,000)					
		2000	2001	2002	2003	2004	2005
Thailand	63,079,765	18,617/32 (30)	139,355/245 (224)	114,800/176 (185)	63,657/75 (101)	39,135/48 (62)	44,765/82 (72)
Krabi	377,954	152/0 (40)	1,209/7 (320)	1,853/10 (490)	378/0 (100)	260/0 (69)	714/0 (184)
Phuket	270,438	39/0 (14)	170/0 (63)	503/1 (186)	205/0 (76)	115/0 (43)	163/2 (57)
Phang-Nga	239,401	99/1 (41)	366/0 (153)	984/0 (411)	236/0 (99)	108/0 (45)	234/0 (98)
Surat Thani	920,283	530/2 (58)	2,351/0 (256)	4,963/15 (539)	843/1 (92)	725/1 (79)	1,802/4 (192)

Source: Department of Disease Control, Ministry of Public Health, Thailand.

only DEN-2 serotype, whereas double infections with two serotypes (DEN-2 and DEN-3) were detected in individual mosquitoes collected in Phuket (1) and Phang-Nga (3).

A total of 124 blood specimens were collected from some DHFpatients admitted to hospitals in the study areas (Table 3). These included 77 samples from Krabi, 31 from Surat Thani, 10 from Phuket and 6 from Phang-Nga. Overall, DEN-2 (36.3%) and DEN-1 (34.7%) were the most prevalent serotypes found in this study, followed by DEN-4 (20.1%) and DEN-3 (8.9%). Only a single serotype of dengue virus was detected in each patient. As shown in Table 3, all four serotypes of dengue viruses were detected in blood samples of DHF patients from Krabi, but only DEN-2 serotype (100%) was found in those obtained in Phuket. In Krabi, the prevalence of DEN-1 (32.5%) was equal to DEN-4 (32.5%) whereas DEN-2 (26%) and DEN-3 (9%) were less frequent. Three dengue serotypes DEN-1 (54.8%), DEN-2 (32.3%) and DEN-3 (12.9) were found in samples collected from Surat Thani, and only DEN-2 (83.3%) and DEN-1 (16.7%)were detected in samples from Phang-Nga.

The annual incidence and deaths due to DHF in Thailand (whole country), Krabi, Phuket, Phang-Nga and Surat Thani from 2000 to 2005 are presented in Table 4. During the year 2000, the incidence of DHF in Thailand, including all the four provinces was relatively low (58/ 100,000). Two severe epidemics of DHF occurred in Thailand, with 139,355 reported cases and 245 deaths in 2001, and 114,800 reported cases and 176 deaths in 2002. As a result, the DHFincidence rates were 224/100,000 in 2001, and 185/100,000 in 2002, respectively. There after, the incidences and deaths due to DHF in Thailand and those four provinces dramatically declined in 2003 and 2004, but increased again in 2005.

Discussion

A number of studies have been reported the isolation and detection of dengue virus in field populations of *Ae. aegypti* in Thailand (Watts *et al*, 1985; Rojanasuphot *et ai*, 1988; Thavara *et al*, 1996; Tuksinvaracharn *et al*, 2004). In 1978 and 1979, Watts *et al* (1985) failed to isolate any dengue virus from several thousand specimens of *Ae. aegypti* larvae, pupae and adult males collected from houses in Bangkok in which one or more persons had recent dengue virus infection. However, DEN-2 virus was eventually isolated from dengue patients and individual *Ae. aegypti* females collected in dwellings of DHF patients (Watts *et al*, 1985). Following that, Rojanasuphot *et al* (1988) isolated DEN-1 virus from 5 pools out of 365 pools (1.4%) of *Ae. aegypti* mosquitoes collected from study sites in Rayong between 1983 and 1984. On Ko Samui (an island in the gulf of Thailand in the province of Surat Thani) during a high epidemic of DHF (497/100,000), 5 out of 6 pools (83.3%) of *Ae. aegypti* females were positive for dengue viruses, however serotypes of the viruses were not identified (Thavara *et ai*, 1996). Recently, Tuksinvaracharn *et al* (2004) found an infection of DEN-3 in pooled *Ae. aegypti* mosquitoes collected from communities in Bangkok during the dry season.

Our study constitutes the first report of double infection with two different serotypes of dengue viruses found in field-caught individual *Ae. aegypti* males and females and *Ae. albopictus* females. This phenomenon could have occurred due to multiple feedings of mosquitoes on two different dengue-infected personsor a single blood meal taken from a person with a double infection with two different seretypes of dengue viruses. This event is possible in highly endemic areas where two or more serotypes of dengue viruses are circulating simultaneously. It is thus possible for a human to get a double infection with two different serotypes of dengue viruses from a single bite of the *Ae. aegypti* female infected with two serotypes of dengue viruses. Gubler *et al* (1985) reported a case of natural concurrent primary infection with two serotypes of dengue viruses, DEN-1 and DEN-4, during a 1982 outbreak in Puerto Rico. This patient presented with only mild symptoms of dengue infection without hemorrhagic manifestations, however, this illness was uncommon and not similar to many other single-serotype dengue infections found in the same area at that time. It is generally thought that concurrent infection in a person with two different serotypes of dengue viruses may cause severe disease. This hypothesis may be true for patients who get secondary dengue virus infection, as found in earlier studies (Halstead *et al*, 1970; Vaughn *et al*, 2000; Nisalak *et al*, 2003). Disease severity in DHF correlates with secondary dengue virus infection, high viremic titers and is associated with rapid virus clearance (Vaughn *et al*, 2000).

The presence of dengue single or double infection in field-caught individual *Ae.* a*egypti* males in the present study provides clear evidence for transovarial transmission occurring in the natural environment in the study areas. Based on experimental results obtained from laboratory infections, Rosen (1987) suggested that *Ae. albopictus* male mosquitoes naturally infected with dengue virus may acquire infection vertically with no sexual transmission of dengue virus from female to male mosquitoes. This phenomenon was found with *Ae. aegypti* in our findings. Transovarial transmission of dengue viruses in mosquito vectors takes place naturally and may be able to maintain the viruses in the environment during dry periods when the population of mosquito vectors is scarce (Rosen *et al, 1983).* Detection of dengue virus in *Ae. albopictus* mosquitoes collected from Ko Samui, Surat Thani has been reported previously (Thavara *et al,* 1996). In contrast to previous studies, Watts *et al* (1985) failed to isolate dengue virus from *Ae. albopictus* mosquitoes collected in the field in Saraburi between 1978 and 1979. Our present study, thus, adds to previous reports of dengue virus infection found in field-caught male *Ae. albopictus* mosquitoes in southern Thailand. The success of dengue virus detection in field-caught male *Ae. albopictus* mosquitoes in the present study and that of Thavara *et al* (1996) may be due to the sensitivity of the detection method used, as well as other relevant factors, such as the mosquito collection procedure, specimen preparation, period of investigation and study sites. It is interesting to note that a relatively high infection rate of dengue viruses (21-60 %) was found in *Ae. albopictus* mosquitoes even though its populations were extremely low. We suspect that the incidence of infection of dengue viruses in *Ae. albopictus* mosquitoes in the rainy season is high. *Ae. albopictus* mosquitoes are usually abundant in the rainy season (Thavara *et al,* 2001 a) and widespread in many provinces in southern Thailand, where the natural habitats of the mosquitoes, such as fruit orchards and rubber and palm plantations are prominent (Thavara *et al,* 2001 b). We suggest expanding control methods for *Ae. albopictus* mosquitoes in southern Thailand.

Until recently, only one serotype of dengue virus has been detected or recovered from a single patient (Rojanasuphot *et al*, 1988; Nisalak *et al,* 2003; Anantapreecha *et al,* 2005). However, a single case of natural infection with two serotypes of dengue virus, DEN-1 and DEN-4, was reported by Gubler *et al* (1985). As to the occurrence of the serotypes of dengue virus found in this study, the findings are similar to a previous study (Rojanasuphot *et ai,* 1988) which found that DEN-2 (45%) was the predominant serotype among 51 subjects isolated for dengue viruses in DHF patients admitted to the Rayong Provincial Hospital between 1980 and 1984, followed by DEN-1 (31.4%). DEN-3 (11.8%) and DEN-4 (11.8%). Anantapreecha *et al* (2005), in 2,715 confirmed specimens of dengue patients collected from six hospitals scattered throughout Thailand from 1999 to 2002, found that 45% were infected with DEN-1, followed by DEN-2 (32%), DEN-3 (19%) and DEN4 (5%).The prevalences of the dengue serotypes obtained from these studies are different due to various factors, such as the sensitivity of the technique used for viral detection, sample collection, period of investigation and study site. It is obvious that all four dengue serotypes circulate continuously in Thailand with fluctuations in the dominant serotype from place to place and year to year. Nisalak *et al* (2003) pointed out that each serotype of dengue virus constitutes a distinct influence on disease severity and nature of the dengue epidemic. They found that DEN-1, DEN-2 and DEN-3 were associated with moderately severe dengue epidemic years (annual incidence rate 134 - 175 per 100,000), but DEN-3 was associated with severe dengue years (annual incidence rate >175 per 100,000). DEN-4 may need pre-existing heterotypic dengue antibodies for replication or to create clinical manifestations (Nisalak *et ai*, 2003). In Thailand, dengue disease incidence has fluctuated over time, increasing from 9/100,000 in 1958 to 72/ 100,000 in 2005, with the highest incidence of 325/100,000 in 1987. Moderately severe epidemics occurred in 1984, 1985, 1989, 1990 and 1997, whereas severe epidemics took place in 1987 and 1998 (Nisalak *et ai*, 2003). The years 2001 and 2002 also had severe epidemics. The Ministry of Public Health, Thailand, has set a DHF threshold for each province of 50/100,000 or lower. This goal has been difficult to achieve in many provinces of Thailand. Since an effective dengue vaccine is not yet available. Control strategies have included integrated vector surveillance and control for *Ae. aegypti* and *Ae. albopictus,* monitoring of dengue virus in mosquito vectors and in dengue patients, and the use of geographic information systems (GIS) and remote sensing (RS) to analyze high risk areas and predict dengue epidemics.

Our findings, to our knowledge, are the first report of double infection with two different serotypes of dengue viruses, DEN-2 and DEN-3, in field-caught *Ae. aegypti* females and males, *Ae. albopictus* females and dengue virus infection found in field-caught *Ae. aegypti* males in Thailand. This study reveals the role of transovarial transmission of dengue viruses in field populations of DHF vectors and elucidates circulation of dengue viruses in vectors in the natural environment in endemic areas. The finding of the ability of DHF vectors to transmit dengue virus transovarially is valuable to the health officers and the public in the development of more effective strategies to control DHF and vectors. The incidence of multiple serotypes of dengue viruses found in field populations of *Ae. aegypti* and *Ae. albopictus* in the same area raises the high possibility of a DHF epidemic. These findings provide greater understanding into the relationship of mosquito vectors, virus transmission and DHF epidemiology in endemic areas.

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Laboratory and Semi-Field Evaluation of Mosquito Dunks® against *Aedes aegypti* and *Aedes albopictus* Larvae (Diptera: Culicidae)

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Abstract: Laboratory bioassays and semi-field studies were conducted on the efficacy and longevity of Mosquito Dunks® (7,000 ITU/mg *Bti*) in order to determine the concentration-response relationship and the effectiveness on the potency of the *Bti* product against *Aedes* mosquito species based on the WHO protocol standard methods and to determine the longevity of release for this product against *Ae. aegypti* mosquito larvae in water storage containers. This bio-potency study with the late 3rd instar larvae of *Ae. aegypti* and *Ae. albopictus* was carried out according to WHO standard protocols. The six concentrations of the *Bti* product used in each test were replicated 4 times with 25 mosquito larvae. Probit analysis was then used to determine the LC_{50} and LC_{95} which was equated with dosages of 1.02 and 1.86 ppm for *Ae. aegypti;* and 0.39 and 0.84 ppm for *Ae. albopictus*, which reveals a potency of 382.95 and 303.74 ITU/mg, respectively. The semi-field evaluation of this product in 200-liter earthen jars against 3rd instar larvae of *Ae. aegypti* showed satisfactory control of greater than 80% at 11 weeks post-treatment.

Introduction

Currently, dengue fever is considered to be the most important arboviral disease of humans in terms of its public health impact (Gubler, 1989). The disease incidence and distribution have steadily increased with more than 2 to 3 billion people at risk of infection, and an estimated 20 million dengue cases annually (WHO, 1997). *Aedes aegypti* (Linnaeus) and *Ae. albopictus* (Skuse) serve as the primary and secondary vectors, respectively. Unlike the mosquitoes that cause malaria, dengue mosquitoes bite during the day. Although the viruses are related, antibodies obtained after infection with one serotype are not cross-protective for the other serotypes (Beaty and Marquardt, 1996). At present, dengue control measures include the use of chemicals for larvicide and space spraying, personal protection, health education and source reduction with the aim of immediate removal of infected mosquitoes. Insecticide use still remains a major component of any control strategy, especially during an outbreak. Chemicals frequently used are those belonging to the organophosphate and pyrethroid classes of insecticides (WHO, 1997). With the current trends in dengue incidence worldwide and without an effective vaccine or treatment, it is expected that the widespread use of insecticides will continue. This practice will likely lead to the selection of resistant strains, rendering current insecticides less effective, leading to the need to identify replacement control strategies. *Bacillus thuringiensis* var. *israelensis (Bti)* can be used to prevent these vectors from breeding (Marin/Sonoma Mosquito and Vector Control District, 2005). Controlled semi-field studies show that *Bti* can be effective for about 7 to 12 weeks (Mulla *et al*, 2004; Vilarinhos and Monnerat, 2004) in undisturbed conditions. We conducted laboratory investigations to determine the concentration-response relationship and the effectiveness of a *Bti* briquette against *Aedes* mosquito species based on WHO protocol standard methods and determined the longevity of the slow-release version of this product against the *Ae. aegypti* mosquito larvae in water storage containers.

Materials and methods

Laboratory study

Laboratory bioassays, based on the bioassay method for the titration of *Bti* preparations with IPS82 standard (World Health Organization Collaborating Center for Entomopathogenic *Bacillus*) were used to determine the effectiveness of the Mosquito Dunks® briquette [(AI 7,000 ITU *Aedes aegypti*/mg), Summit Chemical, Baltimore, MD, USA] against laboratory reared *Ae. aegypti* and *Ae. albopictus* larvae. The lyophilized powder of the reference standard IPS 82 (*Bti* strain 1884, standard titrating 15,000 ITU *Aedes aegypti*/mg) was used to compare with the *Bti* product. *Ae. aegypti* (strain BKK1) and *Ae. albopictus* late 3rd instar larvae were provided by the insectary, Department of Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS). The tested larvae were starved for 24 hours before and during the test to minimize variability due to nutritional and metabolic conditions. *Bti* briquettes were ground and sieved several times before the bioassay test. In the assays, larvae were introduced into plastic cups containing 150 ml of test concentration. Bottled water was used for testing, as it is chlorine-free. Each bioassay involved 6 concentrations, 4 replications, and 25 larvae. A total of 600 larvae were tested with the standard

IPS 82 and the *Bti* product and a total of 100 larvae for the control. A numbers of range-finding bioassays with widely spread exposure concentrations were conducted. Based on these tests, the results were used to determine a narrow range of concentrations for a more precise bioassay. The numbers of surviving larvae were counted at 24 hours and 48 hours. Death of mosquito larvae was determined as a complete lack of movement, even with gentle prodding with a probe. When pupation occured, the pupae were removed and their numbers excluded from the calculations. If more than 5% of the larvae pupated, the test was invalidated because larvae do not ingest 24 hours before pupation. Many larvae may have survived because they were too old.

Semi-field study

A semi-field evaluation was conducted at the research station of the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health in Bang Bua Thong district, Nonthaburi Province, Thailand beginning in December 2004 for 12 weeks. Eight earthen jars (200 l/jar) were filled with water from the domestic water supply. Twenty-five laboratory-reared 3rd instar larvae of *Ae. aegypti* (from the NIH's insectary) were added to each jar. A quarter piece of Mosquito Dunks® (approximately 65 g) was then added to each jar. There were 4 treatment and 4 control jars. New batches of larvae were added weekly to the jars over a period of 12 weeks. Alive remaining mosquitoes were counted 1 week after each treatment. To count without disturbing the larvae, and to assess of adult emergence, pupal skins were recorded from the jars with a syringe and a small fish net were used for this semi-field experiment. Pupal skins floating on the water surface inside the jar wall were removed without disturbing the water. Pupal skins were placed in the water in white plastic trays and counted (Mulla *et al*, 2004). All tested jars were placed in a shaded test area and covered with Celocrete sheets. Covers were in place continuously, except during the addition of larvae and during assessment of efficacy once a week for 2-3 hours each time. Placement of covers prevented UV light and wind-borne debris from entering and other mosquitoes from laying eggs inside the tested jars.

Analysis of data

Tests with control mortality greater than 10%, or any pupation greater than 5%, were discarded. A probit model was used to analyze the mortality of *Ae. aegypti* larvae as a function of *Bti* concentration. Probit regression analysis which the slope, LC_{50}, LC_{95} and their 95% confidential intervals (CI) were obtained. The toxicity (ITU/mg) of the *Bti* product was determined according to the following formula:

$$\text{Potency (}Bti\text{ product)} = \frac{LC_{50}\text{ (standard)}}{LC_{50}\text{ (}Bti\text{ product)}} \times \text{Potency (standard)}$$

The survival rate (%) and mortality rate (%) were calculated on the basis of the number of pupal skins (indicating adult emergence) compared to the initial number of larvae added. Mortality was not considered in the calculations, since it generally was very low and would not change the results (Mulla *et al*, 2004).

Results

In the laboratory bioassay study, significantly different LC_{50} and LC_{95} values were determined for the *Bti* product tested against both the *Aedes* species (Table 1). *Ae. aegypti* tolerated higher *Bti* concentrations compared to *Ae. albopictus*. The LC_{50} and LC_{95} for *Ae. aegypti* were both 1.86 ppm, giving a potency of 382.95 ITU/mg. The LC_{50} and LC_{95} for *Ae. albopictus* were 0.39 and 0.84 ppm, respectively giving a potency of 303.74 ITU/mg.

During the 12-week semi-field study, we observed 100% adult emergence and 100% larval mortality in the first and second weeks post-treatment. Evaluation of the first batch added on the day of treatment, and the second and third batches added on weeks 1 and 2, respectively, showed 100% mortality (absence of pupal skins). There were some live larvae after week 3. The emergence rate of 7% was seen for weeks 4 and 5. At week 8, the survival rate started to increase. The emergence rates in weeks 10, 11 and 12 were 16, 20 and 23%, respectively, revealing declining efficacy (Table 2). This semi-field study shows that this larvicidal formulation provides long-lasting control of *Ae. aegypti* in water-storage jars under experimental conditions at a quarter of a briquette per 200 liters

Table 1 Twenty-four-hour probit analysis of Mosquito Dunks® against late 3rd instar-larvae of *Ae. aegypti* and *Ae. albopictus.*

Bti product	Mosquito species	LC_{50} (ppm) (95% CI)	LC_{95} (ppm) (95% CI)	Slope (SE)	P[1]
Mosquito Dunks®	*Ae. aegypti*	1.018 (0.976,1.066)	1.864 (1.518,2.360)	6,267 (0.66)	0.9
	Ae. albopictus	0.395 (0.332,0.471)	0.845 (0,551,1.314)	4.982 (0.85)	0.99
IPS 82	*Ae. aegypti*	0,026 (0.022,0,03)	0.114 (0.074,0.178)	2.545 (0.26)	0.99
	Ae. albopictus	0.008 (0.006,0.01)	0.029 (0,018,0.049)	2.891 (0.44)	0.98

P[1] refers to the probability corresponding to maximum likelihood chi-square statistics for goodness of fit of the model

Table 2 The survival and mortality rates of *Ae. aegypti* larvae after treated with *Bti* product for 12 weeks.

Bti product	Weeks post-treat	Adult emergence from pupal skin count post-treatment (weeks)	
		Survival rate (%)	Mortality rate (%)
Mosquito Dunks®	1	0	100
	2	0	100
	3	3	97
	4	7	93
	5	7	93
	6	10	90
	7	8	92
	8	13	87
	9	10	90
	10	16	84
	11	20	80
	12	23	77
Control	1	95	5
	2	98	2
	3	96	4
	4	94	6
	5	97	3
	6	98	2
	7	98	2
	8	94	6
	9	91	9
	10	94	6
	11	90	10
	12	92	8

of water. It also showed a mortality rate of about 90% for 9 weeks. Although the mortality rate declined after weeks 8 post-treatment, satisfactary control of greater than 80% was observed for about 11 weeks. However, water replenishment may cause a reduction in *Bti* efficacy.

Discussion

The *Bti* formulation in this experiment was in the form of a solid briquette of *Bti*, additives and cork, which floated on the water surface. The lethal concentration values obtained from this tested product may differ from other *Bti* formulations, as shown in the Brown *et al* (2001). They evaluated a liquid formulation of *Bti* against *Aedes* mosquito larvae. The liquid product tested on the 3rd instar larvae of *Ae. aegypti* were Cybate, Teknar and VectoBac 12 AS, which showed LC_{95} and LC_{50} values of 0.42, 0.72 l/ha and 0.14, 0.46 l/ha and 0.20, 0.40 l/ha, respectively. They revealed that these differences in efficacy were related to formulation characteristics. The *Bti* toxins per milligram vary between products. For our bioassay, the *Bti* briquette formulation contained both bacterial spores and associated toxins, which are crystals made of protein. The high lethal concentration values occurred probably because the product was ground into fine particles for serial dilution in the laboratory bioassay against IPS82 bacterial standard strain. Grinding and disposal of the cork may have resulted in the loss of toxin crystals, thereby diminishing the potency. The high LC_{50} and LC_{95} values in this bioassay reflect changes in the amount of toxin.

Mulla *et al* (2004) showed the longevity of VectoBac tablets (*Bti* 5%) with 3 regimens of water over a period of 20 weeks at a dose of 1 tablet (0.37 g) per 50 liters of water. The water regimens were full jars, half-full jars, and full jars emptied half way and refilled weekly. At week 12 post-treatment, VectoBac tablets provided excellent control for 98% inhibition of emergence (IE) in the full jars, 96% IE in the half-full jars, and 75% IE in the full jars emptied half way and had water refilled weekly. VectoBac tablets (full jars) showed good control for 16 weeks. A semi-field bioassay in Brazil by Lima *et al* (2005) showed a low persistence of *Bti* product in different weather conditions. They tested 0.2 g of VectoBac WDG per 100 liters of water against the late 3rd instar larvae of *Ae. aegypti* using various types of containers placed in a shaded area: plastic, iron, concreate, and asbestos. They found that during periods of higher temperature (21.5-39.3°C), 70-100% mortality was observed for 1 week, which then declined abruptly thereafter in all types of containers. They also revealed that low persistence of *Bti* was obtained without water replacement. An even lower residual effect of this formulation is expected in house storage conditions, where water is used, then refilled during rainfall.

Heavy use of insecticide in some tropical countries for vector control can enhance resistance in the *Ae. aegypti* population. Tests of different formulations and concentrations of Mosquito Dunks® reveal that this can be used as an alternate option for controlling *Aedes* mosquitoes. The Briquette formulation is an alternative used to overcome the lack of persistence, as it can be used in fast-flowing or turbulent waters, which is one of the major limitations of the *Bti* formulations. It is essential to evaluate this product in dengue vector control in different municipalities.

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Field Evaluation of Deet, Repel Care®, and Three Plant-based Essential Oil Repellents against Mosquitoes, Black Flies (Diptera: Simuliidae), and Land Leeches (Arhynchobdellida: Haemadipsidae) in Thailand

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Abstract: Diethyl methyl benzamide or deet, a commercial plant-based repellent (Repel Care®), and essential oils from 3 species of plants (finger root rhizomes, guava leaves, and turmeric rhizomes), steam distillated and formulated as insect repellents, were evaluated in the field on human volunteers against hematophagous mosquitoes, black flies, and land leeches in Thailand. Field trials were conducted against wild mosquitoes in Bang Bua Thong District, Nonthaburi Province, and in the Thap Lan National Park Headquarters, Nadee District, Prachinburi Province; anthropophilic black flies (Diptera: Simuliidae) at the Forestry Fire Control Station in Doi Inthanon National Park, Chomthong District, Chiang Mai Province; and land leeches (Arhynchobdellida: Haemadipsidae) in the Khao Yai National Park, Pak Chong District, Nakhon Ratchasima Province. The 3 experimental plant-based essential oil formulations as well as Repel Care and deet provided complete protection from mosquito landing and biting for up to 9 h (duration of the experiment). Similar results were obtained with the 5 products against black flies, providing 100% protection for 9 h but 96-82% protection after 10 and 11 h posttreatment. The 5 repellent products also provided 100% protection against land leeches for at least 8 h. This is the 1st report of repellency of plant-based repellents against black flies and land leeches in Thailand. The identification and availability of inexpensive sources of plant-based oils, i.e., finger root rhizomes, guava leaves, and turmeric rhizomes providing long-lasting repellency against blood-sucking organisms are promising leads into commercial production of relatively safe and effective repellents.

Introduction

Blood-sucking insects, such as mosquitoes, black flies, sand flies, and biting midges, pose serious health-related problems for humans, especially in tropical and subtropical regions. Their biting and hovering activity cause annoyance, mild-to-severe allergic reactions, and often transmission of disease agents. These insects at times transmit important human diseases. For example, mosquitoes transmit malaria, filariasis, and dengue hemorrhagic fever; black flies transmit human onchocerciasis; and sand flies transmit leishmaniases (Rozendaal 1997). Protection from insect bites is an important strategy to prevent or minimize the health-related risks caused by these blood-sucking insects, especially in situations where there is no preventive vaccine available against the disease. The use of insect repellents for personal protection is one of the important available measures used against biting insects. The insect repellents currently on the market in Thailand and elsewhere contain mostly diethyl methyl benzamide (deet) and ethyl butylacetylaminopropionate (IR 3535) as active ingredients, whereas a small number of the repellent formulations contain plant extracts, i.e., citronellal, turmeric (Curcuma longa L.), and eucalyptus oil (Thavara *et al*. 2002). Although deet and IR 3535 have demonstrated excellent repellency against mosquitoes in Thailand (Thavara *et al*. 2001), deet has some drawbacks, i.e., it damages plastic objects and has an unfavorable odor (Trongtokit *et al*. 2004). There is also increasing concern about chemicals used as topical repellents by consumers. Toxic and ill effects of deet, especially at high concentrations, have been reported previously (Gryboski *et al*. 1961, Maibach and Johnson 1975, Miller 1982, Roland *et al*. 1985, Snyder *et al.* 1986, Edwards and Johnson 1987, Anonymous 1988, Moody 1989). To discover substitutes for synthetic repellents, studies were conducted on extracts from locally available plants in Thailand that showed a high degree of repellent activity. Citronella oil seems to be the most popular active ingredient of plant origin in various formulations of mosquito repellents (Thavara *et al*. 2002). However, consumers are reluctant to use repellents containing citronella oil because of its strong odor and low repellency (Thavara *et al*. 2002). Although many studies have been conducted on the repellent effects of plant-based products against mosquitoes, none have been conducted against black flies (Diptera: Simuliidae) and land leeches (Arhynchobdellida: Haemadipsidae). In this study, we evaluated and compared the repellent efficacy of 3 experimental herbal repellents formulated from essential oils of finger root rhizomes, guava leaves, and turmeric rhizomes along with a commercial herbal repellent, and a deet formulation against mosquitoes, black flies, and land leeches in Thailand.

Materials and methods

Test repellents: In total, 5 test repellent-3 formulated plant-based essential oils; Repel Cares, a commercial herbal repellent available on the market in Thailand; and deet-were evaluated in this study. Each essential oil from the 3 plants (finger root rhizomes, guava leaves, and turmeric rhizomes) was formulated as 10% (w/w) lotion in absolute ethanol (60%) with additives vanillin (10%), propylene glycol (10%), and polyethylene glycol (10%). Repel Care, the proprietary herbal repellent, consisted of turmeric oil (5%, w/w) and Eucalyptus citriodora oil (4.5%, w/w) as active ingredients. For comparison as standard repellent, deet was formulated as 10% (w/w) lotion similar to the essential oil repellents. The experimental essential oils from the three plant species selected on the basis of preliminary tests showing promise were purchased from Thai-China Flavours and Fragrances Industry Co., Ltd. (Amphur Muang, Nonthaburi, Thailand). These essential oils were extracted by this firm by steam distillation from 3 plant species: ginger root oil from rhizomes of *Boesenbergia rotunda* (L.) Mansf., guava oil from leaves of *Psidium guajava* L., and turmeric oil from turmeric plant rhizomes. Repel Care (produced by Perdya Thai Part., Chachoengsao Province, Thailand) was purchased from a drug store in Nonthaburi Province, Thailand. The technical grade deet (purity 99.3%; produced by API Corp., Osaka, Japan) was provided by Bride Beauty Care Cosmetics Co., Ltd. (Chachoengsao Province, Thailand). These repellent formulations were tested against mosquitoes, black flies, and land leeches in the field.

Field evaluation against mosquitoes: A field site in Bang Bua Thong District, Nonthaburi Province, Thailand (18.48674° N, 98.68145° E) was selected as the experimental site for field evaluation because of its ample developmental sites for various species of mosquitoes in this area. The field tests were conducted in protected locations with minimal wind disturbance where mosquito landing or biting activity was relatively high. Five volunteers (2 females and 3 males, aged 25-61 years) participated in the field evaluations for assessing the repellency of the test repellents against mosquitoes. The volunteers were seated, with both pant legs rolled up to their knees, and each volunteer was treated with a test repellent (2 ml) on one leg (from knee to ankle), whereas the other untreated leg was the control. The surface area of the volunteers' legs that was treated ranged from 710 to 780 cm^2. The different test repellents and rotation of each repellent treatment were applied to each volunteer in each evaluation. Using this procedure, every volunteer was treated with each of the 5 test repellents during 5 separate evaluations. The volunteers were at least 5 m apart, and they collected all of the mosquitoes (individual mosquito per capped plastic vial) landing on or attempting to bite on each leg for a 10 min period. Each exposure and collection period of 10 min was followed by a 20 rain break (moving indoors without rolling down the pant legs) before the next mosquito collection. Therefore, during each hour of the test, there were 2 periods (10 min each) of mosquito collections and 2 breaks (20 min each). The tests were conducted for duration of 9 h each night against night-biting mosquitoes (from 1900 to 0400 h).

The same test procedure was used during 8 h (from 0900 to 1700 h) against day-biting mosquitoes, primarily *Aedes albopictus* (Skuse) at the Thap Lan National Park Headquarters, Nadee District, Prachinburi Province (13.91217° N, 100.390531° E). This national park is located between 300 and 500 m above sea level with small streams meandering through the park. It is a home to the Lan

(Thai name) tree or talipot palm (*Corypha lecomtei* Becc.), which provides a vast number of natural breeding sites in the leaf axils for *Ae. albopictus* during the rainy season (May-October), receiving an annual average rainfall of about 900 mm and average temperature of about 27.5°C.

Wild-caught night and day-biting mosquitoes from both sites were brought to the laboratory and identified to species under a stereomicroscope by using the keys by Rattanarithikul and Panthusiri (1994), Bram (1967), Peyton and Scanlon (1966), and Huang (1977).

The assessment of repellency was based on comparisons of mosquitoes captured on the control (untreated) and treated legs. The percentage reduction of landing and biting rates during each hour of testing was calculated according to Yap et al. (1998) and Tawatsin et al. (2001):

$$\text{Reduction (\%)} = \frac{C - T}{C} \times 100$$

where *C* denotes the number of mosquitoes collected from the control legs, and *T* represents the number of mosquitoes collected from the treated legs.

