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Biology and Ecology Section,
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FOREWORD

The National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand has been involved in spearheading a national effort toward implementing a comprehensive progress of research and training for the prevention and control of a variety of communicable and non-communicable diseases, environmental risks and control of toxic substances. Among these diseases, the vector-borne diseases, such as Dengue Haemorrhagic Fever (DHF), malaria, filariasis and Japanese encephalitis pose major public health problems in Thailand. Control and management of these infections require constant vigilance and administration of comprehensive disease control strategies. As a part of the mission of NIH, medical scientists in the institute are engaged in a variety of research programs focusing attention on the biology, ecology and control of insect vectors, especially mosquitoes. The data and information resulting from their research effort forms a basis for the rational control and management of disease vectors. To make this information readily available, we here compile the published research results gathered by scientists of the Section of Biology and Ecology in cooperation with oversea scientists, especially Professor Mir S. Mulla of the Department of Entomology, University of California, Riverside, California, USA. Most of the scientific information gathered in the research has been published in internationally renowned peer reviewed journals, not widely available in Thailand.

Among the various mosquito disease vectors, *Aedes aegypti* vectoring dengue and dengue haemorrhagic fever in Thailand and other tropical countries has attracted due attention of researchers. An understanding of the seasonal population dynamics of *Ae. aegypti* and its prevalence on Ko Samui has greatly broadened our knowledge regarding this species. Significant finding regarding the oviposition behavior, oviposition attractants and substrate preferences for dengue vectors avail new tools for population measurement and control of these vectors. Another important thrust toward the control of *Ae. aegypti* was the extensive field evaluation of novel insect growth regulators, assessment of the longevity of the currently used and a new temephos formulation as well as

controlled release formulations of a microbial control agent *(Bacillus thuringiensis* var. *israelensis* or Bti). Information on these new tools and strategies will be used in developing future control programs against dengue vectors.

Considerable research was conducted on a common soil bacterium (*Bacillus sphaericus*) formulations against *Culex quinquefasciatus*, filariasis vector, heading in polluted water habitats in urban and rural areas. This microbial agent has exceptional activity and longevity in controlling mosquito larvae in polluted water habitats. One treatment at low dosages produced almost complete control of this species for about two months. However, repeated treatments made in polluted water habitats resulted in a high level of resistance to this microbe. Further research on developing resistance management strategies, came up with a solution involving rotation with another microbial larvicide (Bti) or using mixtures of the two. This vital information will be useful in vector control operation not only in Thailand but also throughout the world.

Personal protection measure using a variety of repellent products is an important part of the strategies available for vector and disease control. In this context, NIH scientists carried out extensive and relevant studies on chemical repellents and their formulations. To broaden the scope of safe and user friendly repellent products, our scientist investigated a variety of natural products of plant origin in which can be incorporated in repellent formulations and mosquito coils. These types of active principles are locally available and possess good margin and commonly public acceptance.

Critical information gained on the longevity, survivorship and gonotrophic cycles of JE vector (*Culex tritaeniorhynchus*) is germane to population phenomena of this vector. An area-wide survey on domestic and peri-domestic cockroaches elucidated the diversity and abundance of cockroaches in houses in different parts of Thailand.

It should be pointed out that the published record compiled here, presents a great deal of applied scientific information gathered in a variety of research projects on disease vectors in Thailand. The information gained in

these NIH research efforts is not only vital and useful for solving vector control problems in Thailand, but it also has relevance and application in other areas of the world.

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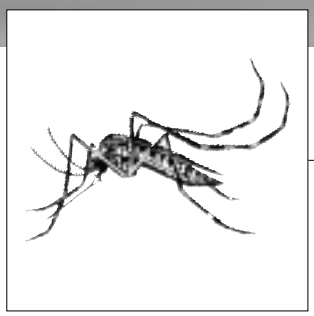

Larval Occurrence, Oviposition Behavior and Biting Activity of Potential Mosquito Vectors of Dengue on Samui Island, Thailand

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Abstract

A 1995 outbreak of dengue haemorrhagic fever (DHF) occurred on Samui Island in Thailand with an incidence of almost 500 cases/100,000 population. To find and develop effective strategies to control this disease through cost-effective vector control programs, entomological studies were carried out on the island between 1996 and 1998. There were two species of DHF vectors, *Aedes aegypti* and *Ae. albopictus* prevailing on the island, and the population of *Ae. aegypti* remained relatively constant throughout the year while the abundance of *Ae. albopictus* increased substantially during the rainy season (May-December) and then declined drastically in the dry season (January-April). The ranges of the three *Aedes* larval indices, Breteau index (BI), house index (HI) and container index (CI) were 93-310, 43-89 and 16-50 respectively. The ceramic or earthen jars both inside and outside the dwellings and concrete water storage tanks (mostly in toilets and bathrooms) served as the main breeding places of *Ae. aegypti* whereas coconut husks and coconut floral spathes found outdoors were the major breeding sites of *Ae. albopictus.* The number of washing water jars, concrete tanks and natural sites infested wtih *Aedes* larvae increased significantly in rainy season, with 60% of ovitraps become positive for *Ae. albopictus* eggs with an average number of 26 eggs/trap in 3 days of setting. There was a complete lack of oviposition by *Ae. aegypti* in outdoor ovitraps (15 m away from the houses). The indoor biting rate ranged from 1.5 to 8.1 mosquitoes/man-hour, while the outdoor rate was between 5 and 78 mosquitoes/man-hour. Of the indoor biting mosquitoes, 75.4% were identified as *Ae. aegypti* and 99% of the outdoor ones were *Ae. albopictus.* The diel biting activity of *Aedes* during the period from 0800 h to 1700 h in the

houses was higher in the morning than in the afternoon period, with a low prevalence between 1300 h and 1400 h.

Keywords

Dengue vectors, *Aedes aegypti, Aedes albopictus,* ecology, biting, oviposition

Introduction

Samui Island is an important tourist attraction in Thailand and is visited by many foreign and local tourists. It is estimated that about 700,000 people visit this island each year. The visitors coming to the island each year amount to approximately 35 times of the local population. The tourists as well as the indigenous people suffer equally from mosquito bites and register complaints regarding mosquitoes with the local authorities. The mosquitoes that adversely affect people on Samui are primarily *Ae. albopictus* (Skuse) and *Ae. aegypti* (L.). In addition, these mosquitoes are involved in precipitating dengue outbreaks. An epidemic of DHF occurred on Samui Island in 1966 and 1967 (Winter et al. 1968) where *Ae. aegypti* and *Ae. albopictus* were abundant and widespread and dengue viruses were repeatedly isolated from both species (Gould et al. 1968, Russell et al. 1968, Russell et al. 1969). Recently, in 1995, there was another outbreak of DHF on this island when 159 cases of the disease (497 per 100,000 resident population) were reported and dengue virus was detected in both *Ae. aegypti* and *Ae. albopictus* (Thavara et al. 1996).

In southeast Asia, it has been noted that *Ae. aegypti* has spread throughout urban areas such as Bangkok, and has replaced the competing native species, *Ae. albopictus* (Rudnick and Hammon 1960). *Ae. albopictus* exhibits greater flexibility in various traits than *Ae. aegypti,* such as the choice of oviposition sites (Gould et al. 1968). However, the preferred breeding sites of the species are different and only slight overlap has been noted (Gould et al. 1970). In the past two decades, there has been a dramatic increase in the development of infrastructure, accommodations and facilities for tourism purposes, such as hotels, resorts or bungalows and associated services as well as residential units in various areas around the island. It is believed that these developments have had an impact on the abundance of *Aedes* mosquitoes by providing more habitats for these mosquitoes and thus leading to an increase in the abundance of dengue vectors.

To provide background information on larval occurrence, biting seasonality and oviposition behavior of adult mosquitoes, we studied the role of major developmental sites of larvae using various indices for *Aedes* larvae. We also elucidated the magnitude of oviposition of *Ae. albopictus* on the island using modified ovitraps. The overall objectives were to identify major sources of larval breeding using several indices and to elucidate the oviposition behavior of *Ae. albopictus* and biting activity of both these two important species (*Ae. aegypti and Ae. albopictus*). These studies were carried out on nine occasions over a period of about two years, from March 1996 to July 1998.

Materials and methods

Study sites

Samui Island is the largest of a group of several dozen islands in the Gulf of Thailand, located between 99˚ 38' and 100˚ 7' east longitude, and 9˚ 20' and 9° 45' north latitude. It is one of the districts of the Surat Thani province in southern Thailand, with an area of 277 km^2. The island is quite mountainous with 54% of it covered with mountains. The island is divided into seven administrative subdistricts, consisting of 39 villages with a local population of 32,814, a density of 144 people/km^2. Normally each year, two tropical monsoons (i.e., southwest and northeast monsoon) dominate the climate of Samui Island. The onset of the first monsoon starts in May while that of the second begins in November. As a result of these monsoons, the annual average rainfall for Samui Island is over 1000 mm each year.

To carry out the proposed studies, one representative village per each of the seven subdistricts was selected. At least 50 premises (mostly residential) in each village were selected randomly to carry out the entomological surveys. In general, each village consists of a mix of residential areas, coconut plantations, fruit orchards and/or forested areas. The residential areas are of two types, residential houses and shop houses. Most of the bungalows are built in the residential areas in the villages around the island to supply economical accommodations for the tourists who would like to stay for long periods of time. Coconut palms constitute the most common landscape tree around residential and touristic structures, and the palm stands interspersed with grasses and small bushes between and around the trees. The residential areas also have small fruit orchards consisting of many kinds of fruit trees, such as rambutan, lansat,

durian, magosteen and sapodilla trees. The mountainous areas (not studied) are wooded areas and jungles. Although there is a piped water supply in most residential and commercial areas of the island, many water-storage containers are still kept in and around each house for collection and storage of rain as well as domestic water. To supplant the precarious source of domestic water supply, the local people catch and store rainwater in small to large jars and tanks. Moreover, the local people prefer using rainwater to the piped water. These multitude of water storage containers provide preferred developmental sites for *Aedes* species.

Entomological Studies

Larval occurrence: The entomological studies were carried out on the island during nine survey periods in March and July 1996, January, March, May, June, July 1997, and January and July 1998. We were not able to gather entomological data during the heavy monsoon season (August to December of each year) because of heavy rains and also fiscal year financial constraints at that time of the year. Populations of *Aedes* mosquito larvae were determined by visual larval survey techniques (Service 1976), and expressed as indices giving the percentage positive for each parameter. There indices were House Index (HI): the percentage of houses positive for larvae, Container Index (CI): the percentage of water-filled containers positive for larvae, and Breteau Index (BI): the number of positive containers per 100 houses. In each period of the nine occasions of the study, at least 50 houses in each of the selected village in each of the seven subdistricts were surveyed for both indoor and outdoor *Aedes* mosquito breeding places. The outdoor larval surveys were conducted within 15 m of the houses.

For species composition during one study in July 1996, mosquito larvae collected from both indoor and outdoor containers form 137 houses randomly chosen from among the seven villages were identified to species. From the larvae collected from these sources, 411 fourth instar larvae were mounted on slides for species identification.

A separate study was also carried out in July 1996 to get information on the outdoor breeding potential of *Aedes* mosquitoes in natural sites. About one thousand individual natural breeding sites, such as coconut husks and coconut floral spathes (both hold water) on the ground around the island were examined randomly for larval prevalence and species identification.

***Outdoor oviposition*:** Because of scanty information on the oviposition activity of *Aedes* outdoors on Samui Island, we examined the breeding potential of *Aedes* in July 1996 in four villages of the island by using modified ovitraps of Pratt and Jacob (1967). The ovitraps used were 450 ml-capacity flower pots (9 cm high and 10.5 cm in diameter at the top) that had no drain holes. Eighty ovitraps filled with 300 ml of rain water were set 15 m away from the houses on the ground in shady areas protected from intense rain and wind. The inside of each ovitrap was lined with a strip of white filter paper for mosquito oviposition. Three days after their placement, the paper strips were collected and examined for *Aedes* eggs. If eggs were present, they were counted under stereomicroscope. For species compositions, the egg strips were brought to the laboratory and after conditioning for a week were hydrated and the eggs hatched. The larvae were reared to the adult stage and identified to species.

Biting activity: In order to ascertain the biting activity pattern of *Aedes* mosquitoes on Samui Island, two studies were carried out. In the first study, mosquito biting activity was assessed both indoors and outdoors on 9 occasions in 1996, 1997 and 1998. Human volunteers were used for collection of mosquitoes. Three volunteers captured mosquitoes indoors for 20 min, each volunteer collecting in two dwellings in each village. The three volunteers thus collected mosquitoes in six dwellings in each village and then moving to the next village. The collectors indoors usually situated themselves in dark areas of the rooms where most biting activity occurs (personal observations). The collectors bared their legs between the knee and the ankle and collected all landing and biting mosquitoes individually in vials. Similarly, three volunteers were used in outdoors collections, the volunteers stationed themselves 15 m away from the dwellings. Each outdoor collector captured mosquitoes landing or biting on their legs outside each of two houses for 20 minutes each. In total, six indoor and six outdoor captures (sites selected randomly) were made in each village. After finishing the capture and collection in one village, the volunteers moved on to another nearby village where they made similar collections in each of the seven villages. The landing-biting captures were carried out form 0800 to 1200 h, and the collections for the total seven villages were completed in two consecutive days. Collected mosquitoes were visually identified, as there were only two species, *Ae. aegypti* and *Ae. albopictus* present. The data of all biting and landing activity for all the

houses, indoors and outdoors of all seven villages were pooled and reported as biting rate of mosquitoes indoors and outdoors for each of the nine observations. To relate the extent of biting activity to rainfall, we obtained rainfall data for January 1996 to December 1998 from the Samui Meteorological Station.

In the second study, landing and biting rates were assessed one time in Mae Nam village in July 1996. The biting activity of *Aedes* mosquitoes was assessed indoors only using three volunteers, one in each of three houses determined to have high larval and adult populations. Each volunteer was stationed in a dwelling and collected landing-biting mosquitoes continuously on both bared legs for a 20-minute period with a 10-minute break. Thus two 20-minute collections were made by each volunteer in each house per hour. The collections were made from 0800 to 1700 h. All biting mosquitoes collected by the three volunteers in the three houses were pooled and the biting rate calculated per each hour. The collected mosquitoes were sexed (significant number of males also landed), identified and the biting rate was based on female mosquitoes only.

Data analysis.

The mean number of containers positive for *Aedes* larvae per house in each survey was compared by a one way ANOVA; if significance was observed, the mean number was then compared by Duncan's Multiple Range Test. The number of each type of containers found positive for *Aedes* larvae was compared between dry and rainy season by Student's *t*-test. Before analysis, the data were transformed to $\sqrt{x+0.5}$ prior to statistical comparisons. The accepted level of significance for all comparisons was $P \leq 0.05$. Analysis was carried out using the SPSS program for windows version 9.0.

Results

Larval occurrence

A total of 18,937 containers from 3,233 houses on Samui Island were inspected for *Aedes* larvae during the nine study occasions between March 1996 and July 1998. Of these containers, a total of 7,514 containers situated in and around 2,425 houses were infested with *Aedes* larvae. As can be seen from the data, *Aedes* larval prevalence as expressed by BI, was greater than 100 during the study period, except that in March 1996 it was only 93 (Table 1).

The other two larval indices, HI and CI, showing percentage positive ranging from 43 to 89 and 16 to 50, respectively, for these two indices. These indices were also lowest in March 1996 as was the case with BI. Unlike BI, the HI and CI indices were relatively constant over the entire study period.

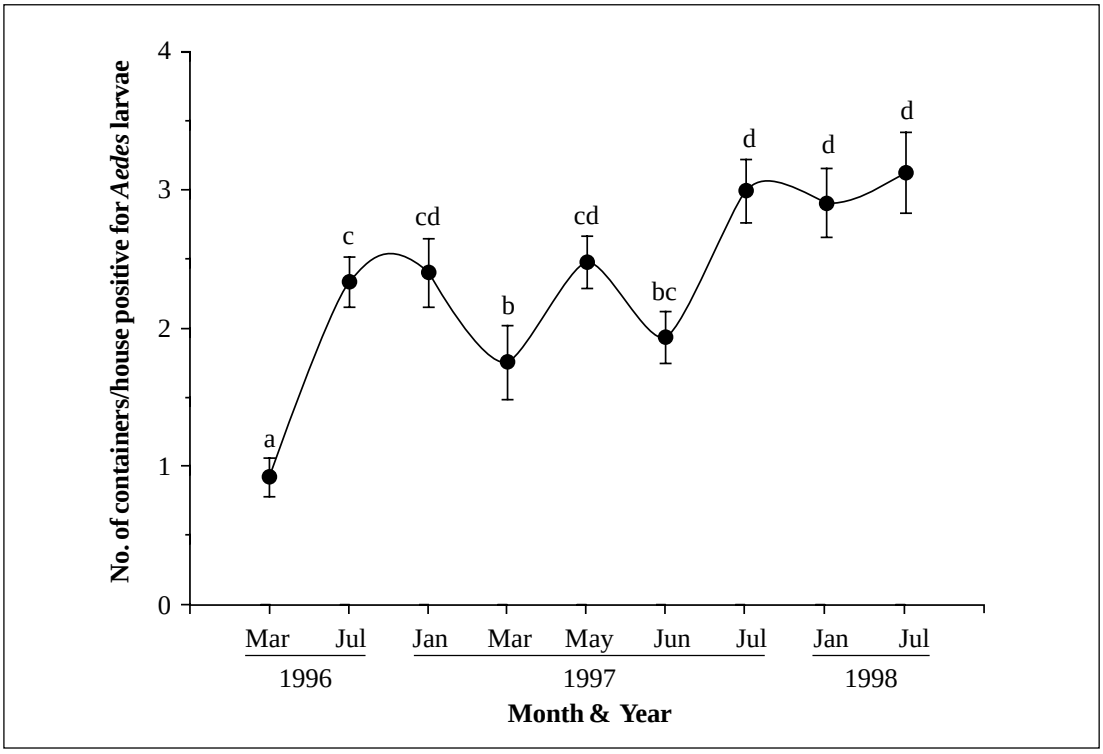

Fig. 1. Mean number (+S.E.) of containers positive for** Aedes **larvae on Samui Island, Thailand, on 9 occasions from March 1996 to July 1998. Means followed by same letter are not significantly different form each other at the 0.05 level.

The mean number of containers (indoors and outdoors) infested with *Aedes* larvae per house as shown in Figure 1 revealed a relatively stable level of larval occurrence over the study period with some fluctuations, with an especially a low value at the beginning of the study in March, 1996. The average number of all types of containers positive for *Aedes* larvae found per house in 1996, 1997 and 1998 was 1.6, 2.3 and 3.0, respectively. The low average number in 1996 is due to the very low value obtained at the outset of the study in March 1996. The average number of positive containers per house in 1997 and 1998 was essentially the same.

Table 1. Larval abundance indices of** Aedes **mosquitoes on Samui Island, Thailand, between March 1996 and July 1998.