Field evaluation against black flies: Field tests were conducted in Doi Inthanon National Park located in Chiang Mai Province (14.35383°N, 101.95089°E). This park is one of the most scenic and natural tourist attractions in Thailand. It is a rugged mountainous area with varying elevations of up to 2,565 m above sea level. There are numerous waterfalls and streams flowing throughout the park, providing breeding habitats for black flies. Doi Inthanon National Park receives an annual average rainfall of about 2,000 mm. It provides a wide range of vegetation with deciduous forests, submontane evergreen and montane evergreen forests, native pines, and wild flowers, including a large number of orchid species, and it is considered to have the richest flora and fauna in Thailand. It is also a well known area for black fly abundance in Thailand among locals and tourists. The abundance of black flies has been reported in many sites at different altitudes from 400 to 2,460 m above sea level (Kuvangkadilok *et al*. 1999). After searching various locations for black fly abundance and biting activity, we selected a study site at the Forestry Fire Control Station (860 m above sea level). This site harbored relatively high populations of black flies during the preliminary surveys. The same 5 volunteers in mosquito repellent tests participated in field evaluation of the test repellents against black flies. Although the repellent test procedure was similar to that conducted against mosquitoes, the duration was 11 h (from 0730 to 1830 h) because the black flies are active and bite during the day. The collected black flies (one individual per capped plastic vial) were brought to the laboratory and identified to species under a stereomicroscope by using the keys by Takaoka and Suzuki (1984), Takaoka and Davies (1995), and Takaoka and Choochote (2004). The reduction in landing and biting activity was calculated as described for mosquitoes.

Field evaluation against land leeches: The evaluation against land leeches was conducted along a hiking trail near the park headquarters in Khao Yai National Park, Pak Chong District, Nakhon Ratchasima Province (latitude 14.98515° N, longitude 101.47476° E). This park includes plateaus with elevations ranging from 200 to 1,351 m above sea level, which slope toward the south and east. The park area is about 2,168 km^2 and is covered by dry evergreen forest, mixed deciduous forest, dry dipterocarp forest, and grassland. During the southwest monsoon, rain is heavy between May and October, with average annual rainfall ranging from 2,200 to 3,000 mm. The climate in Khao Yai National Park is relatively cool with an average temperature of 23°C. Preliminary surveys revealed abundance of land leeches in Khao Yai National Park (Ngamprasertwong 2001). The same repellent test procedure and assessment technique used against mosquitoes were used for land leeches. However, instead of sitting, the volunteers walked for 10 rain along the hiking trail in 1-hour intervals and collected attached land leeches on both legs (one treated with repellent and the other untreated as control). The collected land leeches (one individual per capped plastic vial) were brought to the laboratory and identified to species under a stereomicroscope using the keys by Moore (1927, 1935). The tests were conducted for 8 h (from 0900 to 1700 h). To confirm the repellent efficacy, every hour postrepellent application, a land leech was placed on the treated area of each volunteer and left for 3 rain, and its behavior examined. The percentage of reduction in attachment or crawling was calculated in the same manner as for mosquitoes and black flies.

Statistical analysis: The percentage of reduction for each hour of the testing period was transformed to log (x + 1) for analysis of variance (Yap *et al*. 1998). A 1-way analysis of variance followed by Duncan's multiple range test was used to compare the mean repellency of the test repellents against mosquitoes, black flies, and land leeches. A *P* value of equal or less than 0.05 was used to denote statistically significant differences among the treatments.

Results and Discussion

Mosquitoes: At Bang Bua Thong, 3,830 mosquitoes in total belonging to 11 species were collected on untreated legs of the volunteers. *Culex vishnui* Theobald represented the majority (77.1%) of mosquitoes collected. Other species attracted to the volunteers were *Culex quinquefasciatus* Say (13.8%), *Culex gelidus* Theobald (3.4%). *Culex tritaeniorhynchus* Giles (1.6%), *Mansonia indiana* Edwards (1.3%), and *Aedes aegypti* (L.) (1.2%). The remaining 5 species - *Aedes albopictus*, *Anopheles barbirostris* Van der Wulp, *Anopheles umbrosus* Theobald, *Armigeres* *subalbatus* (Coq.), and *Mansonia annulata* Leicester-constituted less than 1% each of the total collected (Table 1). Repellency tests in the field at Bang Bua Thong by using the 5 repellent compositions yielded excellent results (Table 2). The 3 plant-based essential oil repellent compositions (finger root, guava, and turmeric) were similar to Repel Care and deet in providing 100% protection during the entire testing period of 9 h ($P < 0.05$). The testing of these products beyond the 9 h was not continued. This duration of protection from mosquito bites with these repellents is satisfactory for use under field conditions. It is noteworthy to obtain long-lasting protection with compositions of plant essential oils equaling that of an effective commercial synthetic repellent such as deet. The plant essential oils are cost-effective, locally available, and pleasant to use by consumers.

Table 1 Mosquito species and overall number collected from untreated human volunteers at Bang Bua Thong, Nonthaburi, Thailand (April 23-2 May, 2005).

Species	Total	collected %
Ae. aegypti	46	1.2
Ae. albopictus	11	0.3
An. barbirostris	6	0.15
An. umbrosus	6	0.15
Ar. subalbatus	27	0.7
Cx. gelidus	130	3.4
Cx. quinquefasciatus	529	13.8
Cx. tritaeniorhynchus	61	1.6
Cx. vishnui	2,953	77.1
Ma. annulata	11	0.3
Ma. indiana	50	1.3
Total	3,830	100

The total collection from untreated legs for 20 min in each hourly testing period was relatively constant, ranging from 280 to 550 mosquitoes (Table 2). By converting this value to an hourly attack rate, the total collection ranged from 33.6 to 66 mosquitoes/person-h.

As for the repellents tested against day-biting *Aedes* mosquitoes in the field, 5,486 mosquitoes in total (5,313 from control and 173 from treated areas) were collected (Table 3). All collected mosquitoes were identified, and only 2 species were found: *Ae. albopictus* (99.9%) and *Ar. subalbatus* (0.1%). However, only *Ae. albopictus* were collected from the treated legs of volunteers. The biting rates of mosquitoes in this study were relatively high, ranging from 59.2 to 126.7 mosquitoes/person-h (Table 3). Overall, the 4 plant-based repellents and deet provided excellent repellency against *Ae. albopictus* for 8 h. Complete protection

Table 2 Relative repellency (percentage of biting reduction) of test repellents and total number of night-biting mosquitoes collected in each period postapplication at Bang Bua Thong, Nonthaburi Province, Thailand (April 23-2 May 2005).

	Reduction of mosquitos bites (%) posttreatment[1] (h)								
Repellents (%)	**1 (1900-2000)**	**2 (2000-2100)**	**3 (2100-2200)**	**4 (2200-2300)**	**5 (2300-0000)**	**6 (0000-0100)**	**7 (0100-0200)**	**8 (0200-0300)**	**9 (0300-0400)**
Finger root oil (10)	100	100	100	100	100	100	100	100	100
Guava oil (10)	100	100	100	100	100	100	100	100	100
Turmeric oil (10)	100	100	100	100	100	100	100	100	100
Repel Care® (9.5)[2]	100	100	100	100	100	100	100	100	100
Deet (10)	100	100	100	100	100	100	100	100	100
Total mosquitoes[3]	305	335	520	435	550	460	505	440	280
Average biting rate (no./ person-h)	36.6	40.2	62.4	52.2	66	55.2	60.6	52.8	33.6

1 There was no significant difference in repellency among test repellents ($P > 0.05$).
2 Consisting of turmeric oil 5% (w/w) and *E. citriodora* oil 4.5% (w/w).
3 Total number of mosquitoes collected from control areas of 5 volunteers in 5 runs of this evaluation, and no mosquitoes were collected from the treated areas of volunteers during of this study.

Table 3 Relative repellency (percentage of biting reduction) of test repellents and total number of night-biting mosquitoes collected in each period postapplication at Bang Bua Thong, Nonthaburi Province, Thailand (June 11-17, 2005).

	Reduction of mosquitos bites (%) posttreatment[1] (h)							
Repellents (%)	**1 (0900-1000)**	**2 (1000-1100)**	**3 (1100-1200)**	**4 (1200-1300)**	**5 (1300-1400)**	**6 (1400-1500)**	**7 (1500-1600)**	**8 (1600-1700)**
Finger root oil (10)	100	100	100	100	100a	100a	94.7a	93.5a
Guava oil (10)	100	100	100	100	100a	93.9c	91.9a	90.9a
Turmeric oil (10)	100	100	100	100	96.2b	94.2bc	93.3a	76.1c
Repel Care® (9.5)[2]	100	100	100	100	100a	96.9ab	92.4a	91.8a
Deet (10)	100	100	100	100	100a	97.3a	90.2a	85.5b
Total collected mosquitoes (control)[3]	605	613	493	540	498	870	733	963
Total collected mosquitoes (treated)[4]	0	0	0	0	5	28	48	93
Average biting rate[5] (no./ person-h)	72.6	73.6	59.2	64.8	66	107.8	93.7	126.7

1 Means in each column followed by the same letter are not significantly different ($P > 0.05$, Duncan's multiple range test).
2 Consisting of turmeric oil 5% (w/w) and *E. citriodora* oil 4.5% (w/w).
3, 4 Total number of mosquitoes collected from control areas of 5 volunteers in 5 runs of this evaluation.
5 Average biting rate in each period was calculated from total number of mosquitoes collected from treated and untreated (control) areas.

6 h postapplication was obtained from finger root oil; 5 h postapplication from guava oil, Repel Care, and deet; and 4 h postapplication from turmeric. The repellents containing finger root oil, guava oil, and Repel Care provided equivalent repellency ($P < 0.05$) of more than 90% reduction of mosquito bites at the last period of testing (8 h posttreatment), whereas deer and turmeric oil provided significantly less repellency ($P < 0.05$) of about 85.5% and 76.1%, respectively.

In general, plant-based extracts and products have been evaluated primarily for larvicidal, adulticidal, and other types of activities against mosquitoes. According to the comprehensive review of Sukumar et al. (1991), plant-based products in 29 of 344 plant species have been tested for repellency against mosquitoes. In our previous studies using released mosquitoes in a large room, we obtained 5-h protection from mosquito bites by using steam distilled oil extracts (turmeric, kaffir lime (*Citrus hystrix* DC.), citronella grass (*Cymbopogon winterianus* Jowitt), and hairy basil (*Ocimum americanum* L.)) with 5% vanillin (Tawatsin *et al*. 2001). The extent and duration of protection from mosquito bites in our current study are similar or greater than those obtained from neem, *Azadirachta indica* A. Juss, extracts (Sharma *et al*. 1993), *Kaemferia galanga* L. extracts (Choochote *et al*. 1999), aromatic turmeric (*Curcuma aromatica* Salisb.) extracts (Pitasawat *et al*. 2003), and celery (*Apium greveolens* L.) seed extracts (Choochote *et al*. 2004, Tuetun et al. 2004). In extensive field studies, Trongtokit et al. (2004) observed repellency and almost 100% protection with gel formulation of clove oil or clove plus makaen oil, equaling that yielded by 20% deer formulation. From the information available in the literature and our own studies, it is inferred that some plants contain a variety of bioactive principles exhibiting repellent action against mosquitoes. Formulation technology using adjuvants and extenders can greatly increase the repellent activity of plant-based products. It is our opinion that plant-based products in tropical and developing countries will provide cost-effective and locally available safe alternatives to synthetic repellents against mosquitoes.

Black flies: In total, 2,582 black flies were collected from untreated legs of volunteers during the test periods in the Doi Inthanon area (Table 4). Only two *Simulium* species were collected: *Simulium nigrogilvum* Summers constituted more than 99% of the collections, whereas *S. chumpornense* Takaoka and Kuvankadilok constituted less than 1% of the collections. However, the collected black flies that landed or bit the treated areas of volunteers after 10 h posttreatment were *S. nigrogilvum* only. Choochote (2004) reported 45 species of black flies found in Thailand of which 41 species (91.1%) were in northern Thailand. Of these, 41 species (68.9%) were from Doi Inthanon National Park. At the Forestry Fire Control Station (current study site), at least 18 species of swarming and hovering adult black flies were collected over different periods of the year by sweeping net around human hosts (Choochote 2004). In our repellent study, *S. nigrogilvum* constituted 99% of the collected black flies, whereas those netted by Choochote in 2004 were mostly *S. askoae* (89-93.7%) and *S. nigrogilvum* (5.5-8.9%). This difference could be because of seasonality, different methods of black fly capture, or the behavior of *S. nigrogilvum*, which prefers to land and attack humans more directly than does *S. asakoae*. Choochote (2004) also pointed out that *S. asakoae* usually form swarms and hover around humans without landing or biting. Rarely, blood-engorged *S. asakoae* were collected by sweeping net despite the large collection made by netting. There were more engorged specimens of *S. nigrogilvum* collected by the sweeping method. This phenomenon was reported by Takaoka *et al.* (2003) who found that among 305 black flies (217 *S. nodosum*, 86 *S. asakoae*, and 2 *S. nigrogilvum*) obtained by net sweeping from a human bait, bloodfeeding on humans was identified only in *S. nodosum* and *S. nigrogilvum*. Although there are no human onchocerciasis cases in Thailand, natural infections with filarial larvae resembling Onchocerca suzukii in *S. nodosum* were reported previously (Takaoka *et al*. 2003). Unidentified filarial larvae in *S. nigrogilvum* and *S. asakoae* also were reported previously (Fukuda *et al*. 2003). Identification of natural infections of filarial larvae in black flies in Thailand warrants further investigation.

Table 4 The relative repellency (percentage of biting reduction) of test repellents and total number of black flies collected in each period postapplication at Doi Inthanon National Park, Chiangmai, Thailand (February 19-26, 2005).

	Reduction of mosquitos bites (%) posttreatment[1,2] (h)					
Repellents (%)	**1(0730-0830)**	**2(0830-0930)**	**3(0930-1030)**	**91530-1630)**	**10(1630-1730)**	**11(1730-1830)**
Finger root oil (10)	100	100	100	100	91.0a	82.1a
Guava oil (10)	100	100	100	100	96.2a	87.0a
Turmeric oil (10)	100	100	100	100	90.5a	90.4a
Repel Care® (9.5)[3]	100	100	100	100	94.9a	92.8a
Deet (10)	100	100	100	100	96.6a	91.8a
Total collected blackflies[4]	571	639	94	80	411	787
Average biting rate (no./ person-h)	67.4	76.8	1.4	9.6	49.2	94.5

[1] No data are presented between 4 and 8 h posttreatment because no black flies were collected because of the lack of biting activities during these periods.

[2] There was no significant difference in repellency among test repellents ($P > 0.05$).

[3] Consisting of turmeric oil 5% (w/w) and *E. citriodora* oil 4.5% (w/w).

[4] Total number of black flies collected by 5 volunteers in 5 runs of this evaluation.

As against mosquitoes, the 4 plant-based repellent products and deet proved highly effective in repelling black flies (*S. nigrogilvum*) and protecting treated body parts from their bites (Table 4). Complete protection (100%) was observed for all repellents for 9 h posttreatment. After 10 and 11 h posttreatment, the level of protection decreased but still affording a high level of protection (>82%). During these two last assessment periods when flies landed on treated areas, there was no significant difference among the treatments ($P > 0.05$).

As noted in Table 4, no black flies were collected from controls during 4 to 8 h posttreatment (1030-1530 h). In addition to these periods, flying and biting activity was relatively low during 3 (0930-1030) and 9 (1530-1630) h posttreatment (Table 4). Two biting activity peaks were observed, one in the morning (0730-0930 h) and the other in the afternoon (1630-1830 h). With the onset of decreasing light intensity (sunset at 1830 h), flying and biting activities stopped.

Extensive and numerous studies have been conducted on synthetic repellents against black flies (Schiefer *et al*. 1976, Schreck *et al*. 1979, Das *et al*. 1985, Robert *et al*. 1992, Debboun *et al*. 2000). However, there are virtually no reports on the activity of plant-based products against black flies. It is of great interest to note that the 4 plant-based repellent preparations and deet tested in this study provided similar protection from landing and biting by *S. nigrogilvum* and *S. chumpornense*. This is the 1st field study on the evaluation of plant-based essential oils against black flies.

Because of some of the undesirable properties of deet, it is encouraging to note that there are effective plant-based repellent products against anthropophilic black flies. In addition, the ethanolic preparation of the 3 experimental plant-based extracts and commercial product of plant origin had no objectionable characteristics and produced no ill effects to the volunteers using them.

During the course of our repellent evaluations against black flies, volunteers experienced allergic reactions from black fly bites on the untreated legs, arms, and elbows as well as any other untreated exposed parts of the body. These reactions included swelling, itching, mild allergic irritations, or chronic dermatitis that may require medical attention (Gudgel and Grauer 1954). Salivary secretions containing anticoagulants or other materials injected into host tissues during bloodfeeding caused the allergic reactions. The reactions manifested due to the bites of black flies in our study persisted for a few days or longer, varying in degree depending on the individual (Figure 1). Most of the volunteers in this study recovered completely within a week after taking antihistamines or application of antipruritic cream. However, some individuals showed strong allergic reactions persisting for months.

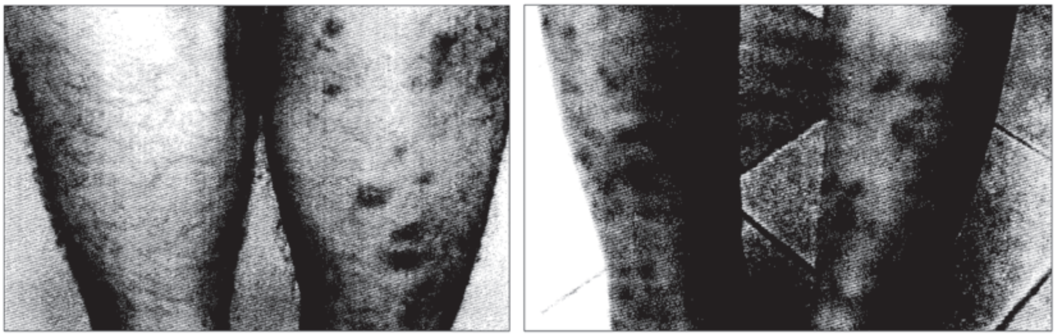

Figure 1 Dermatological syndromes (raised red spots with central depressions at the site of the puncture) caused by the bites of *S. nigrogiluum* on volunteers. (Left) A male volunteer with the left leg treated with repellent and the right leg as control showing numerous bites, symptoms lasting for months. (Right) Chronic scabs and scars on both legs of a female resident receiving bites of black flies for prolonged period. These symptoms without treatment last for months.

Land leeches: In total, 652 land leeches were collected. Only one species of the genus *Haemadipsa* was collected and has not been identified to species. This species is similar to *Haemadipsa* sp.2 as described by Ngamprasertwong (2001). Regarding repellency, the 3 plant-based essential oil repellents, Repel Care, and deet provided 100% protection during the 8 h testing period ($P < 0.05$). None of the land leeches made contact with any of the treated areas of the volunteers, whereas all of the land leeches were collected from the untreated legs only. Land leeches primarily seek blood from warm-blooded animals. They attach themselves on a host to suck blood when the host passes through the vegetation where land leeches rest and quest for hosts. In this study, some land leeches also attempted to attach themselves on the treated areas of the volunteers, but they detacfied from the areas immediately when their anterior suckers touched the treated areas. This behavior showed excellent contact repellent activity of the 5 test repellents. Additional tests by placing a land leech for 3 min on the treated area of each volunteer also confirmed" repellency and toxicity of the 5 test repellents during the entire 8 h postapplication. Interestingly, no land leeches sucked blood or engorged on the treated area of volunteers, and all the leeches died within 3 rain of exposure on the treated surfaces. During the exposure period, the land leeches could not attach normally, lost their capability to move, and secreted a mucous fluid around their bodies. These remarkable effects occurred on exposure to all test repellents. It is evident that land leeches are highly sensitive to repellents containing essential oils and deet 8 h or longer after treatment. This study shows for the 1st time that the 3 plant-based essential oil repellents not only have repellent effects but also exhibit contact toxicity. In summary, the 3 experimental plant-based repellents formulated from essential oils of finger root rhizomes, guava leaves, and turmeric rhizomes; the commercial herbal repellent Repel Care; and deet provided complete protection against a number of mosquito species for up to 9 h in the field. These plant-based repellents also demonstrated a high degree of repellency against black flies *S. nigrogilvum* and *S. chumpornense*, similar to deet for up to 11 h posttreatment in the field. In addition, the 5 test repellents provided complete protection (100% repellency) against land leeches (*Haemadipsa* sp.2). No adverse effects were detected in any of the volunteers from using all 5 tested repellents.

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Genetically Modified Mosquitoes: a New Strategy to Control Mosquito Borne Diseases

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Abstract: Mosquito borne diseases remain a major human and animal health concern in many areas of the world. Chemical control strategy faces a number of problems including the development of insecticide resistance in mosquitoes, the impact of insecticides on human and animal health and the enironment and the constant inability to control mosquitoes effectively. Advances in the knowledge about mosquito-pathogen relationships and the molecular biology of mosquitoes make it now possible to produce mosquito strains that are unable to transmit various parasites or viruses. Strategies of using the genetically modified mosquitoes to control mosquito borne diseases are discussed here.

Introduction

Mosquito-borne diseases are still a major human and animal health problem in many countries. Diseases transmitted by mosquitoes include malaria, filariasis, yellow fever, Japanese encephalitis and dengue fever. The presence of multi-drug resistant strains of the pathogen has led to difficulty in controlling the diseases. The development of novel vaccines also faces the problem of antigenic variation in the pathogens. Chemical control used to be the primary strategy for controlling mosquito-borne diseases, but concerns about the impact of the available compounds on human and animal health and the environment. together with the development of insecticide resistance in mosquitoes, has limited the usefulness of this approach (Taksinvarajarn *et al*., 2004; Thavara *et al*., 2006). Through advances in our knowledge of mosquito-pathogen relationships and the molecular biology of the mosquito, it is possible to produce mosquito strains that are incapable of transmitting various parasites or viruses (Collins and lames, 1996; Beemtsen *et al*., 2000). There are a number of molecules which, when expressed or introduced into mosquitoes, are able to block the transmission of pathogens. For example, monoclonal antibodies directed against a circumsporozoite protein of the avian parasite, *Plasmodium gallinaceum*, blocks sporozoites from entering the salivary glands of the mosquito, *Aedes (Ae.) aegypti.* when injected into the hemolymph (Warburg *et al*. 1992). Expression of a 12 amino-acid peptide SMD that binds specifically to the midgut lumen and distal salivary gland. lobes of mosquitoes or a bee venom phospholipasc (PLA2) in *Anopheles (An.) stephensi* mosquitoes strongly reduces the number of the developing rodent parasite, Plasmodium berghei, in the midgut of these transgenic mosquitoes (Ito *et al*., 2002; Moreira *et al*., 2002). More recently, expression of RNA interference against the dengue virus type 2 in the midgut of genetically modified *Ae. aegypti* mosquitoes interrupted the dengue type 2 replication in the transformed mosquitoes (Franz *et al*., 2006). Expression of similar molecules in genetically transformed mosquitoes could lead to the production of strains that can be used in the control of transmission of pathogens (Coates *et al*. 1999; James, 2002).

Requirements for creating genetically modified mosquitoes

Genetic transformation is defined as the uptake and expression of exogenous DNA by cultured cells or whole organisms. Gene transfer may be transient, reflecting the episomal state of the introduced DNA, or stable following the integration of DNA into the chromosome (Besansky *et al*., 1992). Genetic manipulation depends on the successful solution to four separate but independent problems: (1) the delivery of DNA into the mosquito embryo, (2) the efficient and stable integration of DNA into chromosome, (3) a promoter to control expression of the exogenous gene and (4) a suitable marker gene that encodes a dominant phenotypic trait.

DNA delivery

The problem of introducing DNA into mosquito embryos has been overcome by the development of microinjection techniques. The technique for microinjection of mosquito embryos was based on that used to introduce DNA into the developing embryo of *Drosophila (D.) melanogaster* (Rubin and Spardling, 1982). The microinjection technique has been carried out using *An. gambiae* (Miller *et al*., 1987), *Ae. triseriatus* (McGrane *et al*., 1988). *Ae. aegypti* (Morris *et al*., 1989), *An. stephensi* (Catteruccia *et al*., 2000) and *Culex (Cu.) quinquefasciatus* (Alien *et al*., 2001).

To facilitate gcrmline transformation, the exogenous DNA must be introduced into the developing embryo prior to pole cell formation (approximately 90-120 minutes after mosquito eggs have been laid. Fig. 1). The introduced plasmid will be incorporated into the developing pole cells and will integrate into the chromosomal DNA. The integrated DNA sequences may then be expressed throughout the somatic tissue of subsequent generations

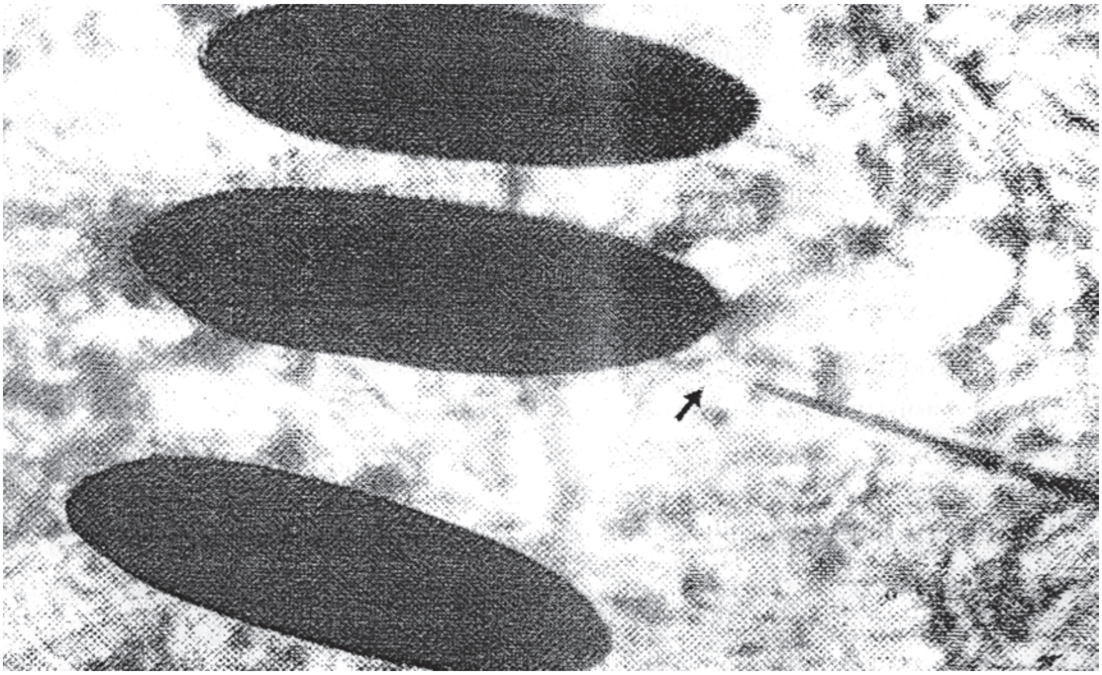

Figure 1 Microinjection of Aedes aegypti embryos: The embryos were oriented with their posterior aligned DNA solution was injectioned into the embryos via acapillary needle (arrow).

DNA integration

Transposable elements (transposons) are DNA segments that can insert themselves in a genome. They can be maintained in a variety of related genomes, serve as an agent of chromosomal insertion, deletion or rearrangement, and provide the basis for the transformation of somatic or germ cells (Garza *et al.*, 1991). Class II transposable element or DNA element, a group which consists of elements that transpose DNA to DNA directly are widely used for genetic transformation including mosquito transformation. The class II transposable elements are characterized by their small inverted terminal repeats of less than 100 base pairs bordering an internal transposase-encoding sequence which functions in a DNA-only mechanism of transposition (McDonald, 1993).

Exogenous DNA has been successfully introduced into the mosquito germ line using the P element transposon and some integration events have been stable over multiple generations (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989). However, in all cases, the efficrency of integration has been at least 10-fold lower than expected from *Drosophila* even though survival rates following injection were comparable. It is now accepted that the P element is not a functional transformation vector in non-drosophilid insects in that in phylogentically restricted (Handler *et al.*, 1993; To date. the elements which have been used effectively to transform mosquitoes are the *Mos 1 (mariner), Hermes, Minos and piggyBac* (O'Brochta *et al.*, 2003).

Promoters

The promoter is crucial to express the introduced gene (transgene) in cooperation with the transcriptional apparatus of the host. The application of species-specific promoters could provide the benefits of increase levels and control of gene expression. Numerous groups have cloned a variety of both constitutively expressed (the promoters are those active in an cells at all times) and tissue, sex and stage-specific genes from the mosquitoes *An. gambiae* and *Ae. aegypti*, including those for vitellogenin (Romans *et al.*, 1995), α glucosidase, α amylase, the female-enriched protein D7, and the *Apyrase (Apy)* gene from female *Ae. aegypti* salivary gland (James *et al.*, 1989; 1991; Smart *et al.*,1995). The *Maltase-like 1 (Mal 1)* and the *Apy* gene promoters have been successfully used to express the reporter gene luciferase *(luc)* in both cultured cells and in *Ae. aegypti* embryos (Coates *et al.*, 1999). More recently the hexamerin promoter from the mosquito *Ochlerolatus atropalpus* has heen used to drive expression of the *luc* reporter gene in *D. melanogaster* (Jinwal *et al.*, 2006) and it. seems to be a sex-, stage- and tissue-specific promoter.

Reporter genes and Selectable markers

Marker genes are one of the most important factors in genetic transformation. The choice of suitable marker genes remain critical for detection transformants. A dominant genetic marker or a recessive marker and a suitable mutant recipient are essential. The marker must be suitable for the detection of a relatively rare event. If no suitable mutant strains are available, then the choice is usually limited to a marker that confers resistance to a toxic chemical or to one that, when expressed, confers an easily scored visible phenotype (Ashburner *et al.*, 1998).

The use of an antibiotic or insecticide resistance strategy to select transgenic insects can lead to a generation of false positives due to, for example. the breakdown of the insecticide or antibiotic. Furthennore, genuine transfonnants can be lost if the level of expression of the transgenc is not sufficient to confer antibiotic or insecticide resistance to the transgeoic individual (Pinkerton *et al.*, 2000).

Eye color marker genes appear to be the most useful markers to select transformants for several reasons: they generally produce unequivocal phenotypes. the transformants are easily identifiable and are viable under normal laboratory conditions. The genes involved in eye color biosynthesis fall into three classes: genes required for ommochrome biosynthesis, genes required forpteridine biosynthesis and genes required for both pathways. Typically, the last class gives an unpigmented (white) eye. The *white* genes of *Drosophila*, *An. gambiae* (Kaiser *et al.*, 1982; Besansky *et al.*, 1995), *Ceritatis capitata* (Zweibel *et al.*, 1995), *Lucilia cuprina* (Garcia *et al.*, 1996). Batrocera tryoni (Bennett and Frommer,

1997) and *Ae. aegypti* (Coates *et al*., 1997) are members of this class. In some cases a similar phenotype may also be caused by a mutation in the ommochrome or pteridine pathways as in *An. gambiae* (Mukabayire *et al*., 1996) or *Ae. aegypti* (Bhalla, 1968), where color metabolites of only one pigment class are present. Mutations in early biochemical steps of ommochrome or pteridine biosynthesis can be especially valuable as they may well be non-autonomous at the cellular level. This means thal neither tissue-specifie transgene expression nor expression at a high level is required for complementation (Ashburner *et al*., 1998). This is the case. for example. with the rosy (xanthine dehydrogenase). *cinnabar* (kyneurenine 3hydroxylase) and *vermilion* (tryptophan oxidase) mutants of *Drosophila*. The *cinnabar* (*cn*$^{+}$) gene from *D. melonogaster* has been cloned (Warren *et al*., 1996) and has been successfully used to complement the white-eye phenotype of the *kynurenine hydroxylase*-*white* (*kh*^{w}) strain of *Ae. aegypti* (Corne1 *et al*., 1997; Coatcs *et al*., 1998; Jasinskiene *et al.*, 1998; Siriyasatien. 2000; Sethuraman and O'Brochta. 2005).