	1996		1997					1998	
Indices	Mar	Jul	Jan	Mar	May	Jun	Jul	Jan	Jul
HI[a]	43	82	77	62	80	74	87	89	83
CI[b]	16	43	44	37	42	42	50	46	38
BI[c]	93	234	240	175	248	194	302	292	310

HI[a] : Percentage of houses positive for *Aedes* mosquito larvae.

CI[b] : Percentage of containers positive for *Aedes* mosquito larvae.

BI[c] : Number of containers postive for *Aedes* mosquito larvae per 100 houses.

It is important to note that the washing water storage jars and concrete water storage tanks (in bathrooms and toilets) were the main breeding sites of *Aedes* larvae inside and adjacent to the houses in both dry and rainy season (Figure 2). On the other hand, flower vases and pot saucers, drinking water storage jars, ant guards and natural sites (adjacent to houses) served as minor breeding sites during both seasons. The number of washing water storage jars, concrete tanks and natural sites infested with *Aedes* larvae was significantly greater in the rainy season than in the dry season ($P < 0.05$). In contrast, there were no significant differences in the number of infested drinking water storage jars, ant guards and flower vases & pot saucers between the dry and rainy seasons ($P > 0.05$).

As to the species cohabitation of larvae in water storage containers and natural sources based on 411 larvae collected from containers inside and around 137 houses, some containers had one or the other or both species (*Ae. aegypti*, *Ae. albopictus*). Among the water containers sampled, 55% were infested with *Ae. aegypti* alone, 35% with only *Ae. albopictus*, and 10% with both species. It was also found that either *Ae. aegypti* or *Ae. albopictus* or both these species. cohabited with other mosquito species, such as *Culex quinquefasciatus* Say or *Toxorhynchites splendens* Wiedemann, but the latter two species occurred in very small numbers. Another important finding during the course of this study was that *Ae. aegypti* larvae were found only in artificial containers, whereas those of *Ae. albopictus* were distributed more widely in various kinds of oviposition places, both natural and artificial sites.

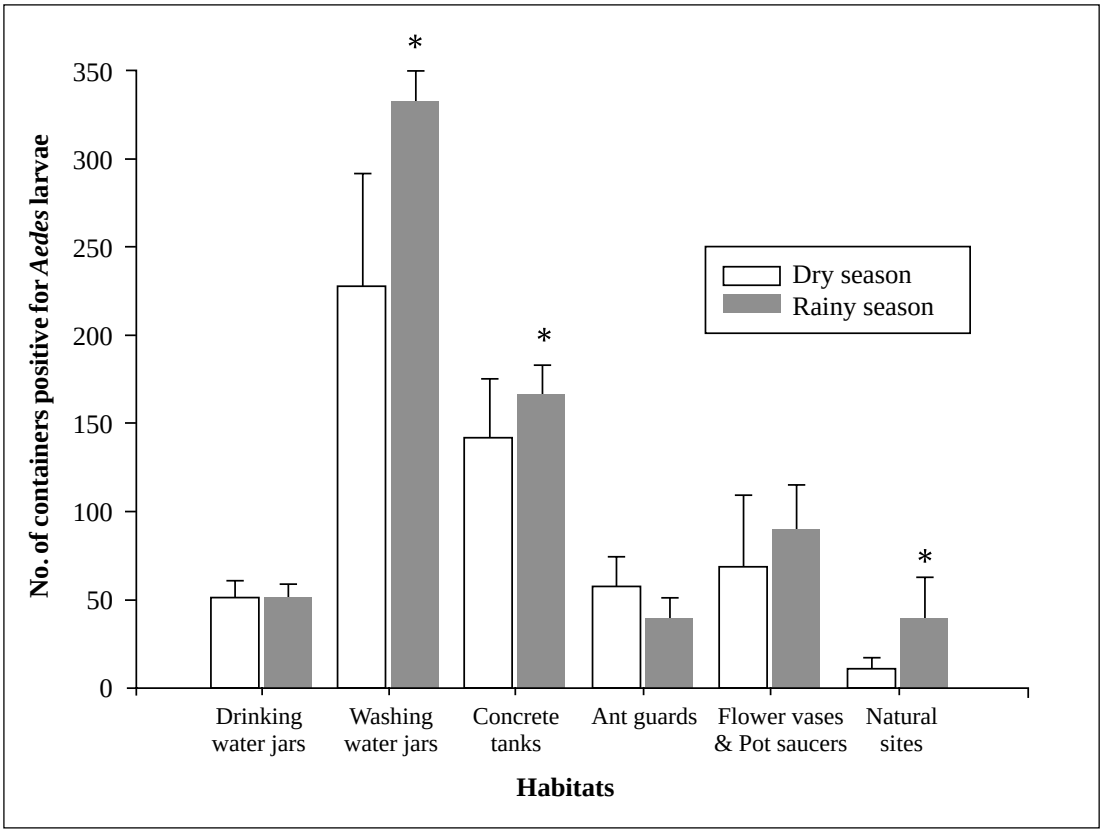

Fig. 2. Average number (+S.E.) of containers infested with** Aedes **larvae per observation according to seasons on Samui Island, Thailand. Means of the containers in dry season were computed from the four observations conducted beween January and March, whereas those in rainy season were computed from the five observations carried out between May and July. Asterisks indicate significant differences of the means of each habitat between dry season and rainy season at the 0.05 level.

As far as the developmental sites of *Ae. albopictus* are concerned, most of the breeding potential of this species was noted to be outdoors. Of the almost 1000 outdoor natural breeding sites surveyed around the island, it was found that approximately 45% of the 623 coconut husks and 10% of 360 coconut floral spathes inspected were routinely infestes with *Ae. albopictus* larvae.

Outdoor oviposition

To examine the outdoor breeding potential in artificial containers and natural sources, ovitrap assessment was made in outdoor premises. The oviposition activity showed that 60% of the 80 ovitraps were infested with *Ae. albopictus* eggs. The average number of eggs deposited on filter paper strips

was 26 eggs per trap per 3 days with range of 11 to 59. Of the eggs collected in ovitraps about 40% of the eggs hatched out on first hydration. The larvae reared to the adult stage all belonged to *Ae. albopictus*. These results clearly indicate that outdoor artificial and natural breeding sources provide suitable habitats for the oviposition and breeding of *Ae. albopictus.* There were no adult *Ae. aegypti* resulting from the larvae hatched from the eggs laid in the outdoor traps during this study, indicating that this species propagates in water storage containers located either indoors or just within 15 m outside the residences.

Biting activity

The data of seasonal adult mosquito biting activity as shown in Figure 3 clearly indicate that the biting activity rate of indoor biting mosquitoes, comprised mostly of *Ae. aegypti* remained relatively constant throughout the nine occasions of this study. However, the population of outdoor biting mosquitoes mostly or exclusively *Ae. albopictus* usually increased dramatically in the rainy season, especially in July 1998. Over the entire study period, the indoor biting rates of *Ae. aegypti* ranged from 1.5 to 8.1 mosquitoes/man-hour, whereas the outdoor rates of *Ae. albopictus* were between 5 and 78 mosquitoes/man-hour. Although there was little or no rainfall in March 1996 and 1997 (dry season), both *Aedes* mosquitoes were still found to be biting at the study sites, *Ae. albopictus* occurring at much lower numbers than in the rainy season. It is interesting to note that even though the breeding sites of *Ae. albopictus* were severely restricted during those dry periods than those of *Ae. aegypti*, the number of adult *Ae. albopictus* was about five times greater than that of *Ae. aegypti* in March 1996, but somewhat lower than that of *Ae. aegypti* in March 1997, *Ae. aegypti* breeds in artificial containers indoors and outdoors close to the dwellings and their numbers are not affected by lack of rainfall because these containers are usually filled with tap or well water even in the dry season. The natural sites outdoors (breeding sources of *Ae. albopictus*), however, are essentially devoid of water in the dry season. In terms of spatial biting activity, the indoor biting mosquitoes were composed of 75.4% *Ae. aegypti* and 24.6% *Ae. albopictus*, while the outdoor biting adults (15 m away from the houses) were 1% *Ae. aegypti* and 99% *Ae. albopictus*. Thus, it is quite obvious that *Ae. aegypti* is primarily an endophagic mosquito, rarely biting outside while *Ae. albopictus* is primarily an exophagic mosquito

but can be found in significant numbers biting indoors. From the data in Figure 3, it appears that *Ae. albopictus* exhibits much higher biting rates than *Ae. aegypti* in the rainy periods. This marked difference is the result of extensive breeding sources outdoors that become productive in the rainy season.

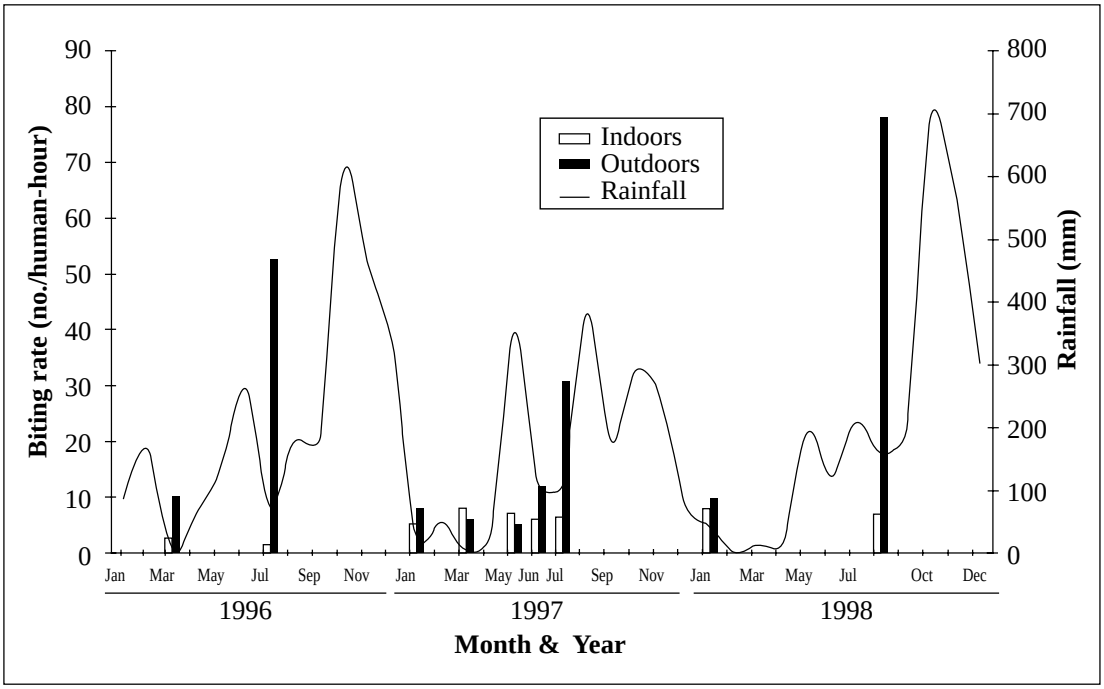

Fig. 3. Adult index (biting rate) of** Aedes **mosquitoes in relation to rainfall on Samui Island, 1996-1998.

According to the results we have obtained on the diel biting activity of mosquitoes indoors, it was found that the biting activity of *Aedes* mosquitoes was higher in the morning hours than in the afternoon period, with a low biting activity between 1300 h and 1400 h (Figure 4), the sun usually rising at about 0600 h and setting around 1800 h in the month of July. The biting rate in the morning period was almost twice as high as that in the afternoon period. The *Aedes* mosquitoes collected in this indoor biting study were identified as 80.2% *Ae. aegypti* and 19.8% *Ae. albopictus.*

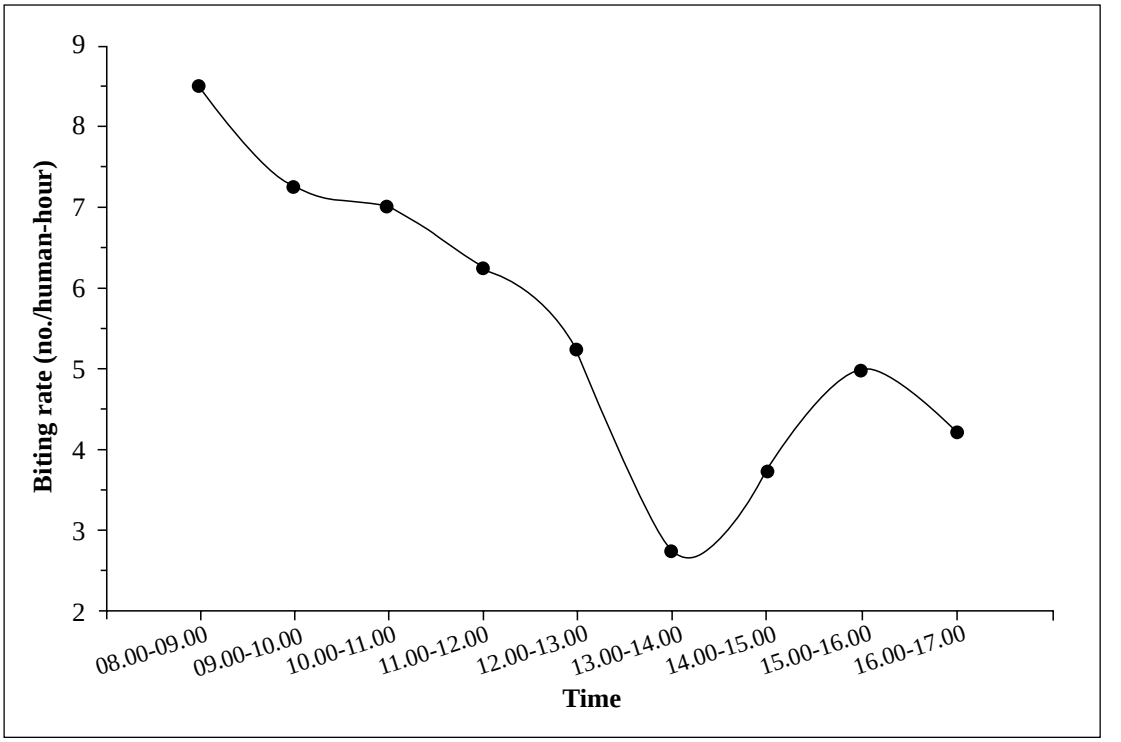

Fig. 4. Daytime biting activity** Aedes **mosquitoes assessed indoors on Samui Island, July 1996.

Discussion

As demonstrated in this as well as previous studies, Samui Island is infested with both *Ae. aegypti* and *Ae. albopictus*. However, *Ae. aegypti* populations as indicated by their seasonal biting activity found here, prevail throughout the year at a relative constant level because their major breeding places are human-made water storage containers which are regularly filled with water, even during the dry season. *Ae. albopictus*, on the other hand, breeding primarily in natural developmental sites outdoors, were noted to be markedly depressed in dry seasons when the developmental sites were mostly dry. With an increasing trend of housing developments, resorts and tourist facilities and effective solid waste management that do not allow water-catching coconut husks and spathes to accumulate on the premises, it would be expected that the breeding potential for *Ae. albopictus* may decline in the future, because these new developments will lead to the elimination or reduction in natural and artificial developmental sites over time. In some highly urbanized areas of Southeast Asia such as Bangkok and Manila because of intensive developments,

Ae. aegypti has replaced *Ae. albopictus* (Hammon et al. 1960), while this trend has not yet materialized in Samui Island. Over three decades ago, Gould et al. (1968) pointed out that *Ae. albopictus* might be displaced by *Ae. aegypti* as has happened in high urbanized areas of Southeast Asia. At the present time it is not conceivable that Samui Island might become highly urbanized in the foreseeable future. This scenario will continue to maintain the presence of *Ae. albopictus* at least for a few decades. From this and previous studies (Gould et al. 1968, 1970), it is clear that *Ae. aegypti* and *Ae. albopictus* have coexisted and currently coexist on Samui Island. However, the overlapping of preferred breeding sources in our study (10%) was considerably higher than that found by Gould et al. (1970). Favorable conditions leading to the high density and activity of these two vectors on Samui Island are created by the long rainy season form May to December and the water use patterns of the residents. Gould et al. (1970) mentioned that the abundance of *Ae. aegypti* and *Ae. albopictus* during the epidemic of DHF in 1967 on Samui Island related directly to the amount of rainfall. With regard to the water use patterns, the islanders prefer rain and well water to piped water for their drinking and cooking purposes, and for this reason rain and well water are always stored in jars and concrete tanks, which are the most favorite breeding sites of *Ae. aegypti*. However, in these types of containers situated indoors or just outside houses, few larvae of *Ae. albopictus* were noted. As evidenced by the Breteau Index values found in this study, *Ae. aegypti* has increased since March 1996 at the beginning of the study, showing an upward trend until the end of the study in July 1998, when the index was approximately six times higher than that set by the Ministry of Public Health for the national goal (BI of *Ae. aegypti* ≤ 50) in the DHF control program. Therefore, it is urgent to develop and implement effective control strategies against these vectors on the island. As found in our studies, over two-thirds of all infested containers were ceramic or earthen jars and concrete water storage tanks. Because of their large volume and presence of heavy larval populations, these two major types of breeding sources should be given due consideration and they should be subjected to rigorous control measures.

Although 1% temephos granular formulation (Abate SG) is usually the treatment of choice for the control of *Aedes* larvae in artificial containers, this kind of treatment in domestic potable water supplies is always rejected by the

residents. Therefore, other acceptable strategies, for example, physical or biological control measures, such as larval source reduction by dwellers or the use of larvivorous predators should be encouraged as alternatives. Although the use of *Bacillus thuringiensis israelensis* (Bti) as a biological control agent against *Ae. aegypti* larvae has been carried out in Thailand, its high cost and the unacceptability of treatments to dwellers are some of the major impediments. Also, the currently available formulations of Bti are short lived and are not cost effective for use in DHF vector control.

It should be pointed out that Samui Island is one of the largest coconut plantation areas in Thailand resulting in large amounts of coconut husks strewn over the landscapes on the island. It is estimated that over one million coconut husks exist on the island at any given time. These coconut husks will eventually be burned to make charcoal for commercial purposes, but before their burning, invariably, they are kept outdoors near the houses for considerable period of time and are usually infested by *Ae. albopictus*, especially in the rainy season. These husks are considered to be one of the highly productive breeding sites for *Ae. albopictus*. On the basis of information gethered on the potential outdoor breeding sites, using ovitraps simulating natural breeding sources, approximately 60% of the ovitraps were infested with *Ae. albopictus* eggs, yielding up to 59 eggs in a trap. No *Ae. aegypti* oviposition was noted in these ovitraps set outdoors. This may be due to numerous breeding sites available for *Ae. aegypti* indoors. The coconut husks have the potential to produce a huge number of *Ae. albopictus* on the island, especially in the rainy season. On the other hand, other natural breeding sites, such as coconut floral spathes or fruit peels are also considered to provide potential natural breeding habitats for *Ae. albopictus*. These natural breeding sites are more difficult to control than artificial containers, but for disease and pest control, it will be necessary to reduce or possibly eliminate these sources of vector species. To cope with these problems, environmental management tactics and the use of ultra low volume (ULV) spraying of pyrethroid insecticides, Bit or the combination of pyrethroids an Bit (Yap et al. 1997) are some of the options for area-wide control of *Ae. albopictus* larvae in coconut husks as well as other natural breeding sites on the island. The former method (environmental management) can be economically administered by the villagers themselves under the guidance of health officers while the latter technique may be performd regularly by the health officers once a month or more often if necessary.

During our study, it was quite rare to find *Ae. aegypti* biting outdoors, constituting only 1% of the biters, while *Ae. albopictus* bit at higher frequency inside the houses (24.6%). These findings indecate that the feeding behavior of both species as found on Samui Island by Gould et al. (1968, 1970) has not changed over the years, *Ae. aegypti* is still endophagic while *Ae. albopictus* is primarily exophagic. The corresponding months to our study (January - March), the biting rates of *Ae. aegypti* and *Ae. albopictus* by Gould et al. (1970) were 0.2-1.5 and 0.4-1 mosquitoes/man-hour, whereas those obtained in our study were 6-10 and 2.6-8.1 mosquitoes/man-hour, respectively. This marked difference leads us to believe that the numbers of both species have increased over time. It is interesting to note that in our study *Ae. albopictus* was found to be biting more indoors (24.6%) than that reported (0.1%) by Gould et al. (1970). Because of the high breeding potential and significant biting activity, *Ae. albopictus* should be considered as an important vector of DHF on Samui Island. In addition, during the morning period, especially from 0800 h to 0900 h, is the critical period for blood feeding of both *Aedes* mosquitoes. This time window is therefore a high risk period for transmission of dengue virus from mosquitoes to humans. The high biting risk period in the morning dictates use of bednets and other personal protection measures for protecting infants and children from mosquito bites.

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Dengue Vector Mosquitoes at a Tourist Attraction, Ko Samui, in 1995

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Abstract

On Ko Samui, Thailand there were two epidemics of dengue hemorrhagic fever (DHF) in 1966 and 1967, followed by endemics up to 1994. *Aedes aegypti* and *Aedes albopictus* were the vectors. From January to July 1995, 51 cases of DHF were reported, out of these were many foreigners who still suffer from dengue fever and return home with negative impression. We carried out an entomological survey around the island and collected the mosquitoes to detect dengue virus by digoxigenin-cDNA probe. The data revealed that *Aedes agypti* and *Aedes albopictus* still were abundant and some were infected with dengue virus. Visual larval survey indices (HI, CI and BI) were 90.4, 61.3 and 301.3 respectively. Biting rate (BR) of *Aedes* mosquitoes was high, the average indoor and outdoor BR were 9.7 and 100.8 mosquitoes/man-hour. From 13 pools of mosquitoes, 8 strains of dengue virus were detected (61.5%). The results may encourage the local authorities to improve vector surveillance and control before the famous island becomes an unplesant island.

Keywords

Dengue, vector, mosquitoes, Ko Samui, *Ae. aegypti*, *Ae. albopictus*

Introduction

Dengue hemorrhagic fever (DHF) has been recognized as a serious disease in Thailand since 1958. It is caused by dengue virus, which has four serotypes and is transmitted by *Aedes aegypti* (Scanlon 1965). On Ko Samui there were epidemics of DHF in 1966 and 1967; *Aedes aegypti* and *Aedes albopictus* were found to be abundant and responsible for transmission of dengue virus (Gould et al. 1968; Russell et al. 1968; 1969). Then, there have been endemics up to 1994. Although there are two species of vectors in Ko Samui (Gould et al. 1970), vector surveillance and control are rather poor. From January to July

1995 there were 51 cases of DHF on the island, including foreign tourists. This report presents data from an entomological survey and determination of dengue virus in collected mosquitoes from Ko Samui in 1995, with a view to providing a basis for improvement of vector control.

Materials and methods

Study sites

Ko Samui is the biggest of a group of several dozen islands in the southern Gulf of Thailand and a part of Surat Thani Province. It is about 19 km long and 16 km wide, with an area of about 304 km^2 and a population of about 32,000. Many regard it as the biggest "coconut island" in the world. The climate of Ko Samui is dominated by the tropical monsoons. Climatologic features (precipitation, humidiy and temperature) recorded at Ko Samui weather station from July 1994 to June 1995 are shown in Figure 1. In July 1995, larval and adult *Aedes* surveys were performed in the 6 villages of Ang Thong, Ma Ret and Na Muang subdistricts (Figure 2).

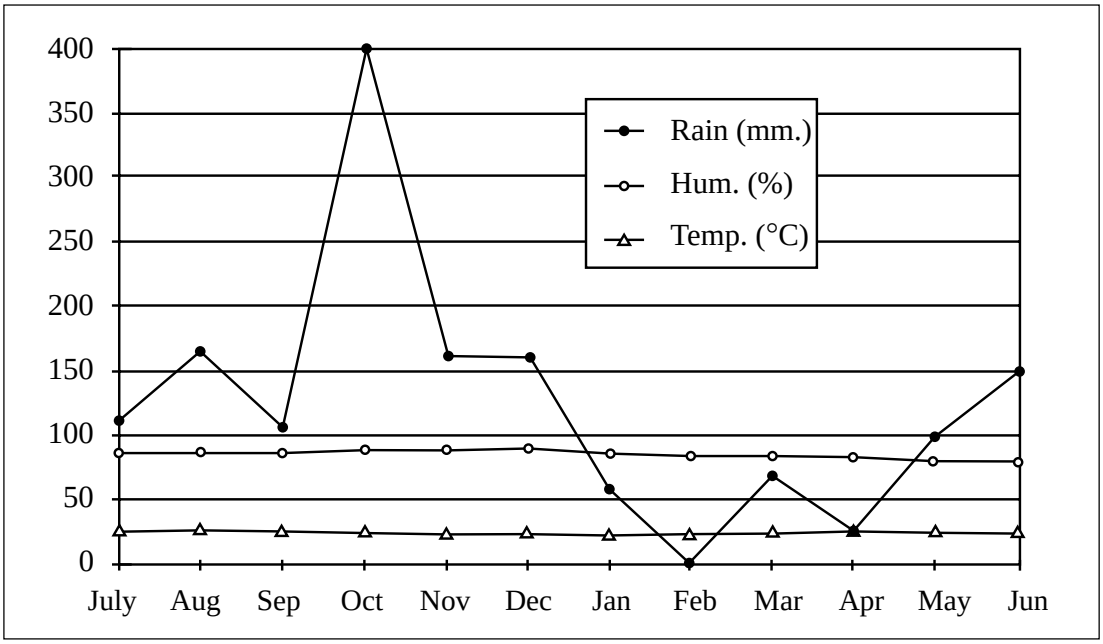

Figure 1. Cilmatologic features of Ko Samui (July 1994-June 1995).

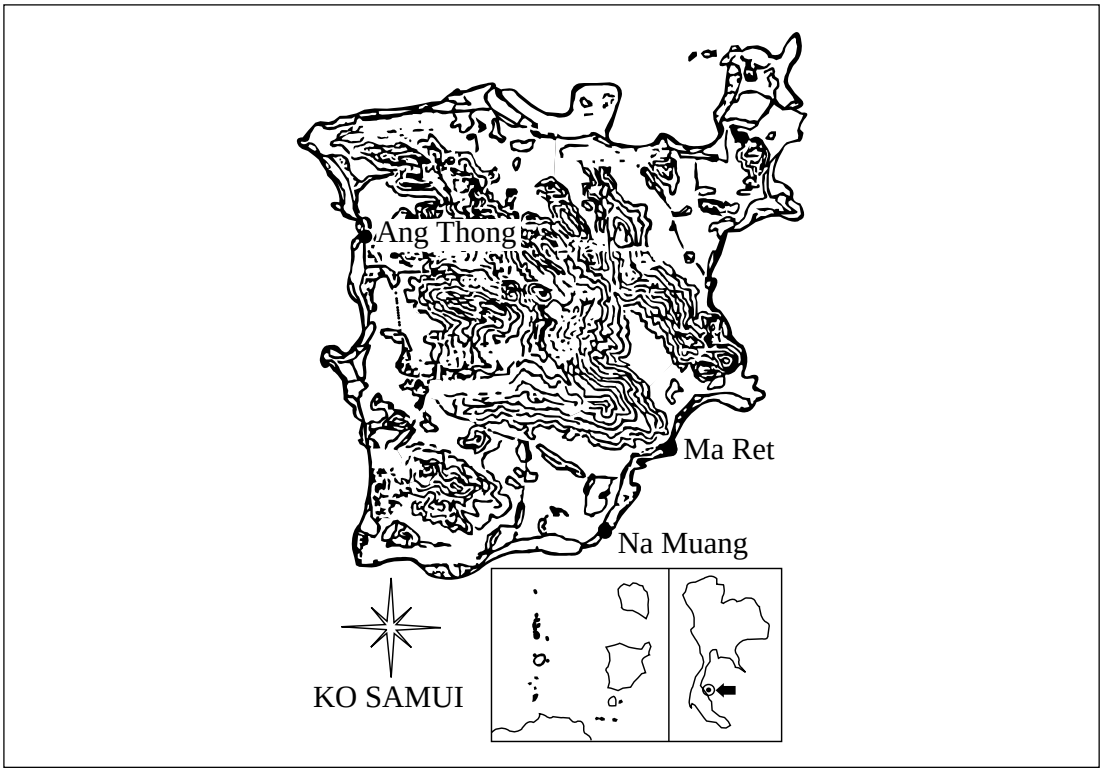

Figure 2. Map of Ko Samui showing study sites.

Mosquito collection methods

The indices of populaiton density of *Aedes* mosquitoes were determined by visual larval survey technique and the collection of adult mosquitoes by human-bait collection according to WHO methods in patients' houses and neighborhoods, as well as other suspected infected areas both indoors and outdoors on the island. The collected mosquitoes were counted for landing and biting rates and identified with respect to species, then reared ~ 24 hours until their stomachs were empty, pooled by collection site, kept in liquid nitrogen and brought back to the laboratory for determination of viruses.

Detection of dengue virus

Dengue RNA was extracted from collected mosquitoes by phenol/ chloroform technique, then hybridized with digoxigenin labeled cDNA probe (Dig-cDNA probe) developed by Guangxi Health and Anti-Epidemic Center, China (Liu et al. 1992). Dig-cDNA probe, 190 base-pairs long is specific for the 4 serotypes of dengue virus. Sensitivity and specificity of this probe are ~

90% and 70% respectively (Liu, unpublished). The details of this method will be presented in a separate paper.

Results

A total of 285 mosquitoes from 6 locations of 3 subdistricts on Ko Samui were identified : 132 *Aedes aegypti*, 140 *Aedes albopictus* and 13 *Armigeres subalbatus*. The biting rate of *Aedes albopictus* outside the houses was 7.2-194.4 mosquitoes/man-hour. Biting rates of both species of *Aedes* inside the houses was 5.8-15.5 mosquitoes/man-hour (Table 1). We could collect both species inside the houses; however, most were *Aedes aegypti*, whereas outside were only *Aedes albopictus.* The data from our surveys confirmed that *Aedes aegypti* was endophilic and *Aedes albopictus* was exophilic. Results from visual larval survey showed that there was high density of *Aedes* mosquito larvae with 85.7-93.8% house index (HI) 54.4-72.2% container index (CI) and 227-419 Breteau index (BI) (Table 2). From 13 pools of mosquitoes, 8 strains of dengue virus were detected by Dig-cDNA probe (Table 3).

Table 1. Biting and landing rate of** Aedes **mosquitoes from human-bait collections on Ko Samui, July 1995.

Location	BR[a] (mosquito/man-hr)		LR[b] (mosquito/man-hr)	
	Indoor	Outdoor	Indoor	Outdoor
Ang Thong	7.7	ND	10.9	ND
Ma Ret	5.8	7.2[c]	8.4	8.5
Na Muang	15.5	194.4[d]	17.7	194.4
Average	9.7	100.8	12.3	101.5

ND : Not done, because of rain.

[a] : Biting rate = The number of female *Aedes* mosquitoes.

[b] : Landing rate = The total number of *Aedes* mosquitoes.

[c] : Coconut plantations, 150 meters far from houses.

[d] : A fruit orchard, 150 meters far from houses.

Table 2.** Aedes **larva index from visual larval survey on Ko Samui, July 1995.

Location	HI[e]	CI[f]	BI[g]
Ang Thong	85.7	72.7	227
Ma Ret	91.7	54.4	258
Na Muang	93.8	56.8	419
Average	90.4	61.3	301.3

[e] : House Index = The number of house positive for *Aedes* per 100 houses.

[f] : Container Index = The number of container positive for *Aedes* per 100 container.

[g] : Breteau Index = The number of container positive for *Aedes* per 100 houses.

Table 3. Detection of dengue virus by Dig-cDNA probe.

Species	No. of tested pools	No. of positive pools	% positive
Ae. aegypti (female)	6	5	83.3
Ae. aegypti (male)	1	0	0
Ae. albopictus	5	3	60.0
Armigeres spp	1	0	0
Total	13	8	61.5

Discussion

There was a high density of *Aedes aegypti* and *Aedes albopictus* on Ko Samui according to WHO Density Figure (Anonymous 1972) and some were infected with dengue viurs. Differences in the preferred habitats and behavior of these two species led to more frequent vector contact with man and made the island a risk area to degue fever than the other areas with only *Aedes aegypti*. We were able to detect the dengue virus in both species of *Aedes* and to confirm that both are implicated in the transmission of dengue viruses on Ko Samui as in the epidemic of 1966-1976 (Gould et al. 1968; 1970). Thus, dengue fever is a threat to the local people and to visitors, for the island is an international tourist attraction. Using rapid detection methods to monitor the dengue virus in vector mosquitoes is important for surveillance and control of dengue epidemics since they provide tools for assessment of infection rate and hence for epidemic forecasting.

In our surveys we found that the biting behavior of *Aedes* mosquitoes was different form those in the other places. Usually the biting peaks of *Aedes aegypti* in the rainy season are at 0900-1100 hour and 1300-1700 hour (Yasuno and Tonn 1970), but the biting peak of these mosquitoes on Ko Samui appeared

to be different and the outdoor biting rate of *Aedes* spp (100.8) was higher than the indoor biting rate (9.7), and different from the results of the survey by Gould et al. (1968). Therefore, it is worthwhile to undertake further ecological studies there. The major controlling measure of dengue fever is reducing the density of vector mosquitoes if possible eliminating them. It is suggested that efficient control methods should be applied on Ko Samui, because the average BI on Ko Samui was 301 and the result of Chansang et al. (1993) revealed that areas with average BI of over 100 are at risk for dengue infection (Chansang et al.1993).

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Evaluation of Attractants and Egg-Laying Substrate Preference for Oviposition by *Aedes albopictus* (Diptera: Culicidae)

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Abstract

Evaluation of oviposition attractants and substrate preferences of *Aedes albopictus* was carried out under laboratory and field conditions. To obtain candidate oviposition substances we used a water rinse of 3 mollusk species: blood cockle (*Anadara granosa*), carpet shell (*Paphia undulata*), sea mussel (*Mytilus smaragdinus*), and the giant tiger prawn (*Penaeus monodon*). The rinse water of carpet shell and giant tiger prawn showed higher attractiveness for ovipostion than the other candidate attractants. The filter paper substrate received fewer eggs than the other two substrates. There was no significant difference between the mean number of eggs laid on hardboard paddles and sponge sheets. The hatching rate of *Ae. albopictus* eggs laid on hardboard paddles was higher than those from the filter papers and sponge sheets. The sponge had lethal effects on *Ae. albopictus* eggs, and very few eggs laid on sponge hatched. In field experiments, evaluation of attractiveness of carpet shell rinse in ovitraps lined with sponge sheet as egg-laying substrate was carried out in various habitats and different areas of Thailand. The mean number of eggs in traps containing carpet shell rinse was significantly higher than those laid in rainwater traps. These studies reveal that the carpet shell and giant tiger prawn rinses are sources of oviposition attractant for *Ae. albopictus* under both laboratory and field conditions and could possibly be used as attractant in surveillance and control.

Keywords

Oviposition attractants, mosquitoes, *Aedes albopictus*, oviposition substrates, ovitraps

Introduction

Aedes albopictus (Skuse), the Asian Tiger Mosquito, is a vector of dengue haemorrhagic fever (DHF), and is capable of breeding in a wide range of container types and water holding habitats. In Thailand, *Ae. albopictus* has been found in forested habitats ranging in elevation from 450 to 1,800 m as well as in a variety of other habitats in rural and suburban areas (Scanlon and Esah 1965, Gould et al. 1970, Thavara et al. 1996). Ubiquitous breeding sites, such as tree holes, coconut shells, fruit peels, water jars, unused and discarded tires and boats holding water have been found to contain *Ae. albopictus* larvae. Because of the diverse breeding sites of *Ae. albopictus*, especially in the forested areas, they may be hard to reach to monitor larval populations. Detection and measuring mosquito abundance through their egg-laying activities using ovitraps is the most common surveillance or sampling method for this and some other *Aedes* mosquitoes, especially *Ae. aegypti* (Service 1992). Yap et al. (1995) pointed out the importance of ovipostion site preferences in planning vector control programs against *Aedes* mosquitoes. However, information on oviposition attractants for *Ae. albopictus* is rather limited at the present time. Sucharit et al. (1980) studied the oviposition behavior of *Ae. aegypti* and *Ae. albopictus* to be influenced by their own larval holding water or that of other species. They found that larval holding water of *Ae. albopictus* significantly increased oviposition by *Ae. aegypti*, but there was no oviposition attractancy for *Ae. albopictus*. Thavara et al. (1989) demonstrated that *Ae. albopictus* (*Ae. aegypti* absent) prefer to lay eggs in the field in containers with conditioned water that was left outside for a long period and with a stable flora together with the immature stages of this species. The present study was carried out to investigate a range of attractant materials and egg-laying substrates for oviposition of *Ae. albopictus* under both laboratory and field conditions.

Materials and methods

Laboratory evaluations

Mosquito colony

Ae. albopictus mosquitoes used in this study were taken from the colony maintained in the insectary of the Biology and Ecology Section, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. The colony was established from eggs collected from

Samui Island, Surat Thani Province, in 1989 by the authors. To obtain larvae, a filter paper substrate with conditioned attached eggs was submerged in water for four hours in a plastic tray (23x32x5 cm) containing 500 ml of deionized water. After hatching, larvae were fed with mouse-food powder (~ 0.5 g at a time) twice a day until completion of larval development. All pupae were removed daily and placed in a beaker with 300 ml of deionized water and then kept in a screen cage (30x30x30 cm) for adult emergence. The mosquito colony was kept in an environmentally controlled insectary, temperature of 26±2 °C, relative humidity of 70±10% and photoperiod of 14L:10D. A piece of cotton wool soaked in 10% sugar syrup was placed in each cage to provide food for the adults. Three days post-emergence, restrained 1-month-old mice were provided for adult females for blood feeding for four hours during daytime. Thereafter, the females were fed with mouse blood at 3-day intervals after the first feeding. A few days after blood meal, the gravid mosquitoes were allowed to lay their eggs in a 400 ml beaker, containing 200 ml of deionized water, the inside of which was lined with a strip of filter paper (Whatman No.1) 27-cm long and 6-cm wide for oviposition. The water level in the beakers reached the middle of the filter paper strip. After oviposition, the filter paper strips were removed and dried at room temperature (25-29 °C) for three days in order to facilitate embryonic development and conditioning of the eggs under dry conditions.