The Enhanced Green Fluorescent Protein (EGFP) gene from the jelly fish *(Aequorea victoria)* has been used as a marker in both cultured mosquito cells (Zhao and Eggleston, 1999) and in mosquitoes *Ae. aegypti* (Pinkerton *et al*., 2000) and *An. stephensi* (Catteruccia *et al*., 2000). Recently, the Ds Red gene from the coral Discosoma has been cloned (Fradkov *et al*., 2000) and introduced into transgenic *An. stephensi* mosquitoes (Catteruccia *et al*., 2003). The EGFP and the Ds Red genes appear to be stably integrated into the genome as they have been inherited over at thirty generations without detectable loss of the fluorescent marker or evidence of transposon mobilization (Catteruccia *et al*., 2003). The EGFP and Ds Red genes are now said to be the universal markers for transgenic insects. The benefit of these genes is that microinjection can be perfonned using wild type mosquitoes and it is easy to identify the transformants by examination under a fluorescence microscope (Berghammer *et a1*., 1999).

Strategies for using genetically modified mosquitoes to control mosquito borne diseases

With the microinjection technique now developed and the avability of a number of transposable elemen based vector systems, the tissue specific promoters and the universal marker genes, it is possible routinely to introduce exogenous DNA into mosquitoes. If the gene encoding for anti-pathogens can be introduced and expressed in mosquitoes as a part of a transposable element-based expression construct, it may be possible to block the development of pathogens in mosquitoes: Several studies have reported the success of creating transgenic mosquito strains which are resistant to pathogens. Expression of an SM1 or a bee venom phospholipase PLA2 in *An. stephensi* mosquitoes in a midgut specific manner strongly reduced the number of the developing rodent parasite, *Plasmodium berghei*, in the midgut of these transgenic mosquitoes (Ito *et al*., 2002; Moreira *et al*., 2002). Allen and Christensen (2004) demonstrated the expression of the GFP under the control of the flight muscle specific *act88F* gene in *Cu. quinquefasciatus mosquitoes*. The GFP was expressed specifically in the flight muscle of the transformed mosquitoes, and this is the part of the strategy to engineer genetically modified mosquitoes refractory to filarial parasite development. More recently, expression of RNA interference against the dengue virus type 2 under the control of a bloodmeal specific promoter, in the midgut of genetically modified *Ae. aegypti* mosquitoes was able to interrupt dengue virus type 2 replication in the transformed mosquitoes (Franz *et al*., 2006).

Genetically modified mosquitoes in natural populations

Once transgenic mosquitoes have been created, it is necessary to consider the problems likely to be faced in applying the technology in experimental and natural populations. There arc several issues that must be addressed.

Fitness cost

Fitness cost is defined as the relative success with which a genotype transmits its genes to the next generation (Marrelli *et al*., 2006). There are two major components of the fitness cost, survival and reproduction. which can be evaluated by analyzing several parameters, such as fecundity, fertility, larval biomass productivity, developmental rate, adult emergence, male ratio and mating competitiveness. Fitness of genetically modified mosquitoes can be reduced from the negative effect of transgene products such as fluorescent markers and antipathogen proteins or from insertional mutagenesis after a transposition event (Catteruccia *et al*., :2003; Marrelli *et al*., 2006). Catteruccia *et al*. (2003) demonstrated that the reductions of fitness of the transgenic *An. stephensi* mosquitoes is caused by the expression of an exogenous gene and the mutations from the insertion of the transgene. The fitness cost of transgenic *Ae. aegypti* mosquitoes has been examined by Irvin *et al*. (2004), the results show that fitness of transgenic mosquitoes were reduce significantly compared to not-transgenic mosquitoes The fitness of the genetically modified mosquitoes should therefore be evaluated under laborator conditions for planning release strategies.

Stability

Stability of the integrated gene is a crucial issue for application, in either the mass-rearing factory or in the field. The problems posed by these two environments differ greatly, since in the former there will be control over the genotype while in the field in will not be the case except in particular cases of the field release of sterile transformants. In the field, the release of fenile transformants may lead to potential interactions between the transgene and other genes or transposable elements that will affect stability (Asburner *et al*., 1998). The stability of transformants should therefore be tested under experimental contions before any release can be completed.

Safety

There are other concerns that will dictate the use of transgenes in mass-rearing factory conditions. Transgenes that might be considered safe under laboratory conditions will have to facc severe regulatory tests before being cleared for use in the factory or in the field. The problem of containment needs to be addressed. Transgenes requiring selection with a toxic agent or conferring pesticide resistance should not be used.

Field objective

The objectives of field release will differ among target organisms but will include eradication, population suppression and population replacement. However, there are some general principles. Firstly. the need for markers for field-release organisms, enabling the unambiguous distinction between transformants and the endemic population. Secondly, a consideration of the consequences of a successful field release of another transgenic strain of the same species. Some designs may be essentially "one-shot", for example, any release of a mobile transgene that leads to the evolution of a transposition suppression may prevent any subsequent release of a transgene carried by the same vector (Ashbumer *et al*., 1998).

For target species where population replacement is an objective there is an urgent need for research on how this may be achieved. One possible mechanism is meiotic drive. This describes any event occurring during meiosis, by which one particular chromosome is recovered preferentially over its homologue. Such a mechanism has been demonstrated in *Ae. aegypti* when linkage to the M^D locus was used to drive the marker gene, re (red eye) into a caged population (Wood *et al*., 1977). Transposable elements, the possible basis of a gene transfer system, also have potential as drive mechanisms. The efficiency of a transposon-mediated drive mechanism is such that it is theoretically possible to spread even and unfavorable traits through a population, before natural selection can act on it, in spite of the sometime deleterious consequences of transposition itself (Ribiero and Kidwell, 1994). The rapid spread of a transposable elements through a natural population has already been demonstrated by the P element. which has invaded populations of *D. melanogaster* worldwide within the past fifty years (Anxolabehere *et al*., 1988), having invaded the species from the distantly related *D. willistoni*. Another advantage of transposable elements is that some have shown themselves capable of transferring across species boundaries (Houck *et al*., 1991). However, the impact of releasing fertile transgenic mosquitoes in the gene pool of the natural population needs to be fully assessed.

Risk evaluation and public acceptance

The release of any transgenic organism into the field will be governed by local and national regulatory agencies. Any proposal to release will generate concern among both the general public and pressure groups. Both the must be addressed earlier, rather than later, if field release is the intended endpoint of technology development (Hoy, 2003).

Conclusion

Advances in our knowledge of the molecular biology of mosquito vectors and mosquito-pathogen relationships, allow us to create transgenic mosquitoes that are refractory to thc pathogens. Although the stability of these transgenic mosquitoes in a caged population can be made, the fitness of the transgenie mosquitoes is diminished compared to wild type mosquitoes. Once the problem of the fitness of transgenic mosquitoes has been overcome, thc safety and effect of releasing these transgenic mosquitoes into environment needs to be fully assessed. Before contemplating release of transgcnic mosquitoes containing active transposable elements.one must be aware of the possibility of horizontal transmission of the transgene non-target species. Public acceptance of the release of transgenic mosquitoes must also be considered.

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Analysis of Salivary Gland Proteins of the Mosquito *Armigeres subalbatus*

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Abstract: Quantitative studies of total salivary gland protein of *Armigeres subalbatus* mosquito revealed that the total salivary gland protein increased dramatically during the five days after emergence as adults. The amount of salivary gland protein of female and male mosquitos at day five after adult emergence were on the average 11.55 and 1.32 µg/pair gland respectively. SDSPAGE studies showed that salivary gland protein profiles of *Armigeres subalbatus* demonstrated 9 major polypeptide bands of 68, 65, 60, 55, 40, 30, 28, 21, and 15 kDa. The 21 and 65 kDa bands were found only in the distal lateral region of the mosquito salivary gland and were depleted after the female mosquito took a blood meal.

Introduction

Mosquito-borne diseases still remain a major health problem in both human and veterinary sectors. Diseases transmitted by mosquitos include malaria, dengue hemorrhagic fever, Japanese encephalitis, yellow fever, and filariasis. The pathogens are transmitted to a vertebrate host when the female mosquito takes a blood meal. Many pathogens take up residence in the mosquito salivary glands before being transmitted to a new vertebrate host. In addition, the mosquito saliva may enhance or facilitate infectivity (Ribeiro, 1995; Osorio *et al*, 1996; Edwards *et al*, 1998). Mosquito salivary gland extracts contain α-glucosidases and α-amylases that initiate the digestion of carbohydrates present in dietary carbohydrate sources and other enzymes and peptides involved in blood feeding and ingestion such as anticoagulants, vasodilators, and platelet aggregation inhibitors (Ribeiro and Francischetti, 2003). The saliva also contains molecules that provoke a humoral and cellular immune response in the vertebrate host (Peng *et al*, 1995; Peng and Simons, 1997; Malafront *et al*, 2003).

The proteins present in the salivary glands of several mosquito species have been investigated (Mellink and van Zenben, 1976; Poehling, 1979; Al-Ahdal *et al*, 1990; Marinotti *et al*, 1990; Andrews *et al*, 1997; Saliman *et al*, 1999; Nascimento *et al*, 2000; Moreira *et al*, 2001; Jariyapan and Harnnoi, 2002; Suwan *et al*, 2002), however, little is known of *Armigeres subalbatus*, a major vector of a heart dog filaria, *Dirofilaria immitis*. In Thailand, *Ar. subalbatus* is the most common early morning and early night biting mosquito and is found throughout the country especially in the rural areas. It feeds on both human and domestic animals (Srinivas *et al*,1994) . The study on salivary gland protein of *Ar. subalbatus* mosquito has never been reported. Therefore, in this study the salivary gland proteins of *Ar. subalbatus* were determined and analysed. Our initial finding on the salivary gland proteins showed significant reduction of some major proteins after blood feeding.

Materials and methods

Maintenance of *Ar. subalbatus* mosquitos

Ar. subalbatus mosquitos were maintained in an insectary of the Department of Medical Sciences, National Institute of Health, Bangkok, Thailand. Conditions were set at 28°C ± 1°C at 80% ± 5% relative humidity under 12/12 hours light/dark photo-period. Adults were supplied with a damp cotton wool pad contained 10% sucrose solution as a carbohydrate source. For blood feeding, female mosquitos were allowed to feed on anesthetized mice for 30 minutes. Groups of mosquitos were reared simultaneously from the same cohort of eggs. Adult mosquitos aged 1 to 5 days after emergence were used.

Salivary gland dissection

Mosquitos were anesthetized on ice and salivary gland dissection was performed as described by Suwan *et al* (2002). Mosquito salivary glands were transferred to a microcentrifuge tube containing a small volume of PBS (phosphate buffer saline solution) and kept at -70°C until used.

Protein quantification

Amount of total mosquito salivary gland protein was determined using a Bio-Rad Protein Assay (Bio-Rad) following the manufacturer's instruction. Two pairs of female or 10 pairs of male *Ar. subalbatus* salivary glands at day 1, 3, and 5 after emergence were used in this study. Each determination was repeated 3 times.

SDS-PAGE analysis and protein staining

SDS-PAGE was performed according to Laemmli (1970) and the proteins were silver stained using a Silver Stain kit (Bio-Rad) according to the manufacturer's instruction.

Results and Discussion

The salivary glands of adult *Ar. subalbatus* are paired organs, located in the thorax. The female salivary glands display difference in structure when compared to the male ones. The female gland is composed of two identical lateral lobes and a shorter and wider median lobe (Figure 1). The lateral lobes can be divided into two regions, proximal and distal. The male gland consists of three morphologically homogenous lobes and is approximately one-fifth size of the female (Figure 1). Morphological pattern of *Ar. subalbatus* adult salivary glands followed the same pattern as described for *Ae. aegypti* (Mellink *et al*, 1976; Poehling, 1979), *Ae. albopictus* (Marinotti *et al*, 1996), *Ae togoi* (Jariyapan and Harnnoi, 2002), *Cx. pipiens* and *Ae. caspius* (Saliman *et al*, 1999). Total protein contents of male and female mosquito salivary glands were determined. Table 1 demonstrates the average amount of total salivary gland protein content in male and female mosquitos at various times after emergence. Total salivary gland protein content of male mosquito on day 1 after emergence was 0.44 ± 0.10 µg/gland pair and increased to 1.32 ± 0.14 µg/ gland pair at day 5. In females, total salivary gland protein content of mosquito at day 1 after emergence was 1.85 ± 0.53 µg/ gland pair and increased dramatically to 11.55 ± 1.71 µg/gland pair at day 5. Comparison of protein content between male and female mosquitos at day 5 showed that a pair of male glands contained approximately 10% of that found in female ones. Similar results were found in *Cx. pipiens* (Saliman *et al*,1999) and *Ae. togoi* (Jariyapan and Harnnoi, 2002).

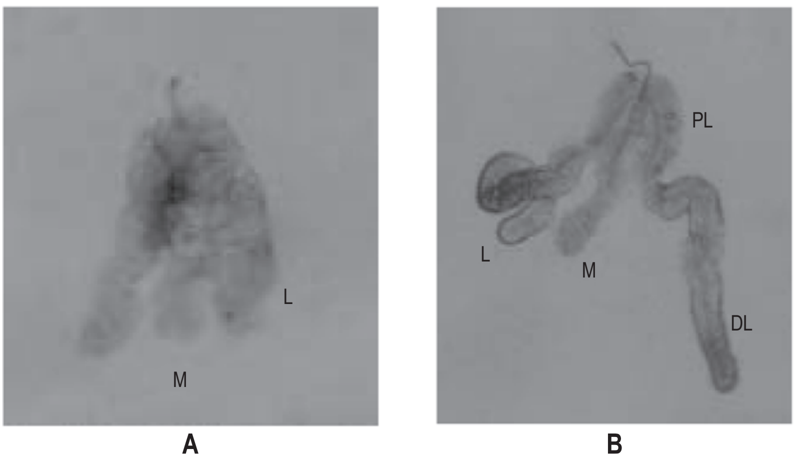

Figure 1 Salivary glands of *Armigeres subalbatus* mosquito. A: a male salivary gland; B: female salivary gland, M: median lobe; L: lateral lobe; PL: proximal region of lateral lobe; DL: distal region of lateral lobe. The photographs were taken using an Olympus (AH) microscope at 100x magnification.

Table1 Total salivary gland protein contents of male and female *Armigeres subalbatus* mosquitos after the adult emergence.

	Post emergence (day)					
	Male			Female		
	1	3	5	1	3	5
Protein content (μg/gland pair)[A]	0.44 ± 0.10	0.49 ± 0.14	1.32 ± 0.14	1.85 ± 0.53	6.53 ± 0.78	11.55 ± 1.71

A = mean ± SD; n = 10

SDS-PAGE analysis of the proteins present in the salivary glands of male and female mosquitos at day 5 of emergence was performed. The analysis of both female and male salivary gland proteins revealed the presence of at least 9 major and several minor protein bands (Figure 2). Protein bands with estimated molecular masses of 68, 60, 55, 40, 30, 28 and 15 kDa were found in salivary glands of both sexes whereas the 65 and 21 kDa protein bands were observed only in females.

The different morphological regions of the female salivary glands displayed distinct protein profiles (Figure 3). The 65 and 21 kDa proteins appeared predominantly in the distal-lateral lobe. The protein profile of the female proximal-lateral lobe was similar to that of the male salivary gland. The protein profiles at 0, 6, 24, and 48 hours after a blood meal was also analyzed (Figure 4). Immediately after blood feeding, the 65 and 21 kDa protein bands were barely detected, but both proteins started to appear gradually 6 hours later and returned to the unfed level in 48 hours. Further investigations in molecular, biochemical, and immunological aspects of *Ar. subalbatus* salivary glands will provide information for better understanding of the role of mosquito salivary gland proteins in blood feeding and disease transmission.

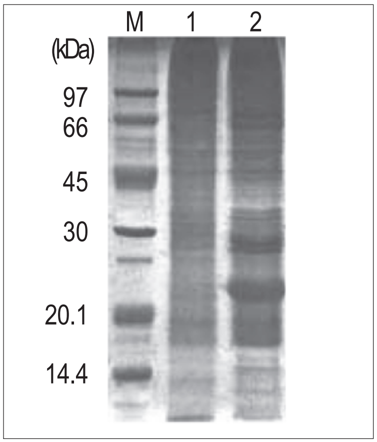

Figure 2 Protein electrophoresis pattern of salivary glands of adult male and female *Armigeres subalbatus* at day 5 after adult emergence. Ten pairs of sugar fed female salivary glands (lane 2) and 15 pairs of male salivary glands (lane 1) were submitted to SDS-PAGE in a 12% polyacrylamide gel followed by silver staining. Molecular mass markers (M) are in kilodalton (kDa).

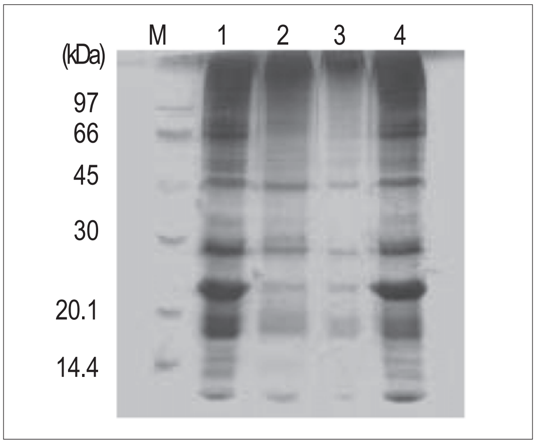

Figure 3 Electrophoretic profile of polypeptides from female *Armigeres subalbatus* mosquitos salivary gland lobes. Proteins were separated on a 12% SDS-PAGE gel and silver stained. Lane 1,ten whole female salivary glands; lane 2, twenty proximal lateral lobes; lane 3, ten median lobes; lane 4, twenty distal lateral lobes. M: molecular weights markers of sizes (kDa) indicated on the left side of the picture.

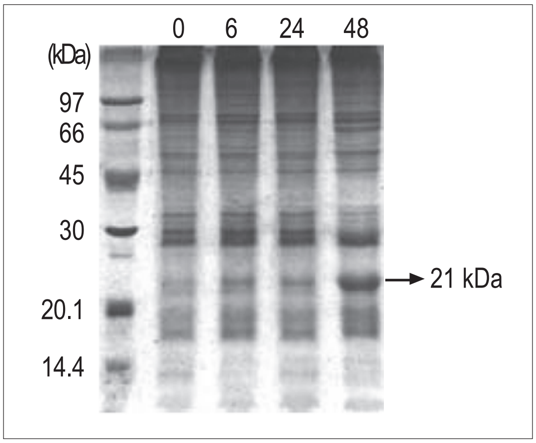

Figure 4 Protein electrophoretic profile of salivary glands of blood fed *Armigeres subalbatus* mosquitos. Ten pairs of salivary glands were dissected from blood fed mosquitos at 0, 6, 24 and 48 hours after a blood meal and submitted on 12% SDS-PAGE and silver stained. M: molecular weight markers of sizes (kDa) indicated on the left side of the picture. Number at the top indicate hours after a blood meal.

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Decrease of Mosquito Salivary Gland Proteins after a Blood Meal: An Implication for Pathogenesis of Mosquito Bite Allergy

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Abstract: Salivary gland protein profiles of *Aedes aegypti* (L) and *Culex quinquefasciatus* (Say) pre- and post-blood feeding were analyzed. SDS-PAGE studies before blood feeding of *Ae. aegypti* demonstrated 8 major polypeptide bands of 20, 35, 37, 42, 45, 47, 70 kDa and a high molecular weight band > 118 kDa, whereas those of *Cx. quinquefasciatus* demonstrated 9 major polypeptide bands of 20, 26, 36, 38, 45, 47, 49 kDa and 2 high molecular weight bands > 118 kDa. After a blood feeding, salivary gland polypeptides of *Ae. aegypti* at 35,37,45,47, 70 kDa and high molecular weight band > 118 kDa were depleted, while the polypeptide bands of 20, 26, 36, 38 kDa were depleted in *Cx. quinquefasciatus*. The presented study suggests that these major polypeptides were introduced into vertebrate hosts when a mosquito took a blood meal, Further investigation in molecular, biochemical and immunological aspects of these salivary gland polypeptides may provide information for better understanding in the role of these proteins in mosquito bite allergy.

Introduction

Dennal allergy to mosquito bites is a common problem worldwide. Although in most cases of mosquito bites elicit mild symptoms such as cutaneous reactions, systemic reactions including generalized urticaria and angioedema, rhinitis, conjunctivitis, asthma have been documented[l,3]. Anaphylactic shock following mosquito bites also has been reported[4]. These reactions are caused by proteins in the mosquito saliva and involved in IgE, IgG_1 and IgG4 responses and lymphocyte proliferation[5,6].

Mosquito saliva contains α-glucosidases and α-amylases that initiate the digestion of carbohydrates present in dietary carbohydrate sources and other enzymes and peptides involved in blood feeding and ingestion such as anticoagulants, vasodilators, and platele taggregation inhibitors[7,8] The saliva also contains molecules that provoke a humoral and cellular immune response in the vertebrate host[9,11]. Although salivary glands of several mosquito species have been investigated[7,12-20], changes of salivary gland protein post blood feeding using SDS-PAGE was demonstrated only in *Armigeres* (Ar.) *subalbatus* (Coquillett) mosquito[20].

In Thailand, *Aedes* (Ae.) *aegypti* (L.) and *Culex* (Cx.) *quinquefasciatus* (Say) mosquitoes are the most important mosquito species distributed throughout the country. *Ae. aegypti* is the most important endophagic, daylight-bite mosquito and plays a major role of dengue virus transmission. *Cx. quinquefasciatus* is exophagic, night-bite mosquito found mainly in urbanized areas. Mosquito bite allergy is a common problem found in clinical practice especially in children. Despite this, only a few reports in which modem laboratory techniques. have been applied to the study of mosquito allergy in Thailand[21]. In the present study the authors would like to determine the major polypeptides which were related to blood feeding of *Ae. aegypti* and *Cx. quinquefasciatus* by SDS-PAGE. This would provide crucial information for further investigation in mosquito bite allergy.

Materials and methods

Mosquito rearing

Ae. aegypti and *Cx. quinquefasciatus* mosquitoes were raised in an insectary at the Experimental Animal Unit, Faculty of Medicine, Chulalongkorn University. Briefly, after the emergence as adults, the mosquitoes were reared in insectariums at 28°C ± 1°C, 80% ± 5% relative humidity under 12/12 hours light/dark photo-period. Adults were supplied with a damp cotton wool pad which contained 10% sucrose solution as a carbohydrate source until used.

Mosquito blood feeding

Female mosquitoes were allowed to feed on anaesthetized mice for 30 minutes. Groups of mosquitoes were reared simultaneously from the same cohort of eggs. Adult mosquitoes aged 4 to 5 days after emergence were used.

Mosquito salivary gland extraction

Mosquito salivary gland extracts were prepared from 5 days old female mosquitoes. Mosquitoes were anaesthetized on ice and salivary gland dissection was performed as in the method described by Suwan *et al*. (2002)[18]. Mosquito salivary glands were then transferred to a microcentrifuge tube containing a small volume of PBS (phosphate buffer saline solution) and kept at -70°C until used.

SDS-PAGE Analysis

SDS-PAGE was performed according to Laemmli (1970)[22] and the proteins were stained using a Coomassie Brilliant Blue (PhastGel™ Blue®)

according to the manufacturer's instruction. Twenty pairs of mosquito salivary glands were used for each sample. and each experiment was repeated three times.

Results

Morphology of mosquito salivary glands

The salivary glands of female *Ae. aegypri* and *Cx. quinquefasciatus* are paired organs, located in the thorax. The gland is composed of two identical lateral lobes and a shorter and wider median lobe. The lateral lobes could be further divided into two regions, proximal and distal. Salivary glands of these two mosquito species are undistinguishable morphologically (data not shown).

SDS-PAGE Analysis

SDS-PAGE analysisof salivarygland proteins of female *Ae. aegypti* mosquito pre-blood feeding demonstrated 8 major polypeptide bands of 20, 35, 37, 42,45,47, 70 kDa and a high molecular weight band > 118 kDa. After a blood meal, the depletion of major peptide bands of 35, 37, 45, 47, 70 kDa and high molecular weight band > 118 kDa was observed (Figure 1).

Study in *Cx. quinquefasciatus* found 9 major polypeptide bands of 20, 25, 36, 38, 45, 47, 49 kDa and 2 high molecular weight hands > 118 kDa, the polypeptide bands of 20, 26, 36 and 38 kDs were depleted after a blood feeding (Figure 2).

Discussion

Morphology of *Ae. aegypti* and *Cx. quinquefasciatus* from the present study is similar to the pattern described for *Ae. aegypti*[13,14], *Ae. albopictus* (Skuse)[23], *Ae. togoi* (Theobald)[19], *Cx. pipiens* (L), *Ae. caspius* (PallasY1S),and *Ar. subalbatus*[20].The female gland is composed of two identical lateral lobes and

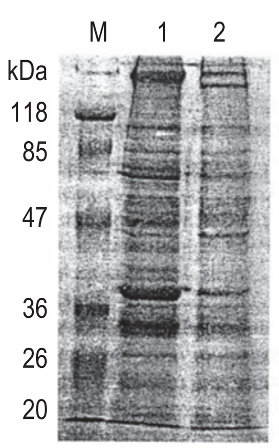

Figure 1 Protein electrophoretic profile of salivary glands of *Aedes aegypti* mosquitoes. Proteins were separated on a 12% SDS-PAGEgel and Commasse Brilliant Blue stained. Lane I, twenty pairs of salivary glands of female mosquitoes at day 5 after emergence (sugar feeding); Lane 2, twenty pairs of salivary glands of female mosquitoes dissected immediatelyaftera bloodmeal; M: Molecular weights markers of sizes (kDa) indicatedon the left side of the picture

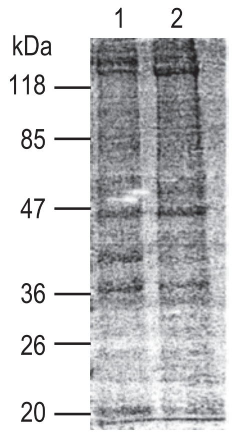

Figure 2 Protein electrophoretic profile of salivary glands of *Culex quinquefasuJtus* mosquitoes. Proteins were separated on a 12% SDSPAGEgel and CommasseBrilliantBlue stained. Lane 1, twenty pairs of salivary glands of female mosquitoes at day 5 after emergence (sugar feeding); Lane 2, twenty pairs of salivary glands of female mosquitoes dissected immediately after a blood meal; Molecular weights markers of sizes (kDa) indicated on the left side of the picture

a shorter and wider median lobe. The lateral lobes could be further divided into two regions, proximal and distal.

The salivary gland protein profile of *Ae. aegypti* and *Cx. quinquefasciatus* mosquito showed a different pattern. The different protein. profiles are found not only in different species but also in the same mosquito species. Study by Moreria *et a1*. demonstrated that *Anopheles darlingi* (Root) mosquito collected from different geographical regions of Brazil showed some differences in pattern of salivary gland protein profile(17). In the present study the authors demonstrated the salivary gland protein profile of *Ae. aegypti* and *Cx. quinquefasciatus* which originally were collected from Bangkok and maintained at the insectary of the National Institute of Health, Department of Medical Sciences, Nonthaburi, Thailand.

Decreasing of major peptide bands of 35, 37, 45, 47, 70 kDa and a high molecular weight band > 118 kDa in *Ae. aegypti* and 20, 26, 36 and 38 kDa in *Cx. quinquefasciatus* indicate that these polypeptide proteins were released to vertebrate hosts while female mosquitoes took a blood meal. Therefore, these salivary gland proteins may cause mosquito bite allergy in human. Hudson *et al*. (1960) demonstrated that mosquito saliva was a source of antigens which produced typical bite reaction in man(24) and Peng *et al*. (1996) showed that recombinant 37 kDa protein in *Ae. aegypti* was shared by all five *Aedes* species and also *Cx. quinquefasciatus* mosquito(5). In the present study the authors also found the 37 kDa salivary gland protein in *Aedes aegypti* mosquito and this protein was depleted after blood feeding.

Nasciomento *et al*. (2000) and Malafronte *et al*. (2003) demonstrated that salivary gland proteins of *Cx. quinquefasciatus* mosquito had 2 major polypeptide bands of 28.3 and 35.7 kDa, which induced immune response in mice(11,16). In the present study the authors also showed 36 kDa polypeptide band that related to blood feeding. But were unable to demonstrate depletion of the 28.3 kDa polypeptide protein in the present study.

At present, laboratory diagnosis of mosquito bite allergy using the commercial mosquito extracts prepared from whole mosquitoes are not standardized for diagnosis of mosquito allergy(25). In order to improve the precision of diagnosis of mosquito allergy, purified mosquito saliva should be developed. The present study provides data of salivary gland proteins, which related to blood feeding. Therefore, these proteins may be related to mosquito bite allergy. Further study of these purified or recombinant salivary gland proteins would help physicians to diagnose mosquito bite. allergy more accurately.

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Sequential Release and Residual Activity of Temephos Applied as Sand Granules to Water-Storage Jars for the Control of *Aedes aegypti* Larvae (Diptera: Culicidae)

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Abstract: Two long-term experiments were carried out on the release profile and efficacy of temephos 1% GR (sand granules) against *Aedes aegypti* larvae in water-storage containers. In the first experiment, the efficacy of temephos 1% GR enclosed and tied in a muslin cloth and placed in water at the bottom of 200 L earthen water-storage jars was studied by exposing the packets for four to nine wk in one set of jars and then transferring them sequentially to new sets of jars four times successively. Temephos released slowly from the granules, the magnitude of release being adequate in the initial period of two to three wk after treatment. Following this period, the efficacy of the granules increased substantially where 92 – 100% inhibition of emergence even at the lowest dosage of 1g/100 L (0.05 mg/L AI) was obtained for about another five mo or longer. On removal of the packets from a given set of jars, the released residues remaining in the jars and water lasted a maximum of one to six wk post-removal depending on the magnitude of prior release into the jars. This experiment provided clear evidence that temephos is released slowly over a long period of time in water-storage jars. In the second experiment, we compared the efficacy of temephos 1% GR at 1 and 10 g (0.05 and 0.5 mg/L AI) per 200-L water in jars painted and unpainted on the inside. The efficacy in the painted jars, although high, was consistently lower than that in the unpainted jars, where 99 - 100 % control of larvae was achieved at both rates for a minimum of five mo after treatment. On the basis of this experimental evidence, it is desirable to study the efficacy of lower dosages of temephos than those currently used in *Ae. aegypti* control programs. The use of controlled release formulations or sachets that are retrievable during cleaning and washing will be more practical and desirable. Both of these interventions will make the program more cost effective.

Introduction

Temephos 1% GR (sand granules) has been used for the control of *Aedes aegypti* (L.) larvae for many years in Thailand and elsewhere (Bang *et al*. 1972, Carvalho and da Silva 2000, Weber *et al*. 1987). The efficacy of this formulation was studied in detail in Thailand in the late 1960s and early 1970s (Bang and Tonn 1969ab, Bang *et al.* 1972). On the basis of their findings, temephos sand granules (1%) were recommended for use in *Ae. aegypti* control programs and are currently used at the standard dosage of 10 g/100 L water (1 mg/L AI). Recently, Mulla *et al*. (2004) studied the efficacy of 1% sand granules and a new granular formulation of temephos (1% Zeolite) at the dosage of 10 g/100 L each in 200-L capacity earthen water-storage jars painted on the inside for ease of counting live larvae, pupae, and pupal skins. Both formulations yielded close to 100% control (inhibition of emergence) for over six mo under three different water-use conditions. Even though temephos 1% GR has been used for over 30 years in Thailand, there is no published information on the release pattern of temephos from granules applied to water-storage jars. Although temephos has low solubility in water (0.03 mg/L at 25°C), it is not clear whether temephos release occurs in a short time or is it released over a long period of time from granular formulations under field conditions. This question is often posed and still remains unanswered. To shed light on the efficacy and continuous release of temephos over time, we carried out the present study where temephos release, as reflected in the level of larval control and adult emergence over time, was determined by sequential transfers of temephos granules in packets from one set of jars to another. Efficacy was determined in each sequence both in the presence of temephos granule packets in a given set of jars as well as after removal and transfer of the packets to the succeeding sets of jars. A second experiment was carried out to compare efficacy of temephos sand granules (1%) added to water in painted and unpainted water-storage jars. In our previous study (Mulla *et al*. 2004), we tested two temephos granular formulations in painted jars only, using three water-use regimens. At that time, we were not able to determine the effect of the alkyd resin paint used on the release and efficacy of temephos as compared to unpainted jars. In order to compare the release of temephos and efficacy of sand granules, we did a comparative study of painted versus unpainted jars into which sand granules were sprinkled loosely where they sank to the bottom.

MATERIALS AND METHODS

Two experiments were carried out in the current study. In the first experiment, sequential release of temephos from 1% sand granules contained in a packet and applied to water in earthen water-storage jars was determined. In the second experiment, activity and long-term efficacy of temephos GR applied loosely (not in packets) to the jars was compared in earthen water-storage jars painted and unpainted on the inside. Since we got an unexpectedly low release of temephos from granules contained in packets during the first two mo after application, we ran a third experiment using the same amounts of GR in packets to re-evaluate the release pattern over 56 d period post-application. Packets in this experiment remained in the initially treated jars without transfer to other sets. Experimental set-up and design and evaluation procedures developed in our previous studies (Mulla *et al*. 2004) were closely followed.

Sequential release

Earthen jars (200-L capacity) were filled with domestic water and set for a wk before treatment. The depth of water in the jars was 62 cm. The jars were treated with packets of 1, 5, and 10 g temephos 1% GR (Larvifos 15/30 mesh, lot 5B030822-2, Ikari Trading Co. Thailand) on August 27, 2003. The treatments were challenged weekly by adding cohorts of 25 third-instar laboratory-reared larvae of *Ae. aegypti*. Prior to the addition of larvae, 0.5 g larval food (ground-up mouse pellets) was added to each jar. Additional food was given on a monthly basis. Water level in the jars was replenished monthly. The jars were covered with sheets of celocrete protecting the jars from intrusion of extraneous material and penetration by UV light. The jars were set on a concrete slab under a roof, but the area was completely open on all sides. Water temperature was monitored throughout the test by submerging a minimum-maximum thermometer in one of the jars. To determine sequential release and longevity of temephos from the sand granules, quantities of 1, 5, and 10g per jar to yield three concentrations (0.05, 0.25, and 0.50 mg/L AI) were confined in muslin cloth and the pack of granules was tied with cotton string and lowered to the bottom of the jars. A small pebble was enclosed in the packets to facilitate sinking of the granule packets to the bottom of the jars. Sand granules without temephos were placed in control jars. A long string of cotton was attached to the knotted end of the packets to facilitate lowering and lifting the packets during treatment and transfer to a new set of jars. The treatments and control were replicated four times. In total, 80 identical jars were used in this experiment.

For assessing the activity and longevity of the treatments, 25 third-instar laboratory-reared larvae of *Ae. aegypti* were introduced into each jar, usually on weekly basis. Assessment of inhibition of emergence (IE %) was carried out by counting (five-seven d post-addition of larvae) pupal skins floating on the surface of water. Due to the high temperature of water in the jars, third-instar larvae completed development to adult emergence in five d. Pupal skins were removed by syringes to white plastic trays with water, where the skins were readily visible and counted. The pupal skins were removed in totality for each cohort before placement of larvae of the next cohort. The inhibition of emergence (IE %) was calculated by comparing the number of pupal skins to the original number of larvae added. Correction for control mortality was not made as the mortality in the controls was usually 10% or less while that in most of the treatments and at most intervals was 100%. The data are presented in tables and, to reduce the tabular data, we have omitted some cohorts where the results of the omitted cohorts were identical to either the immediately preceding or succeeding cohorts or both. This omission does not impair the strength of the data presented and the trends noted. Two types of assessment of the efficacy of temephos were made in this experiment. The first assessment involved determining the efficacy of temephos 1% GR when the granules packets were in the jars. This was carried out for 28-67 d in jars with granule packets present in the jars in the 5 sequences. The packets were transferred (four transfers) from one set of jars to another, but the efficacy of residual temephos was also determined in jars without the packets for three to seven wk in each sequence. This second type of assessment was made on the longevity of released and absorbed or adsorbed temephos in the jars after removal of the packets. This assessment will provide valuable information following removal of the source of applied temephos granules due to washing or cleaning of the jars.

Longevity in painted and unpainted jars

A total of 24 glazed-clay jars (200 L capacity) was used in this experiment of which 12 jars were painted with alkyd resin paint (Glipton G100, synthetic resin high gloss enamel, TOA Paint Co., Bangkok) as in our previous study (Mulla *et al*. 2004) and 12 jars were left unpainted. The jars were arranged on a concrete slab under a roof. The jars were covered with celocrete sheets, preventing extraneous materials and UV light from entering the jars. Water temperature was measured throughout the test by submerging a minimum maximum thermometer in one of the jars. Temephos 1% GR at two rates (1 and 10 g/jar (0.05 and 0.5 mg/L AI) were sprinkled loosely on the top of water in each treated jar where the granules sank to the bottom. The same granules (Larvifos) as used in the previous experiment were used here. Control jars (both painted and unpainted) were treated with blank sand granules. Each treatment was replicated four times.

After filling the jars from a domestic water supply, they were set for a week before treatment. At the outset, each jar was provided with 0.5 g powdered larval food. Prior to treatment, each jar was stocked with 25 third-instar laboratory reared larvae of *Ae. aegypti*. Subsequent larval cohorts were added at weekly intervals. Assessment of activity was made by counting pupal skins (indication of successful emergence) five to seven d post-addition of larvae. Due to the high temperature of water, third-instars were able to complete development to emergence of adults in five d. The overall efficacy and longevity of temephos were determined by calculating the inhibition of emergence (IE %) on the basis of pupal skins in treatments versus the number of larvae added. Mortality in controls was not considered in these calculations, as the control mortality was usually 10% or less while in the treatments it was generally 95-100%. In total, 21 cohorts of larvae were used over a period of five mo after treatment. For reducing the tabular data, information on some cohorts is not presented where the deleted results were similar to the immediately preceding or succeeding cohorts or both.

Re-evaluation of temephos GR in packets

To determine the release of temephos 1% GR enclosed in packets, a third experiment was run for 56 d, using 1, 5, and 10 g packets per jar, where the packets remained in the treated jars over the entire period. The experiment was run for 48 d. Procedures of assessment were the same as in the previous two experiments.

Results and Discussion

Temephos, an organophosphate larvicide, has been used for the control of *Ae. aegypti* in Thailand for over 30 y, since it was first evaluated in water-storage containers (Bang and Tonn 1969ab, Bang *et al*. 1972). The current dosage used in the *Ae. aegypti* control program in Thailand is 10 g of 1% sand granules per 100 L water in water-storage containers. It is not clear whether temephos releases quickly in water-storage containers and is absorbed or adsorbed to particles and sides of the containers and then released back slowly or is it released slowly over time. To answer this crucial question, the current study was carried out.

Sequential release

In this study we wanted to provide evidence for the release pattern of temephos from sand granules and its longevity after release into the water jars, by sequential transfers of the same packets of temephos granules to new sets of jars four times over a period of about eight mo. Efficacy of granule packets transferred sequentially and the longevity and residual activity of released temephos into the jars after removal of packets were studied over time. Minimum-maximum water temperatures during the test period were 28-30°C and 31-34°C, respectively.

In the first sequence, the granule packets were left in the first set of jars for 28 d, where temephos release was moderate, yielding low to moderate level of efficacy (IE %) at the two lower dosages (Table 1). The highest level of control was 88% at the 10 g/jar dosage. As a whole, during the initial period of two mo, the magnitude of release, although adequate, was not high as compared to the release later on in the experiment as reflected in almost complete control of emergence. This low level of initial release during the first two mo is in contrast to the high level of release (giving IE 100% within a wk or two of application of loose granules) as found in the second experiment here (see Table 6) and in our previous studies (Mulla *et al*. 2004). The low level of initial release in the present experiment could be due to uncontrolled experimental conditions. We therefore ran another experiment using the same three dosages of granules enclosed in packets and placed continuously in the jars for 56 d. The release of temephos was assessed in the same manner as in the previous experiment. In this experiment, the release was moderate for 19 d post-treatment, but the treatment gave 89 to 100% IE at the two high dosages during the 26 to 56 d post-treatment (tabular data omitted). This clearly indicates the low level of release in the previous experiment was due to experimental error. The results of the second experiment are in agreement with those in the following experiment and those of Mulla *et al*. (2004).

Since the release of temephos was not high in the first sequence jars of the first experiment, the residual activity of released temephos was also not adequate and only short-lived. The maximum IE% was 70% at the highest dosage for one wk (data not shown) after removal of the packets, declining precipitously during the second and third wk (Table 1).

After transfer of the granule packets into sequence II jars, moderate to high level of control (IE 86-97%) at the highest dosage was achieved for 28 d of observations post-transfer of packets into these jars (Table 2). However, on and after 43d post-transfer into sequence II jars, the extent of release and control increased markedly in all three dosages (IE 90- 100%) on day 43 and 50 post-transfer into sequence II jars (Table 2), where the lowest dosage (1 g/jar) yielded IE 92-99%. On removal of granule packets from these jars, the residual activity of released temephos was higher than before in the jars (IE 95-100%) at all three dosages for one wk only (Table 2). The activity of temephos, however, was very low (IE 12-43%) during the next two wk of evaluation. These results show that adequate release in sequence II jars occurred six wk after placement of the packets in the first sequence jars.

After transfer of granule packets to sequence III jars, the magnitude of release and efficacy (IE 96-100%) in these jars was studied for 53 d, where the IE% ranged from 91-100% in the presence of granule packets during the whole period (Table 3). After removal of packets (after 53 d) from sequence III jars, the residual activity of released temephos remained very high (IE 92-100%) at the two higher dosages but lower (75%) at the lowest rate for a period of 39 d post-removal and IE 88-95% at the two high dosages and 66% at the lowest dosage, 46 d post-removal (Table 3). We did not test for residual activity after this period, but it appears from the low as well as the two higher dosages that activity was declining.

The transfer of granule packets from sequence III to sequence IV jars still exhibited a very high level of quick release and activity of temephos after the transfer (Table 4). The IE% ranged from 97 to 100% in the three dosages for a period of 38 d or longer, when the packets were removed and transferred to sequence V jars (Table 4). After removal of granule packets from sequence IV

Table 1 Sequential release and residual activity of temephos when temephos sand granules (1%) were applied as packets to water in 200 L earthen jars and challenged with cohorts of 3rd instar larvae of *Ae. aegypti*. Bang Bua Thong, Nonthaburi, Thailand. Sequence I treated Aug 27, 2003.

Sequence I jars	Granules g/jar	mg/L AI	Pupal skins	EI* (%)
		1st cohort assessed 7 d post-treatment (Sept 3, 2003)		
1A	1	0.05	4.75	81
1B	5	0.25	5.00	80
1D	10	0.50	5.50	78
1C	0	0.00	24.00	4
		3rd cohort assessed 21 d post-treatment (Sept 17, 2003)		
1A	1	0.05	7.00	72
1B	5	0.25	7.00	72
1D	10	0.50	3.00	88
1C	0	0.00	22.75	9
		4th cohort assessed 28 d post-treatment (Sept 24, 2003)**		
1A	1	0.05	16.50	34
1B	5	0.25	16.75	33
1D	10	0.50	5.50	78
1C	0	0.00	22.50	10
		2nd cohort assessed 14 d post 1st removal (Oct 8, 2003)		
1A	1	0.05	22.50	10
1B	5	0.25	22.00	12
1D	10	0.50	14.75	41
1C	0	0.00	23.25	7
		3rd cohort assessed 21 d post 1st removal (Oct 15, 2003)		
1A	1	0.05	21.00	16
1B	5	0.25	20.75	17
1D	10	0.50	18.00	28
1C	0	0.00	23.50	6

* Data for cohort 2 with packets and cohort 1 without packets omitted. For explanation see text.

** Packets of temephos granules removed from sequence I (A-D jars) and placed in sequence II (2A-2D jars) on Sept 24, 2003 when the pupal skin count was taken. Both A-D (without packets) and 2A-2D (with transferred packets see Table 2) were evaluated.

Table 2 Sequential release and residual activity of temephos when temephos sand granules (1%) were applied as packets to water in 200 L earthen water storage jars and challenged with cohorts of 3rd instar larvae of *Ae. aegypti.* Bang Bua Thong, Nonthaburi, Thailand. Second sequence. (Sequence I treated Aug 27, 2003).

Sequence II jars*	Granules g/jar	mg/L AI	Pupal skins	EI** (%)
		1st cohort assessed 7 d post 1st transfer (Oct 1, 2003)		
2A	1	0.05	14.00	44
2B	5	0.25	13.50	46
2D	10	0.50	3.50	86
2C	0	0.00	23.50	6
		4th cohort assessed 28 d post 1st transfer (Oct 23, 2003)		
2A	1	0.05	11.50	54
2B	5	0.25	5.50	78
2D	10	0.50	0.75	97
2C	0	0.00	22.50	10
		5th cohort assessed 43 d post 1st transfer (Nov 5, 2003)		
2A	1	0.05	0.25	99
2B	5	0.25	0.25	99
2D	10	0.50	1.00	96
2C	0	0.00	22.50	10
		6th cohort assessed 50 d post 1st transfer (Nov. 12, 2003) ***		
2A	1	0.05	2.00	92
2B	5	0.25	0.00	100
2D	10	0.50	2.50	90
2C	0	0.00	23.00	8
		1st cohort assessed 7 d post 2nd removal (Nov 19, 2003)		
2A	1	0.05	1.25	95
2B	5	0.25	0.00	100
2D	10	0.50	0.75	97
2C	0	0.00	22.50	10
		3rd cohort assess 28 d post 2nd removal (Dec 8, 2003)		
2A	1	0.05	22.00	12
2B	5	0.25	18.75	21
2D	10	0.50	15.75	35
2C	0	0.00	22.50	10

* Packets of granules from sequence I (A-D jars) transferred to sequence II (2A-2D jars) on Sept 24, 2003.

** Data for cohort 2 and 3 before and cohort 2 after removal of packets omitted. For explanation see text.

*** Packets of granules from Sequence II (2A-2D jars) transferred to Sequence III (3A-3D jars) on Nov 12, 2003. Both sequence II jars (Table 2) and sequence III jars (Table 3) were evaluated.

Table 3 Sequential release and residual activity of temephos sand granules (1%) when applied as packets in 200 L earthen water storage jars and challenged with cohorts of 3rd instar larvae of *Ae. aegypti.* Bang Bua Thong, Nonthaburi, Thailand. Third sequence (1st sequence treated Aug 27, 2003).

Sequence III jars*	**Granules g/jar**	**mg/L AI**	**Pupal skins**	**EI** (%)**
		1st cohort assessed 7 d post 2nd transfer (Nov 19, 2003)		
3A	1	0.05	0.00	100
3B	5	0.25	0.00	100
3D	10	0.50	1.00	96
3C	0	0.00	23.25	7
		3rd cohort assessed 26 d post 2nd transfer (Dec 8, 2003)		
3A	1	0.05	3.00	88
3B	5	0.25	0.00	100
3D	10	0.50	2.25	91
3C	0	0.00	24.00	4
		6th cohort assess 53 d post 2nd transfer (Jan 5, 2004)		
3A	1	0.05	0.25	99
3B	5	0.25	0.00	100
3D	10	0.50	0.00	100
3C	0	0.00	23.50	6
		1st cohort assessed 12 d post 3rd removal (Jan 12, 2004) ***		
3A	1	0.05	0.75	97
3B	5	0.25	0.00	100
3D	10	0.50	0.00	100
3C	0	0.00	23.00	8
		5th cohort assessed 39 d post 3rd removal (Feb 9, 2004)		
3A	1	0.05	5.75	77
3B	5	0.25	0.00	100
3D	10	0.50	2.00	92
3C	0	0.00	23.75	5
		6th cohort assessed 46 d post 3rd removal (Feb 16, 2004)		
3A	1	0.05	8.50	66
3B	5	0.25	1.25	95
3D	10	0.50	4.50	82
3C	0	0.00	24.00	4

* Packets from sequence II (2A-2D jars) transferred to sequence III (3A-3D jars) on November 12, 2003.

** Data for cohort 2, 4 and 5 before removal and cohorts 2, 3 and 4 post-removal are omitted. For explanation see text.

*** Packet transferred to sequence IV (4A-4D jars) on Dec 31, 2003.

jars, the residual activity of released temephos remained very high (IE 94-100%) at all three dosages for up to two w post-removal (Table 4). However, IE% declined precipitously to 62% and 49% after three and four wk (data not shown) at the low dosage, but remained high at the other two dosages. Likewise, the activity declined to IE 73% and 94% at the 5 and 10 g/L dosages 35 d post-removal

Table 4 Sequential release and residual activity of temephos granules (1%) when applied as packets in 200 L capacity earthen jars and challenged with cohorts of 3rd instar larvae of *Ae. agypti*. Bang Bua Thong, Nonthaburi, Thailand. Fourth sequence (1st sequence treated Aug 27, 2003).

Sequence IV jars*	Granules g/jar	mg/L AI	Pupal skins	EI** (%)
		1st cohort assessed 5 d post 3rd transfer (Jan 5, 2004)		
4A	1	0.05	0.50	98
4B	5	0.25	0.50	98
4D	10	0.50	0.75	97
4C	0	0.00	23.00	8
		6th cohort assessed 38 d post 3rd transfer (Feb 9, 2004)		
4A	1	0.05	0.25	99
4B	5	0.25	0.00	100
4D	10	0.50	0.00	100
4C	0	0.00	24.00	4
		1st cohort assessed 7 d post 4th removal (Mar 10, 2004)***		
4A	1	0.05	0.50	98
4B	5	0.25	0.00	100
4D	10	0.50	0.00	100
4C	0	0.00	24.00	4
		3rd cohort assessed 14 d post 4th removal (March 22, 2004)		
4A	1	0.05	1.50	94
4B	5	0.25	0.25	99
4D	10	0.50	0.25	99
4C	0	0.00	24.75	1
		6th cohort assessed 35 d post 4th removal (April 12, 2004)		
4A	1	0.05	14.00	44
4B	5	0.25	6.75	73
4D	10	0.50	1.50	94
4C	0	0.00	23.00	8

* Packets transferred to sequence IV on December 31, 2003.

** Data for cohorts 2, 3, 4 and 5 pre-removal, and 2, 4 and 5 post-removal are omitted. For explanation see text.

*** Packets transferred to sequence V on March 3, 2004.

(Table 4). It is evident that even in the absence of temephos granules, the previously released temephos persisted in the jars providing excellent control for an additional five-six wk at the highest dosage. It should be noted, however, that in actual practice under constant water use conditions, the residues may be diluted and not expected to yield adequate control for five-six wk post-removal or loss of granules.

The granule packets when removed and transferred for the fourth time to sequence V jars, yielded 96-100% IE for several weeks of assessment (Table 5). After about five wk in this sequence, the granule packets were removed and disposed of and the experiment terminated. Activity of the released temephos in sequence V jars after removal of packets was monitored for three wk only, and the

Table 5 Sequential release and residual activity of temephos granules (1%) when applied as packets to 200 L earthen water-storage jars and challenged with cohorts of 3rd instar larvae of *Ae. aegypti.* Bang Bua Thong, Nonthaburi, Thailand. Fifth sequence (First sequence treated Aug 27, 2003) .

Sequence V jars	Granules g/jar	mg/L AI	Pupal skins	EI** (%)
		1st cohort assessed 7 d post 4th transfer (Mar 10, 2004)		
5A	1	0.05	0.00	100
5B	5	0.25	0.00	100
5D	10	0.50	0.00	100
5C	0	0.00	24.50	2
		5th cohort assessed 33 d post 4th transfer (April 5, 2004)***		
5A	1	0.05	1.00	96
5B	5	0.25	0.00	100
5D	10	0.50	0.00	100
5C	0	0.00	23.00	8
		1st cohort assessed 7 d post 5th removal (April 12, 2004)		
5A	1	0.05	1.25	95
5B	5	0.25	0.00	100
5D	10	0.50	0.00	100
5C	0	0.00	22.75	9
		3rd cohort assessed 21 d post 5th removal (April 26, 2004)***		
5A	1	0.05	4.75	81
5B	5	0.25	2.25	91
5D	10	0.50	1.50	94
5C	0	0.00	22.50	10

* Packets transferred to sequence V jars on March 3, 2004.

** Data for cohorts 2, 3, 4 before removal of packets and cohort 2 after removal omitted. For explanation see the text.

*** Packets removed from sequence V on April 5, 2004 and disposed.

residues were high enough to yield 91% to 100% IE at the two high dosages. The test was then discontinued (April 26, 2004) eight mo from the start (August 27, 2003) of the experiment.

From this and the third experiment, it is clear that temephos is released slowly from sand granules over a long period of time. It is equally important to note that once released in adequate quantities, it remains in the jars and yields excellent control of larvae for three - six wk after removal of the granule packets depending on the extent of prior release. These findings point out the possibility of employing temephos granules in retrievable formulations which can be recovered during washing and cleaning of water-storage containers with temephos dosages lower than those currently used in operational control programs. Removable and retrievable formulations in packets or sachets can be reused and will have decided advantages over placement of granules as loose particles in the jars. During the process of cleaning and washing water-storage jars, loose granules are washed out and thus are lost, while granules in a retrievable form can be removed and transferred to the same jars after washing or to other untreated jars. This scheme will diminish the waste of scarce and costly formulations and will extend control program capacity to treat large numbers of jars which often go untreated due to insufficient amounts of the formulation being available to users.

Residual activity in painted versus unpainted jars

In this experiment, temephos 1% GR was added to waterstorage jars at the dosages of 1 g and 10 g per 200 L of water equaling 0.05 and 0.5 mg/L AI respectively and challenged with 21 weekly cohorts of larvae over a period of five mo. Here, we present results for 10 cohorts only, omitting the remaining 11, where the results were similar to those of the immediately preceding and succeeding cohorts to the omitted ones. The minimum-maximum temperatures of water during the test period were 28-30°C and 31-33°C respectively. At both dosages, release of temephos in painted jars was moderate to high throughout the duration of the experiment, which lasted for about five mo. The inhibition of emergence in the painted jars ranged from 51% to 98% (mostly in the 80th percentile) at the 1 g/jar dosage in the 21 cohorts of larvae added during this period (Table 6). The inhibition of emergence was consistently higher (IE 83% to 99%) at the higher dosage than at the low dosage in the painted jars. The inhibition of emergence overall was lower in the painted jars as compared to the unpainted jars. It seems that temephos released in painted jars is absorbed and bound in the paint layers, releasing into the water slowly. In contrast, the activity of temephos granules in our previous study, when we used 2x (20 g/jar) the current high dosage, was much higher in jars painted than here (Mulla *et al*. 2004). The difference is probably caused by the higher dosage used in that earlier study.

The magnitude of release in the unpainted jars was consistently very high in both dosages. In every cohort (a total of 21 cohorts) of assessment, the inhibition of emergence was 98% to 100%, most of the times the IE was 100% in both dosages (Table 6). It seems that even at the 1 g / 200 L water, which is 1/20 of the recommended dosage used in the control program, the level of control was about 100% for up to five mo or longer. In view of these findings, it will be desirable to consider studies in the field on reducing the currently used dosage to 2-5 g/200 L of water, providing material to treat many other water-storage containers than is currently possible.

The longevity and efficacy of temephos 1% GR in unpainted jars found here are identical (over five mo) to what was found in our earlier studies in painted

Table 6 Residual activity of temephos (1%) sand granules applied to painted and unpainted earthen water-storage jars, challenged with cohorts of 3rd instar larvae of *Ae. aegypti* at intervals post-treatment. (Treated November 12, 2003).

Granules g/jar	mg/L AI	Painted jars: Jars	Painted jars: Pupal skins	Painted jars: EI (%)	Unpainted jars: Jars	Unpainted jars: Pupal skins	Unpainted jars: EI (%)
		1st cohort assessed 7 d post-treatment (Nov 19, 2003)*					
1	0.05	G	0.50	98	L	0.25	99
10	0.50	I	0.25	99	K	0.25	99
0 (control)		H	22.50	10	J	24.00	4
		5th cohort assessed 40 d post-treatment (Dec 29, 2003) *					
1	0.05	G	4.75	81	L	0.00	100
10	0.50	I	2.00	92	K	0.00	100
0 (control)		H	24.25	3	J	24.00	4
		11th cohort assessed 82 d post-treatment (Feb 9. 2004) *					
1	0.05	G	5.75	77	L	0.00	100
10	0.50	I	4.25	83	K	0.00	100
0 (control)		H	22.75	9	J	24.00	4
		19th cohort assessed 145 d post-treatment (April 12, 2004) *					
1	0.05	G	7.75	69	L	0.50	98
10	0.50	I	1.50	94	K	0.00	100
0 (control)		H	23.00	8	J	22.50	10
		21st cohort assessed 161 d post-treatment (April 26, 2004) *					
1	0.05	G	8.25	67	L	1.00	99
10	0.50	I	4.25	83	K	0.00	100
0 (control)		H	23.50	6	J	23.50	6

* Cohorts 2, 3, 4, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, and 20 are omitted as the results of these were similar to those immediately preceeding or succeeding these cohorts.

jars (Mulla *et al*. 2004). However, this level of activity and longevity are not to be expected in community-wide *Ae. aegypti* control programs. As pointed out by Thavara *et al*. (2004) many factors, especially water use patterns, influence the longevity and efficacy of chemical control agents in water-storage jars.

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Mention of specific companies or products does not imply that they are recommended or endorsed by WHO in preference over others not mentioned.

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Cockroaches

Repellent Activity of Essential Oils against Cockroaches (Dictyoptera: Blattidae, Blattellidae, and Blaberidae) in Thailand

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Abstract: Seven commercial essential oils extracted from the plant species *Boesenbergia rotunda* (L.) Mansf., *Citrus hystrix* DC., *Curcuma longa* L., *Litsea cubeba* (Lour.) Pers., *Piper nigrum* L., *Psidium guajava* L. and *Zingiber officinale* Roscoe, and naphthalene as a control, were evaluated for repellent activity against the three cockroach species *Periplaneta americana* (L.), *Blattella germanica* (L.) and *Neostylopyga rhombifolia* (Stoll) under laboratory conditions. The essential oil derived from *Citrus hystrix* showed the best repellency over other candidate essential oils and naphthalene. The essential oil of *Citrus hystrix* exhibited complete repellency (100%) against *P. americana* and *B. germanica*, and also showed the highest repellency (among the essential oils tested) of about 87.5% against *N. rhombifolia* under laboratory conditions. In the field, *Citrus hystrix* essential oil formulated as a 20% active ingredient in ethanol and some additives provided satisfactory repellency of up to 86% reduction in cockroaches, mostly *P. americana* and *N. rhombifolia* with a residual effect lasting a week after treatment. *Citrus hystrix* essential oil has good potential for being used as a cockroach repellent. Further improvements in efficacy and residual activity may be realized with appropriate formulations.

Introduction

Cockroaches have the potential to mechanically carry and transmit many pathogens, such as bacteria, viruses, fungi, protozoa and helminthes (Cochran, 1982). They also serve as potential carriers of the causes of bacterial diarrhea and nosocomial infections in hospitals (Agbodaze and Owusu, 1989; Fotedar *et al*, 1991). There is ample evidence that substances produced by cockroaches are involved in producing allergic symptoms (Kongpanichkul *et al,* 1997; Pumhirun *et al,* 1997). At least 12 species of cockroaches have been reported in Thailand to date and the American cockroach is the most common cockroach found in dwellings in various provinces of Thailand (Tawatsin *et al,* 2001). Sriwichai *et al* (2002) confirmed the abundance of the American cockroach in all types of buildings in Bangkok, especially private residences, and found that German cockroaches were significantly predominant in grocery establishments.

Several chemicals were studied for repellent effects against cockroaches, such as N, N-diethylphenylacetamide (Prakash *et al,* 1990), methyl neodacanamide, propyl neodecanamide, methyl neotridecanamide, alkyl and aryl neoalkanamides (Steltenkamp *et al*, 1992), citral and eugenol (Vartak *et al,* 1994). Research regarding cockroach repellents, especially those derived from plant extracts, is quite limited at this time. Recently, the essential oil of catnip (*Nepeta cataria* L.) was reported as having repellency against adult male German cockroaches (Peterson *et al,* 2002). Up to the present time, no studies have reported evaluation of repellents against cockroaches in Thailand. The present study was initiated to study the repellent activity of seven essential oils, extracted from local plants of Thailand, against three cockroach species under laboratory conditions. The most promising essential oil was then further evaluated for repellency against cockroaches in the field.

Materials and methods

Essential oils for laboratory and field evaluation

Seven commercial essential oils (100%) were evaluated for repellent activity against three cockroach species (the American cockroach, the German cockroach and harlequin cockroach) under laboratory conditions. These essential oils were derived from lesser galanga (*Boesenbergia rotunda* (L.) Mansf.) rhizomes, kaffir lime (*Citrus hystrix* DC.) leaves, turmeric (*Curcuma longa* L.) rhizomes, Litsea (*Litsea cubeba* (Lour.) Pers.) fruits, black pepper (*Piper nigrum* L.) fruits, guava (*Psidium guajava* L.) leaves and ginger (*Zingiber officinale* Roscoe) rhizomes. These oils were selected for this study because the plants are commonly available in Thailand and the oils are available commercially. These oils were purchased from Thai-China Flavours and Fragrances Industry Co, Ltd, Nonthaburi Province, Thailand. Naphthalene was used as a control since it is commonly used as cockroach repellent.

The essential oil of kaffir lime leaf was selected for further evaluation against cockroaches in the field because of its high efficacy under laboratory conditions. This oil was prepared at various concentrations in ethanol [5, 10, 20, 50 and 100% (undiluted)] and tested against two cockroach species (the American cockroach and the German cockroach) under laboratory conditions. For field testing, the essential oil was then formulated as 20% (w/w) liquid repellent in ethanol with some additives.

Test cockroaches

Three cockroach species used in laboratory repellent tests were laboratory-reared *Periplaneta americana* (L.) (the American cockroach), *Blattella germanica* (L.) (the German cockroach) and *Neostylopyga rhombifolia* (Stoll) (Harlequin cockroach). These cockroaches have been reared according to the standard protocols of the Biology and Ecology Section, National Institute of Health, Thailand, and maintained in the insectary of the institute. The colonies were maintained in the insectary under ambient temperature (24-30°C) and humidity (70-90% RH). Adult *P. americana* (aged 3-5 months), *B. germanica* (aged 6-8 weeks) and *N. rhombifolia* (aged 3-5 months) were employed for repellent testing under laboratory conditions. Both males and females of each cockroach species were used in the laboratory tests.