Attractants for ovipositions

The hypothesis that mollusks may be producing odors that attract mosquitoes originated from preliminary observations on the attractancy of mollusks to adult mosquitoes. After purchasing some carpet shells, we put them in a bucket of water to wash and clean them for cooking but observed numerous adult mosquitoes hovering over the bucket. This stimulated our interest in testing several mollusks for *Ae. albopictus* ovipositional activity. Three mollusk species: *Anadara granosa* Linnaeus (blood cockle), *Paphia undulata* Bom (carpet shell), *Mytilus smaragdinus* Chemnitz (sea mussel) and one prawn species: *Penaeus monodon* Fabricius (giant tiger prawn) were used for obtaining oviposition attractant agents for *Ae. albopictus* in tests conducted under laboratory conditions. The three mollusk species were caught in the sea whereas the giant tiger prawn was cultured in an aquaculture farm. The fresh

marine animals were purchased from a local market in Nonthaburi for obtaining attractants before start of each test. One kg of each species was submerged in one L of distilled water in a plastic tray and left for 30 minutes. The animals were then removed by netting from the trays and the rinse waters were used as candidate sources of attractants. During the netting most, if not all, of the sediments were removed. The rinse water was used immediately in oviposition bioassays.

For oviposition testing, 250 *Ae. albopictus* gravid females (aged 5-7 days, 3-4 days after blood meal) were released in a mosquito screen cage (40x40x40 cm), where five white plastic cups (15-cm high and 12 cm rim-diameter) were placed for oviposition. Each cup was filled with 300 ml of one of the five test waters: blood cockle rinse, carpet shell rinse, sea mussel rinse, giant tiger prawn rinse, and distilled water control. These cups were randomly located in a circle of 15-cm diameter. The inside of each cup was lined with a strip of white filter paper (Whatman No.1, 7-cm long and 6-cm wide) where the water level reached the middle of the strip for mosquito oviposition. A piece of cotton wool soaked in 10% sugar syrup was placed in the cage to provide food for the adult mosquitoes. The cage was kept for 48 h in an environmentally controlled room with photoperiod (14L:10D), relative humidity (70±10%) and temperature (26±2 °C). After oviposition for two days, all oviposition cups were taken from the cage and the strips were removed and dried at room temperature (25-29 °C) for one day. The numbers of eggs deposited in each strip were then counted using a stereomicroscope. Each experiment was carried out in one cage for a total of nine replications.

Egg-laying substrate preferences

Filter papers, sponge sheets and hardboard paddles were studied for oviposition by *Ae. albopictus*. The white filter paper (Whatman No.1) and pale-yellow sponge sheets (approximately 2 mm thick, mostly used as shoulder pad in lady garments) were 27 cm long and 6 cm wide, whereas the dark-brown hardboard paddles (made from compressed sawdust) were 12 cm long and 2 cm wide. All were placed in cups containing distilled water. The filter paper strip and sponge sheet lined the inside of the cup where the water level reached the half way mark. The paddle was clipped to the inside of the trap with one half submerged and the other half out of water.

For oviposition, 50 *Ae. albopictus* gravid females (aged 5-7 days, 3-4 days after blood meal) were released into each of three small mosquito screen cages (30x30x30 cm) where three white plastic cups (15 cm high and 12 cm rim-diameter) were used as before for oviposition. Each cup was filled with 300 ml of distilled water and supplied with different substrates (i.e., filter paper, sponge sheet, and hardboard paddle). The water level reached the middle of the filter paper and sponge sheet strip, whereas the hardboard paddle was clipped to the inside of the cup with the rough side exposed for mosquito oviposition. The oviposition cups were randomly located in a 15-cm diameter circle. A piece of cotton wool soaked in 10% sugar syrup was placed in the cage to provide food for adult mosquitoes. The cage was kept for two days in an environmentally controlled room with photoperiod (14L:10D), relative humidity of 70±10% and temperature of 26±2O °C. The numbers of eggs deposited on various substrates in the cups were counted under a stereomicroscope. The substrates containing eggs were dried at room temperature for three days for embryonic development and conditioning of the eggs, and then each substrate was submerged in water for four h in a plastic tray (23x32x5 cm) containing 500 ml of deionized water for hatching observations. Two days after hatching, the numbers of larvae hatched from each substrate were counted and recorded. Each experiment was carried out in three cages on seven occasions for a total of 21 replications.

Laboratory evaluations of ovitraps with carpet shell rinse water as attractant and sponge sheet as substrate

Because the carpet shell rinse and sponge sheet substrate received greater ovipositional activity, we wanted to test these two factors jointly. An ovitrap using carpet shell rinse water as an attractant and dechlorinated water as control with the sponge sheet as egg-laying substrate was evaluated for ovipositional activity under laboratory conditions. The test procedures used were similar to the study of egg-laying substrate preferences for ovipositions and hatching observations as described above. Each experiment was carried out in four cages on nine different occasions for a total of 36 replications.

Field evaluations

Evaluations of the attractiveness of carpet shell rinse were carried out in the field by using modified ovitraps of Pratt and Jacob (1967). The ovitraps used were 450-ml capacity black flower-pots (9 cm high and 10.5 cm in diameter at the top) that had no drain holes. A total of 120 ovitrap pairs (i.e., one trap filled with 300 ml of carpet shell rinse and the other trap with 300 ml of rainwater) were set at a time in various habitats, including an orchard, palm plantation, rubber plantation, waterfall area, and a public park on the hill, in four southern provinces: Surat Thani, Nakhon Si Thammarat, Trang and Songkhla. These traps were set on the ground in shady areas protected from intense rain and wind. The carpet shell rinse was prepared as described in the laboratory studies and the fresh rinse was used in the ovitraps. The inside of each ovitrap was lined with a strip of the sponge sheet (27x6 cm) for mosquito oviposition. Three days after their placement in the different field sites, the sponge sheet strips were collected, dried, and examined for *Aedes* eggs. If eggs were present, the eggs were counted under a stereomicroscope about two or three days after drying. For species compositions, the egg strips were brought to the laboratory and after conditioning for a week were flooded and the eggs hatched. The larvae were reared to the adult stage and identified to species. These field trials were carried out on three occasions once a month in July 1998, April and July 1999.

Data analysis

The numbers of *Ae. albopictus* eggs obtained from various attractants, substrates and hatching rates were transformed to $\sqrt{x+0.5}$ to normalize the data prior to statistical analysis. They were compared for mean numbers using the one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test. For the laboratory and field trials of ovitrap using carpet shell rinse water as an attractant and sponge sheet as egg-laying substrate, the *t*-test analysis was used to compare treatments with controls. The accepted level of significance for all comparisons was $P \leq 0.05$. Analysis was carried out using the SPSS program for windows version 10.0 (SPSS 1999).

Results

Laboratory evaluations of oviposition attractants

The average numbers (± S.E.) of mosquito eggs in cups holding rinse water from blood cockle, carpet shell, giant tiger prawn, sea mussel and distilled water (control) were: 1,081 ± 60, 2,604 ± 128, 2,455 ± 94, 1,655 ± 104 and 861 ± 102, respectively. There was no significant difference between mean numbers of eggs collected from the carpet shell and the giant tiger prawn rinses ($P > 0.05$), but both received significantly higher number of eggs than the sea mussel, the blood cockle rinse and distilled water ($P < 0.01$). There was also no significant difference between the blood cockle rinse and distilled water ($P > 0.1$), but both were significantly lower than the sea mussel ($P < 0.05$).

Egg-laying substrate preferences

The substrate preference study revealed that *Ae. albopictus* preferred to lay eggs in the cups provided with sponge sheets and hardboard paddles over the filter paper strips used in laboratory colonies. The mean (± S.E.) number of eggs collected was: 169.7 ± 21.6, 277.9 ± 27.5 and 364.1 ± 40.9 for filter paper, sponge sheet and hardboard paddle, respectively. The mean number of eggs collected from filter papers was significantly lower than those collected from the hardboard paddles and sponge sheets. However, there was no significant difference between the mean numbers of eggs collected from hardboard paddles and sponge sheets. The average hatching rate of *Ae. albopictus* eggs obtained on the hardboard paddles was higher than the hatches on the filter papers and sponge sheets, with mean (± S.E.) hatching of 54 ± 3.7%, 31.6 ± 2.8%, and 3.7 ± 0.8%, respectively. Significant differences in hatching rates from each other were observed among the three substrates ($P < 0.01$).

Evaluation of carpet shell attractant and sponge sheet ovitraps

An experiment was carried out to compare oviposition of *Ae. albopictus* in carpet shell rinse water and dechlorinated tap water using sponge sheets as egg-laying substrates. The results showed that *Ae. albopictus* laid significantly more eggs in carpet shell water about twice as many eggs as in dechlorinated tap water ($P < 0.001$), the mean numbers (± S.E.) of eggs collected were 854 ± 86 and 425 ± 55, respectively. As in the previous hatching rate study, the hatching rate of eggs laid on sponge sheets was very low and there was no

significant difference between the hatching rates of the eggs obtained from both carpet shell rinse water and dechlorinated tap water ($P > 0.05$). The mean hatching rates (± S.E.) of *Ae. albopictus* eggs obtained from the ovitraps containing carpet shell rinse water and dechlorinated tap water were 5.3 ± 1.2% and 4.2 ± 1.0%, respectively.

Field evaluations

We used a total of 360 ovitrap pairs, with each pair was filled with 300 ml of either carpet shell rinse or rainwater was used. The substrate for oviposition was sponge sheet. In the two treatments run on three occasions (120 ovitrap pairs in each occasion), the positive rate of oviposition in the ovitrap having carpet shell rinse was higher than that of the rainwater in all instances. A total of 10,588 eggs were collected from 243 positive traps filled with the carpet shell rinse, whereas a total of 6,606 eggs was obtained from 222 positive traps filled with rainwater (control). In the group of traps with carpet shell rinse, the eggs found in each trap ranged from a single egg to 225, whereas those in the control group ranged from a single egg to 175. The mean number of eggs (43.6 ± 2.8) collected in the traps containing carpet shell rinse was significantly higher than that of the control group (29.8 ± 2.0) containing rainwater only ($P < 0.01$). In hatching experiments, very few *Ae. albopictus* larvae hatched from eggs collected by sponge sheets in the traps. Hatching rates of the eggs obtained from the traps containing carpet shell rinse and rainwater were equal and less than 1%. This again showed the lethal effects of the sponge sheets as before. All the hatched larvae reared to adults were identified to be *Ae. albopictus*.

Discussion

The carpet shell and giant tiger prawn rinses increased the numbers of *Ae. albopictus* eggs collected in the ovitraps under both laboratory and field conditions. The oviposition attractants in these rinses may be chemical stimuli released from the carpet shell and giant tiger prawn. This is the first report on the ovipositional activity of water rinse obtained by submergence of carpet shell and giant tiger prawn in water. It would be interesting to chemically identify the specific compound(s) responsible for attractant activity in these rinses. Nonetheless, the carpet shell or giant tiger prawn rinses could be used as an

effective attractant sources in ovitraps used in monitoring and management of *Ae. albopictus* populations.

The sponge sheet had two interesting features that should encourage its use as an effective egg-laying substrate. First, although the sponge sheet collected lower numbers of eggs than did the hardboard paddle, there was no significant difference between the two means. This may imply that the sponge sheet is equally effective in receiving eggs and it could be used as the egg-laying substrate instead of the hardboard paddle. Second, the sponge sheet demonstrated highly significant inhibitory effect on the hatching of *Ae. albopictus* eggs as compared to the other substrates. This lethal effect on eggs will be an important strategy for use in the control of *Ae. albopictus* mosquitoes because it requires no insecticidal treatments. This novel strategy offers a viable option for sampling *Ae. albopictus* that also kills them, with a mortality ranging from 95 to 99%.

An ovitrap with a combination of the carpet shell rinse as the attractant and the sponge sheet as the egg-laying substrate could constitute a lethal ovitrap system for *Ae. albopictus* mosquitoes in the field. This type of trap could reduce mosquito populations substantially as it could induce more oviposition of the mosquitoes in baited ovitraps in natural sites and subsequently, due to the lethal effects of the substrate, very few eggs laid in the traps would hatch. Further research is needed to clarify these aspects of this novel lethal trap system.

In conclusion, these studies reveal that the carpet shell and giant tiger prawn rinses exhibit a good level of attractiveness for oviposition of *Ae. albopictus* under both laboratory and field conditions. If the attractant principles can be improved by blending and concentration, the strategy could provide a viable and practical tool for use in surveillance and management of *Ae. albopictus* populations. The rinses may contain one or more chemical stimuli inducing ovipositon in *Ae. albopictus*. Further research for identifying the specific compound(s) responsible for attractancy in the carpet shell and giant tiger prawn rinses is warranted. As for the egg-laying substrate study, the sponge sheet showed excellent hatching-inhibition effects against *Ae. albopictus* eggs under both laboratory and field conditions. With further refinement of this technique, it could become an effectively lethal ovitrap for *Ae. albopictus* without the use of insecticides. Further studies on the factors causing hatching-inhibition of eggs on the sponge sheet could lead to the development of new strategies for reducing the hatch of eggs of *Ae. albopictus.*

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Climatic and Social Risk Factors for *Aedes* Infestation in Rural Thailand

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Abstract

An intense epidemic of dengue haemorrhagic fever in 1998 prompted the Thai government to investigate the feasibility of focalized vector *(Aedes aegypti)* control programmes. We tested for correlations of three indices of *Aedes* larval abundance (housing index, container index and Breteau index) against 38 socio-economic and four climatic variables. Availability of public water wells, existence of transport services and proportion of tin houses were positively associated with larval indices. Private water wells, health education, health insurance coverage, thatched houses and use of firewood for cooking were negatively associated. These probably represent both direct effects on breeding sites (private vs. public wells decrease necessity to store water, and health education may encourage breeding site removal), and more general effects of health-related attitude, housing quality and remoteness from urban areas. Indices were positively associated with daily minimum temperature, an increase in precipitation from the previous month (reflecting the onset of the rainy season) and daily maximum temperatures of approximately 33-34 °C. The associations were used to derive statistical models to predict the rank order of larval indices within the study area (Spearman's correlation coefficients = 0.525-0.554). The study provides a rational basis for identifying possible social interventions, and for prioritizing previously unsurveyed villages for further monitoring and focalized vector control.

Keywords

Dengue, climate, socio-economic, risk factors, risk map, *Aedes*

Introduction

Aedes mosquitoes transmit dengue virus, causing both classical dengue fever and potentially fatal dengue haemorrhagic fever (DHF). The first reported epidemic of DHF occurred in southeast Asia in 1953 (Gubler 1997). The disease has subsequently expanded in distribution to become a major public health problem throughout the tropics, with about 3,700,000 cases worldwide between 1956 and 1995. Approximately one-third of these cases were reported from Thailand (Halstead 1997). This represents a large disease burden for patients, and high financial costs of medical treatment and vector control for the Thai Government (Okanurak et al. 1997). In 1998, Thailand experienced and exceptionally intense epidemic of DHF: 112,488 cases (23.3% increase from 1997) and 415 deaths (64.0% increase).

In the continued absence of an effective vaccine, efforts to control dengue are generally through control of the principal vector *Aedes aegypti*, and the secondary vector *Ae. albopictus*. Control measures targeting larvae in and around houses (i.e. source reduction and larvicide application) are usually considered more effective than adulticidal aerosols, which show poor penetration to *Aedes* resting sites (Reiter & Gubler 1997). Thailand operates integrated vector control. A community-level health education campaign has been conducted from late 1980s (Swaddiwudhipong et al. 1992a,b). Since 1992, the Ministry of Public Health (MOPH) and the Ministry of Education have integrated information about dengue into the primary school curriculum, and since 1998, vector control and health education specialists have worked throughout the country, applying larvicide and fogging during the epidemic season.

However, limited financial and human resources restrict further expansion of this costly government programme. There is a need to focus vector control interventions on those areas that are most at risk, and to recommend social and environmental changes that will have the greatest effect on vector abundance, and therefore (presumably) disease incidence.

Both climatic and socio-economic risk factors are known to affect the abundance of *Aedes* mosquitoes. Temperature has been shown to affect population biology in the laboratory (Rueda et al. 1990; Tun-Lin et al. 2000), while models based on precipitation, temperature and atmospheric moisture explain much of the intra-annual variation in *Aedes* abundance (Moore 1985;

Focks et al. 1993a,b) and dengue incidence (Focks et al. 1995; Jetten & Focks 1997). In addition to these climatological factors, cultural and socio-economic factors, particularly housing, may effect vector abundance and disease transmission (Kuno 1995; Tun-Lin et al. 1995). As vector control programmes are heavily focussed on community involvement in environmental modification, it would clearly be an advantage to identify, and subsequently modify, community level behavioural and housing risk factors for *Ae. aegypti.*

Risk factor investigations have generally been restricted to detailed studies of characteristics of breeding sites recorded in specifically entomological surveys, and concentrated on urban areas. In order to be of practical value in Thailand, it is needed to carry out risk factor analysis and prioritization based on the more generally available socio-economic indicators (i.e. not requiring expensive additional surveys). Rural areas, which have become the important sites of dengue transmission in Thailand since the late 1960s (Jatanasen & Thongcharoen 1993), should be included in such analyses, not least because of their economic importance as tourist centres. The present study tests for statistical associations between climatic and socio-economic variables and three indices of mosquito abundance, in order to identify risk factors which could be used to identify high-risk villages, and could potentially be modified to decrease risk. It then generates models to predict abundance in unsampled villages, and tests their accuracy against independent observations.

Materials and methods

Study area

Thailand is composed of 76 provinces, which in turn consist of approximately 900 districts, 7,000 subdistricts and 60,000 villages. The present analysis included 18 provinces in the northern region of the country (Figure 1). A computerized map of Thailand, TDS Pro plus® (scale 1:250,000, representing borders for 875 districts registered in November 1998), was used as the basis for geographical analysis using the geographical information system software, Mapinfo® version 4.