Laboratory tests

A stainless steel square-box (50x50x15 cm, with the top open) was employed in the repellent tests. All four walls of the box were smeared with Vaseline to prevent escape of cockroaches. A piece of filter paper (Whatman No.1, 50x50 cm) was marked by a pen to divide it into 2 equal parts (treated and control areas) and then placed at the bottom of the box. The test repellent (1.25 ml) was applied (equal to dosage of 10 ml/m^2) by placing drops from a pipette on the treated area to cover the treated portion of the paper, whereas the control area was untreated. Naphthalene, the control standard, is solid. Two pieces of naphthalene (1 g each) were placed together as a treatment on the treated side. Each set of containers of food and drink for the cockroaches was placed at both sides of the box (treated and control areas). Twenty adult cockroaches (10 males and 10 females) were anesthetized with CO_2 and released into the box at the central point. The box was then placed in a Peet Grady chamber (180x180x180 cm) surrounded by cloth curtains to keep a dark environment and to prevent disturbances from surroundings. The cockroaches located in the treated and control areas were carefully observed and counted at 48 hours after treatment. Repellency against the cockroaches was calculated with the following equation:

$$\text{Repellency (\%)} = \frac{100 - [T \times 100]}{N}$$

where T stands for the number of cockroaches located in the treated area and N stands for the total number of cockroaches used. The average repellency was calculated from the values obtained in six replicates.

Field evaluations - village-scale trials

The essential oil of kaffir lime (*Citrus hystrix*) leaves was selected for further evaluation in the field because it showed the highest repellent activity against the three cockroach species tested under laboratory conditions. Field evaluation of the repellent formulated from the essential oil of kaffir lime leaves was carried out in Pitsanulok Province, Thailand. Three villages in rural areas were selected where the experiment was carried out. Kok Makham Yai Village of Wang Thong District (51 houses) and Wang Itok Village of Bang Rakam District (36 houses) were designated as the treatment sites, whereas Bang Saphan Village of Wang Thong District (50 houses) was designated as the control site. All sites were surveyed for cockroach species and densities using sticky traps (HOY HOY, produced by Earth Chemicals, Japan) before and after treatment. This sticky trap has been shown to be an effective tool for cockroach surveys in the field (Tawatsin *et al*, 2001). The sticky trap is a simple device that can be folded into a trapezoid paper-house (10x15x3 cm), having four entrances for cockroaches. The sticky area for catching cockroaches inside the trap is about 9.5x15 cm. Cockroaches are lured into the trap by built-in attractants located in the middle of the sticky area. At least 36 houses in each experimental site were randomly sampled for cockroaches by placing two sticky traps in the kitchen of each house and left there for one night. The cockroaches caught in each trap were identified by species following the handbook of domiciliary cockroach species in Thailand (Asahina, 1983) and other relevant references (Cornwell, 1968; Bell, 1981; Cochran, 1982, 1999) and then counted. Three days after the preliminary survey, the test repellent was applied at a dosage of 10 ml/m^2 by a hand-trigger windowsprayer on the floor in the kitchen area in each house of the two treated sites, whereas the houses in the control site were untreated. The treatment was carried out only once in each house of the two treated sites. To assess the degree of cockroach infestation, the sticky traps were again placed in the kitchens of each house at the three experimental sites and left there for one night. Then, all the traps were collected and the cockroaches caught in each trap were identified by species and counted. Assessment of the cockroach densities and species in each house at the three experimental sites was carried out on three additional occasions at 6-, 9- and 12-days post-treatment.

Another field evaluation of the repellent formulated from the essential oil of kaffir lime leaves was conducted in Bang Khaen District, Bangkok, Thailand. Two urban communities (one treated and one control site) were selected for this evaluation. Bang Khaen-1 (the treated site) included 44 houses, whereas Bang Khaen-2 (the control site) consisted of 37 houses. The evaluation carried out in the field in Bangkok was similar to that in Pitsanulok as described above. However, the assessments were carried out once a week for four weeks post-treatment during the course of this study. After each weekly assessment, the repellent was reapplied at the same dosage in the previously treated areas in each house of the treated sites.

The average number of collected cockroaches per house (mean no.) and standard error of the mean (SE) were calculated for each study site in each assessment in the field. The percentage reduction in cockroach number following treatment at each treated site was calculated by Mulla's formula (Mulla *et al*, 1971):

Reduction (%) = $100 - [(C_1/T_1) \times (T_2/C_2)]100$

where: C1 = average number of cockroaches per house at the control site (pre-treatment) ,

T_1 = average number of cockroaches per house at the treated site (pre-treatment),

C_2 = average number of cockroaches per house at the control site (post-treatment),

T_2 = average number of cockroaches per house at the treated site (post-treatment).

These values, mean SE and percentage reduction (%), are presented in the figures.

Data analysis

Comparison of repellency among test repellents was carried out employing the one-way analysis of variance (ANOVA) with Duncan's multiple range test. All differences were considered significant at $p < 0.05$.

Results

Laboratory repellency

The essential oil of *Citrus hystrix* provided complete repellency (100%) against *P. americana* cockroaches, and a high degree of repellency was also obtained from the essential oils of *Psidium guajava* (95%) and *Boesenbergia rotunda* (90%) (Table 1). Moderate levels of repellency were derived from the essential oil of *Litsea cubeba* (88.3%) and *Zingiber officinale* (85%). The

Table 1 Repellency of essential oils and naphthalene against *P. americana, B. germanica and N. rhombifolia* cockroaches in the laboratory.

Plant essential oils/chemical	Mean repellency* (%) ± SE		
	P. americana	*B. germanica*	*N. rhombifolia*
Boesenbergia rotunda	90 ± 2.6 c	95 ± 1.8 b	60.8 ± 7.7 bc
Citrus hystrix	100 ± 0.0 a	100 ± 0.0 a	87.5 ± 5.3 a
Curcuma longa	80 ± 1.8 e	95 ± 1.3 b	50.8 ± 8.0 c
Litsea cubeba	88.3 ± 3.3 cd	90 ± 2.9 c	67.5 ± 8.6 b
Piper nigrum	80 ± 1.3 e	95 ± 1.8 b	58.3 ± 7.0 bc
Psidium guajava	95 ± 1.8 b	100 ± 0.0 a	56.7 ± 9.4 bc
Zingiber officinale	85 ± 2.2 d	95 ± 1.3 b	70.0 ± 8.1 b
Naphthalene	80 ± 1.8 e	85 ± 2.9 c	65.0 ± 9.2 bc

* Repellency against the same species (in the same column) followed by the same letter is not significantly different from each other ($p \geq 0.05$, by one-way ANOVA and Duncan's multiple range test).

essential oil of *Curcuma longa* and *Piper nigrum* exhibited repellency equal to that of naphthalene (80%).

The essential oils of *Citrus hystrix* and *Psidium guajava* showed excellent repellency (100%) against *B. germanica* cockroaches in the laboratory (Table 1). However, high degrees of repellencies against cockroaches were also obtained from the essential oils of *Boesenbergia rotunda* (95%), *Curcuma longa* (95%), *Piper nigrum* (95%), *Zingiber officinale* (95%) and Litsea cubeba (90%). It is interesting to note that all the essential oils in this experiment provided better repellencies than did the standard repellent naphthalene (85%).

Regarding the repellent tests against *N. rhombifolia* cockroaches, the essential oil of *Citrus hystrix* exhibited the highest repellency (87.5%) of the tested repellents, whereas the essential oil of *Curcuma longa* provided the lowest repellency of about 50.8% (Table 1). Moderate repellencies against *N. rhombifolia* were obtained from the essential oils of *Zingiber officinale* (70%), followed by those of *Litsea cubeba* (67.5%), naphthalene (65%), *Boesenbergia rotunda* (60.8%), *Piper nigrum* (58.3%) and *Psidium guajava* (56.7%). There were no significant differences among repellencies obtained from this group.

The repellency of essential oil of *Citrus hystrix* at various concentrations against *P. americana* and *B. germanica* is shown in Fig 1. The 50% concentration and undiluted essential oil provided excellent repellency from 95 to 100% against *P. americana* cockroaches. The essential oil at 20% concentration exhibited a moderate level of repellency at an average of about 83.5%. The essential oil diluted to 10% and 5% showed lower repellency against *P. americana*, about 60% and 58.5%, respectively. Regarding repellency against *B. germanica*, the 50% concentration and the undiluted essential oil of *Citrus hystrix* also provided

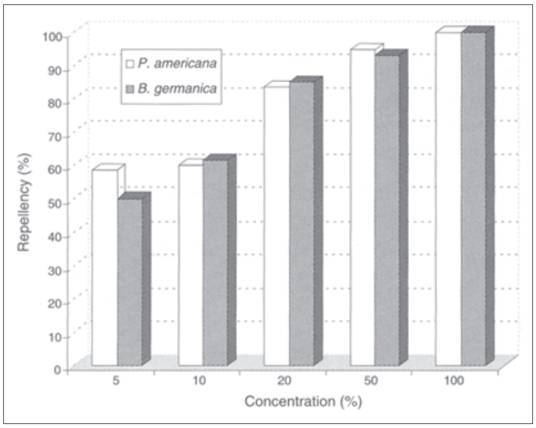

Figure 1 Comparison of repellency of essential oil of kaffir lime *(Citrus hystrix)* leaves at various concentrations in ethanol against *P. americana* and *B. germanica* cockroaches under laboratory conditions,

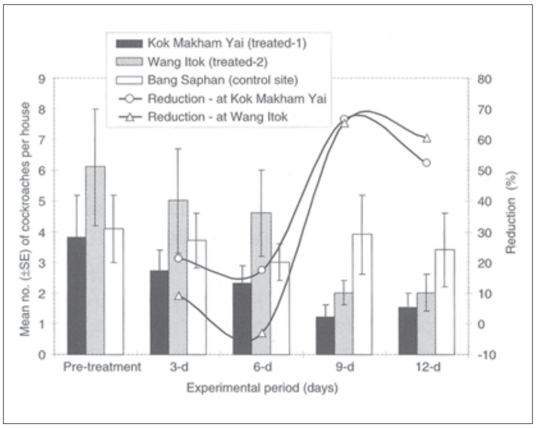

Figure 2 Field evaluation of formulated repellent containing 20% essential oil of kaffir lime *(Citrus hystrix)* against cockroaches conducted in three villages of Pitsanulok Province, Thailand.

excellent repellency (93-100%), whereas the essential oil at 20% concentration showed an average repellency of about 85%. The results against *B. germanica* were similar to those against *P. americana*, the essential oil diluted to 10% and 5% exhibited repellency of 61.5% and 50% against the *B. germanica* cockroaches, respectively.

Field repellent test against cockroaches in Pitsanulok Province

Kok Makham Yai Village (the 1st treated site).

A total of 51 houses in the first treated site (Kok Makham Yai Village) were surveyed and a total of 194 cockroaches were found in 31 houses (60.8% positive) during the preliminary inspection before treatment. The average number of cockroaches collected at Kok Makham Yai Village prior to treatment was 3.8 cockroaches/house (Fig 2). Three days after treatment with the test repellent (20% *Citrus hystrix*), 138 cockroaches were caught from 21 houses (41.2% positive). The average number of captured cockroaches dropped to 2.7 cockroaches/house with a reduction of about 21.3% using the formula of Mulla *et al* (1971), taking both the treated and control populations (Fig 2). Subsequently, 19 houses (37.3%) were positive for 119 cockroaches during the inspection at 6 days after treatment. An average of 2.3 cockroaches/house was obtained in this assessment with 17.3 % reduction (Fig 2).

The number of houses infested with cockroaches declined to 17 houses (33.3% positive) at 9 days post-treatment with only 59 cockroaches found in this survey. As shown in Fig 2, the mean number of captured cockroaches dropped to 1.2 cockroaches/house with the reduction reaching a peak of 66.8%. Finally, 20 houses (39.2%) were positive for a total of 76 cockroaches in the last period of survey (12 days post-treatment). The reduction remained at 52.4% with an average of 1.5 cockroaches/house (Fig 2).

There were seven species of cockroaches found at Kok Makham Yai Village during the five surveys taken during the course of this study. The predominant species was *P. americana* (42.8-84.9%), followed by *N. rhombifolia* (13.5-34.2%), *P. brunnea* Burmeister (0.7-30%), *Pycnoscellus surinamensis* (Linneaus) (1.7-18.9%), *Supella longipalpa* (F.) (0.8-10.3%), *B. germanica* (0.7-7.9%) and *P. australasiae* (Fab.) (0.5-2.6%).

Wang Itok Village (the second treated site).

The preliminary survey conducted at Wang Itok Village (the second treated site) revealed that 26 (72.2%) of 36 houses were infested with a recovery of 221 cockroaches. The average number of cockroaches captured at Wang Itok Village before treatment was 6.1 cockroaches/ house (Fig 2). A total of 20 houses (55.6%) were positive for cockroaches 3 days after treatment with the test repellent (20% *Citrus hystrix*) and 179 cockroaches were captured. An average of 5 cockroaches/ house was obtained in this survey with a reduction rate of 9.2% according to Mulla's formula compared to pre-treatment at the control site (Fig 2). The numbers of houses infested with cockroaches decreased to 18 houses (50% positive) during the survey carried out 6 days post-treatment with a total of 166 cockroaches. The average number of cockroaches captured in this assessment was 4.6 cockroaches/house, a reduction of 3.1% (Fig 2).

In the survey conducted 9 days after treatment, 16 houses (44.4%) were positive for cockroaches and only 72 cockroaches were collected. An average of 2 cockroaches/house were found in this survey. The reduction reached a peak of about 65.5% (Fig 2). Twelve days after treatment, the number of houses infested with cockroaches decreased to 11 houses (30.6% positive) with 73 cockroaches captured. As seen in Fig 2, the average number of collected cockroaches was about the same as the previous survey (~2 cockroaches/house), however, the reduction declined to 60.5%.

Eight species were identified from cockroaches caught from Wang Itok Village from five surveys in this study. *P americana* (47.587%) was the predominant species found in all surveys, followed by *N. rhombifolia* (7.3-31.7%), *S. longipalpa* (2.2-18.1%), *B. lituricollis* (Walker), *B. germanica*, *P. brunnea*, *Nauphoeta cinerea* (Olivier) and *Py. surinamensis* were found less than 2%.

Bang Saphan Village (control site).

Cockroach surveys were also carried out in Bang Saphan Village (control site) during the same period as the trial in Kok Makham Yai Village and Wang Itok Village. On a preliminary survey, 32 out of 50 houses (64%) were positive for cockroaches. A total of 206 cockroaches were caught in this survey with an average of 4.1 cockroaches/house (Fig 2). Three days after the preliminary survey, the number of houses infested with cockroaches declined to 21 houses (42%) and 187 cockroaches were caught (average 3.7 cockroaches/house). Later, 25 houses (50%) were positive for cockroaches in the survey conducted 6 days after the preliminary survey and 150 cockroaches were captured during this inspection. An average number of 3 cockroaches/house was obtained in this inspection.

A total of 197 cockroaches were found in 24 houses (48% positive) in the control site in the fourth survey carried out nine days after the preliminary survey, with an average of 3.9 cockroaches/house. Finally, 24 houses (48%) were still positive with a total of 168 cockroaches at the last inspection (12 days after the preliminary survey). The average number of captured cockroaches remained at 3.4 cockroaches/house (Fig 2).

Seven species of cockroaches were collected from Bang Saphan Village (control) during five surveys in this study. These included *P. americana* (34.5-70%), *N. rhombifolia* (24-60.7%), *S. longipalpa* (2.4-16.3%), *B. germanica* (0.5-4.6%), *P. brunnea* (1-2%), *P. australasiae* (0.6-1 %) and *Py. surinamensis* (0.5-1 %).

Field repellent test against cockroaches in Bangkok

Bang Khaen-1 Community (the treated site).

A total of 28 (63.6%) out of 44 houses were positive for cockroaches in the pre-treatment survey carried out in the treated site (Bang Khaen-1 Community) and 268 cockroaches were collected in this survey with an average number of 6.1 cockroaches/house (Fig 3). One week after treatment with the test repellent (20% *Citrus hystrix*), although the number of houses infested with cockroaches still remained at 28 (63.6%) similar to that of the pre-treatment survey, the number of collected cockroaches had declined to 131. As a result, the average number dropped to 3 cockroaches/house with a 35.4% reduction. The number of infested houses decreased slightly to 25 (56.8%) in the survey conducted two weeks post-treatment and 107 cockroaches were captured. In this inspection, an average number of 2.4 cockroaches/house was obtained, whereas the reduction rate increased to 52.4% (Fig 3).

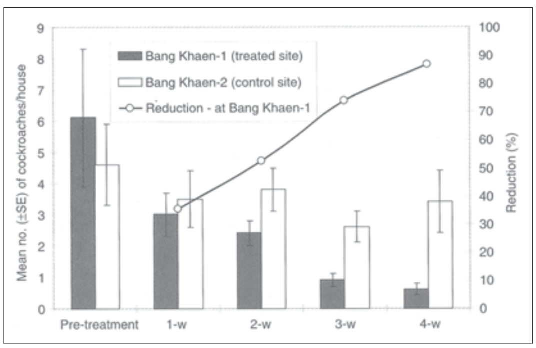

Figure 3 Field evaluation of formulated repellent containing 20% essential oil of kaffir lime (*Citrus hystrix*) leaves applied weekly against cockroches in two urban communities of Bang Khaen District, Bangkok, Thailand.

In the survey carried out three weeks after treatment, the number of houses positive for cockroaches declined to 13 (29.5%) and 40 cockroaches were collected. As shown in Fig 3, the average number of 0.9 cockroaches/

house was achieved with a reduction of about 73.9%. Finally, the number of houses infested with cockroaches remained at 10 (22.7%) in the final inspection conducted four weeks post-treatment and 27 cockroaches were captured in this survey. An average number of 0.6 cockroaches/house was found in this assessment with a high reduction rate of 86.7% (Fig 3).

There were only five species of cockroaches collected from the five surveys carried out at Bang Khaen-1 Community. These were *P. americana* (28-80%), *P. brunnea* (12.5-47.8%), *N. rhombifolia* (0.7-23.4%), *B. germanica* (3-15.3%) and *S. longipalpa* (0-4.7%).

Bang Khaen-2 Community (control site).

The cockroach surveys were also carried out in Bang Khaen-2 Community (the control site) during the same period as the study in Bang Khaen-1 Community for comparison. The results of pre-treatment survey showed that 26 (70.3%) out of 37 houses were infested with cockroaches and 169 cockroaches were caught. An average of 4.6 cockroaches/house was obtained from this assessment (Fig 3). One week after the preliminary survey, 20 houses (54.1 %) were positive with a total of 129 cockroaches (average 3.5 cockroaches/house). Subsequently, it was found that 27 houses (73%) in the control site were infested with 142 cockroaches during the inspection at two weeks after the preliminary survey with an average of 3.8 cockroaches/house. In the third week post-treatment, the inspection revealed that 15 houses (40.5%) were positive for a total of 95 cockroaches and the average captured cockroaches declined to 2.6 cockroaches/house. Finally, 21 houses (56.8%) in the control site were found positive for cockroaches with 125 cockroaches collected in the last inspection. The average number of cockroaches at this assessment increased to 3.4 cockroaches/house.

Six species were identified from cockroaches captured in Bang Khaen-2 Community during the course of this study. These were *N. rhombifolia* (26.1-77.9%), *P. americana* (13.7-69%), *S. longipalpa* (5.8-13.9%), *P. brunnea* (4.9-8.4%), *B. germanica* (4.7%) and *N. cinerea* (0-3.2%).

Discussion

The test method used in this study was developed by the Biology and Ecology Section, the National Institute of Health, Thailand, since no standard method for evaluation of cockroach repellent had been established elsewhere. It was selected because of its reliability among several attempts that had been made previously. The laboratory repellency results indicated differences in susceptibility to volatile chemicals derived from essential oils among the three species of test cockroaches. *B. germanica* was the most sensitive species in this study, followed by *P. americana*, *N. rhombifolia*. All the essential oils in this study provided equal or better repellencies against *P. americana* and *B. germanica* than naphthalene (80%). Naphthalene is the most common chemical used as a cockroach repellent. However, naphthalene is hazardous to humans. Humans exposed for long periods to naphthalene by inhalation, ingestion or dermal contact may develop hemolytic anemia, damage to the liver, or neurological damage in infants (ATSDR, 1995).

The regulation for cockroach repellent products in Thailand states there must be a minimal repellency of 80% against *P. americana*. The seven essential oils tested in this study therefore qualified against both *P. americana* and *B. germanica*. However, *Citrus hystrix* was the most effective repellent tested providing complete repellency (100%) against both *P. americana* and *B. germanica*, and the highest repellency (among all the essential oils tested) of about 87.5% against *N. rhombifolia*. It was selected for further evaluation in the field at a dosage of 20% *Citrus hystrix*.

In the field, the cockroach repellent formulated from *Citrus hystrix* essential oil (20%) showed satisfactory repellency in the treated areas in both Pitsanulok Province and Bangkok. In Pitsanulok, the repellent activity reached its peak at nine days post-treatment at both treated sites (66.8 and 65.5% reduction). Therefore, we assessed repellent activity with weekly applications in the field evaluation carried out in Bangkok. We found that weekly surveys and re-applications of repellent were practical and effective. Although the repellent could not repel cockroaches completely, it could substantially reduce the numbers of cockroaches as well as the numbers of houses infested with cockroaches in the treated sites compared to the control sites. The infestation rate and number of cockroaches captured in the treated site in Bangkok declined substantially, especially two weeks post-treatment. This could be due to the accumulated residual activity of the repellent that was applied weekly. The reduction of cockroaches and infestation rates in the treated sites may have been partially affected by trap catching; however, this factor is minor as seen in the results of the control site.

In this study, we used Mulla's formula to assess the degree of reduction in cockroach number for each treated site following the treatment with the assumption that the treated and control sites were uniform in regard to factors contributing to changes in cockroach populations (Mulla *et al*, 1971). In practice, this formula was powerful for assessing the level of reduction of cockroaches in this study as it compares the number at the treated site and the control site both pre- and post -treatment. According to this formula, no reduction occurs in the cockroach numbers because of treatment if the factor $[(C_1/T_1) \times (T_2/C_2)]$ is greater than 1. This phenomenon appeared once in the treated site at Wang Itok Village (6 days post-treatment). In fact, it occurred because the average number of cockroaches post-treatment at the treated site (T_2) was greater than that of post-treatment in the control site (C_2)

It is interesting to note that people at the treated sites mostly accepted this repellent and some residents acknowledged that mosquito-biting activity was reduced at night in the treated areas along with the cockroach reduction. These results indicate the potential for the use of the *Citrus hystrix* essential oil as a cockroach repellent in the future. This is the first study of repellents derived from plant extracts against cockroaches in Thailand. More research is needed to develop more effective formulations. These may include long-lasting formulations using micro-encapsulation techniques and combinations with other essential oils for synergistic effects.

Research regarding repellents against cockroaches is limited at this time, especially in agents derived from plant extracts. Recently, Peterson *et al* (2002) investigated the repellent activity of catnip essential oil (*Nepeta cataria*), two purified isomers of nepetalactone and deet (N,N-diethyl-methylbenzamide) against male German cockroaches (*B. germanica*) in a choice-test arena and found that E,Z -nepetalactone was the most active of the compounds tested, being significantly more active than equivalent doses of the essential oil, Z,E-nepetalactone, or deet. Other studies have evaluated chemicals for repellent effects against cockroaches. N,Ndiethylphenylacetamide (OEPA), at a dosage of 0.5 mg/cm^2 showed residual repellency against *P. americana, B. germanica* and *S. longipalpa* for 4, 3 and 2 weeks, respectively (Prakash *et al,* 1990). Steltenkamp *et al* (1992) demonstrated that alkyl and aryl neoalkanamides with a total carbon number between 11 and 14 exhibited highly repellent effects against male *B. germanica.* In addition, methyl neodecanamide, propyl neodecanamide and methyl neotridecanamide were also found highly repellent against females and nymphs of *B. germanica,* and male *P. americana* (Steltenkamp *et al,* 1992). It is interesting to note that these chemicals showed relatively specific repellency against certain species, sexes and developmental stages of cockroaches. Vartak e*t al* (1994) showed that citral and eugenol were effective as repellents against *P. americana* under laboratory conditions when used at the dosages of 25-100 mg per 4 x 4 cm filter paper. However, none of these chemicals is currently marketed as a commercial repellent product against cockroaches.

In conclusion, the essential oil derived from *Citrus hystrix* exhibited complete repellency (100%) against *P. americana* and *B. germanica*, and also showed the highest repellency (among all essential oils tested) of about 87.5%, against *N. rhombifolia* under laboratory conditions. In addition, the repellent containing 20% *Citrus hystrix* essential oil formulated in ethanol and some additives also showed satisfactory repellency yielding up to 86% reduction in cockroaches, mostly *P. americana* and *N. rhombifolia,* in field tests with residual effects for a week after treatment. The present study reveals the potential for

Citrus hystrix essential oil to be used as a cockroach repellent. Further improvements in efficacy and longevity are expected with appropriate formulations.

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Population Dynamics of the German Cockroach, *Blattella germanica* (Linnaeus), from Markets in Urban Areas of Thailand

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Abstract: A study on population dynamics of the German cockroach using modified jar traps was investigated in twelve Bangkok markets from March 2005 to March 2006. The selected market areas were conducted monthly during the study period. The mean numbers of the total cockroaches caught per trap were significantly different. The two highest peaks of the German cockroach were in July (4.54 ± 1.78) and August (4.21 ± 1.63) whereas the two lowest peaks of the cockroach were in December (0.37 ± 0.14) and January (0.43 ± 0.17). The highest catch of the German cockroach was the large nymphal stage (the 5^{th} and 6^{th} instars). The highest number of the German cockroaches was in a poor sanitary market in low population density zone of Bangkok (4.62 ± 0.68) whereas none of the German cockroach was caught from a good sanitary market in high population density zone of Bangkok. Moreover, the cockroaches caught from the groceries (3.02 ± 0.19) were significantly higher than those caught from the butcher shops (0.61 ± 0.04) and vegetable shops (1.52 ± 0.13) in the twelve Bangkok markets.

Introduction

Cockroaches are an important group of insect pests in human environment. Cockroaches are not only an annoying pest but are also capable of transmitting pathogens to human such as viruses, bacteria, protozoa, fungi and helminthes (Brenner 1995). Moreover, they generally give displeasure and an impression of dirtiness. When they walk across food and utensils, they may leave food containing many pathogens that cause enteric fever, food poisoning, and other human diseases (Fotedar et al. 1991, Rivault et al. 1993, Brenner 1995, Tachbele et al. 2006). Cockroaches are also important components of house dust allergen (Ross and Mullins 1995, Pumhirun et al. 1997, Sarinho et al. 2004).

At least ten species of cockroaches could be found in urban environment of Thailand. These include *Blattella germanica*, *B. lituricollis*, *Periplaneta americana*, *P. australasiae*, *P. brunnea*, *P. fuliginosa*, *Neostylopyga rhombiofolia*, *Nauphoeta cinerea*, *Pycnoscelis surinamensis* and *Supella longipalpa* (Benjapong et al.1997, Tawatsin et al. 2001, Sriwichai 2001, Damsuwon 2003). From the previous reports, the German cockroach, *B. germanica*, was the common species that infested in urban areas in Thailand. Prior to this study, there are a few documents existed on the population dynamics of cockroaches in markets which are public place and many foods are stayed. The objective of this experiment was to investigate the population dynamics of the German cockroaches in the market areas of Bangkok, Thailand.

Materials and methods

The study was conducted monthly in twelve Bangkok markets from March 2005 to March 2006. Bangkok is the capital of Thailand with a population of 5.6 million and 3,592 persons per square kilometers. Cockroach population dynamics was investigated by modified jar traps. A modified jar trap was a plastic cup (8 cm in diameter and 11 cm in height) with the sticky crepe paper fixed around the cup for enabled cockroaches to enter. The upper two-fifth of the inner surface of the jar trap was coated with a thin film of petroleum jelly (Vaseline®), cockroaches once entering trap lured by food were unable to climb out. Following the technique reported by Wileyto and Boush (1983), ground peanut and cat food saturated with beer were used as baits in this study. Twenty modified jar traps filling with baits were placed randomly in 3 kinds of shops in each market such as butcher shops (fresh pork, beef, and chicken), vegetable shops (fresh vegetable), and groceries (rice, seasoning, garlic, red onion, bean, dried shrimps, and dried squids). The traps were placed next to walls and near equipments in each shop for 24 hours. The traps were collected and taken to the laboratory, where the cockroaches were identified and counted. The number and stage of the German cockroach were recorded. The nymphal stages were divided into three class sizes such as small nymphs were the 1^{st} and 2^{nd} instars, medium nymphs were the 3^{rd} and 4^{th} instars, and large nymphs were the 5^{th} and 6^{th} instars. The temperature and relative humidity were recorded using thermo-hygrometer. Descriptive statistics were used to present as number, percentage, mean, and standard error. Mean numbers of the German cockroaches caught per trap were compared and analyzed. All data were checked for normal distribution. If any data was normal distribution, ANOVA with LSD analysis ($p < 0.05$) was used. But, if any data was not normal distribution, *Mann-Whitney U*-test ($p < 0.05$) was used instead. All analyses were performed using SPSS 12.0 version (SPSS 2003, Cary, N. C., U.S.A.).

Results

I. Cockroach species in Bangkok markets

A total of 11,944 cockroaches were trapped from 12 Bangkok markets during the investigation. The seven species of cockroaches, belonging to six genera, caught from 12 Bangkok markets were *Blattella germanica* (46.12%), *Nauphoeta cinerea* (5.16%), *Neostylopyga rhombiofolia* (0.03%), *Periplaneta americana* (45.30%), *P. brunnea* (2.20%), *Supella longipalpa* (0.04%), and *Symploce pallens* (1.15%). The *B. germanica* were trapped at the highest number in Bangkok markets in this study.

II. Population dynamics of the German cockroaches in Bangkok markets

The German cockroaches showed the fluctuation during the study period. The results showed that the mean numbers of the total cockroaches caught per trap were significantly different (F = 17.062; df = 12, 3107; $p < 0.0001$). The mean number of cockroaches was highest in July, followed by August, June, September, May, April, October, March 2005, November, March 2006, February, January, and December, respectively. The two highest peaks were in July (4.54 ± 1.78) and August (4.21 ± 1.63) whereas the lowest peaks were in December (0.37 ± 0.14) and January (0.43 ± 0.17). Even though the mean numbers of cockroaches caught per trap in July and August were not significantly different, the mean in July was significantly higher than the other months (LSD; df = 3107; $p < 0.05$). During the period of study, the mean numbers per trap of cockroaches started increasing in April (1.49 ± 0.58) and rapidly decreased in September (2.33 ± 0.87) (Table 1, Figure 1).

The result in Table 2 presented the mean numbers (per trap) of all stages of the German cockroaches caught from Bangkok market from March 2005 to March 2006. Mean numbers of all stages of the German cockroaches were significantly different during the study period (F = 17.733; df = 3, 3116; $p < 0.0001$). The highest mean number was the large nymphal stage (12.93 ± 1.43) whereas the lowest mean was the small nymphal stage (3.11 ± 0.49). Although the mean numbers of the large nymphal stage (12.93 ± 1.43) and adult cockroaches (11.94 ± 1.14) caught per trap were not significantly different, the mean of the large nymphal stage was significantly higher than that of the other cockroach stages (LSD; df = 3116; $p < 0.05$) (Table 2).

Mean numbers of all stages of the German cockroaches showed the same pattern. The data from Table 1 showed the two highest peaks in July and August. The mean numbers per trap of the German cockroaches started increasing in April and the mean rapidly decreased in September. The means decreased close to zero in December and January, especially in December and January the mean numbers of the small nymph were zero. The curves of mean numbers of the total number, adult, large nymph, medium nymph, and small nymph of the German cockroaches, the average temperature (°C), and the average relative humidity (%) from twelve Bangkok markets were presented in Figure 2.

Table 1 Mean (± SE) number of the German cockroaches caught per trap from twelve Bangkok markets from March 2005 to March 2006 (S = small nymph, M = medium nymph, L= large nymph, A = adult, and T = total number).

Mean number (per trap) of the German cockroaches

	March 2005	April	May	June
S	0.05 ± 0.02	0.12 ± 0.05	0.23 ± 0.11	0.18 ± 0.07
M	0.21 ± 0.09	0.35 ± 0.14	0.43 ± 0.17	0.64 ± 0.27
L	0.35 ± 0.14	0.52 ± 0.20	0.83 ± 0.32	1.04 ± 0.40
A	0.45 ± 0.17	0.50 ± 0.19	0.76 ± 0.30	0.90 ± 0.34
T	1.06 ± 0.42	1.49 ± 0.58	2.25 ± 0.89	2.76 ± 1.08

	July	August	September	October
S	0.59 ± 0.26	0.39 ± 0.15	0.22 ± 0.08	0.12 ± 0.04
M	1.00 ± 0.42	1.03 ± 0.44	0.42 ± 0.15	0.26 ± 0.09
L	1.65 ± 0.62	1.60 ± 0.61	0.87 ± 0.33	0.58 ± 0.20
A	1.30 ± 0.48	1.19 ± 0.44	0.83 ± 0.31	0.45 ± 0.16
T	4.54 ± 1.78	4.21 ± 1.63	2.33 ± 0.87	1.41 ± 0.50

	November	December	January 2006	February
S	0.05 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.01
M	0.14 ± 0.05	0.02 ± 0.01	0.04 ± 0.02	0.10 ± 0.09
L	0.24 ± 0.09	0.11 ± 0.04	0.13 ± 0.05	0.18 ± 0.07
A	0.40 ± 0.18	0.24 ± 0.09	0.26 ± 0.09	0.23 ± 0.08
T	0.83 ± 0.32	0.37 ± 0.14	0.43 ± 0.17	0.54 ± 0.21

	March
S	0.03 ± 0.01
M	0.12 ± 0.04
L	0.30 ± 0.10
A	0.25 ± 0.09
T	0.70 ± 0.25

Table 2 Mean (± SE) number (per trap) of the German cockroach stages caught from twelve Bangkok markets from March 2005 to March 2006 (S = small nymph, M = medium nymph, L= large nymph; and A = adult).

Months	Cockroach stages			
	S	M	L	A
March 2005	0.05 ± 0.02	0.21 ± 0.09	0.35 ± 0.14	0.45 ± 0.17
April	0.12 ± 0.05	0.35 ± 0.14	0.52 ± 0.20	0.50 ± 0.19
May	0.23 ± 0.11	0.43 ± 0.17	0.83 ± 0.32	0.76 ± 0.30
June	0.18 ± 0.07	0.64 ± 0.27	1.04 ± 0.40	0.90 ± 0.34
July	0.59 ± 0.26	1.00 ± 0.42	1.65 ± 0.62	1.30 ± 0.48
August	0.39 ± 0.15	1.03 ± 0.44	1.60 ± 0.61	1.19 ± 0.44
September	0.22 ± 0.08	0.42 ± 0.15	0.87 ± 0.33	0.83 ± 0.31
October	0.12 ± 0.04	0.26 ± 0.09	0.58 ± 0.20	0.45 ± 0.16
November	0.05 ± 0.02	0.14 ± 0.05	0.24 ± 0.09	0.40 ± 0.18
December	0.00 ± 0.00	0.02 ± 0.01	0.11 ± 0.04	0.24 ± 0.09
January 2006	0.00 ± 0.00	0.04 ± 0.02	0.13 ± 0.05	0.26 ± 0.09
February	0.03 ± 0.01	0.10 ± 0.09	0.18 ± 0.07	0.23 ± 0.08
March	0.03 ± 0.01	0.12 ± 0.04	0.30 ± 0.10	0.25 ± 0.09
Overall mean (per trap)[1]	3.11 ± 0.49 [a]	7.33 ± 0.99 [b]	12.93 ± 1.43 [c]	11.94 ± 1.14 [c]

[1] Means with different letters are significantly different at $p < 0.05$, ANOVA with LSD.

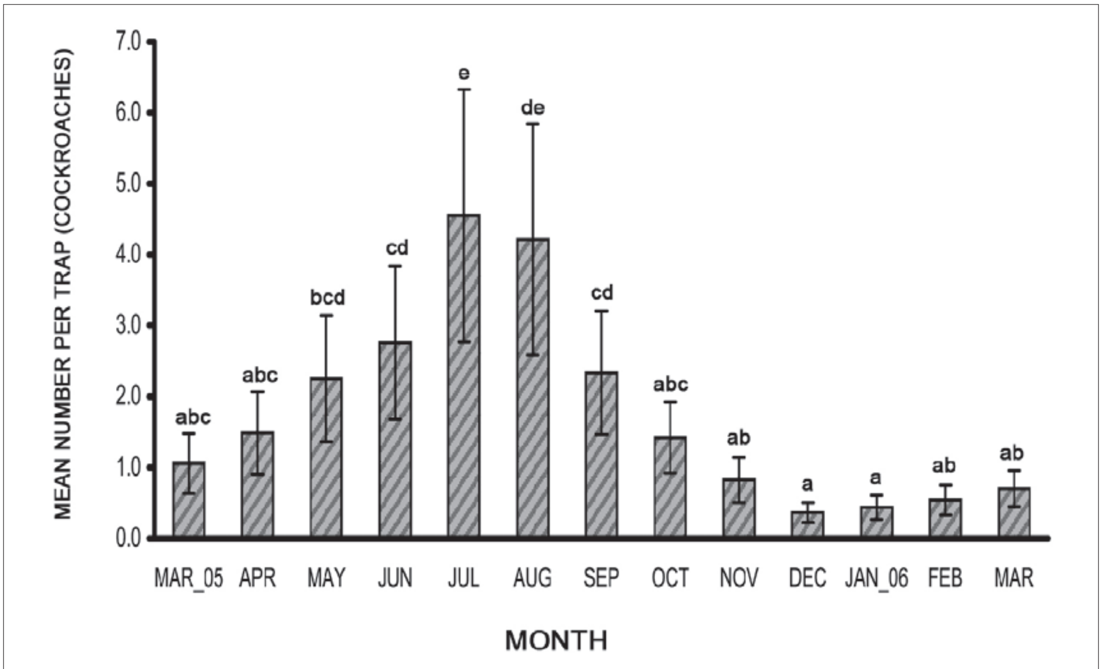

Figure 1 The overall mean (± SE) number (per trap) of the German cockroaches caught from twelve Bangkok markets from March 2005 to March 2006. The bar graphs with different letters are significantly different at $p < 0.05$, ANOVA with LSD.

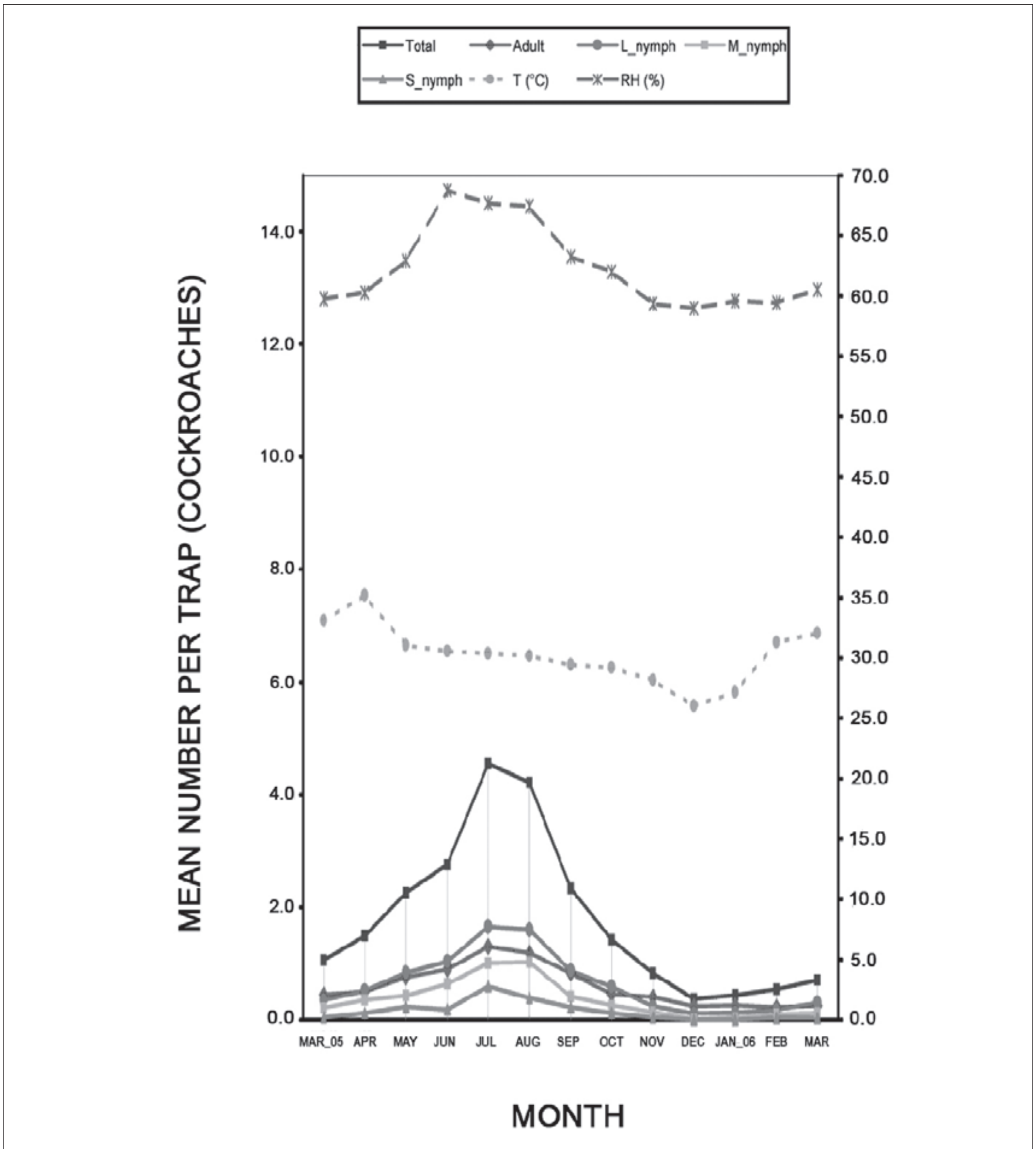

Figure 2 Mean number of the total number, adult, large nymph (L), medium nymph (M), and small nymph (S) of the German cockroaches in relation to temperature (°C) and relative humidity (%) in twelve Bangkok markets. Each point on a curve represents a mean number per trap in each month during the study period.

Mean numbers (per trap) of the German cockroaches in all studied markets showed the same pattern. The two highest peaks were in July and August whereas the two lowest peaks were in December and January. The mean numbers per trap of the cockroaches started increasing in April and rapidly decreased in September. The mean numbers were close to zero in December and January (Figure 3). The results showed that the overall mean numbers (per trap) of the German cockroaches caught in twelve Bangkok markets were significantly different (F = 21.771; df = 11, 3107; p < 0.0001). The highest mean was in market number 12 (4.62 ± 0.68) followed by market number 6 (4.55 ± 0.72), market number 7 (3.64 ± 0.48), market number 4 (1.77 ± 0.23), market number 10 (1.69 ± 0.23), market number 9 (1.53 ± 0.25), market number 1 (1.27 ± 0.17), market number 8 (1.01 ± 0.15), market number 5 (0.42 ± 0.08), market number 2 (0.41 ± 0.07), market number 11 (0.26 ± 0.05), respectively whereas the lowest was in market number 3 (0.00 ± 0.00). Although the mean numbers of the German cockroaches caught per trap in market number 12 and market number 6 were not significantly different, the mean number in market number 12 was significantly higher than, that in any other markets (LSD; df = 3107; p < 0.05) (Figure 4).

The results in Table 3 presented the average numbers of the German cockroaches in 3 kinds of shops in the Bangkok markets. Overall, the mean numbers of the German cockroaches caught per trap were significantly different during the study period (F = 76.36; df = 2, 465; p < 0.0001). The highest mean was in groceries (3.02 ± 0.19) followed by vegetable shops (1.52 ± 0.13) and butcher shops (0.61 ± 0.04), respectively. In November, the mean numbers in grocery (1.19 ± 0.09) and vegetable shops (0.67 ± 0.05) were not significantly different. However, the mean numbers in groceries were significantly higher than, that in vegetable and butcher shops in the rest of the months (LSD; df = 465; p < 0.05).

Table 3 Mean (± SE) number (per trap) of the German cockroaches caught per trap in three kinds of shop in Bangkok markets from March 2005 to March 2006 (B = butcher shop, V = vegetable shop, and G = grocery).

Months	B	V	G
March 2005	0.58 ± 0.04^{a}	0.86 ± 0.06^{a}	1.67 ± 0.12^{b}
April	0.36 ± 0.02^{a}	0.79 ± 0.06^{a}	3.15 ± 0.24^{b}
May	1.03 ± 0.07^{a}	1.65 ± 0.12^{a}	3.90 ± 0.30^{b}
June	0.60 ± 0.04^{a}	1.39 ± 0.10^{a}	5.98 ± 0.46^{b}
July	1.49 ± 0.11^{a}	5.07 ± 0.39^{b}	6.65 ± 0.51^{c}
August	1.15 ± 0.08^{a}	4.56 ± 0.35^{b}	6.49 ± 0.49^{c}
September	0.93 ± 0.07^{a}	1.24 ± 0.09^{a}	4.63 ± 0.35^{b}
October	0.43 ± 0.03^{a}	1.43 ± 0.11^{b}	2.26 ± 0.17^{c}
November	0.58 ± 0.04^{a}	0.67 ± 0.05^{b}	1.19 ± 0.09^{b}
December	0.04 ± 0.00^{a}	0.30 ± 0.02^{b}	0.71 ± 0.05^{c}
January 2006	0.19 ± 0.01^{a}	0.40 ± 0.03^{b}	0.68 ± 0.05^{c}
February	0.21 ± 0.01^{a}	0.58 ± 0.04^{b}	0.79 ± 0.06^{c}
March	0.29 ± 0.02^{a}	0.60 ± 0.04^{b}	1.17 ± 0.09^{c}
Overall mean (per trap) [1]	$\mathbf{0.61 \pm 0.04^{a}}$	$\mathbf{1.52 \pm 0.13^{b}}$	$\mathbf{3.02 \pm 0.19^{c}}$

[1] Means with different letters in the same row are significantly different at p < 0.05, ANOVA with LSD.

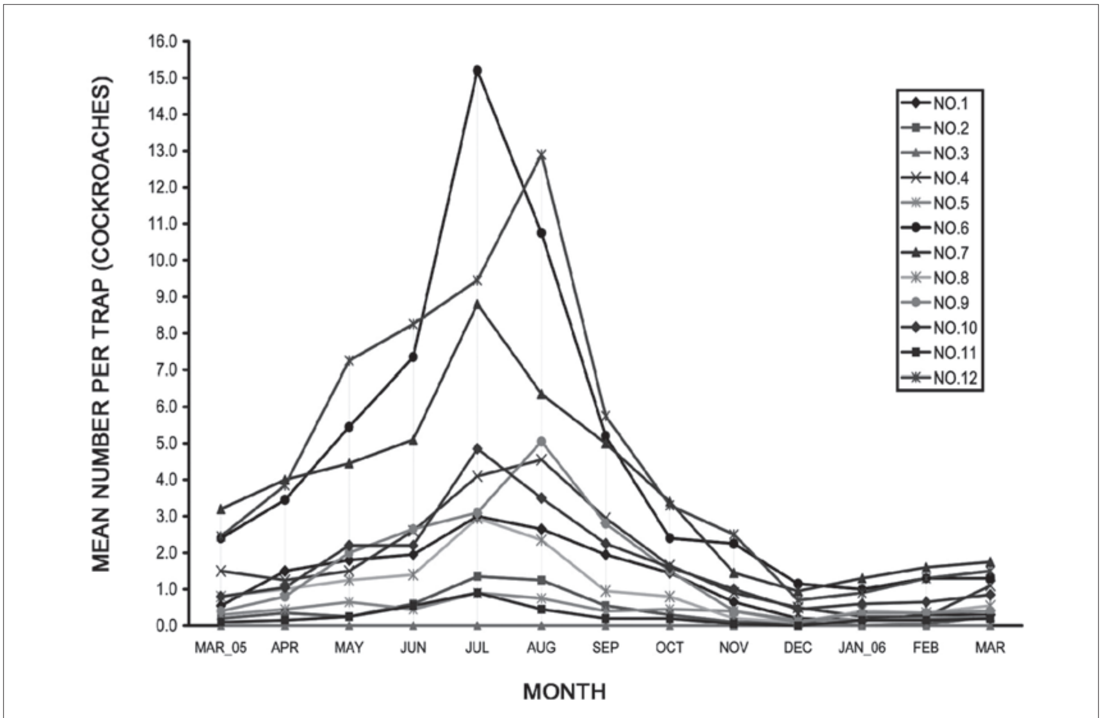

Figure 3 The curves of mean numbers of the German cockroaches caught per trap from twelve Bangkok markets. Each point on a curve represents a mean number (per trap) in each month during the study period.

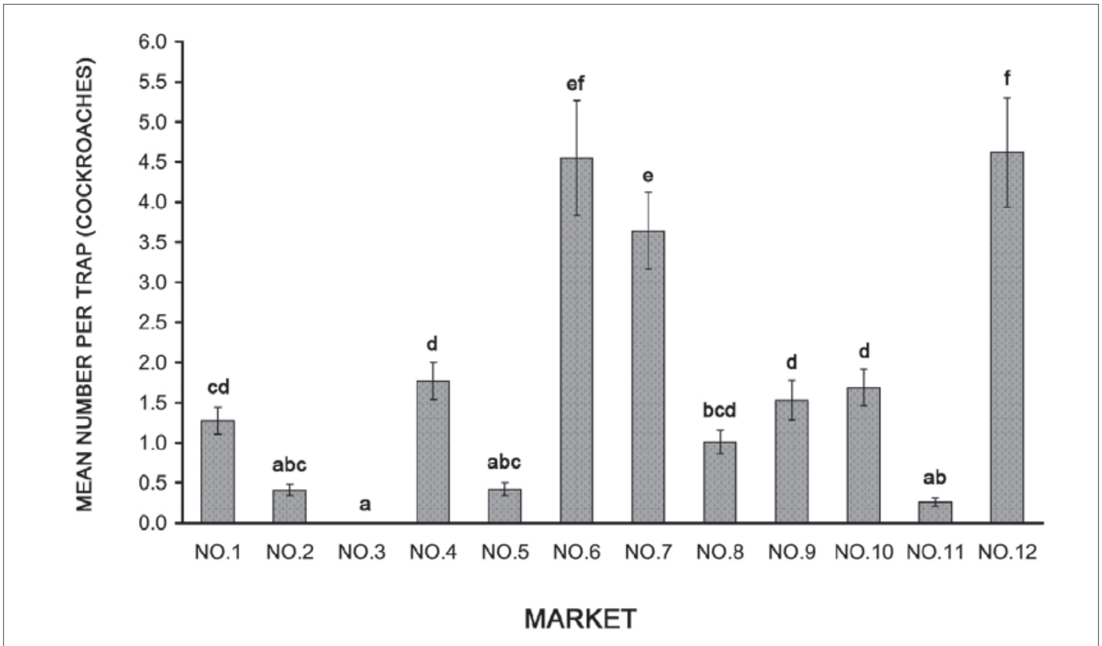

Figure 4 Comparison of mean numbers (± SE) of the German cockroaches caught per trap from twelve markets from March 2005 to March 2006. The bar graphs with different letters are significantly different at $p < 0.05$, ANOVA with LSD.

Discussion

A total of 11,944 cockroaches were trapped from the twelve Bangkok markets. They consisted of seven species, belonging to six genera, such as *Blattella germanica*, *Nauphoeta cinerea*, *Neostylopyga rhombiofolia*, *Periplaneta americana*, *P. brunnea*, *Supella longipalpa*, and *Symploce pallens*. The German cockroach, *B. germanica*, were caught in the largest number (46.12 %), followed by *P. americana* (45.30 %). This result is similar to those reported earlier by Lee et al. (1993), Benjapong et al. (1997), Tawatsin et al. (2001), Sriwichai (2001), Damsuwon (2003), Lee et al. (2003), and Pai et al. (2005). The former reports showed that the *B. germanica* and *P. americana* were dominant cockroach species in urban environments in Malaysia, Republic of Korea, Taiwan, and Thailand. Additionally, this is the first record of *Sy. pallens* in Thailand. This species had only been recently reported as a domiciliary pest (Lee and Lee 2000) and it has been reported as a new domiciliary pest in Malaysia (Lee et al. 2000).

During the 13-month-study, the German cockroaches were abundant throughout the year that may be because of their short life cycle leading to rapid population recovery (Ross and Mullins 1995). Other causation were the proper temperature (average degree of temperature: minimum 26.02 ± 0.11 °C; maximum 35.17 ± 0.02 °C), the suitable humidity (average percentage of humidity: minimum 59.00 ± 1.48 % RH; maximum 68.75 ± 0.97 % RH), suitable harborage, and the presence of food and water in the markets. The two highest mean number peaks of the German cockroaches caught per trap were in July and August whereas the two lowest peaks were in December and January. The German cockroaches found in July and August in a large number indicated that the German cockroaches had high reproductive activity. On the other hand, the small number found in December and January suggests that the German cockroaches had low reproductive activity of in these months. The temperature and relative humidity may be the important factors influencing on the cockroach reproduction (Ross and Mullins 1995). In this study, the large nymphs of the German cockroach were trapped in the highest number followed by adults, medium nymphs, and small nymphs. The large nymphs and the adults indicated that they had high foraging activity (Cloarec and Rivault 1991, Ross and Mullins 1995). The adult males had more foraging activity than the females and the females with oothecae, respectively (Metzger 1995). While the small nymphs were found in a small number implied that they had the less foraging activity than the other stage of the German cockroaches (Cloarec and Rivault 1991, Ross and Mullins 1995). In addition, the low weight of the small nymphs might be another reason for the lowest number trapped. It might be because of the small nymphs escaped from the slippery inner surface of the jar traps (Wang and Bennett 2006).

The nymphal stages were found in the highest number in the population of

Bangkok markets, this result was consistent with the prior studies (Owens and Bennett 1983, Ross and Muliins 1995). However, the nymphals were found at 66.19 % of the German cockroach population in the field of this study while the nymphal stages of populations growing under optimal conditions in laboratory were found at 80 % (Ross and Mullins 1995). It may be because of the external factors including pathogens, predators, and physical environments.

Market number 3 was classified in class 1 market while market number 12 was classified in class 2 market. In this study, the German cockroaches found in market number 12 with the largest number whereas none of the German cockroaches was caught from the market number 3. The suitable temperature, proper humidity, poor sanitation, infrequent cleanliness, and the old structure which make several cracks and crevices of the market number 12 may be the reason of the high population of the German cockroaches in this market. In contrary, none of the German cockroaches was caught in market number 3 may be because of the unsuitable humidity, the occurrence of predator such as cat, and the good sanitary practice of the vendor such as clean and clear materials on the shelf after finishing their work in everyday. Although none of the German cockroaches was caught, other species of cockroaches were caught in this market such as *Na. cinerea*, *Ne. rhombiofolia*, *P. americana*, *P. brunnea*, and *Sy. pallens*. It might be because *P. americana*, and *P. brunnea* usually inhabit in the more moist condition than the German cockroaches (James and Harwood 1969, Sriwichai 2001, Damsuwon 2003).

The catch number of the German cockroaches in the grocery was statistically higher than that in the vegetable shop and butcher shop. It may be because the groceries have many crevices and corners for their harborage. The infrequently cleanliness was another reason that a large number of the German cockroaches were caught in this shop. From the observation, when the butcher shop owners finished their work, they collected their meat and cleaned their shop daily. The vegetable shop owners also collected their vegetable from shelf and they cleaned their shop sometimes. However, the grocery shop owners also collected their goods, covered their shelf with plastic sheets but they cleaned their shop infrequently. It is similar to the previous report by Appel (1995) that the German cockroaches preferred a warm microhabitat and dark harborage for their shelter.

In conclusion, the German cockroaches were found in various types of places in the markets. They were abundant throughout the years in these markets that may be because these markets had a lot of food and water, proper temperatures, suitable humidity, good harborage, and infrequent cleanliness. Thus, the German cockroach control in the Bangkok markets should be done by integration between the sanitary control, such as removal of the food supply and elimination of the shelter of the cockroaches, and chemical control measures (Gold 1995, Koehler *et al*. 1995).The chemical control measures should be done at night because the German cockroaches are nocturnal insects (Ross and Mullins 1995). Moreover, the effective chemical control should be applied in December and January because the two lowest peaks of this cockroach species shown in these two months.

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Flies

Mitochondrial DNA - Based Identification of Some Forensically Important Blowflies in Thailand

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Abstract: Accurate identification of insects collected from death scenes provides not only specific developmental data assisting forensic entomologists to determine the postmortem interval more precisely but also other kinds of forensic evidence. However, morphological identification can be complicated due to the similarity among species, especially in the early larval stages. To simplify and make the species identification more practical and reliable, DNA - based identification is preferentially considered. In this study, we demonstrate the application of partial mitochondrial cytochrome oxidase I (COI) and cytochrome oxidase II (COII) sequences for differentiation of forensically important blowflies in Thailand; *Chrysomya megacephala, Chrysomya rufifacies and Lucilia cuprina* by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The PCR yields a single 1324 bp-sized amplicon in all blowfly specimens, followed by direct DNA sequencing. Taq$^{\alpha}$I and VspI predicted from the sequencing data provide different RFLP profiles among these three species. Sequence analysis reveals no significant intraspecific divergence in blowfly specimens captured from different geographical regions in Thailand. Accordingly, neighbor-joining tree using Kimura's 2-parameter model illustrates reciprocal monophyly between species. Thus, these approaches serve as promising tools for molecular identification of these three common forensically important blowfly species in Thailand.

1. Introduction

Estimation of postmortem interval (PMI) is crucial for time of death determination. Some physiological changes such as body cooling, lividity, and mechanical excitability of skeletal muscle have been used to calculate the early postmortem period. However, such postmortem changes may not be a reliable parameter for approximation of PMI after 48-72 h. After decomposition is initiated, developmental period from hatching to larval stage of insects collected from corpses is the best evidence for PMI estimation [1].

Blowflies play a role in the early decomposition process because of their great abundance and behaviours including feeding and egg laying on corpses. Unsurprisingly, blowfly larvae are usually collected from death scenes and also determined as the best entomological indicator for PMI estimation. The challenging step for forensic entomologists is larval species identification because many morphological similarities among closely related species make difficulties in definite differentiation. A taxonomic key covering all immature stages of common forensically important insect species in all geographical regions is yet unavailable. Although electron microscopy-based identification of some chrysomyine larval stages has been proposed [2], it is not practical and requires many special skills for sample preparation. Alternatively, rearing larvae to the adult stage followed by traditional identification based on the adult morphological characteristics can be performed, but rearing is a time-consuming procedure. Moreover, specimens may be killed or damaged before arrival at the laboratory.

Several studies using DNA-based identification of some forensically important blowfly specimens have been reported [3-7]. These molecular tools can overcome many difficulties associated with morphological problems as previously described. At present, mitochondrial DNA (mtDNA) is preferably applied for forensic investigations because greater abundance in tissues, when compared with nuclear DNA (nuDNA), makes it easier for extraction even from small amount of sample [6]. In addition, because of its strictly maternal inheritance and no genetic recombination, mtDNA haplotype is a good candidate for evolutionary and population genetics study. Especially, mitochondrial cytochrome oxidase I and II (COI-COII) genes are suitable as molecular markers because relatively a high degree of genetic variation in this region has been reported [6,7].

Chrysomya megacephala and Chrysomya rufifacies are the most common blowfly species in all locations of Thailand and have been extensively documented from forensic cases in both urban and forested areas [8]. *Lucilia cuprina* is another species of forensic interest since it can be found in both urban and forested locations, although less frequently than *Chrysomya* species. Interestingly, it has also been reported that this blowfly species can be found with *C. megacephala* and *C. rufifacies* in the same cadavers [8]. It should be mentioned, however, that molecular identification and genetic relationships of these three blowfly species of forensic importance have not been reported before. Therefore, our approach is to demonstrate the utility of mitochondrial cytochrome oxidase genes for identification purpose and phylogenetic analysis of these three common forensically important blowflies.

2. Materials and methods

2.1. Specimens collection

Adult blowfly specimens were collected from various regions of Thailand including Bangkok, Phitsanulok, Chiang-Mai, Tak, Chumphon and Buri-Ram (Figure 1) using fly-trap method as previously described [9]. Briefly, pork viscera were used as baits for trapping blowflies in a large plastic bag for 24 h. Flies were anesthesized in a freezer at -20 °C for 1 h and then identified morphologically under a stereomicroscope (SZX9, Olympus, Tokyo, Japan), by reference to the taxonomic key of Crosskey and Lane [10]. The identified flies were stored in 70% ethanol and kept at 4 °C until use.

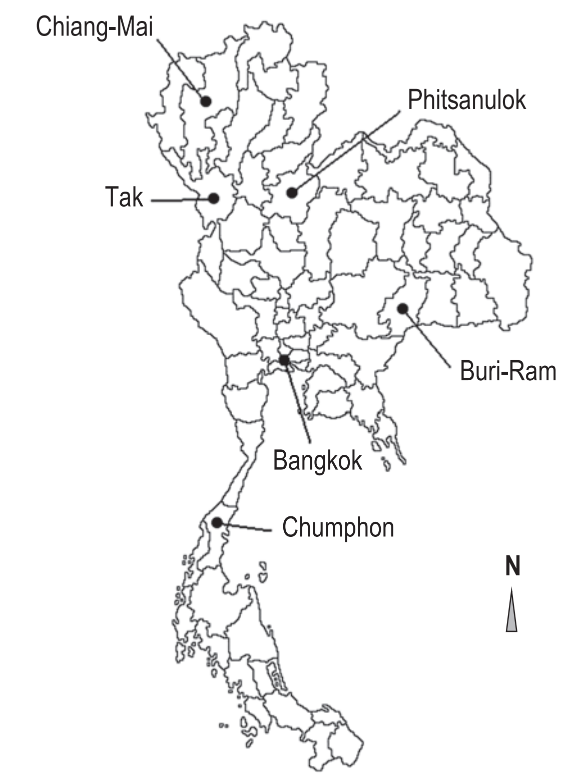

Figure 1 Map of Thailand, showing the locations for collection of blowfly specimens.

2.2. DNA extraction

Total DNA was prepared from thorax and legs of the fly specimens using QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. The extracted fly DNA was eluted in 200 µl of elution buffer and kept at -20 °C for long term storage. The fraction of extracted DNA was spectrophotometrically quantitated and diluted to 50 ng/µl prior to PCR amplification step.