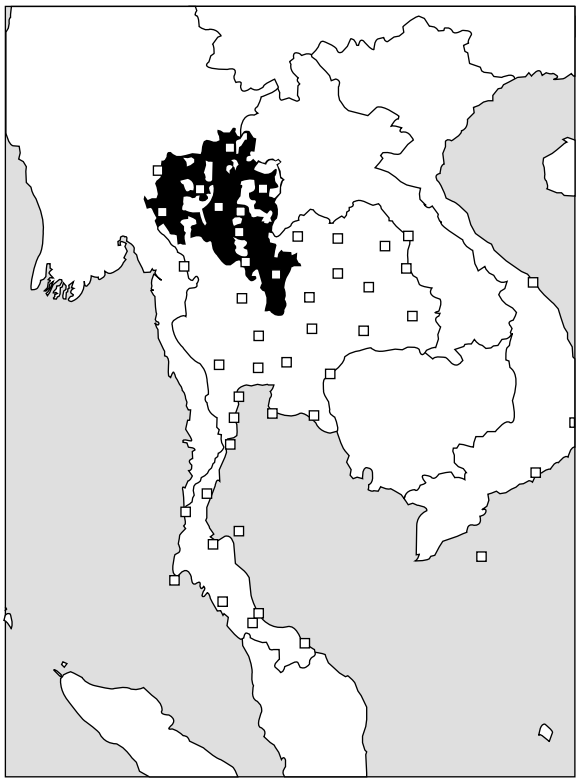

Figure 1. Coverage of the entomological survey and weather stations used in the analysis. Shading indicates 91 districts where entomological surveys were conducted. Squares denote the locations of weather stations in Thailand and Vietnam used in the analysis.

Vector abundance data

Bi-monthly household surveys of *Aedes* larvae were conducted by trained community volunteers from 1992 to 1996 in 91 districts in northern Thailand, under the supervision of the Communicable Disease Center (CDC) of Thailand (Suwonkerd & Prachakwong 1996) (Figure 1). The survey teams recorded House Index (HI): percentage of houses with containers positive for *Aedes* (subgenus *Stegomyia*) larvae or pupae, Container Index (CI): percentage of positive water-holding containers, Breteau Index (BI): number of positive containers per 100 houses. The volunteers did not have to differentiate *Ae. aegypti* and *Ae. albopictus*. However, in Thailand larvae and adults captured outdoors are usually *Ae. albopictus*, while those found indoors are almost exclusively *Ae. aegypti* (Thavara et al. 2001), as in other countries (Rodhain & Rosen 1997). These indices are therefore considered to mainly reflect the abundance of *Ae. aegypti.*

Entomological data are reported to the vector control authority at the village level. Observations were registered to the subdistrict name because villages in Thailand sometimes change their names. In total, 1092 entomological records were available (367 in 1994; 489 in 1995; 236 in 1996).

Climatological data

Daily Surface Observation Data From 1994 to 1998 was purchased from the US Department of Commerce, National Oceanic and Atmospheric Admisiatration. Monthly averages of minimum and maximum temperatures, and total precipitation were calculated for 144 weather stations on the Indo-China peninsula, for the period January 1994 to December 1998. Weather stations were excluded if any variable was missing for more than 10 days in any month. Data from 48 stations (44 in Thailand; four in Vietnam) remained eligible for analysis (Figure 1). The climate in each district was estimated by interpolating the data from all enrolled weather stations to the geographical centre for each district, using the Inverse Distance Weighting (IDW) method (reviewed by Roberts et al. 1993). To validate the reliability of IDW, monthly means of daily maximum and minimum temperatures, and precipitation, were interpolated to the location of each of the 48 weather stations by using the other (47) weather stations. The correlation coefficients (r) between interpolated and actual values were: 0.791 for mean maximum temperature; 0.885 for mean minimum temperature; 0.644 for mean precipitation (n = 2880). IDW gave a better fit than an alternative technique of interpolation, weighing by inverse of the exponential of distance.

The following climatic variables from the month prior to each entomological survey, used as explanatory variables:

- Mean maximum temperature (°C), and its quadratic term.
- Mean minimum temperature (°C), and its quadratic term.
- Mean precipitatioln (mm/day).
- Increase of mean precipitation before 2 months (mm/day).

Quadratic terms were introduced to allow the fitting of non-linear relationships between larval indices and temperature, as excessively low or high temperatures are known to decrease mosquito fitness (Rueda et al. 1990; Clements 1992; Tun-Lin et al. 2000).

In addition, one sine and one cosine term with annual periodicity were included as explanatory variables to account for any stable seasonal variation other than that already explained by climatic factors (Montgomery et al. 1990).

Socio-economic data

Information on a wide variety of socio-economic characteristics has been collected biannually, for every region in Thailand, excluding Bangkok. These data are based on questionnaire surveys, applied to each 'village office' by provincial representatives from governmental ministries, under the initiative of the National Rural Development Committee (NRDC). Each district has a central subdistrict ('municipality'), which was excluded from this survey. The collected information is then compiled into NRDC 'rural database' at the Information Processing Institute for Education and Development. In our analyses, the rural database of 1994 was used to represent conditions in 1994/ 1995, and the 1996 survey was used to represent 1996 conditions. In total, 121,267 villagelevel records were available (60,133 villages in 1994; 61,134 in 1996).

Of 249 items in the questionnaire, 38 variables (Table 1) were selected based on the following criteria: scale or dichotomous items; items for which more than 95% of all the records have meaningful values; items which are defined only with objective terminology (e.g. such words as 'sufficient' or 'hygienic' were rejected as subjective); items which do not contain monetary value (because of the difficulty in adjusting for inflation between different census years); items for which duplicated independent translations of the questionnaire were consistent; items which cannot be calculated automatically by combining other items (e.g. the number of concrete houses from the total number); items for which all the records are within a meaningful range (e.g. between 0% and 100% for proportional data). Only the total size of population was used to represent demography. Summary values of these 38 variables were calculated for each subdistrict and district.

Statistical analysis

Stata 5.0 was used to analyse the relationships between climatological and socio-economic predictors, and *Aedes* abundance at the subdistrict level (dictated by the resolution of the entomological data). The entomological indices

were transformed to approximately normal distributions, by using aresine (square root) transformation for HI, and CI, and taking the natural logarithm of BI (Campbell 1989). A total of 1092 records (covering 12 provinces, 91 districts and 115 subdistricts) were linked to socio-economic and climatic variables.

To allow robust and easily interpretable multivariate analysis, we first used univariate analysis to pre-select amongst the 38 socio-economic indices above. As each subdistrict was surveyed several times, random effect regression was applied, with subdistrict identity being assigned as the random effect parameter, to account for similarities within each subdistrict in both these analyses, and subsequent multivariate analyses. Socio-economic indices which showed a significant association ($P < 0.05$) with all the entomological indices were retained for the multivariate analysis. All the climatic variables (or variable pairs), and the sine/cosine terms, showed a significant association with all three entomological indices.

The pre-selected socio-economic, climatic and sine/cosine variables were used as predictor variables in random effect multivariate linear regression. The least significant explanatory variables were omitted in a stepwise fashion until all remaining variables explained a significant proportion of the variance (backward Wald's test). Odd-numbered entomological records (n=546) were used to build predictive models for each entomological index. These models were then applied to the remaining even-numbered records ('validation data set'). To assess the fit of the model, predicted and observed values were compared using Sperman's rank correlation coefficient (r). Rank correlation is used rather than the correlation of absolute values, both because it is not affected by the transformation used in the present study, and because the main aim of our models is to rank different regions in order of priority for vector surveillance and control programmes. Finally, the statistical significance of the variables selected by Wald's test and the fit of multivariate models were examined by reversing the data set for model building and that for validation.

Risk map

The resulting models were applied to socio-economic indices since 1994 and climatic values of July 1995 (i.e. during the epidemic season), to generate a risk map of *Aedes* abundance for 183 districts in 18 provinces in northern

Thailand (including 91 districts included in the original survey). Districts that were present in TDS but absent in the 1994 rural database, were left blank. As the predictions are based on a socio-economic database that excludes one central (usually the most urban) municipality in each district, the map represents the risks in rural areas.

Table 1. Socio-economic indices tested for association with larval indices.

Number, per total population	Number, per total families	Proportion of all villages	Number, per total houses	Number, per total area
1. Public water wells	13. With multiple occupations	18. With religious buildings	34. Population	37. Population
2. Private water wells	14. Involved in farming	19. With rice bank	35. Tin houses	38. Houses
3. Population with school education	15. Not owning own farms	20. With animal bank	36. Thatched houses	
4. Population with junior high school education	16. Participation in weekly religious rites	21. With public health office		
5. Population with senior high school education	17. Participation in weekly traditional rites	22. With kindergarten		
6. Population with higher education		23. With primary school		
7. Illiterate population 14-50 years of age		24. With high school		
8. Population with health insurance		25. With community centre		
9. Participations in occupational training in last 2 years		26. With library		
10. Participations in ethics education in past 2 years		27. With rubber factory		
11. Participations in military exercise in past 2 years		28. With occupational school		
12. Partcipations in health education in past 2 years		29. With post office		
		30. With road to district centre		
		31. With transport to district centre		
		32. Using firewood for cooking		
		33. Located in the national forest		

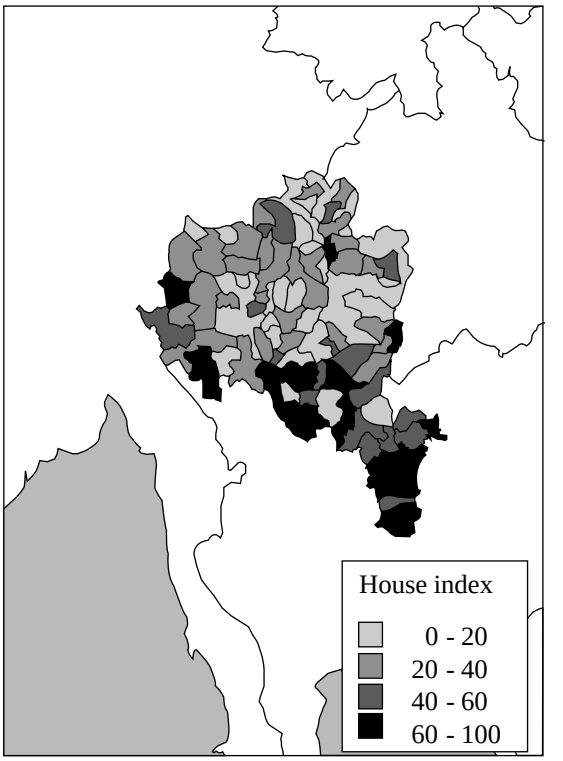

Figure 2. Observed infestation of** Aedes**, represented as House Index (HI), in the surveyed region. HI observed between May and September in 1994-1996 was averaged for each district.

Results

Entomological data

Figure 2 shows the spatial variation in the abundance of *Aedes* averaged from the records taken during the epidemic season (May-September) between 1994 and 1996. HI, CI, and BI had highly skewed distributions: HI (mean 38.3, range 0-99.9); CI (mean 11.9, range 0-80.7); BI (mean 95.2, range 0-7.50). The transformations described above approximately normalized the distributions: HI (mean 0.650, range 0-1.54); CI (mean 0.315, range 0-1.12); BI (mean 3.96, range -0.511-6.62).

Temporal variation

Temporal variations of climate and entomological indices are shown in Figure 3a, b. The climatic variables are the means of 11 weather stations located in the surveyed area (Figure 1). As all indices are highly correlated with each other ($r > 0.83$), only HI is shown.

Socio-economic indices in univariate analysis

Public water wells (item one in Table 1), transport services (31), and tin houses (35) were all significantly positively correlated with each of HI, CI and BI. Private water wells (2), health insurance (8), ethics education (10), health education (12), religious rites (16), rice bank (19), kindergarten (22), primary school (23), community center (25), library (26), use of firewood (32), thatched houses (36) and house density (38) were all significantly negatively correlated.

Multivariate analysis

Table 2 shows those variables that remained in the minimum adequate model for each *Aedes* index and the fit of each model, expressed as Spearman's rank correlation coefficient (r) between the actual and predicted values for validation data set. Potential positive risk factors were tin houses (for HI, CI and BI), public water wells and transport services (for HI and CI). Factors associated with negative risk were: private water wells, health insurance, health education, use of firewood, thatched houses.

Indices were positively associated with minimum temperature, and the increase in precipitation from 2 months to 1 month before. The relationship with maximum temperature was non-linear for each index. Differentiation of the expression combining the linear and quadratic temperature terms (Kuhn et al. 2002) indicated the various indices reached maxima at very similar temperature values: 33.2 °C for HI; 33.2 °C for CI; 34.2 °C for BI.

When the data set for model building and that for model validation were reversed, thatched houses did not remain as a statistically significant variable for HI and CI, suggesting that this variable is not as robust as other variables in Table 2. However, the fits of the predictive models were similar even without this variable (r = 0.619-0.637), and all other risk factors remain significant.

Table 3 shows mean and standard deviations for the significant socio-economic variables (measured at district level in the 1996 rural database), both for the original 91 entomological survey districts, and for all 183 districts in 18 provinces in northern Thailand. Socio-economic characteristics in the survey districts were broadly similar to those elsewhere, suggesting that they provide a reasonable basis for generating predictive models for elsewhere in the northern region.

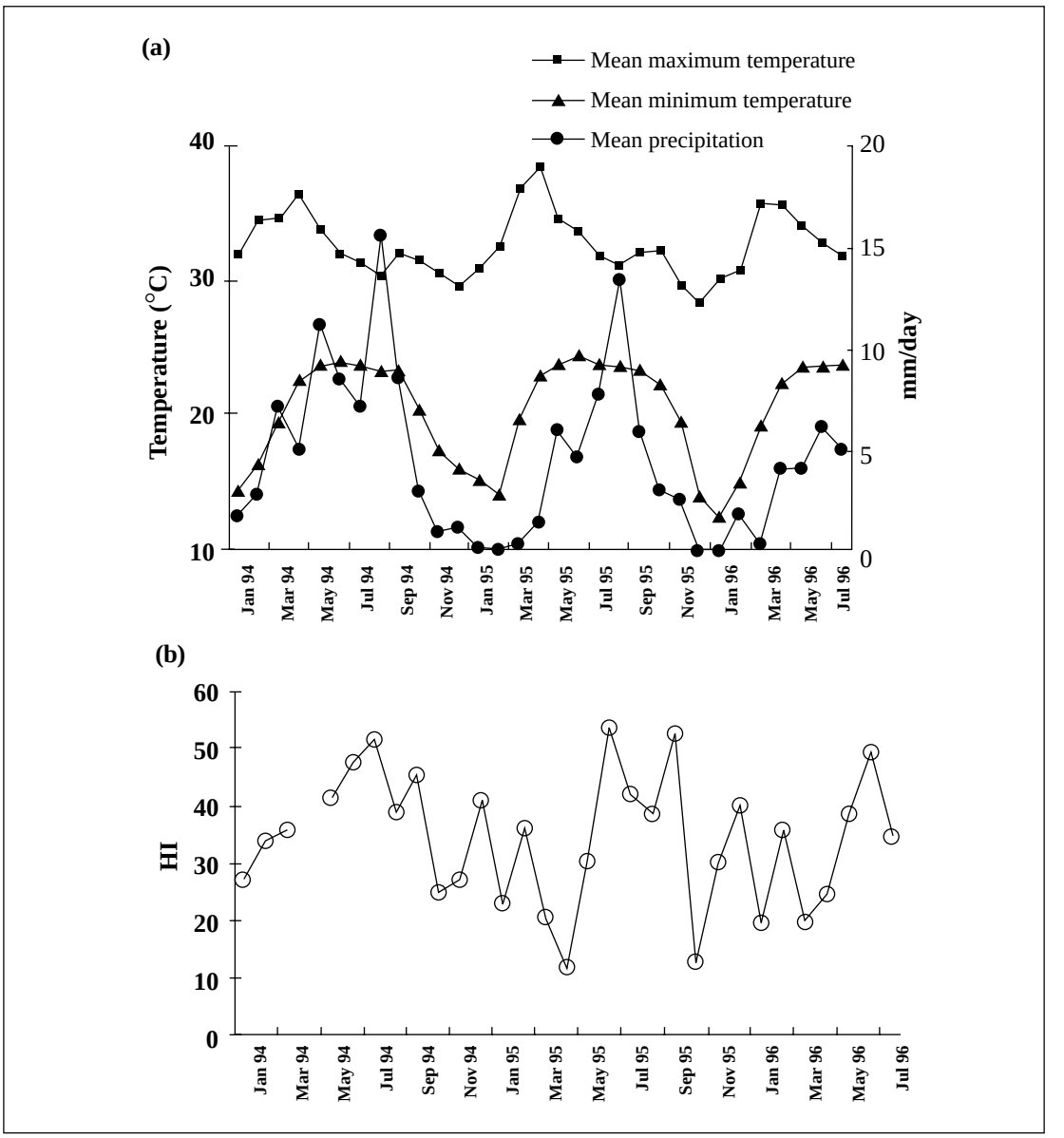

Figure 3. Temporal variation of climate and House Index (HI). (a) mean maximum temperature, mean minimum temperature and mean precipitation, averaged from the monthly summary records of 11 weather stations within the surveyed area. (b) HI averaged from all entomological surveys.

Risk map

The spatial variation of *Aedes* mosquitoes predicted by the final model is shown for 18 northern provinces in Figure 4. In general, southern provinces within the region have higher values of HI.

Discussion

Socio-economic risk factors

Considering the strict conditions applied in identifying socio-economic risk factors, the associations are statistically robust. However, biological judgement and caution are necessary in interpreting whether each represents a direct causal relationship. Among the positively correlated variables, the proportion of tin houses may be only an indirect indicator, as such houses are often found in poorer regions and slums, including housing for transient workers. They, therefore, tend to have generally unhygienic conditions (e.g. irregular garbage collection, promoting *Aedes* breeding sites) and poor house design (e.g. absence of window screens and water supply), both of which may promote *Aedes* proliferation. The association with transport services probably reflects the fact that *Ae. aegypti* have a limited flight range, and their dispersal is largely assisted by human movement (e.g. transportation of larvae in used tries) (Hawley et al. 1987). Public water wells, however, are very likely to have a direct causal link, as they necessitate water storage in individual containers inside houses. Concern that neighbours might consume the water in the public well may also encourage water storage in greater quantities, for longer period.

Amongst the factors which are apparently protective, coverage of health insurance and health education are likely to reflect general commitment to healthly behaviours within both the local community (e.g. co-operation in vector control programmes), and on behalf of local health authorities (e.g. regular garbage collection); many of those not covered by health insurance are engaged in informal occupations (Mongkolsmai 1997), and hence may pay less attention to community hygiene. Similarly, the use of firewood for cooking may indicate remoteness from urban centres and therefore reduced passive transport of vectors, while the proportion of thatched houses may possibly reflect greater traditional knowledge about hygiene. The availability of private water wells is probably directly protective, as they would reduce the necessity to store water in containers.

Climate

The associations with the different temperature variables confirm that *Aedes* populations are generally favoured by higher temperatures (shown by the positive relationship with minimum temperature), provided they do not

exceed harmful upper limits (defined by the optimum maximum temperatures of 33.2-34.2 °C for the various entomological indices). This suggests that global warming may decrease vector populations in warmer regions that are currently close to these limits. It is therefore likely to lead to changes in the endemic ranges of mosquito-borne diseases, rather than necessarily leading to expansion in all areas (Rogers & Randolph 2000).