2.3. PCR amplification

Using the total DNA as a template, partial COI-COII region was amplified. Two oligonucleotide primers including forward primer (5′-CAGCT-ACTTTATGAGCTTTAGG-3′) and reverse primer (5′-GAGACCATTACT-TGCTTTCAGTCATCT-3′) were designed following previous studies [3,7]. The amplification reaction was set up in a final volume of 25 µl, containing 150 ng of extracted DNA, 0.4 µM of each primer, 2.5 mM of $MgCl_2$, 200 µM of dNTPs and 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad CA, USA). The PCR reactions were performed in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystem, Foster city, CA, USA) using the condition as follows: the initial denaturation (94 °C for 3 min); the subsequent 5 cycles consisted of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 1.5 min; followed by 25 cycles of 94 °C for 45s, 60 °C for 45 s and 72 °C for 1.5 min; and the final extension at 72 °C for 10 min. Aliquots of the amplicons were detected on 1% agarose gel electrophoresis.

2.4. DNA sequencing and restriction patterns prediction

In order to verify unique restriction sites for PCR-RFLP, the PCR products were purified from agarose gel by using a Perfectprep® Gel Cleanup kit (Eppendorf, Germany) following the manufacturer's instructions. Direct DNA sequencing was performed by using ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Applied Biosystems Division, Foster City, CA). Determination of the nucleotide sequences was performed and analyzed in both directions using the same forward and reverse primers used in PCR step to ensure that variations of nucleotide sequences were not due to sequencing errors. The reaction products were analyzed with an automated ABI PRISM® 310 Genetic Analyzer (PerkinElmer, Foster City, CA). The resulting sequences were used for prediction of species-specific restriction sites by using the NEBcutter V2.0 web-based program (available at http://tools.neb.com/NEBcutter2/index.php). From restriction prediction data, two restriction endonucleases ($Taq^{\alpha}I$ and VspI) were chosen for restriction fragment length polymorphism (RFLP).

2.5. RFLP

The PCR products were digested in separate reaction with Taq$^{\alpha}$I and VspI (New England Biolabs, Ipswich, USA). Reaction mixture was composed of approximate 500 ng of PCR product, 1 µl of 10 × appropriate buffer, 2 units of restriction enzyme and DNase-free water to final volume of 10 µl. The mixture was incubated overnight at 65 °C and 37 °C for Taq$^{\alpha}$I and VspI, respectively. The restriction products were electrophoresed through 6% native polyacrylamide gel electrophoresis 6% gel concentration (T), 3.3% crosslinking (C), 1 × TBE buffer (89 mM Tris- base, 89 mM Boric acid, 2 mM EDTA, pH 8.3), run at 100 V for 60 min [11], followed by ethidium bromide staining and visualized on a Gel Doc EQ system (Bio-Rad, CA, USA).

2.6. Sequence analysis and phylogenetic tree construction

Nucleotide sequences were prepared and analyzed using Chromas Lite version 2.01 (http://www.technelysium.com.au) and BLAST search (http://www.ncbi.nlm.gov/BLAST) for species identification. All partial nucleotide sequences of COI-COII genes obtained from this study were submitted to the GenBank database and assigned accession numbers as FJ153258-FJ153278. Sequences were aligned by using the Clustal W algorithm implemented in the BioEdit Sequence Alignment Editor v. 6.0.7 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Percentage of G+C contents and sequence identity matrix were also calculated using the BioEdit program. Phylogenetic tree based on the COI-COII sequences was constructed by neighbor-joining method using the Kimura's 2-parmeter model implemented in the MEGA© version 3.1 [12] and the tree was tested by 1000 bootstrap replicates. Bootstrapping values indicate percentage support for grouping by random resampling of the data.

3. Results

3.1. Amplification of mitochondrial COI-COII genes

Result obtained from PCR amplification and agarose gel electrophoresis revealed that total DNA extracted from thorax and legs of an adult fly was appropriate enough to serve as a template for amplification of mitochondrial COI-COII genes. The specific primers used in this study yielded the PCR-amplified product of 1.3 kbp in length for all blowfly species tested as shown in Figure 2.

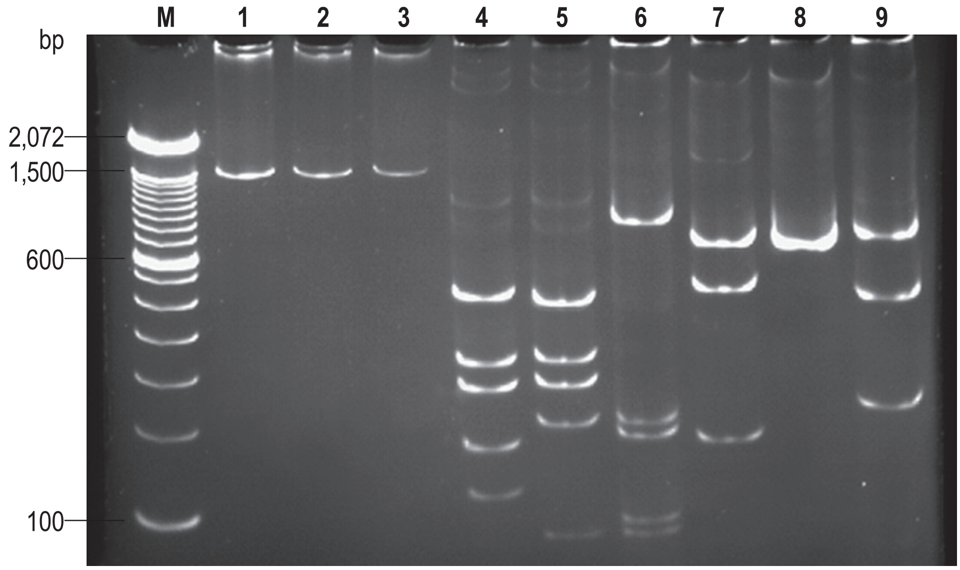

Figure 2 The 6% native polyacrylamide gel shows different PCR-RFLP patterns of COI-COII amplicons digested with two restriction endonucleases. From left to right as follows: undigested PCR products from *C. megacephala, C. rufifacies* and *L. cuprina,* respectively (lane 1-3); *C. megacephala/Taq* $^{\alpha}$I (lane 4); *C. rufifacies/Taq* $^{\alpha}$I (lane 5); *L. cuprina/Taq* $^{\alpha}$I (lane 6); *C. megacephala/Vsp*I (lane 7); *C. rufifacies/Vsp*I (lane 8); *L. cuprina/Vsp*I (lane 9). Lane M is 100 bp DNA standard marker.

3.2. Identification and distribution of blowfly species in Thailand

In this study, 20 blowflies were collected from 6 different regions of Thailand (Figure 1) including Bangkok (Center), Chiang-Mai, Tak, Phitsanulok (Northern), Buri-Ram (Eastern) and Chum-phon (Southern). Based on the sequence analysis and BLAST search of partial COI-COII sequences (1200 bp in length), the blowflies could be classified into 3 species: *C. megacephala*, *C. rufifacies* and *L. cuprina*. These bioinformatics results were consistent with the taxonomic key-based identification (data not shown). From the 20 blowflies collected, 12 specimens (from all 6 regions of Thailand) were identified as *C. megacephala*; 5 specimens were of *C. rufifacies* (isolated from Bangkok, Chiang-Mai and Phitsanulok), whereas the remaining 3 specimens (collected only in Chiang-Mai) were classified as *L. cuprina* (Table 1).

Table 1 Isolate code, sex, location and accession number of each blowfly used in this study

Blowfly species	Sex	Location	Accession no.
Chrysomya megacephala			
A01	♂	Tak	FJ153258
A02	♀	Chiang-Mai	FJ153259
A03	♂	Chiang-Mai	FJ153260
A04	♂	Chiang-Mai	FJ153261
A05	♂	Chiang-Mai	FJ153262
A06	♀	Buri-Ram	FJ153263
A07	♀	Buri-Ram	FJ153264
A08	♀	Phitsanulok	FJ153265
A09	♂	Phitsanulok	FJ153266
A10	♂	Chumphon	FJ153267
A11	♂	Chumphon	FJ153268
A12	♀	Bangkok	FJ153269
Chrysomya rufifacies			
B01	♀	Bangkok	FJ153270
B02	♂	Bangkok	FJ153271
B03	♀	Phitsanulok	FJ153272
B04	♀	Chiang-Mai	FJ153273
B05	♂	Chiang-Mai	FJ153274
Lucilia cuprina			
C01	♀	Chiang-Mai	FJ153275
C02	♀	Chiang-Mai	FJ153276
C03	♂	Chiang-Mai	FJ153277
Musca domestica (Outgroup)			
D01	♂	Bangkok	FJ153278

3.3. Discrimination of blowfly species in Thailand based on PCR-RFLP

According to the sequence analysis of partial COI-COII genes, the restriction sites and suitable restriction enzymes were selected by using web-based bioinformatics program (NEBcutter V2.0). Two restriction enzymes (Taq$^{\alpha}$I and VspI) were used in separate reactions in order to generate distinct RFLP patterns and thus be applied for discrimination among different blowfly species. Digestion with Taq$^{\alpha}$I and VspI yielded different RFLP patterns in each species as summarized in Table 2. The representative of restriction patterns for each species is shown in Figure 2.

Table 2 Comparative RFLP patterns among three blowfly species

Blowfly species	*Taq* $^{\alpha}$I digestion	*Vsp*I digestion
Chrysomya megacephala	30, 111, 156, 264, 307 and 456 bp	4, 12, 160, 495 and 653 bp
Chrysomya rufifacies	30, 81, 186, 264, 307 and 456 bp	12, 653 and 659 bp
Lucilia cuprina	30, 81, 86, 177, 188 and 762 bp	210, 450 and 664 bp

3.4. Sequences analysis and comparison

Multiple-alignments of 20 partial mitochondrial COI-COII sequences were performed by using Clustal W implemented in the BioEdit Sequence Alignment Editor version 6.0.7. Analysis of the mean G+C contents revealed slight differences between species, ranging from 28.95% (*C. megacephala*), 28.26% (*L. cuprina*) and 27.37% (*C. rufifacies*). Sequence comparisons between the same species showed that percentage of sequence similarity ranged from 99.7 to 100 (mean 99.91%), 99.6 to 100 (mean 99.72%) and 99.7 to 99.9 (mean 99.80%) for *C. megacephala*, *C. rufifacies* and *L. cuprina*, respectively. The result indicated that there was no significant divergence of partial mitochondrial COI-COII sequences within the same species isolated from different geographical regions in Thailand. On the other hand, sequence comparisons among different blowfly species revealed that percentage of sequence identity between *C. megacephala* and *C. rufifacies* ranged from 93.0 to 93.3 (mean 93.18%), whereas similarity between *C. megacephala* and *L. cuprina* ranged from 60.0 to 60.2 (mean 60.04%) and that between *C. rufifacies* and *L. cuprina* ranged from 60.0 to 60.3 (mean 60.13%). The interspecific sequence comparison indicated that the partial mitochondrial COI-COII sequence provided effective data for discrimination among these three blowfly species in Thailand. Table 3 shows the identity matrix representing the similarity between each individual sequences.

3.5. Phylogenetic analysis

Phylogenetic tree based on COI-COII nucleotide sequences of blowflies from several worldwide geographical areas including available data from GenBank was constructed by neighbor-joining (NJ) method with the Kimura's 2-parmeter model implemented in the MEGA® version 3.1 and the tree were tested by 1000 bootstrap replicates (Figure 3). *Musca domestica* was used as the outgroup of the phylogenetic tree. According to the tree, all of the *C. megacephala* isolates clustered together showing no significant differentiation between different regions. *C. megacephala* and *C. rufifacies* could be well separated although they belonged to the same genus, implying that the COI-COII sequence was useful for identification of these congeneric species. All of the *C. rufifacies* isolates formed a single cluster with branches indicating minor nucleotide variations between the same species. *L. cuprina* was clearly separated from *C. megacephala* and *C. rufifacies* and all 3 isolates were clustered together.

Table 3 Identity matrix reveals the percentage of partial COI-COII sequence similarity between each isolate

Code	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	B01	B02	B03	B04	B05	C01	C02	C03
A01																				
A02	99.8																			
A03	99.8	100																		
A04	99.8	100	100																	
A05	99.8	100	100	100																
A06	99.8	100	100	100	100															
A07	99.8	100	100	100	100	100														
A08	99.8	100	100	100	100	100	100													
A09	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9												
A10	99.8	100	100	100	100	100	100	100	99.9											
A11	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.9										
A12	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.9	99.8									
B01	93.1	93.3	93.3	93.3	93.3	93.3	93.3	93.3	93.2	93.3	93.2	93.2								
B02	93.1	93.3	93.3	93.3	93.3	93.3	93.3	93.3	93.2	93.3	93.2	93.2 9	99.8							
B03	93	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93	93.1	93	93	99.6	99.6						
B04	93.1	93.3	93.3	93.3	93.3	93.3	93.3	93.3	93.2	93.3	93.2	93.2	99.8	100	99.6					
B05	93	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93	93.1	93	93	99.6	99.8	99.6	99.8				
C01	60.1	60	60	60	60	60	60	60	60	60	60.1	60	60	60.1	60.2	60.1	60.1			
C02	60.2	60.1	60.1	60.1	60.1	60.1	60.1	60.1	60	60.1	60.2	60	60.1	60.2	60.3	60.2	60.2	99.9		
C03	60.1	60	60	60	60	60	60	60	60	60	60.1	60	60	60.1	60.2	60.1	60.1	99.7	99.8	
D01	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	58.4	58.4	58.3	58.4	58.3	53.3	53.2	53.4

A01-A12 (*Chrysomya megacephala*); B01-B05 (*Chrysomya rufifacies*); C01-C03 (*Lucilia cuprina*) and D01 (*Musca domestica*)

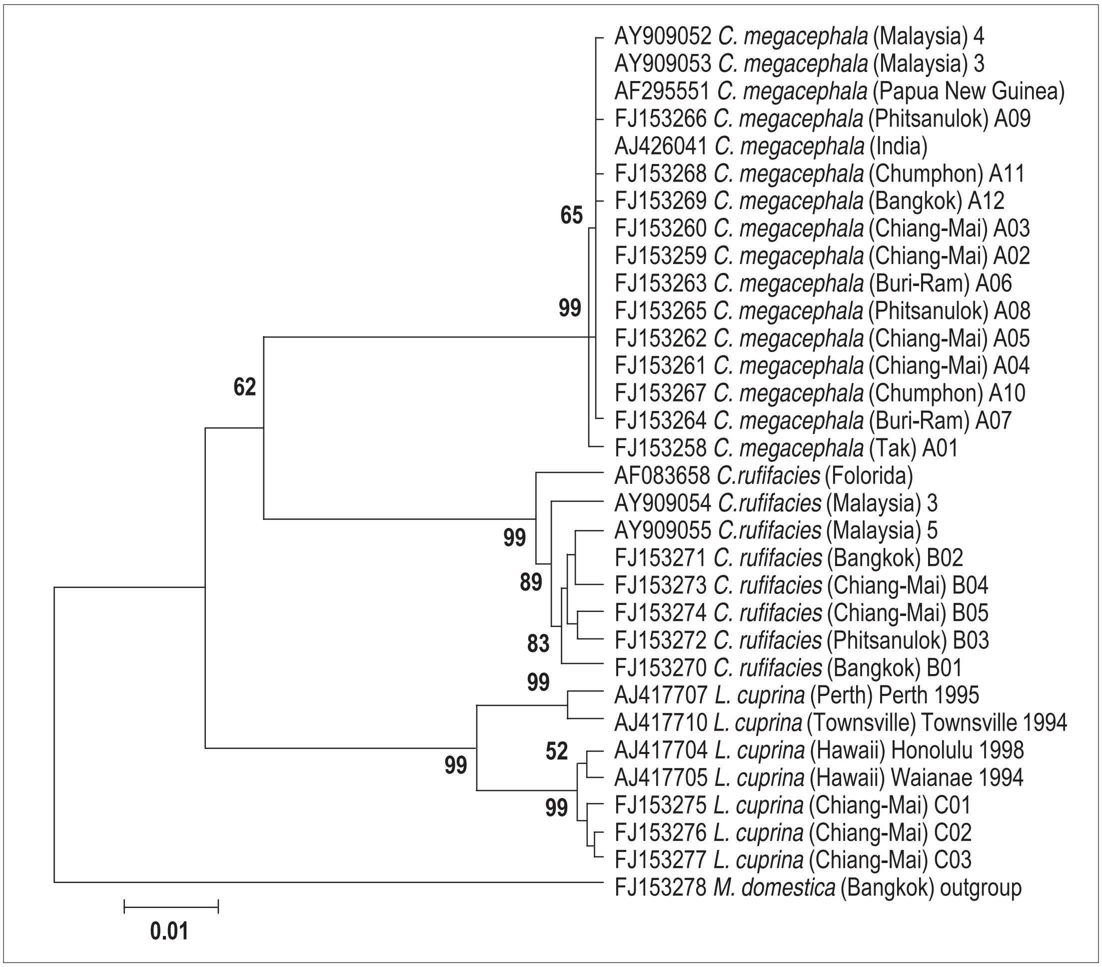

Figure 3 The neighbor-joining tree using Kimura's 2-parameter model illustrating phylogenetic relationships among three blowfly species and one housefly outgroup, based on the COI-COII nucleotide sequences data.

4. Discussion

In accordance with previous reports [13-15], *C. megacephala* is the most common blowfly species and more easily captured than other species, throughout Thailand. Boonchu et al. [9] found that *C. megacephala* (96.3%) represented the majority of all species whereas *C. rufifacies* (0.2%) was rarely captured by this trapping method. A possible explanation for their abundance is their capacity to utilize a wide range of ecological niches throughout Thailand. Additionally, each blowfly species may be differentially attracted by the baits used. Sukontason et al. [8] reported that only *L. cuprina*, not *L. sericata*, was found in forensic cases in Thailand and, to our knowledge, *L. sericata*, the sister species of *L. cuprina*, has never yet been reported in forensic entomology cases in Thailand.

Although PCR-RFLP used to be a preferred method for polymorphism demonstration, this method has some problems since restriction sites can mutate or get lost despite no morphological change. In addition, some authors state that identification by this strategy will be accurate when enough samples of overall flies in the taxa are done as reference profiles [16,17]. However, RFLP serves some advantages for forensic personnel, by partly assisting in the exclusion of some fly species and for screening purposes.

In this study, we demonstrated the value of PCR-RFLP in differentiating three blowfly species, *C. megacephala*, *C. rufifacies* and *L. cuprina*. The 6% native polyacrylamide gel showed good resolution for discriminating two small fragments (81 and 86 bp) for *L. cuprina* by Taq$^{\alpha}$I digestion. On the other hand, VspI digestion provided two larger fragments (653 and 659 bp) overlapping like a single band on the gel, for *C. rufifacies*.VspI also demonstrated different profiles between *C. megacephala* and *L. cuprina*. Very small fragments (<50 bp) often disappear in Figure 2 due to low ethidium bromide fluorescence intensity and possible diffusion during electrophoresis. All samples after digestion by Taq$^{\alpha}$I and VspI showed similar restriction profiles within same species (data not shown). This result showed that intraspecific polymorphism was not observed here by RFLP. Ethidium bromide-stained polyacrylamide gel techniques demonstrate that the different species-specific restriction profiles are suitable to apply in routine forensic laboratory tests. Although the PCR-RFLP can differentiate these three blowfly species, fly specimens collected from the death scene could be of other closely related species [16-18], a situation which could potentially lead to misidentification errors.

To distinguish sister species more effectively, phylogenetic analysis has been widely employed [16-19]. From Figure 3, maximum parsimony phylogeny constructed from global data (available in GenBank) reveals reciprocal monophyly between species. Table 3 also shows that intraspecific variation was just detected ≤0.3% for *C. megacephala*, ≤0.4% for *C. rufifacies* and ≤0.3% for *L. cuprina*, supporting monophyletic pattern. However, Wells et al. [16-18] have emphasized the importance of using a broad enough genetic database of all relevant species as an essential key for accurate species identification by phylogenetic analysis of COI sequence. In addition, phylogenies based on different test loci may be discordant. Stevens et al. [19] found that phylogenies constructed from worldwide *L. cuprina* and L. sericata were paraphyletic using COI but each monophyletic for 28S ribosomal RNA gene. Therefore, the pitfalls of this study are small sample size and lack of genetic database of close relatives at different test loci for validating the methods. However, the current study successfully demonstrates not only the application of mitochondrial cytochrome oxidase genes for species identification, but also provides phylogenetic information for these common forensically important blowflies from several geographical areas of Thailand.

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Advance of Natural Products

Recent Advances in the Development and Application of Natural Products (biopesticides) for the Management of Disease Vectors and Pests of Public Health Importance

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Abstract: At the outset, natural products used in pest control consisted of plant parts, preparations, and bioactive principles. Such plant-based products have been used in the control of phytophagous insects and insects of public health importance for centuries. However, the development and use of phytochemicals attracted considerable attention from researchers and industrial concerns in the last quarter of a century. Examples of major plant-based products used in pest control are pyrethrins, neem constituents, as well as many plant volatile essential oils for repelling haematophagous insects affording personal protection of humans from biting arthropods and noxious insects. Considerable advances have been made in formulating phytochemicals to increase their efficacy, providing protection and acceptability in agriculture and public health.

A major breakthrough in the development and practical use of natural products occurred in the late 1970s, when a spore-forming bacterium producing entomotoxins was discovered. This organism was designated as *Bacillus thuringiensis* ssp. *israelensis* (Bti). The discovery of this organism opened the door for launching efforts to isolate and identify other entomopathogenic organisms. By 1985, large quantities of Bti formulations were employed in mosquito and black fly control programs globally. As research efforts in this area continued, a second bacterium *Bacillus sphaericus* (Bsph) was isolated and found to be highly effective against most mosquito species. This entomopathogen found its way in mosquito control around 1997. Today, both these (Bti and Bsph) microbial control agents are produced by large-scale fermentation process in many countries. Recent advances in formulation technology of both bacteria have increased their usefulness in vector control programs.

In the early 1990s, another soil bacterium known as *Saccharopolyspora spinosa* (actinomycete), was discovered, producing bioactive components known as spinosyn A and spinosyn D. Large-scale fermentation technology has been developed and the product known as spinosad was produced and developed in agriculture and recently for use in public health. It has been found to have considerable activity against insect pests of some 200 major crops and is currently

used in agriculture in many countries. Spinosad has not been used to any great extent in public health as yet. It is anticipated that use of spinosad products will be launched and expanded during 2009/ 2010 in mosquito control programs on a world-wide basis. It should be pointed out that all three microbial control agents have a good margin of safety for mammals, birds and wildlife and are environmentally friendly.

Also in the last quarter century, considerable efforts were directed toward developing plant extracts for vector control especially mosquitoes. In this context plant-based extracts were formulated for the control of larval and adult mosquitoes. Some of the plant parts were used in manufacturing mosquito coils which number into the billions on a world-wide basis. Only a small proportion of coils is manufactured with plant-derived products. A major area for the development of plant based products is the use of plant essential oils which are formulated in various ways for personal protection from the attack of haematophagous insects. A number of such insect repellent products are commercialized in many countries. The public has the perception that plant-based and other natural products are environmentally friendly and safer to use for vector control or apply to human skin as personal protectants than synthetic chemicals.

In this chapter, we will dwell upon the research and development efforts leading to the development, production and application of microbial control agents, and phytochemicals for the control of adult and preimaginal stages of disease vectors, as well as the development and use of plant essential oils for personal protection from anthropophilic insects.

Introduction

In recent times, many terms and expressions have been coined to describe a new and improved breed of pesticides now known as "Biopesticides." Equivalent terms describing this group of agents are: biorational pesticides, biopesticides, biocides, biological pest control agents, natural products, botanicals, and naturalytes (Menn 1999, Mulla 1997). The two terms that have greater use and acceptance are "biopesticides" and "natural products." We have been promoting and holding "The International Conference on Biopesticides" for some 14 years and this conference here in Delhi, India is the 5th such one. These conferences have been quite successful and attendance and research presentations have been increasing. Proceedings of the past four conferences have been published.

Since biopesticides in the broad sense include tools and strategies for many pests and disease problems caused by pathogens and insects, the subject now covers insect pest management, weed control, semiochemicals, antagonistic bacteria and fungi and finally transgenic crop plants or mosquitoes. The core disciplines of biopesticides research have been bearing on the management of crop and public health insects, fungal and bacterial diseases of crops and other injurious agents through the use of "biopesticides" or natural products. This subject covers many other themes as you can gather from thematic subjects in this conference.

Mulla (1997) at the first International Conference on "Biopesticides" defined and characterized "biopesticides". A couple years later, a book edited by Hall and Menn (1999) titled "Biopesticides" presents a comprehensive treatment of this subject. Various experts in the area of "biopesticides" research and development provided chapters and critical reviews of this subject matter. This volume covers topics related to the discovery, isolation, safety, toxicology, development, registration, labeling, commercialization and application of biopesticides in pest control programs. Although the earlier attempts were on finding and developing biopesticides for plant pest control, recent world-wide research efforts have come up with effective and viable "biopesticides" for the control of insects of public health importance.

In this chapter, we will discuss the development and use of microbial control agents and their toxins and plant based products for the management of insects of public health importance. In the past 30 years or so, great strides have been made in discovering and developing entities with novel mode of action that provide viable strategies for solving the major resistance problems encountered in the management and control of medical insects.

Entomopathogenic bacteria-their fermentation products employed in vector control programs

At the advent of synthetic insecticides after world war II, DDT and related compounds were largely used in vector control programs until the emergence of resistance in many target species. The organophosphorous group of insecticides provided substitutes for the organochlorine compounds. Synthetic pyrethroids and insect growth regulators were the next generation of insecticides developed for pest control. Resistance to some or all of these groups appeared soon after use in some areas of the world, but at much slower rate to IGRs. Also the inherent mammalian and wildlife toxicity and environmental contamination problems of synthetics caused disuse of some of these once highly effective insecticides.

By the early 1980s, vector control programs in some areas of the world felt the impact of resistance to insecticides. The World Health Organization at the end of 1970s, launched a world-wide effort to find and develop biological control agents with special emphasis on the isolation, characterization and development of microbial control agents for use in public health. As a result of this international effort many entomopathogenic organisms were isolated and evaluated against disease vectors. In addition to the WHO global initiative, industrial firms in many countries launched their own search to isolate and characterize entomopathogens. At the end of the 1970s, at the peak of resistance in mosquitoes to most synthetic insecticides, the spore-forming bacterium *Bacillus thuringiensis* ssp. *israelensis* (Bti) was discovered. Rapid development, registration and labeling of this microbial agent promoted advances in fermentation, production and formulation technologies. Within 5 years after its discovery its formulations were employed in large-scale mosquito and black fly control programs. The development of Bti was soon followed by the discovery of another spore-forming bacterium *Bacillus sphaericus* (Bsph), which was isolated from black flies from Africa. This bacterium showed exceptional activity against most mosquitoes. Bti was labeled for mosquito control in 1980, while Bsph was registered in 1997. These two microbial control agents and their formulations are used at present in large quantities in mosquito and other vector control programs.

Recently, another soil bacterium *Saccharopolyspora spinosa* (actinomycete) was isolated and studied for activity against insects in agriculture and public health. This bacterium on fermentation produces two bioactive compounds known as spinosyn A and spinosyn D. This product is named spinosad, which is currently used in pest control in agriculture, but it will be launched for use in public health insect control programs in 2009/ 2010. All three bacterial products are developed for the control of larval mosquitoes and other public health insects. We will discuss the development and efficacy of various formulations of the three microbial control agents (Bti, Bsph and spinosad) with details of the efficacy and evaluation of tailor- made formulations for use in a variety of habitats supporting immature stages of mosquitoes. It has been documented that in mosquitoes there is no cross resistance to these biological agents and further that no resistance has emerged to Bti. In some polluted habitats, some mosquito species have developed resistance to *B. sphaericus* after six to twenty applications. A solution to this resistance problem was found quickly, by using a mixture of Bsph and Bti, the later used in small proportions.

Bacterial larvicides

About three decades ago, world-wide research was launched to find, isolate, and develop entomopathogenic bacteria for the control of disease vectors, especially mosquitoes. As a result of this initiative and intensive search, several isolates and strains of two bacterial species which produced parasporal protein toxins showing high level of activity against mosquito larvae were discovered (de Barjac 1978, 1990a, 1990b, de Barjac and Sutherland 1990, Mulla 1991, Weiser 1984,WHO 1985). Although *Bacillus thuringiensis* Berliner had been discovered as an entomopathogen several decades ago, the strains discovered up to the 1970s showed activity against herbivorous insects only. It was not until the late 1970s, that a highly mosquitocidal strain of this bacterium was found. This pathogen later described and designated as *Bacillus thuringiensis* ssp *israelensis* (deBarjac 1978, 1990a) was isolated by Goldberg and Murgalit (1977). This pathogen was studied intensively and developed quickly for the control of mosquito and black fly larvae (de Barjac and Sutherland 1990, Mulla 1990).

Bacillus thuringiensis israelensis (Bti) evaluations against mosquitoes

Soon after its discovery, research on production and formulation technology was initiated by academic institutions, research institutes and the industry. Tremendous advances were made in this regard and a variety of formulations (liquid, powder, WDG, granules, briquettes, pellets, and others) were developed for different habitats and various species. A comprehensive tome on Bti (deBarjac and Sutherland 1990) was published on the very basic and applied aspects of bacterial pathogens. There are many publications covering research on laboratory and field evaluations of Bti and its formulations against various species of mosquitoes and black flies around the world. This information is all contained in this volume of "Bacterial Control of Mosquitoes and Black Flies" (deBarjac and Sutherland 1990). Information on laboratory activity and field efficacy is contained in this volume compiled by Mulla (1990). We will decline from reviewing or analyzing this published information, except to say that significant advances were made in the application of Bti and other microbes for the control of medically important insects. It should also be mentioned that a great deal of research has been conducted on the safety and environmental aspects of Bti (deBarjac and Sutherland 1990, Mulla 1995, Su and Mulla 1999b). For details, one is referred to these publications. We will, however, present and discuss our research findings on the evaluation of Bti formulations against *Aedes aegypti* (dengue vector) larvae breeding in water-storage containers commonly used in developing countries. Most of the other testing and evaluations have been published and contained in deBarjac and Sutherland (1990).

Bti formulations were rigorously subjected to field evaluation in water-storage containers where *Aedes aegypti*, a dengue vector breeds. This entomopathogen when tested in open bodies of water and impoundments as WDG formulation, showed short residual activity (Mulla 1990). However, controlled release formulations were developed which increased longevity of Bti against mosquito larvae. These formulations consisted of briquettes (Fansiri *et al* 2006), donuts, pellets, and tablets. The tablet formulation was tested and evaluated in water-storage containers (200 L of water) against *Ae. aegypti* larvae (Fig 1). The 200 L earthen jar is the most common jar used for water storage in Thailand and elsewhere. This formulation showed exceptional activity and longevity, giving

Figure 1 Placement of 200 L water storage earthen jars and plastic barrels used for testing microbial pest control agents and other insecticides against larvae of the mosquito *Aedes aegypti*, in Thailand.

prolonged control (>80% inhibition of emergence of adults) for about 112 days at the rate 1 tablet (0.37 g 2700 ITU/mg) per 50 L water (Mulla *et al*, 2004). Under the experimental conditions in water-storage containers, various factors such as sunlight, wind, water use practices (removal and addition) and water pollution were controlled, thus obtaining longevity for 90-112 days. Under normal use conditions; however, we expect that the duration of efficacy will be shorter; most likely lasting for 2 months or so, a period which is still adequate from the standpoint of operational control programs. We can get this inference from village trials of a temephos granular formulations applied to water-storage containers where its longevity was 2-3 months (Thavara *et al*, 2004) as compared to the longevity of 6-8 months, using the same formulations in the current experimental set up (Mulla *et al*, 2004).