The observation that the indices did not correlate with the mean precipitation, but with the increase in precipitation from 2 months to 1 month before, suggests that the greatest rate of increase of *Aedes* population coincides with the onset, rather than the peak, of the rainy season (Figure 3a, b). This is probably because eggs of *Aedes*, laid on the inner surface of water containers, hatch when dampened by rising water levels [reviewed by Rodhain & Rosen (1997)]. This is consistent with the observations that the number of DHF cases in Thailand generally starts to rise about 1 month after the onset of the rains (Wellmer 1983), and that *Aedes* proliferate during the first half of the rainy season but not during the latter half (Mogi et al. 1988).

Table 2. Regression coefficients for significant predictors of entomological indices.

n = 1092	asin(HI/100)$^{1/2}$	asin(CI/100)$^{1/2}$	In(BI)
Public water wells	+19.2	+8.81	
Private water wells	-1.74	-0.763	-6.02
Health insurance	-0.136	-0.0650	- 0.487
Health education	-0.779	-0.316	-2.44
Transportation	+0.125	+0.0766	
Use of firewood	-0.0949	-0.0483	-0.538
Tin houses	+0.315	+0.199	+1.37
Thatched houses	-0.355	-0.183	-1.07
Mean maximum temperature	+0.407	+0.262	+2.23
Quadratic term of mean Maximum temperature	-0.00613	-0.00394	- 0.0326
Mean minimum temperature	+0.0187	+0.00978	
Increase in precipitation	+0.0116	+0.00657	+0.0422
Constant	-6.58	-4.30	-33.9
Spreaman's rank Correlation coefficient	0.554(P<0.0001)	0.551(P<0.0001)	0.525(P<0.0001)

Spearman's rank correlation coefficient shows the correlation between the predicted and actual values in the validation data set. Definition and measurement units for socio-economic indices are given in Table 1.

Table 3. Values of significant socio-economic predictors of entomological indices, both within the study area and throughout northern Thailand, based on the 1996 rural database.

	183 districts in northern Thailand		91 districts surveyed for *Aedes* mosquitoes	
	Mean	SD	Mean	SD
Public water wells	0.00360	0.00227	0.00303	0.00157
Private water wells	0.0207	0.0276	0.0149	0.0248
Health insurance	0.418	0.179	0.433	0.180
Health education	0.0477	0.0241	0.0484	0.0249
Transportation	0.747	0.165	0.775	0.166
Firewood	0.604	0.298	0.691	0.251
Tin houses	0.529	0.340	0.465	0.344
Thatched houses	0.0774	0.0949	0.0851	0.107

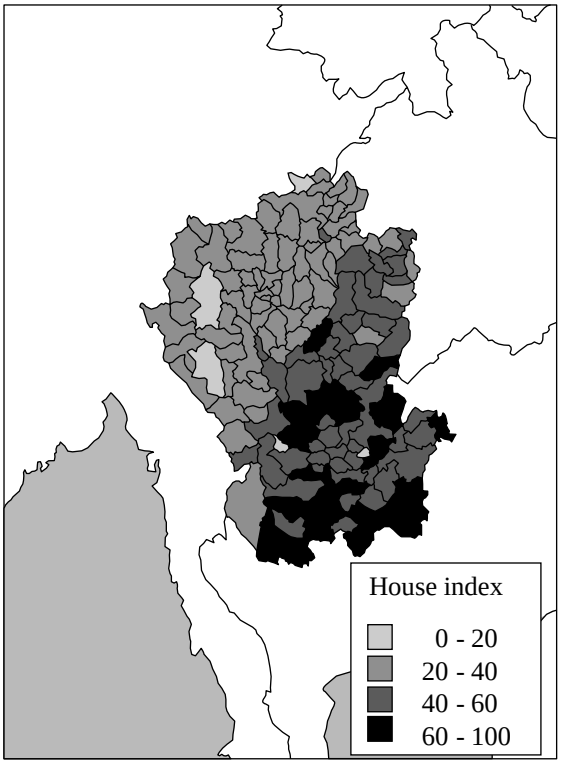

Figure 4. Predicted House Index (HI) of** Aedes **mosquitoes in northern Thailand in July 1995. HI was extrapolated for 183 districts (18 provinces) in northern Thailand using climate data from July 1995. Districts without suitable socio-economic data are left blank.

Risk map

Within the survey area, the derived risk map correlated reasonably well with the spatial pattern observed in actual survillance data (Figure 2 vs. Figure 4), reproducing the tendency of higher HI in lower latitudes. The predictive map also gave a reasonable description of the rank order of observed vs. predicted indices (Spearman's correlation coefficients for all indices >0.5). It should be noted that the predictive map specifically described a risk only in rural areas, as the database used in our analysis lacked information about the central (usually the most crowded and developed) subdistrict. However, as conditions in central subdistricts are likely to correlate with the rural areas surrounding them, the risk map should also have some predictive values for the central subdistricts, and for districts as a whole.

Because the northern half of the study area has higher altitude and cooler climate, rainfall surplus (i.e. rainfall which exceeds evaporation) is higher than in the southern half. In contrast, the southern half of the study area is relatively arid and inspection of the rural database indicates that private water wells are rare there, probably because they are difficult to construct under such conditions. Instead, large earthenware containers are commonly used for water storage (Wellmer 1983). In this case a socio-economic factor (i.e. more frequent storage of water) in turn influenced by a climatic factor (i.e. smaller rainfall surplus) is likely to explain the larger abundance of *Aedes* mosquitoes in the southern part of the study area.

Control implications

The statistical models and risk map presented here have several applications to control programmes aiming to reduce the economic and human costs of this virus in Thailand. Firstly, they indicate specific risk factors that might be targeted in rural development programmes. The finding that public water wells increase risk suggests that their replacement with private wells or water pipelines could effectively supplement direct targeting of breeding sites of *Ae. aegypti*. This could be further investigated with a randomized controlled trial. They also indicate how more general societal changes might affect prevalence of infestation. For example, increasing health education should reduce *Aedes* prevalence, while improved transport services, although clearly desirable for other reasons, may increase risk.

Second, they allow predictions for villages that have not been covered in entomological surveys which are otherwise time-consuming and expensive. Although it would benefit from further refinement, the predictive map derived above provides a rational basis for prioritizing unsampled villages either for interventions, or for further monitoring.

The ultimate aim of risk factor identification and risk mapping for *Aedes* is to control dengue and DHF. Indices such as the number of pupae per person (Focks et al. 2000) may be better indicators of thresholds for sustainable transmission of human disease than the larval indices used here and in many other countries. In addition, these larval indices are imperfect indicators of the number of *Aedes* within a community because they do not take into account variation in larval production between different types of containers. However, the most productive breeding containers are usually large earthen drinking water containers (Kittayapong & Strickman 1993; Thavara et al. 2001), and these containers are commonly used throughout the country. Therefore these larval indices should show a reasonable correlation with the total number of larvae. While this assumption should be tested further, HI is currently used by the Thai CDC as a criterion to prioritize villages for vector control. Our analysis informs decisions about which villages should be prioritized for further surveillance and vector control programme. An unpublished study carried out by the authors indicates that HI was positively correlated with the district-level monthly incidence of DHF in the pre-epidemic season, suggesting that it could usefully contribute to the focalization of control programmes. The relationship between *Aedes* indices and human disease will be explored in detail in a subsequent paper.

Acknowledgements

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Laboratory and Field Evaluation of Novaluron a New Acylurea Insect Growth Regulator against *Aedes aegypti* (Diptera: Culicidae)*

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Abstract

Novaluron, a new chitin synthesis inhibitor type of insect growth regulator, was evaluated in the laboratory and field against larvae of the mosquito *Aedes aegypti* (L.). In the laboratory, the technical material showed a high level of activity against 2nd and 4th instar larvae. The inhibition of emergence (IE) was 100% at concentrations of 0.25 to 1.0 µg/L. Second instars were slightly more susceptible than fourth instars. At the high concentration (1 ppb), most of the mortality occurred in the larvae, but at sublethal concentrations (for larvae) mortality also occurred in pupae and adults (incomplete eclosion). An EC_{10} (emulsifiable concentrate containing 10% active ingredient) formulation of novaluron was evaluated against *Ae. aegypti* in water-storage containers in the field in Thailand. Two tests at high (0.05 to 1 mg/L) and low (1 to 20 µg/L) concentrations were carried out in clay jars (200 L) and plastic pails (75 L). At high concentrations in clay jars, we obtained 86-96% inhibition of emergence (IE) for about 190 d, but the mortality declined at low concentrations after 190 d. At much lower concentrations, in jars and plastic pails in the second experiment, the IE was 80-100% for 2 mo. The highest concentrations of this series (10 µg/L in jars and 20 µg/L in pails) were active for 75 and 68 d respectively, losing efficacy by 82 d post-treatment. In the experiment using low concentrations, all treatments in jars and plastic pails failed to provide adequate control 90 d after treatment. These studies show

* These studies were carried out in collaboration with WHO Pesticide Evaluation Scheme (WHOPES). Mention of specific companies or products does not imply that they are recommended or endorsed by WHO or the University of California, Riverside, in preference over others that are not mentioned.

that novaluron even as EC_{10}, has exceptional long-term activity against *Ae. aegypti* in water-storage containers, with higher concentrations yielding greater long-term control than low concentrations.

Keywords

Aedes aegypti, control, novaluron, IGR, water containers

Introduction

Aedes aegypti (L.) is widely distributed throughout the tropical, subtropical and temperate regions of the world. It is one of the major domestic group of mosquitoes that are pests of man as well as vectors of disease agents. In many areas of the world, this species commands considerable attention in terms of its management and control. The use of larviciding materials is relied upon heavily in controlling this mosquito. The number of options available for mosquito control at the present time are very few. There is a critical need for finding and developing new agents and products for the control of this and other important species of mosquitoes.

In recent years, a new chitin synthesis inhibitor, novaluron, has become available for the control of a variety of insect pests. This material has been noted to have a high level of activity and efficacy against larval Coleoptera, Homoptera, and Lepidoptera, important pests of crops and forestry (Malinowski and Pawinska 1992, Malinowski 1995, Ishaaya et al. 1996, 2001). Because it is very costly to develop and launch a new product for the control of public health insects it is much easier to consider agents and products for development that already have some use in agriculture. Novaluron meets this criterion and it has never been evaluated or tested for activity and efficacy against mosquitoes. In 2002, we carried out extensive laboratory and field studies on this IGR against *Culex* mosquitoes, where novaluron technical material and EC_{10} (emulsifiable concentrate containing 10% active material) exhibited a high level of activity against *Culex* mosquitoes (Su et al. 2003), equaling or surpassing that of diflubenzuron and pyriproxyfen, two commercially available IGRs used in mosquito control programs outside the USA. (Mulla 1974, 1991, 1995).

The current studies were initiated to determine the activity and efficacy of novaluron against larvae of *Ae. aegypti* in the laboratory at Riverside, California, and under field conditions in Thailand. In the field tests, initial as

well as long-term efficacy was studied in water-storage containers for a period of 3 to 6 mo.

Materials and methods

Laboratory tests

Technical material of novaluron (99.4%, NB: 9860726), an acylurea type of chitin synthesis inhibitor, was provided by WHOPES/WHO, World Health Organization, Geneva, Switzerland, and was evaluated against 2nd and 4th instar larvae of *Ae. aegypti*. The procedures for bioassays were those used in previous studies (Estrada and Mulla 1986, Mulla et al. 1985, 1986, 1989). These are briefly described below.

Stock solutions (1%) and serial dilutions of technical material were prepared in acetone. Aliquots of the desired strength dilutions were added to distilled water (200 ml) in cups holding 20 larvae per cup. Five concentrations in the activity range were run, each concentration was replicated 3 times, and control cups were run as well in each test. The experiment was repeated 3 times. A small amount (0.1 g) of rabbit pellets was added as larval food after the larvae were placed in the cups. Water lost to evaporation was replenished every other day. Cumulative mortality of larvae, pupae, and adults was assessed over the developmental period every other day. Adult emergence was assessed by counting and removing pupal skins. Tests were run in a room with photoperiod 16 L: 8D and 30-32°C temperature. Stage-related mortality and final inhibition of emergence (IE) were calculated according to Estrada and Mulla (1986) and Mulla et al. (1989). Percent mortality values obtained at each concentration were subjected to probit-regression analysis using POLO-PC (LeOra Software 1987) to determine IE_{50} and IE_{90} values.

Field evaluation in water-storage containers (Thailand)

Novaluron was evaluated in two experiments in water-storage containers against *Ae. aegypti* larvae. The material used was an EC_{10} (Rimon EC_{10}, BN: 911304) formulation supplied by WHOPES of the World Health Organization, Geneva, Switzerland. The EC_{10} was diluted in distilled water and aliquots of the appropriate dilutions were added to the test units and mixed in the surface. In the first experiment the highest concentration used was 1 mg/L AI, equal to the operational control concentration of temephos. Lower concentrations were also used in this and the second experiment.

Test site and test units

The experiments were carried out in clay water-storage jars (200 L) and plastic pails (75 L), the most commonly used water-storage containers. The evaluation procedures were those used by Mulla et al. (2004). The units were arranged in a block design on a concrete slab covered with a roof (Figure 1). The units were filled with tap water from a domestic water supply. Larval food consisting of ground mouse food was added to the water in the amount of 1 g/ 200 L jar and 0.5 g/75 L pail. The treatments were challenged with successive cohorts of 25 3rd instar larvae/unit before treatment and then on a weekly basis for the duration of the experiment. Larval food was added once a month and water loss was replenished monthly.

Figure 1. Water-storage jars (200 L) and plastic pails (75 L) used in the evaluation of the IGR novaluron against *Aedes aegypti* ***in Nonthaburi, Thailand.***

After the addition of the first cohort, the jars and pails were treated and covered. Assessment of larval survivorship was made 48 h after the addition of 3rd instar larvae by visually counting all live larvae (no pupation at this time) and then assessing adult emergence by counting pupal skins one wk later according to the procedures developed in our previous studies (Mulla et al. 2004). One wk after the addition of larvae, all surviving larvae had pupated and turned into adults or they died as pupae. The resulting pupal skins (reflecting

successful emergence) floating on the surface in the meniscus layer of water were visible and easily counted in jars painted white on the inside or in the pails which were not painted. After this final count, the pupal skins were removed by a syringe or a fish net before the next cohort of larvae was added. Pupal skins persisted for about a wk (Mulla et al. 2004) and reflect a precise level of adult emergence.

Experiment I: high concentrations

The first experiment was carried out in clay water-storage jars (200 L) painted white on the inside for visual counting of live larvae, pupae, and pupal skins. Novaluron EC_{10} was diluted with distilled water and aliquots of appropriate dilutions were added to obtain the desired concentrations. Each concentration and control were replicated 4 times.

One day after they were filled with water, each jar was stocked with 25 2^{nd} or 3^{rd} instar larvae of *Ae. aegypti* from a laboratory colony and larval food was added. The jars were then treated with either 0.05, 0.10, 0.5, 1.0 mg/L AI (ppm), or untreated control. The highest concentration of 1.0 mg/L was equal to that of temephos used as sand granules in the present *Ae. aegypti* control program in Thailand. After the treatment, the jars were covered with 5 mm thick sheets of Celocrete. The efficacy of treatments was assessed with new cohorts (a total of 26 cohorts, data presented on 12) of larvae weekly. To determine the long-term efficacy of the treatments we assessed larval mortality 48 h and emergence of adults (counting pupal skins), 7 d after the addition of larvae. The experiment was terminated 190 d post-treatment when the two lowest concentrations showed low activity, IE being less than 80%.

Experiment II: low concentrations

After recognizing the long residual activity of the lowest concentration of 0.05 mg/L AI in the previous experiment in jars, we realized that in order to find the minimum effective dosage it was necessary to carry out a second experiment using a low range of novaluron concentrations in two types of water-storage containers: clay jars (200 L) and plastic pails (75 L). This experiment was started a little more than two mo after the start of the previous experiment and terminated 90 d post-treatment when the level of the control became lower than 80%.

In the second experiment we employed both the clay jars (200 L) and plastic pails (75 L) (Figure 1). Preparation of the units and procedure for testing were the same as in the previous experiment. The concentrations used in the clay jars were 1.0, 5.0, and 10 µg/L or ppb of AI, and in the plastic pails were 1.0, 5.0, 10.0, and 20 µg/L or ppb of AI. After the addition of 3rd instar larvae, the units were supplied with larval food as in the previous experiment and covered. Assessment of efficacy was carried out as described above, with larval evaluation 48 h and emergence or pupal skins 7 d after addition of larvae. As before pupal skins were counted visually and removed by syringe or fish net before adding a new cohort of larvae. Pupal skins prevailed for one wk (Mulla 2004) and provided a precise count of adult emergence. Additional larval food was given and water loss was replaced monthly. A total of 13 cohorts were used, but data are presented on 10 cohorts, for brevity.

Presentation of data

The data are presented in tabular form where surviving larvae, pupae, and pupal skins are given for each treatment and cohort. Reduction (%) as an indication of larval mortality was based on the number of larvae stocked, while the percent inhibition of emergence (IE or EI%) was calculated from the number of pupal skins on the basis of the initial number of larvae added. Control mortalities were not considered in the calculations as they were generally low in the range of 1-5%.

Results and discussion

Laboratory evaluation

Novaluron technical material was bioassayed against 2nd and 4th instar larvae of *Ae. aegypti* at 5 concentrations (0.01 to 1.0 µg/L) where the test lasted for 18 (2nd) and 14 (4th) d, but most of the mortality occurred in 6-8 d depending on the concentration (Fig. 2, 3, 4, 5). The cumulative mortality increased over time at all concentrations (for larvae). At high concentrations most of the mortality occurred in larvae, while at sublethal concentrations (for larvae), surviving larvae suffered delayed mortality in the pupal and adult stage. The 2nd instars were more susceptible, with IE_{50} and IE_{90} being 0.026 and 0.144 ppb, while these values for the 4th instar were 0.045 and 0.160 ppb respectively. Mortality in the controls was low, 7 and 8% in the 2nd and 4th instars respectively.

Among the chitin synthesis types of IGRs, diflubenzuron has been extensively evaluated in the laboratory and field against *Culex quinquefasciatus*. This compound yielded 100% inhibition of this species at 10 µg/L in the laboratory (Mulla 1974). Another benzoylphenylurea IGR (UC-84572) and diflubenzuron showed excellent activity against *Ae. aegypti* with LC_{50} values of 0.31 and 2.03 µg/L and LC_{90} values of 0.89 and 4.72 µg/L respectively (Ali and Nayar 1987, Mulla 1974). In the present study, novaluron had higher activity than diflubenzuron against 4th instar larvae of *Ae. aegypti*. Novaluron was also more active than pyriproxyfen against 2nd instar larvae of *Ae. aegypti* (Estrada and Mulla 1986)

From the data presented here it is clear that novaluron has a high level of activity against *Ae. aegypti* which is more susceptible to this compound than *Culex quinquefasciatus* (Su et al. 2003). It is also apparent that mortality in various instars and stages is dose dependent, and that the bulk of the mortality occurred in the larval stage as was noted for other chitin synthesis inhibitors IGRs (Mulla 1991, 1995).

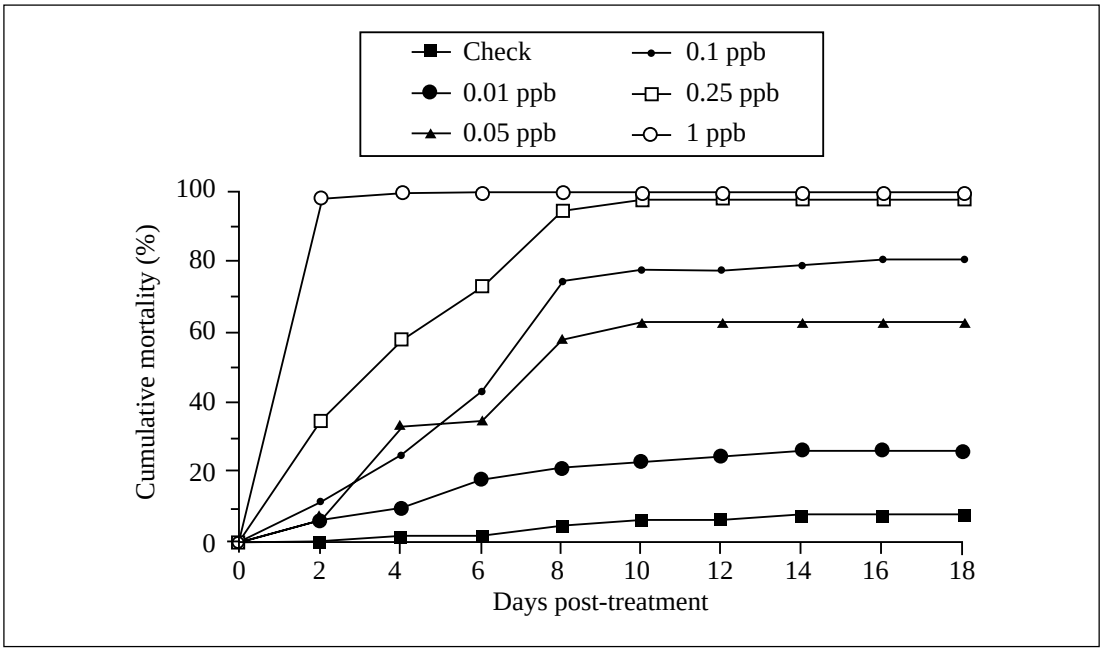

Figure 2. Cumulative mortality of larvae, pupae and adults of** Ae. aegypti **when 2nd instar larvae were treated with novaluron technical material.

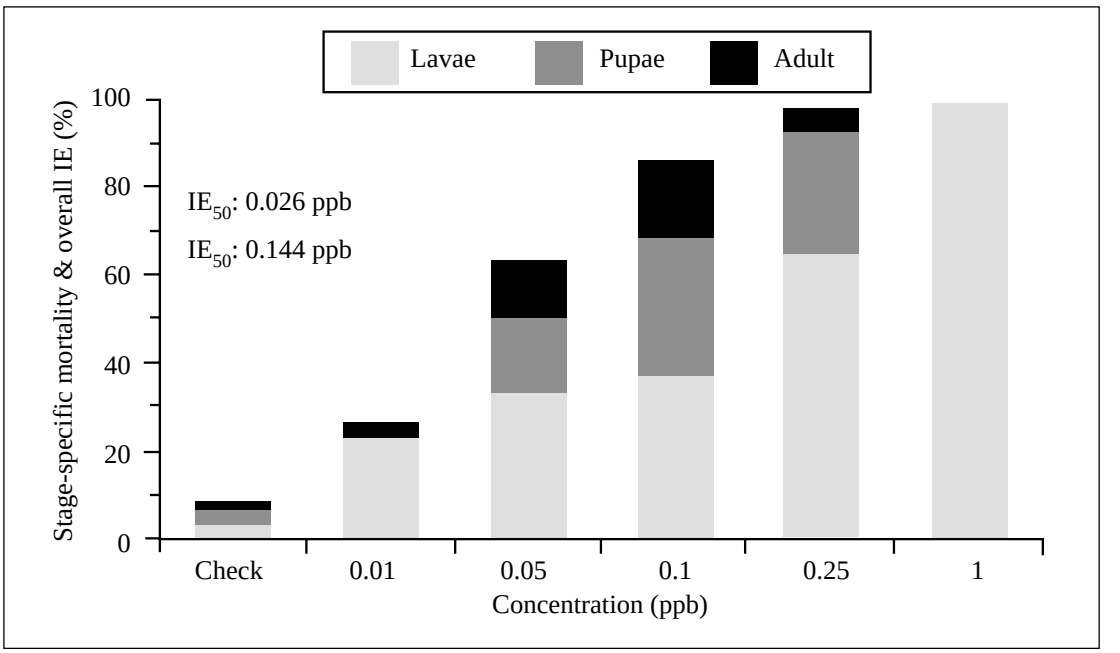

Figure 3. Stage-specific mortality and overall inhibition of emergence of *Ae. aegypti* ***when 2nd instar larvae were treated with novaluron technical material.***

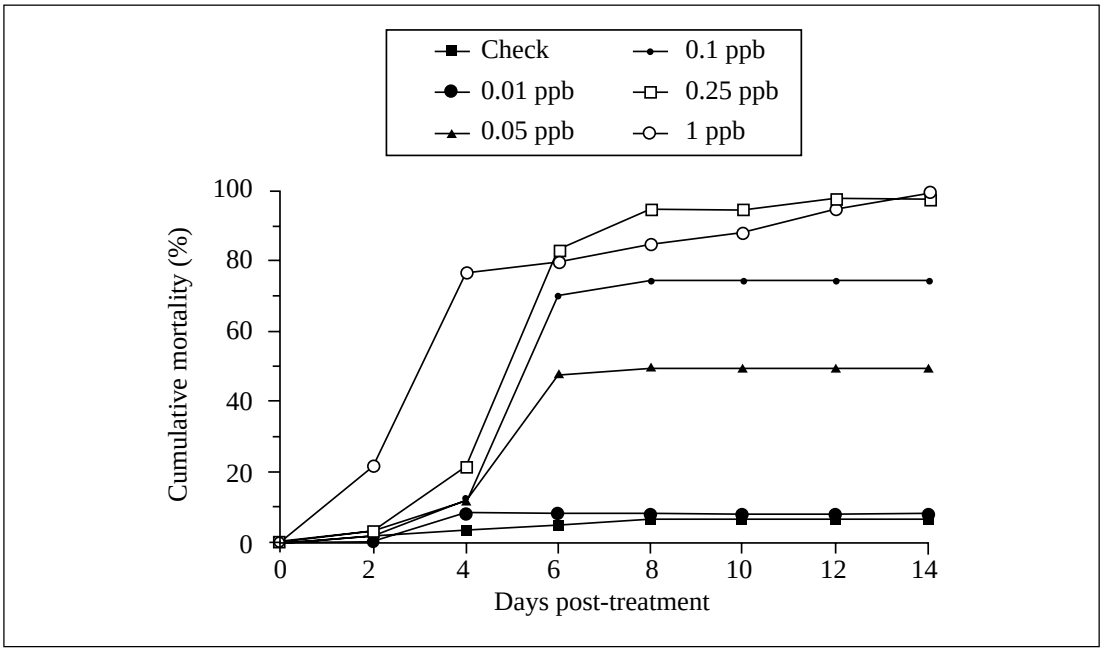

Figure 4. Cumulative mortality of larvae, pupae and adults of *Ae. aegypti* ***when 4th instar larvae were treated with novaluron technical material.***

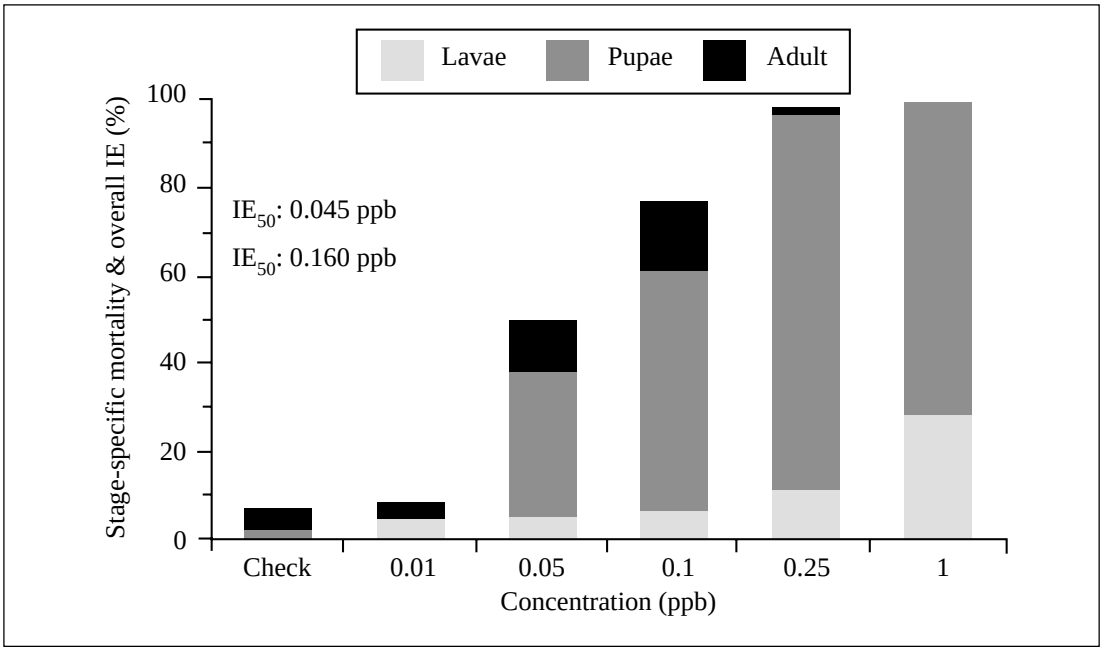

Figure 5. Stage-specific mortality and overall inhibition of emergence of *Ae. aegypti* ***when 4th instar larvae were treated with novaluron technical material.***

Field Evaluation

High concentrations of novaluron (experiment I).

In order to ascertain the residual efficacy of high concentrations (0.05, 0.1, 0.5, and 1.0 mg/L AI) of novaluron, 26 cohorts of *Ae. aegypti* larvae were added to the treated clay water jars (200 L) at weekly intervals over a period of 6 mo (February 25 to August 26, 2002). Larval survivorship was assessed 48 h post-addition of larvae, while pupal skins were counted 7 d post-addition of larvae, by which time most larvae had pupated and emerged as adults. We present data on 10 cohorts only; data on the remaining 16 cohorts are omitted for brevity. Results of the unreported cohorts were similar to that of the corresponding preceding cohorts.

In the first 9 cohorts we used 2nd instar larvae, while in the remaining cohorts 3rd instar larvae (easier to handle and count) were used. We noted little or no difference in the efficacy of novaluron against these two instars. As can be seen, the first cohort showed a high level of larval mortality and IE, but all the concentrations caused a high level of larval mortality (89-99%) and 100% IE in second cohort (Table 1). In cohorts three, four and five (data omitted for all three) larval mortality was very high (78 to 100%), while the IE in cohorts

four and five was 100% at all concentrations. Larval mortality and IE in the control treatment were very low (1-6%) in the first five cohorts.

Table 1. Field evaluation of novaluron (EC_{10}) at high concentrations against 2nd instar larvae of* Ae. aegypti *in water storage jars (200 L) at Bang Bua Thong Field Station, Nonthaburi Province, Thailand, treated February 25, 2002. Assessment of cohorts 1, 2, 6, and 9.

Conc. mg/L	Larvae	Reduction (%)	Pupal skins	IE (%)
	1st cohort, added February 25, 2002			
	48 h post-treatment (2/27/02)		4 d post-treatment (3/1/02)	
0.05	8	92	0	94
0.10	11	89	0	89
0.50	2	98	0	100
1.0	8	92	0	98
Control	100	0	64	0
	2nd cohort added March 1, 2002			
	6 d post-treatment		11 d post-treatment	
	48 h (3/3/02)		7 d (3/8/02)	
0.05	2	98	0	100
0.10	1	99	0	100
0.50	1	98	0	100
1.0	2	98	0	100
Control	97	3	94	6
	6th cohort added March 29, 2002			
	34 d post-treatment		39 d post-treatment	
	48 h (3/31/02)		7 d (4/5/02)	
0.05	0	100	0	100
0.1	6	94	0	100
0.5	1	99	0	100
1.0	0	100	0	100
Control	97	3	98	2
	9th cohort added May 3, 2002			
	55 d of post-treatment		60 d post-treatment	
	48 hr (4/21/02)		7 d (4/26/02)	
0.05	3	97	0	100
0.1	0	100	0	100
0.5	3	97	0	100
1.0	0	100	0	100
Control	93	7	96	4

Larval mortality in cohorts six, seven, eight, and nine was high and the final IE in all these cohorts was 100% (see Table 1, data for cohort seven and eight not presented). Larval mortality and IE in cohorts eleven to fourteen (data omitted) were 100% in all concentrations. Similar results were also noted in cohorts fifteen, sixteen (data omitted for sixteen), and seventeen (Table 2). This trend of complete inhibition of emergence was also noted in cohorts eighteen, nineteen, and twenty (data omitted for all three) which covered the period up to 137 d post-treatment, as well as in cohort twenty one (144 d) where IE was almost 100% in all treated regimens (see Table 2).

Beyond the twenty first cohort we added five more larval cohorts, the last twenty sixth added 183 d post-treatment. In these cohorts, larval mortality declined, indicating a decline in the concentration of novaluron AI in the water in the jars. However, this delayed mortality occurred in all the concentrations and the final IE was close to 100% in all the concentrations in cohorts twenty two, twenty three (data omitted for twenty two, twenty three), and twenty four (Table 3). However, in cohort twenty five, we noted that there was 13 to 14% emergence at the two low concentrations (0.05 and 0.1 mg/L), but the inhibition of emergence was still very high (93 and 96%) at the two high concentrations. The IE declined in all concentrations, in cohort twenty six, 190 d post-treatment (see Table 3). At this time we discontinued further challenging the treatments with new cohorts of larvae as it was evident that the two low concentrations had declined in their efficacy both 175 and 190 d after treatment and that the two highest concentrations also showed a decline at 190 d post-treatment.

From the data, it is evident that novaluron (EC_{10}) has considerable residual activity at 1/20 to 1/10 of the concentration of temephos. At the equivalent concentration of 1 mg/L, it is likely that temephos sand, zeolite granules (Mulla et al. 2004) and novaluron will have similar residual activity for over 6 mo. Using controlled release formulations of novaluron instead of the EC formulation will likely extend the residual efficacy of novaluron in water-storage jars against *Ae. aegypti*. EC formulations in general are short lived with no residual activity. The exceptional residual activity of novaluron in water-storage jars is probably due to the absorption and adsorption of the active ingredient onto organic and inorganic particles and inside surfaces of the jars from where the material is released slowly into the water. The residual activity, to some extent, is dosage dependent, higher dosages exhibiting longer residual activity. We have noted that residual activity for 2-4 mo is sufficient for *Ae. aegypti*

control in small containers (50-200 L capacity) where water is used rapidly, refilled, drained and cleaned frequently. Using higher dosages or controlled release formulations for longer residual activity will be desirable in large water storage containers. In these jars (1000-2000 L+) water is stored for longer periods, and, in practice, water is drained off through a faucet placed 10-15 cm above the bottom and they are filled from the top (Thavara et al. 2004). These jars are infrequently drained and washed; therefore the applied material remaining in the jars could provide control of larvae for 6 mo or longer.

Table 2. Field evaluation of novaluron (EC_{10}) at high concentrations for residual activity against 3rd instar larvae of* Aedes aegypti *in water storage jars (200 L) at Bang Bua Thong, Nonthaburi Province, Thailand, treated February 25, 2002. Assessment of cohorts 15, 17 and 21.

Conc. mg/L	Larvae	Reduction (%)	Pupal skins	IE (%)
	15th cohort added May 31, 2002			
	97 d post-treatment		102 d post-treatment	
	48 h (6/2/02)		7 d (6/7/02)	
0.05	1	99	0	100
0.1	4	96	0	100
0.5	0	100	0	100
1.0	1	99	0	100
Control	96	4	96	4
	17th cohort added June 14, 2002			
	111 d post-treatment		116 d post-treatment	
	48 h (6/16/02)		7 d (6/21/02)	
0.05	19	81	0	100
0.1	11	89	0	100
0.5	0	100	0	100
1.0	0	100	0	100
Control	90	10	93	7
	21st cohort added July 12, 2002			
	139 d post-treatment		144 d post-treatment	
	48 h (7/14/02)		7 d (7/19/02)	
0.05	59	41	0	100
0.1	57	43	0	100
0.5	46	54	2	98
1.0	44	56	3	97
Control	97	3	97	3

Table 3. Field evaluation of novaluron (EC_{10}) at high concentrations for residual activity against 3rd instar larvae of* Aedes aegypti *in water storage jars (200 L) at Bang Bua Thong, Nonthaburi Province, Thailand, treated February 25, 2002. Assessment of cohorts 24, 25 and 26.

Conc. mg/L	Larvae	Reduction (%)	Pupal skins	IE (%)
	24th cohort added August 5, 2002			
	163 d post-treatment		168 d post-treatment	
	48 h (8/07/02)		7 d (8/13/02)	
0.05	59	41	1	99
0.1	50	50	0	100
0.5	31	69	0	100
1.0	22	78	0	100
Control	99	1	99	1
	25th Cohort added August 13, 2002			
	170 d post-treatment		175 d post-treatment	
	48 h (8/15/02)		7 d (8/19/02)	
0.05	27	73	14	86
0.1	24	76	13	87
0.5	11	89	7	93
1.0	9	91	4	96
Control	95	5	95	5
	26nd cohort added August 26, 2002			
	183d post-treatment		190 d post-treatment	
	48 h (8/28/02)		7 d (9/02/02)	
0.05	70	30	26	74
0.1	60	40	21	79
0.5	49	51	12	88
1.0	43	57	10	90
Control	98	2	94	6

Low concentrations of novaluron (experment II)

The experiment on low concentrations (1.0, 5.0, 10.0, and 20.0 µg/L) was run in two water-storage containers (see Figure 1), clay jars (200 L), and plastic pails (75 L) over a period of about 3 mo. To challenge these treatments, successive larval cohorts were added to the containers each week. A total of thirteen cohorts was used in this experiment, but we present data of 10 cohorts, omitting data for cohorts two, three, and six for brevity purposes. In clay jars, the 20 µg/L concentration was not used as this concentration was close to

50 µg/L, the lowest concentration used in the previous experiment. Assessment of efficacy was made in the same manner as in the previous experiment.

Table 4. Field evaluation of novaluron EC_{10} at low concentrations against 3rd instar larvae of* Aedes aegypti *in two types of water storage containers at Bang Bua Thong, Nonthaburi, Thailand, treated May 5, 2002. Assessment of cohort 1, 4, 5, and 7.