In open bodies of water and impoundments, there are a number of other habitats factors which adversely affect the efficacy and longevity of Bti as well as other mosquito larvicides. Depth of water can reduce effectiveness against surface frequenting larvae. Vegetation and plant stand in water also reduces the effectiveness and longevity. Exposure of habitat to sunlight and wind can also reduce the efficacy and longevity of larvicides. Flowing water with currents will required higher dosages as movement of water dilutes the concentration. Mosquito breeding sources with these factors will require higher dosages of Bti and others than habitats not possessing these features. On account of these considerations applications of Bti (WDG) formulations produced mosquito larval control for only 7-12 days with 0.27-0.53 lbs/acre of WDG in open sunlit tubs (Su and Mulla 1999a). Granular formulations similarly used yielded only short-term control.

Bacillus sphaericus (Bsph) evaluations against mosquitoes

The second entomopathogenic bacterium discovered was *Bacillus sphaericus* strain 2362 from black flies in Nigeria (Weiser 1984). Other strains namely 2297 (Sri Lanka), 1593 (Indonesia), C3-41 (China) were also isolated (deBarjac 1990b). All these strains exhibited similar levels of activity against mosquito larvae, with strain 2362 in general showing somewhat higher level of activity (deBarjac and Sutherland 1990, Mulla 1991). None of the strains showed activity against *Aedes aegypti* (breeding in artificial containers) and *Aedes albopictus* breeding in natural containerized water such as tree holes, bromeliads, leaf axels, etc. Therefore, *B. sphaericus* (Bsph) strains were not evaluated against the two container-breeding mosquitoes.

B. sphaericus formulations were extensively tested against culicine and anopheline species in impoundments and open bodies of water. This entomopathogen proved highly effective against culicine mosquitoes in polluted water (deBarjac and Sutherland 1990, Mulla *et al,* 1988, 1997, 1999b, 2001a, 2003 Nicolas *et al,* 1987, Skovemond and Bauduin 1997). It was evaluated against *Culex* and *Anopheles* species throughout the world. The formulations tested were corn grit granules (VectoLex CG) and water dispensable granules (VectoLex WDG) and others. In the volume of deBarjac and Sutherland (1990), there are 3 chapters dealing with evaluation and practical application of *B. sphaericus* formulations. The chapter by L.A. Lacey (10 pages) with persistence and formulations of *B. sphaericus*, the chapter by JM Hougard (12 pages) covers formulations and persistence of Bsph in *Cule*x larval sites in tropical Africa. Another chapter by HH Yap (14 pages) compiles information on efficacy of Bsph formulations against *Aedes, Anopheles, Culex, Mansonia* and *Psoropora* species. We will not evaluate and analyze this information but present some recent studies of our own on Bsph.

We evaluated Bsph formulations against the southern house mosquito *Culex quinquefasciatus* breeding in highly polluted waters in urban areas in Bangkok, Thailand. In surface drainage klongs (with slow moving water in the absence of rain), both GR and WDG (water disposable granules) yielded 14-28 days control of larvae at the dosages of 2 g/m^2 of GR and 0.1-0.25 g/m^2 of WDG. Heavy precipitation increased the volume of water through the klongs and washed away mosquito larvae, resulting in longer control of larvae (Mulla *et al,* 1997). In some stagnant polluted water habitats, Bsph provided *Culex quinquefasciatus* larval control for almost one to two months, a performance not shown by other larvicides, with successive treatments of Bsph (Mulla *et al*, 2001a, 2003). The efficacy and longevity declined due to high levels of resistance and this high level of resistance was managed by using a mixture of Bti and Bsph against resistant larvae (Mulla *et al*, 2003).

The efficacy and longevity of *B. sphaericus* depended on the quality of formulations, species of mosquitoes and factors of habitats. In general, it provided persistent control of *Culex* species in highly polluted water and water not exposed to sunlight and winds. Other factors of habitat that influenced efficacy and longevity were vegetation, dilution by incoming water and solid wastes. The two formulations of Bsph commonly used are corn grit granules and the water dispensable granules (WDG). The effective rate of application are 2-5 lb/acre of the GR formulation and 0.25 – 1.0 g/m^2 of the WDG formulations. These rates may have to be increased in habitats with unfavorable features or where less susceptible species are prevalent.

Saccharopolyspora spinosa – fermentation products

Spinosad is a metabolic and fermentation product of the actinomycete bacterium *Saccharopolyspora spinosa*. This naturally occurring bacterium was isolated from soil in 1988. It was identified as a new species (Mertz and Yao 1990, Thompson *et al*, 1997). Actinomycete bacteria exhibit fungus like characteristics and they are responsible for the decomposition of complex organic materials. In 1989, the most bioactive metabolites from the spinosad formulation broth were identified and designated as spinosyns A & D (Sparks *et al*, 1998). In the mixture, spinosyn A and spinosyn D constitute 85 and 15 %, respectively (Kirst *et al*, 1992). Both spinosyns are non-volatile, slightly soluble in water, and stable under a wide range of temperature and pH (Dow AgroSciences Tech Information).

Mode of Action: Spinosad has a novel mode of action. It alters the formation of nicotine and GABA –gated ion channels and consistently results in neuronal excitation (Salgado 1998, Watson 2001). Spinosad with its novel mode of action is an ideal product for management of resistance. It has shown no cross-resistance to currently used insecticides and can be rotated with all other classes of existing mosquito larvicides (Dow AgroSciences Tech Information). The mode of entry is by ingestion as well as cuticular penetration.

Safety and Ecotoxicology

Spinosad has been registered for use on over 250 crops. It has a good margin of safety for mammals. Short-term toxicity as determined in rats was LC_{50}>3738 to >5000 mg/kg body weight for male and female rats, respectively. Dermal and inhalation tests also showed a high level of safety. It has a good margin of safety for birds and fish and most aquatic macroinvertebrates. It does, however, impact some groups of aquatic crustaceans. Therefore, caution should be exercised in making applications in aquatic habitats where mosquitoes breed and where the sensitive macroinvertebrates might prevail.

Spinosad fate in soil, water, plants, and animals.

Spinosad due to sunlight photolysis, and bacterial action degrade readily. In soil, it dissipates rapidly from soil sampled with half-life of one day or less. Deep in soil, the half-life may be 9 to 17 days. Persistence is influenced by soil type, organic matter, and water flow through. In water, spinosad degrades rapidly; the primary cause of degradation is sunlight photolysis. Half-life in water is estimated to be one day. Residues of spinosad on plant surfaces disappear at a moderate to rapid rate due to sunlight and possibly precipitation. Dissipation half-lives of 2-16 days have been recorded on leaves and fruits. Again, the rate of dissipation is influenced by sunlight and degree of shading, (Dow Agrosciences Tech Sheet).

The fate of residues of spinosad has been studied in animals, both in terrestrial and aquatic animals where they feed on plant material with residues, other animals, or direct absorption from water. The residues were found to metabolize and excreted, and these metabolites do not accumulate in animal tissues. Spinosad residues in water may be acquired by fish, but no significant level of biomagnification has been noted. The residues are metabolized rapidly in fish and are excreted.

From the foregoing, it is quite clear that spinosad has a high margin of safety to mammals, birds, and fish. It is safe to most aquatic biota cohabiting with mosquitoes. This insecticide when used as a mosquito larvicide in aquatic habitats pose little or no environmental risks or bioaccumulation. It is labeled as an environmentally friendly insecticide.

Spinosad – evaluation and efficacy against mosquito larvae

Soon after isolation of the actinomycete bacterium *Saccharopolyspora spinosa* (producing spinosad) from soil, it was cultured and the products were first tested against mosquito larvae (Kirst *et al*, 1992). It was noted that the product had considerable activity against mosquito larvae. Notwithstanding this information, spinosad was first developed for the control of crop insects. It is now registered for insect pest control of some 250 crops. Interest in testing and developing this natural product for mosquito control did not materialize until 2003/2005. We conducted preliminary studies on the activity of spinosad against mosquito larvae in 2000 (Mulla unpublished report). During this period and thereafter numerous studies were carried out on the activity of spinosad technical and formulated materials against mosquito larval in different regions. A flurry of studies followed evaluating various formulations of spinosad against larvae of mosquitoes: *Aedes aegypti, Ae. albopictus, Culex quinquefasciatus, Cx. pipiens, Anopheles albimanus, An. stephensi*, and *An. quadrimaculatus* in the laboratory and field (Bond *et al*, 2004, Cetin *et al*, 2005, Darriet *et al*, 2005, Darriet and Corbel 2006, Paul *et al*, 2006, Romi *et al*, 2006, Thompson and Hutchins 1999). In these studies, various levels of activity were noted in the laboratory against various species. In field studies, efficacy and longevity were dose dependent, species variables, and habitat conditions.

Spinosad against *Culex* mosquito larvae/California

We initiated detailed studies on the evaluation of technical spinosad and SC 120 emulsifiable formulation against larvae of *Cx. quinquefasciatus* in the laboratory in 2005 (Jiang and Mulla 2010). We determined in repeated replicated studies that spinosad showed a high level of activity against the larvae. We determined the LC_{50} and LC_{90} (lethal concentrations killing 50% and 90%) of the exposed larvae. We carried out tests at multiple dosages against 2^{nd} and 4^{th} instar larvae and the mortality was assessed at 24 and 48 hr post exposure. Constant temperature (26-28° C) and photoperiod (14 L:10hr D) were used.

The LC_{50} of the technical material against 2^{nd} instar larvae for 24 hr exposure was 0.021 mg/L AI for the 4^{th} instars it was 0.033 mg/L. The LC_{50} of the SC120 (11.6%) were lower being 0.012 (2^{nd} instar) and 0.014 (4^{th} instar). The LC_{90} for the technical material were 0.051 (2^{nd} instar) and 0.060 (4^{th} instar) and for the SC120 the LC_{90} values were 0.026 (2^{nd} instar) and 0.032(4^{th} instar). The data showed that mortality increased at the 48-hr exposure than the 24 hr exposure for both the 2^{nd} and 4^{th} instars. These tests showed that larvae of *Cx. quinquefasciatus* are quite sensitive to both technical and emulsifiable formulations, the latter being more active than the former. With this data at hand, we got encouraged to take spinosad to simulated field set-ups using standard microcosms and mesocosms supporting natural populations of *Culex* mosquitoes.

Microcosms and mesocosm studies on spinosad/*Culex* mosquitoes

For the simulated field studies, we used the emulsifiable (SC 120, 11.6% AI) formulation. The studies were carried out first in fiberglass tubs (24 tubs, 1 m^2, water depth 30 cm containing 240 L water), followed by experiments in earthen ponds (38 nonvegetated and 64 vegetated; 27 or 37.5 m^2 surface, water depth 30 cm) filled with 8100 L (nonvegetated) or 11250 L (vegetated) of reservoir water. The SC120 was diluted in distilled water and aliquots sprayed on the water surface. The material was applied to obtain multiple dosages, 0.05, 0.1, 0.25 and 0.5 mg/L AI in microcosms and 0.025, 0.05 and 0.1 mg/L for the mesocosms (Figure 2).

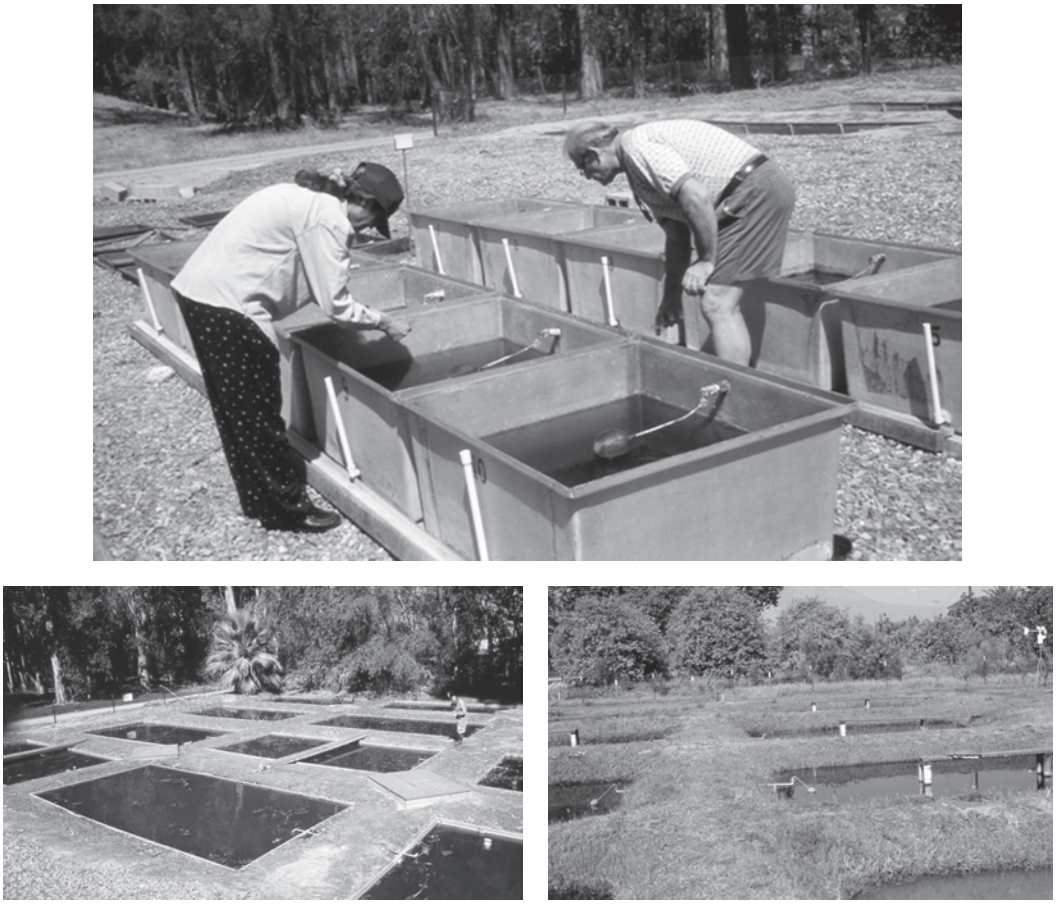

Figure 2 Layout of microcosms (tubs-upper) and mesocosms (lower-ponds without vegetation and ponds with vegetation) for simulated field evaluation of larvicides against mosquitoes at University of California, Riverside, Aquatic and Vector Control Research Facility.

Treated and untreated units (tubs) and ponds (mesocosms) replicated 4 times were sampled for larvae using a standard dipper before and at intervals after treatments. The samples were categorized into early instar larvae (1^{st} and 2^{nd}), late instar, (3^{rd} and 4^{th}) and pupae (Jiang and Mulla 2010).

In the microcosm tests (tubs), we obtained 100% initial control of immatures of 3 species of *Culex* mosquitoes. The longevity of treatments was dose dependent, the higher dosages providing long-lasting control for 21-35 days, depending on the dose. The results were similar in the mesocosm (ponds), where the longevity was also dose dependent. The low dosage (0.025mg/L AI) yielded complete control for 4-7 days, while the 0.05 mg/L AI gave complete control of larvae for 7 days and pupae for 14 days. The highest dosage of 0.1 mg/L AI yielded both larval and pupal control for 14 days.

It should be pointed out that both the tubs and ponds were in open areas (Figure 2) exposed totally to sunlight and wind. As mentioned earlier, sunlight photolysis degrades spinosad rapidly. From these studies, it can be deduced that field application rates of 0.05 to 0.10 lb/acre of active ingredients will be necessary for satisfactory control of *Culex* mosquitoes in open bodies of water. In cases where mosquitoes are breeding in polluted water or habitats with vegetation, higher rates might be necessary. It should be realize that formulation technology can improve the initial activity as well as the longevity of bioactive agents.

Spinosad field activity and longenity against *Aedes aegypti* – Thailand

The activity of spinosad against *Ae. aegypti* in laboratory is similar to *Cx. quinquefasciatus* (Bond *et al*, 2004 and Jiang and Mulla 2010). In 2005/ 2007, we evaluated 3 formulations of spinosad against *Ae. aegypti* in water-storage containers. These studies were carried out under simulated field conditions.

The tests were carried out in 200 L (filled with water) earthen jars or plastic barrels (Fig 1). Spinosad formulations (SC 11.6%, Granules 0.5% and directly applied DT tablets 7.5% AI) were applied to the containers at multiple dosages. Before the treatments 25 *Ae. aegypti* 3rd instar larvae were added to each container and 1 g of ground up larval food added to each and subsequently 0.5 g added weekly. Larval survivorship was assessed using a flashlight. We also assessed the emergence of adult mosquitoes by counting the pupal skins (reflecting adult emergence). The pupal skins float on the surface and they were removed by a suction pipette and counted in a white plastic tray. Pupal skins are easier to remove and count, and their number yielded accurate data on the successful emergence of adults. Inhibition of emergence % (IE%) was calculated based on the number of larvae (25) added to each container(100 larvae in total per 4 replicates).

To determine the efficacy and longevity of each treatment, cohorts of new 25 late instar larvae were added weekly to each container until the IE% reached 80%. The water-storage containers were placed in shaded arenas on a concrete slab roofed over, but open on all sides. The jars were covered either with celocrete sheets aluminum covers or lids with screened area in the center of the lid. They were protected from sunlight, wind, and evaporation. Usually, 3 water regimens were used: full jars all the time, ½ full jars and full jars – ½ removal and refilled weekly. Water loss, which was minor, was replenished monthly.

The results of these experiments are presented here:

Comparisons of Spinosad SC120 and GR 0.5

This experiment was started on March 2, 2005. The SC120 was used at high dosages (8.4, 3.9, 2.23 and 0.89 mg/L AI) and the GR 0.5 used at the realistic dosages of 1, 0.5, 0.25 and 0.1 mg/L AI. The SC120 used at high rates provided almost IE 100% (control) for 104 days at all dosages. The two lower dosages exhausted after 125 days. The GR 0.5 used at realistic dosages gave close to 100% control for 83 days at the 3 lower dosages, the high dosage (1.0 mg/L AI) still producing close to 100% control for 111 days (Mulla, unpublished report)

The second experiment using the SC120 alone at realistic dosages in water-storage jars was started on May 2, 2005. The spinosad doses were 0.5, 0.25, and 0.10 mg/L AI. We used two water regimens, full jars and full jars ½ removed. At these lower dosages, the longevity as expected was low; being 100% control at all 3 dosages for 39 days in both water regimens. The high dosage (0.5 mg/L) still giving 85% reduction for 46 days in full water. Thereafter the level of control declined in all dosage (Mulla 2005, unpublished report)

Comparison of spinosad DT tablets and GR 0.5

The next experiment comparing the DT (directly applied tablet 7.5% AI) and GR 0.5% was started on April 18, 2007. Using the same set up and the same procedures, the dosage for each formulation were 1.0, 0.5 and 0.25 mg/L AI. As before, each treatment and control were replicated 4 times and the treated and untreated jars challenged with 25 *Ae. aegypti* 3rd instar larvae and added weekly until the IE% (control) dropped below 80%. Two water regimens were used, full jars and full jars-1/2 emptied and refilled weekly (Thavara *et al*, 2009).

The DT formulations at all 3 dosages produced 100% control for 13 days in full jars, but the level of 100% control lasted longer for 20 days with the DT highest dosage (1.0 mg/L). The DT at the highest dosage (1.0 mg/L) yielded 79-100% IE for 48 days in full jars, the efficacy declining beyond this period. However, the longevity in jars with water exchange was higher, with 90-100% IE for 62 days (Thavara *et al*, 2009). The manufacturer's recommended rate of 0.5 mg/L AI gave 79-100% IE for 27 days in the water exchange jar. It is evident that water removal and filling facilitates release of the active ingredients from the tablet, which sinks and sets on the bottom of the jar.

All dosages of the GR 0.5 produced IE 90-100% for 34 days in both full jars and full jars with water exchange. The highest dosage (1.0 mg/L) showed longer persistence yielding more than 80% IE for 62 days in both water regiments (Thavara *et al*, 2009). The longevity at the lower dosages was shorter. It appears that the most appropriate dosages of spinosad for *Ae. aegypti* in water-storage containers will be in the range of 0.25 to 1.0 mg/L, higher dosages providing long-lasting control.

Plant-based bioactives for use as repellents and protectants

The use of repellents and protectants containing plant materials and synthetic chemicals is common throughout the world. The most common devices sold for personal protection are mosquito coils and preparations of plant essential oils as repellents against biting insects. It has been estimates that the number of mosquito coils sold world-wide reach to 29 billion, where 95% are used in Asia (WHO 1998). Mosquito coils are used primarily by low-income communities while vaporizing mats and aerosols are used by the middle and high-income sectors. Mulla *et al,* (2001b) noted that in mosquito infested areas of Thailand, the residents spent about U.S. $12.50 to $25 per residence per annum for protection from biting mosquitoes. Similar levels of expenditures were reported for some communities in India (Snehalatha 2003). In addition to these uses, plant products are used as repellents on skin and as larvicides and adulticides, in field situations and residences.

There are numerous publications containing information on plant-based repellents and protectants and it will not be possible to list and review them here. Three publications offer an enormous amount of information on research and development of personal protectants and repellents (Debboun *et al,* 2007, Moore *et al*, 2007, Sukumar *et al,* 1991). These publications have an impressive list of published works on this subject. A considerable amount of research has been carried out on the neem tree (*Azadirecata indica*) and its various products for the control of medically important insects, including reports on the usefulness of neem products as repellents, protectants and deterrents (Mulla and Su 1999). The recent volume published on neem tree by the Neem Foundation (Schmuterer 2002) contains valuable information on the neem tree and the unique products derived from this tree which are used in pest control and many industrial, medicinal, and agricultural enterprises. Bioactive materials from neem tree have multiple modes of action, such as repellency, deterrence, sterility, growth regulation and toxicity (Isman 1999, Mulla and Su 1999, Schmuterer 2002).

The repellent chemicals found in different parts of plants have been identified in some plant extracts. These constituents not only act as repellents but also as feeding deterrents, toxicants, growth regulators, and etc. The major groups of chemical substances, identified are categorized as alkaloids, phenols, terpenoids, and others. Different plant species and their parts yield different groups of chemicals and in varying quantities. Some plants may have one or a few phytochemicals possessing repellent properties, while others may have quite a few principles. The citronella group of grasses originating in India have widespread distribution. These plans have varying amounts of repellent chemicals (mostly terpenoids), but the most abundant repellent chemicals contained are citronella, citronellol, and geraniol. The grass *Cymbopogon nardus* or citronella is the most common species used in commercial blending of repellent products. Several other species of *Cymbopogon* are used in commercially available plant products.

Osimum species (*basilicum* in particular) contains a number of repellent compounds which also act as mosquito larvicides. Species of *Hyptis*, *Mentha* and

Thymus also contain insect repellent principles and they are used in various ways by the local communities. *Tagetes* species have exhibited larvicidal and insecticidal properties, their essential oils act as repellents in some species and not others. *Artimisia* spp have both essential oils which act as repellent and other principles acting as larvicides. The neem tree *Azadiracta indica* has attracted a great deal of attention as a source of agricultural biopesticides. In India, neem tree parts have been used for thousands of years as insect repellents and protectants. In recent years, materials from this plant have been developed for the control of medical insects. The neem tree has about 35 bioactive compounds which show toxicity, causing sterility, decreased fecundity as well as repellency and deterrency. Proper formulations can increase toxicity and repellency of neem and other plant products. Bioactivity is related to concentration of bioactive principles and the extent of release and absorption (Isman 1999, Mulla and Su 1999, Schmuterer 2002).

Lemon eucalyptus extracts from *Corymbia citriodora* (from China), have been the subject of many tests. The essential oil extract was noted to have repellency to mosquitoes, slightly better than most *Eucalyptus* spp. The compound p-menthane-3,8-diol (PMD) was identified as a byproduct. This material was proven to be highly repellent, equaling the repellency of deet (commercial repellent). The Chinese name of this repellent is Quwenling “effective repellent of mosquitoes.”

Essential oils

Essential oils of plants are extracted by steam distillation. They show different levels of repellency against mosquitoes. Moore *et al* (2007) tabulate the protection time for essential oils in extracts of some 37 plants. The protection time increased with concentration. Most of the protection times for 50% concentration was from 0 to 80 minutes. The longest protection time was 88 minutes for *Zanthoxylem limonella* and 60 minutes for each of *Apium graveolens*, *Cymbopogom nardus* and P*ogostemon cablin*. The rest of the plant essential oils showed low levels of repellency. The same authors provide a comparison in protection time among 8 commercial plant based products with Skinsensation (7% deet). Six commercial products were inferior to Skinsensation, while 2 commercial plant products (Bite Blocker and Repel) proved superior to deet. Bite Blocker contains oil of coconut, geranium and soybean (Consep Inc, Bend Oregon) and Repel contains oil of lemon eucalyptus and PMD (Wisconsin Pharmacol Co, Jackson WI). As mentioned earlier, appropriate formulations will greatly enhance the effectiveness of plant essential oils as insect repellents. Gerberg and Novak (2007) list 50 plants and the names of repellent products derived from them. Apparently there are other plants and products used locally that have not been commercialized.

Recent studies on plant based repellents and protectants (Thailand)

A systematic laboratory and field research program is underway at the National Institute of Health, Ministry of Public Health, Thailand. Experimental and commercial plant products with some additives have been evaluated against mosquitoes, black flies, land leeches, and cockroaches. A synopsis of results from these studies during the past 7 years is presented below. Landing counts of blood-sucking organisms on human volunteers were used for field assessment.

Mosquitoes: In the first study (Tawatsin *et al*, 2001) tested volatile oils from plants against three mosquito species determining medium protection time. Essential oils from turmeric, citronella, hair basil with some additives gave essentially the same protection time (6-8 hrs) as deet for *Ae. aegypti*, *An dirus*, and *Cx. quinquefasciatus*. In another field study, the extracts of citronella oil (10-13%), eucalyptus oil (15%), tea tree oil (5%) with some additives provided protection (93-100%) for 4 hrs from night biting mosquitoes (*Culex* and *Mansonia* spp) (Thavara *et al*, 2002). Again, the extent of protection and duration were similar to the standard chemical repellent deet.

In a comprehensive study (Tawatsin *et al*, 2006a) extracted and formulated essential oils from 18 plant species and compared them for protection time with deet and IR 3535 against 4 species of mosquitoes. Chemical composition of the oils for each plant species was determined. The oil yield from the 18 plants ranged from 0.04 to 0.80%. The oils were extracted from leaves, flowers, or rhizomes, depending on the plant. The plant-based products afforded low to medium protection time (0.3 to 2.8 hrs) against *Ae. aegypti* as compared to deet (7.5 hrs) and IR 3535 (6.7 hrs). It seems that *Ae. aegypti* was not sensitive to these plant extracts. On the other hand, all plant extracts with the exception of *Litsia cubeba*, A*glaia odorata*, *Myristica fragrans*, *Psidium guajava*, *Murraya paniculata*, and Z*ingiber officinale*, provided 7+ hrs protection against *Ae. albopictus*, equaling that of deet or IR3535. All 18 plant extracts gave high levels of protection time (7-8 hrs) against *An. dirus*, equaling that of deet or IR3535. Only 4 plant species, yielded around 6 hrs protection time against *Cx. quinquefasciatus*, the remaining 4 species yielded protection time of 7-8 hrs, comparable to the time afforded by the two synthetic chemicals. The plants giving 7.8 to 8 hrs protection against this mosquito were *Piper nigrum* and *Cucurma longa*. It is possible that formulation manipulation might increase activity and duration of plants giving less protection using unformulated material. But this would not be necessary, as the number of active plants currently known is adequate.

A field study with 4 plant-based repellents with some additives was carried out against night-biting (*Culex* and *Mansonia*) and day-biting (*Ae. aegypti* and *Ae. albopictus*) mosquitoes (Tawatsin 2006b). The four plant oils: fingerroot (10%), guava (10%), turmeric (10%), a commercial product Repel Care 9.5%

(turmeric oil 5% and *Eucalyptus citriodora* 4.5%) were compared with deet. All repellents provided 100% protection for 9 hrs. The same products tested on human volunteers against day-biting mosquitoes gave almost 100% protection for 5 hrs and provided 76-93% protection for 8 hrs. In these tests the efficacy of the plant extracts were equal to that of deet against both day and night-biting mosquitoes.

Field tests against black flies:

The four plant-based repellents tested against mosquitoes above (Tawatsin *et al*, 2006b) were tested on human volunteers against black flies (*Simulim nigrogilvum* 99% of the flies collected). All four plant-based repellents gave 100% protection from the bites of black flies for nine hrs, protection decreasing to 90 to 93% after 10 and 11 hrs of application of the repellents. The extent and duration of protection was the same for deet and the plant products.

Field tests against land leeches

Land leeches are severe pests of man and animals in national parks, wilderness, and wetlands. They could be quite a deterrent to outdoor activity and enjoyment of wilderness and open spaces. The same 7 plant-based repellents were tested on human volunteers against land leeches. The leeches were in the genus *Haemadipsa (Haemadipsidae*). We noted that the leeches on contact with repellent treated surfaces shrivelled and died within minutes. All four plant-based repellents and deet provided 100% protection from leeches for 8 hrs (Tawatsin *et al*, 2006b). Not only the products were repellent to leeches but also highly toxic to them. The leeches were highly sensitive to plant-based repellents and these can be used as sprays for clearing leech infestations in trails and wilderness areas. More studies are needed on land leech control.

Tests against cockroaches:

Seven plant-based repellents were tested against the cockroaches *Periplaneta americana*, *Blatella germanica*, and *Neostylopyga rhombifolia* (Thavara *et al*, 2007) in the laboratory. All seven products reduced cockroach populations drastically. The last species being less susceptible than the other two species. Among the seven plants Kaffir lime (*Citrus hystrix*) was the most effective giving the highest repellency (87-100%), higher than that of napthaline used as a standard. *C. hystrix* extracts were formulated (28%) for field tests. In 3 village tests in Pitsanulok province, treatments reduced cockroaches by 50-70% in 9-12 days post-treatment. Similarly, in two areas in Bangkok, Kaffir lime formulations (20%) treatment caused 60-80% reduction in cockroaches. This level of reduction is equivalent to that of some insecticidal treatments. More detailed studies are warranted here.

Efficacy of mosquito coils

Mosquito coils are used in large quantities on a world-wide basis. In most of the coils, the active ingredients consist of synthetic pyrethroids. The use of plant materials in mosquito coils is attracting greater attention. In actual practice, many people in low-income communities burn plant parts to achieve protection from biting insects. This practice serves the same purpose as a manufactured mosquito coil sold on the market.

There have been numerous studies on the efficacy of mosquito coils based on the active ingredients consisting of synthetic pyrethroids. There are very few studies published on the efficacy of mosquito coils containing plant materials. In one study Tawatsin *et al* 2002, incorporated plant materials into the coil and tested them in the field against night-biting mosquitoes. The plant material constituted 25% of the coil. A blank coil was run along with the test coils. In total 9 plants were evaluated. The mosquitoes collected were species of *Culex, Mansonia, Armigeres* and *Anopheles*. All 9 plant materials tested individually provided protection of 50-71% in 3 hrs of testing. Mosquito coils containing leaves of citronella grass showed highest efficacy, while the coils with rhizomes of turmeric were least effective. By comparison, coils containing synthetic pyrethroids showed repellency of 84-86%. It seems that further studies are needed to increase the efficacy of plant-based coils through formulation technology.

Conclusions

In conclusion, it can be said that plant phytochemicals have a multiple mode of action against insects. They can be used for the control of larval and adult mosquitoes. The essential oils extracted from plants with some additives offer a rich source of products that can be used in preparations to repel insects and protect humans form biting insects. They can be employed in mosquito coils or in aerosols and lotions properly formulated for personal protection. Further research is needed to find and develop more active principles and formulations.

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