	Clay jars (200 L)				Plastic pails (75 L)			
Conc. µg/L	Larvae	Reduction (%)	Pupal skins	IE (%)	Larvae	Reduction (%)	Pupal skins	IE (%)
1st cohort larvae added May 5, 2002, day of treatment								
	48 h (5/7/02)		7 d (5/12/02)		48 h (5/7/02)		7 d (5/12/02)	
1.0	0	100	-	100	0	100	-	100
5.0	0	100	-	100	0	100	-	100
10.0	0	100	-	100	0	100	-	100
20.0	-	-	-	-	0	100	-	100
Control	96	4	96	4	90	10	90	10
4th cohort added May 24, 2002, 19 d post-treatment								
	48 h (5/26/02)		7 d (5/31/02)		48 h (5/26/02)		7 d (5/31/02)	
1.0	-		0	100	72	28	3	97
5.0	-		0	100	29	71	1	99
10.0	-		0	100	30	70	2	98
20.0	-		0	100	25	75	0	100
Control	-		95	5	98	2	98	2
5th cohort larvae added May 31, 2002, 26 d post-treatment								
	48 h (6/2/02)		7 d (6/7/02)		48 h (6/2/02)		7 d (6/6/02)	
1.0	90	10	55	45	-		0	100
5.0	72	28	2	98	-		0	100
10.0	74	26	0	100	-		0	100
20.0	-	-	-	-	-		0	100
Control	98	2	97	3	-		98	2
7th cohort larvae added June 14, 2002, 40 d post-treatment								
	48 h (6/16/02)		7 d (6/21/02)		48 h (6/16/02)		7 d (6/21/02)	
1.0	84	16	1	99	81	19	59	41
5.0	71	29	2	98	25	75	0	100
10.0	0	100	0	100	5	95	0	100
20.0	-	-	-	-	3	97	0	100
Control	94	6	95	5	95	5	95	5

Table 5. Field evaluation of novaluron(EC_{10}) at low concentrations against 3rd instar larvae of *Aedes aegypti* ***in water storage containers at Bang Bua Thong, Nonthaburi Province, Thailand, treated May 5, 2002. Assessment of cohorts 8, 9, 10.***

	Clay jars (200 L)				Plastic pails (75 L)			
Conc. µg/L	Larvae	Reduction (%)	Pupal skins	IE (%)	Larvae	Reduction (%)	Pupal skins	IE (%)
8th cohort larvae added June 21, 2002, 47 d post-treatment								
	48 h (6/23/02)		7 d (6/28/02)		48 h (6/23/02)		7 d (6/28/02)	
1	94	6	75	25	95	5	77	23
5	87	13	8	92	68	32	0	100
10	86	14	3	97	34	66	0	100
20	-	-	-	-	23	77	0	100
Control	98	2	98	2	96	4	96	4
9th cohort larvae added June 28, 2002, 54 d post-treatment								
	48 h (6/30/02)		7 d (7/5/02)		48 h (6/30/02)		7 d (7/5/02)	
1	95	5	65	35	93	7	84	16
5	70	30	10	90	60	40	0	100
10	59	31	0	100	24	76	0	100
20	-	-	-	-	7	93	0	100
Control	98	2	98	2	98	2	99	1
10th cohort larvae added July 5, 2002, 61 d post-treatment								
	48 h (7/7/02)		7 d (7/12/02)		48 h (7/7/02)		7 d (7/12/02)	
1	95	5	66	34	91	9	77	33
5	82	18	16	84	66	34	41	59
10	69	31	11	89	39	61	18	82
20	-	-	-	-	8	92	0	100
Control	98	2	98	2	99	1	99	1

The data from cohorts one, and four (Table 4) showed complete inhibition of emergence in the three concentrations used in clay jars. In the plastic pails there was also complete inhibition of emergence for cohort one and IE 97-100% in cohort four in all four concentrations used. The results of cohorts two and three (both omitted) were essentially the same as in cohorts one and four. The initial evaluation of cohort one established that in all treatments in both the jars and the pails, inhibition of emergence was 100% in all novaluron treatments. In the next cohort (five) the inhibition of emergence was 45% at the 1 µg/L in the jars, but the remaining two concentrations for this cohort as

well as all three concentrations for cohort seven in jars, yielded 98 to 100% IE (Table 4). The 45% IE at the low concentration in cohort five in jars was negated by the results of the succeeding two cohorts which produced IE 99% in both cohorts six (data omitted) and seven. In the pails, all four concentrations yielded IE 100% in cohort five, but it was 92% at the lowest concentration in cohort six (data omitted) and 41% in cohort seven (see Table 4). The three higher concentrations in cohorts five, six (data omitted for six), and seven, produced 100% IE. It seems that the lowest concentration (1µg/L) in the pails lost activity in cohort seven, 40 d post-treatment. This lowest concentration in the jars still produced 99% inhibition of emergence in cohort seven, 40 d post-treatment. From the profile of larval mortality it is clear that larval mortality was dose dependent, higher mortality usually occurring at the high dosages as noted in other benzoylurea IGRs (Mulla 1991, 1995). It also seems that efficacy of the lowest dosage declined faster in the plastic pails with partly screened lids than in the jars with solid covers.

The next three cohorts, eight, nine and ten, added 47, 54, and 61 d post-treatment respectively, showed that some of the concentrations in both the jars and pails were losing strength (Table 5). In all three cohorts larval mortality was low at all three concentrations in the jars as well as the pails except at the highest concentration of 20 µg/L in the pails. In cohort eight, the IE was only 25% in jars and 23% in pails at the lowest concentration, further confirming the loss of activity at this concentration. The two high concentrations of five and ten µg/L in jars yielded 90-100% IE in cohorts eight and nine, but the IE declined to 84-89% in cohort ten in the jars. In the pails, the three high concentrations yielded 100% IE in cohorts eight and nine, but the IE declined precipitously at five and ten µg/L in cohort ten, while still realizing 100% IE at the highest concentration (20 µg/L). This clearly showed that efficacy at all dosages except the 20 µg/L had declined in both containers. It is also evident that, as in the previous experiment, larval mortality was dose dependent, high larval mortality occurring at the highest concentration even in cohort ten. As the efficacy at the three lower concentrations declined markedly in cohort ten, we used three additional cohorts to confirm this trend.

In cohort eleven, there was high larval survivorship at all concentrations except the 20 µg/L in the pails, but the next two cohorts (twelve and thirteen) evidenced further decline in larval mortality as well as inhibition of emergence

(Table 6). These three cohorts further confirmed the decline in activity which was noted first in cohort nine at the low concentration in both types of containers. The highest concentrations of 10 µg/L (jars) and 20 µg/L (pails) yielded IE 84% (in jars) and 96% (in pails) in cohort eleven, respectively. Thereafter, (75 and 82 d post-treatment) all concentrations declined in activity.

Table 6. Field evaluation of novaluron (EC_{10}) at low concentrations against 3rd instar larvae of Aedes aegypti in water storage containers at Bang Bua Thong, Nonthaburi Province, Thailand, treated May 5, 2002. Assessment of cohorts 11, 12, 13.

	Clay jars (200 L)				Plastic pails (75 L)			
Conc. µg/L	Larvae	Reduction (%)	Pupal skins	IE (%)	Larvae	Reduction (%)	Pupal skins	IE (%)
11th cohort larvae added July 12, 2002, 68 d post-treatment								
	48 h (7/14/02)		7 d (7/19/02)		48 h (7/14/02)		7 d (7/19/02)	
1	98	2	88	12	99	1	92	8
5	92	8	38	52	88	12	85	15
10	83	17	16	84	70	30	44	56
20	-	-	-	-	64	36	4	96
Control	98	2	97	3	98	2	99	1
12th cohort larvae added July 19, 2002, 75 d post-treatment								
	48 h (7/21/02)		7 d (7/26/02)		48 h (7/21/02)		7 d (7/26/02)	
1	97	3	90	10	92	8	87	13
5	87	13	35	65	68	32	65	35
10	75	25	16	84	63	37	47	53
20	-	-	-	-	44	56	29	71
Control	99	1	99	1	98	2	98	2
13th cohort larvae added July 26, 2002, 82 d post-treatment								
	48 h (7/28/02)		7 d (8/02/02)		48 h (7/28/02)		7 d (8/02/02)	
1	93	7	92	8	95	5	90	10
5	91	9	54	46	94	4	86	14
10	77	23	26	74	76	24	55	45
20	-	-	-	-	59	41	51	49
Control	97	3	97	3	99	1	99	1

From these studies, it is clear that 10 to 20 µg/L AI of novaluron can provide excellent control of *Ae. aegypti* for at least 2 mo. The length of control could likely be increased by using controlled release formulations of this

material. As mentioned before, residual activity for 2-4 mo is more desirable and cost effective in water use containers (50-200 L) rather than aiming for longer periods in small capacity containers. In actual field situations, residual activity for longer periods in small water-storage containers by using higher dosages or tailor-made formulations may not be possible (Thavara et al. 2004) as water use practices of consumption, addition, draining, and washing of treated jars in particular are factors which negate this advantage. In mega water storage containers, using high dosages or controlled-release formulations would be justified, as these jars are infrequently drained, washed, and cleaned.

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Procedures for Evaluation of Field Efficacy of Slow-Release Formulations of Larvicides against *Aedes aegypti* in Water-Storage Containers

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Abstract

In Thailand, water-storage jars, barrels, drums, pails and tanks, constitute vast developmental sites for *Aedes aegypti* (L.) in urban, semi-urban and rural areas. Clay water jars, cement jars and concrete tanks constitute the greatest proportion of artificial containers where *Ae. aegypti* breed. This species is a major vector of the causal agents of dengue and dengue haemorrhagic fever, and vector control by larviciding is one of the main approaches to disease control. At present, temephos sand granules (1%) is used in large-scale community-based larviciding programs. Due to its use over the past 30 years, there is the likelihood that *Ae. aegypti* may have become resistant to this larvicide already. To develop and avail more options for control, we evaluated VectoBac tablets (Bti 5%) and a new formulation of zeolite granular formulation of temephos (1%) and compared them for efficacy with temephos sand granules (1%) in water-storage jars.

In these tests, we used 48 identical glazed clay water-storage jars (200 L) and developed quantitative sampling procedures for larvae, pupae and pupal skins. Three water regimens were used: full jars, half full jars and full jars emptied half way and refilled weekly. The 3 formulations with 3 regimens of water were assessed over a period of 6 months. VectoBac tablets at the dosage of one tablet or 0.37 g/50 L of water provided excellent control for about 112 days in full water jars. In the other 2 water regimens it gave excellent control for 90 days. The 2 temephos formulations at the operational rate of 5 g/50 L were equal in efficacy yielding almost 100% control for more than 6 months. Unlike temephos sand granules, the temephos zeolite granules lacked the objectionable odor characteristic of temephos sand granules. The zeolite granules also had the additional edge by imparting increased clarity to the water, a feature desired by the users. Similarly, Bti tablets imparted marked

clarity to the water in treated jars. Lack of odor and depression of turbidity are important attributes of Bti tablets and temephos zeolite granules.

Keywords

Aedes aegypti, control, Bti tablets, temephos formulations, water-storage containers

Introduction

Aedes aegypti (L.) with its cosmopolitan distribution is an important human pest and serves as the primary vector of dengue viruses in tropical and subtropical regions (Halstead 1966, Russell et al. 1969, Gubler and CastaValez 1991, Thavara et al. 1996). To ward off epidemics of these diseases, greater reliance is placed on area-wide control of this vector, using larvicidal formulations that possess long-lasting residual activity. In Thailand, temephos sand granules (SG 1%) have been used since the early 1970s (Bang and Tonn 1969ab, Bang et al. 1972) in operational community-based control programs at the rate of 5 g/50 L water in water-storage jars, barrels, water concrete tanks, metal and plastic drums and other artificial containers. The efficacy of temephos EC and temephos SG 1% in water-storage containers and jars was studied in late 1960s (Bang and Tonn 1969ab), and they found that temephos SG 1% (1 ppm AI) provided complete control of *Ae. aegypti* for about 2 months in one study and for 13 weeks in another under simulated water use conditions soon after these findings, temephos sand granules (1%) were used in operational control programs. It is now feared that temephos resistance might already be present or would soon emerge in some areas subjected to temephos treatments for many years. Resistance in *Ae. aegypti* to temephos has been reported in the field in Malaysia (Lee et al. 1984, Lee and Lime 1989), the Caribbean (Georghiou et al. 1987), Santa Domingo (Mekuria et al. 1991), and British Virgin Islands (Wirth and Georghiou 1999) and Brazil (Campos and Andrade 2001). In view of the high probability of resistance development to larvicides it is necessary to evaluate and develop new modes of actions and formulations for use as substitutes in case the use of temephos sand granules becomes unacceptable and impractical.

In determining longevity of slow-release formulations of larvicides against *Ae. aegypti* in mega-capacity (200-2,000 L) water-storage containers, no precise quantitative evaluation techniques are available. In most cases,

estimates of living larvae and pupae are made visually with scoring their abundance or assigning positive or negative status. In the latter case, presence of a few larvae in containers will have the same weight as a container with 100s or 1000s of larvae. Such sampling tactics have little or no relationship to the yield of adult mosquitoes which are the vectors. Also such estimates can vary greatly depending on the sampler, size and color of the container and water use practices and water quality parameters. *Ae. aegypti* larvae and pupae are generally distributed throughout the column (100 cm deep or more) of water and most dive down on disturbance and remain down in the container for a long time. Netting and dipping of the larvae and pupae are time consuming and causing significant disturbance of water and deposits in the jars which will alter the release characteristics of the formulations tested as well as influence the absorption profile of the active ingredients on sediments at the bottom and sides of the jars. To overcome these problems we embarked upon developing methods for the precise visual assessment of live larvae, pupae and pupal skins (indicating successful emergence of adults) without disturbing the water, as well as removing pupal skins from the water surface by syringe or fish net for counting. These assessment techniques, especially that of counting total number of pupal skins either visually or by removal, will provide for accurate estimate of the populations and the assessment of the ultimate efficacy and longevity of the 3 slow-release formulations of larvicides: temephos sand granules (SG 1%), an experimental temephos zeolite granules (ZG 1%) and *Bacillus thuringiensis israelensis* tablets.

Materials and methods

Study site

A field research facility for the evaluation of mosquitocidal products and other agents was constructed in 2001 in Bang Bua Thong District, Nonthaburi Province, Thailand. The project design and planning was initiated in September 2001 and the facility was ready for use in February 2002 when these studies were initiated. The facility consists of a concrete slab slanted toward the center with a 25 cm wide, 3-4 cm deep gutter to drain off water during emptying and washing of water-storage jars. The slab was shaded with a Celocrete tile roof (4 m high at the eaves level), the eaves extending about 30 cm beyond the edges of the slab.

Test units and sampling

One hundred new glazed clay jars (200 L) were placed in 4 rows (25 jars/row) with 2 rows on each side of the gutter (Figure 1). This type of jar was the most commonly used by homeowners (Kittyapong and Strickman 1993), responsible for greater portion of *Ae. aegypti* produced. The jars have a capacity of 200 L of water, when fully filled the depth is 62 cm, when filled half way (100 L), water depth is 32 cm. Lids for covering the mouth of the jars were cut from 5 mm thick Celocrete sheets. Covers were in place all the time except during addition of larvae and assessment of efficacy twice a week for about 2-3 hours each time. Placement of covers prevented light (UV) and wind borne debris from entering the jars.

For developing sampling methods, several jars (dark brown color on the inside) were filled with water from domestic water supply and 50 fourth-instar larvae of *Ae. aegypti* were added. To count the larvae visually without disturbing, 3 or 4 samplers independently counted the larvae in the same jars visually with the aid of a flash light. Several attempts were made to count the larvae in full and 1/2 full jars. The counts between individuals were quite variable and it was not possible to count all the larvae that were added. The inside of the jars had dark brown color and this made it difficult to spot and count all still or moving larvae and pupae visually. Therefore, this method of assessment was considered inappropriate. One reason for this difficulty was that larvae and pupae move around actively throughout the water column, and after disturbance dive down and remain at the bottom for a long time.

We also considered the dipping technique but this technique was also considered undesirable as it would disturb the water too much influencing the release and absorption patterns of formulations tested. Dipping disturbed the water and caused larvae and pupae to dive taking a long time to resurface. We then tried netting out (using fish nets) larvae and pupae from the jars, but this technique was time consuming (as they remain at the bottom for a long time) and netting caused disturbance and stirring of the water, which could influence the release and absorption-adsorption characteristics of the insecticides subjected to testing. We also decided against using sentinel cages or bioassaying field water against larvae in laboratory, as these techniques are time consuming. We came up with the idea of painting the inside of the jars with white paint to facilitate visual counting. A white alkid resin paint, (Glipton G 100, synthetic

resin high gloss enamel, TOA Paint Co., Bangkok) was tested in a few jars. Survival of fourth-instar larvae in the painted jars was excellent for 5-7 days of observation. The white background inside the jars made it easy to spot and count all larvae, pupae and pupal skins in the jars visually with or without the aid of flashlights. Therefore, all 100 jars were painted on the inside, applying two coats a day apart. After drying of the paint for a day or two, they were filled with water from a domestic water supply. Additionally, we developed technique for precise assessment of adult emergence by visually counting pupal skins in the painted jars, or removing pupal skins from painted or unpainted jars with small fish nets or a syringe. Pupal skins float on surface, mostly at the minicus level and can be netted out or picked with a syringe without disturbing the water. The netted or syringed pupal skins were placed in water in white plastic trays and counted.

Figure 1. Arrangement of glazed clay jars (capacity 200 L) for investigating the long-term efficacy of slow-release formulations of larvicides against *Aedes aegypti* ***at Bang Bua Thong, Nonthaburi Province, Thailand in 2002.***

To determine the disintegration and sinking of pupal skins over time, we added *Ae. aegypti* pupae (50) to each of 11 unpainted jars and counted pupal skins visually and by netting or syringing them over a 16-day period in each of 3 jars at each interval. The retrieved pupal skins were counted 48 hrs, 7 and 16 days after the addition of pupae to the jars. This test was done to gain information on the length of period during which pupal skins remain intact and floating at the surface of water.

Assessment of efficacy

On February 3-5, 2002, the 100 jars were washed and cleaned, dried, and painted with white synthetic resin high gloss enamel paint. The paint was allowed to dry for 1-2 days before filling the jars with water. The jars were arranged in 4 rows (25 jars/row) on the concrete slab.

On February 6-7, 2002, the jars were filled with tap water, some were filled half way as required by the experimental design. About 1 g of ground up mouse food was added per each jar and 0.5 g/ half-full jar for larvae. The jars were covered and treated on February 8, 2002 after addition of the first cohort of larvae. Additional larval food was added at the rate of 1 g/200 L, and 0.5 g/ 100 L and the water loss was replenished monthly.

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. Survivorship, by counting live larvae (48 hours post exposure), live pupae and pupal skins one week after placement was determined. Pupal skins (floating on surface) were easily counted in white painted jars one week or longer post addition of larvae and this evaluation technique gave a precise estimate of the yield of adults emerging in each jar.

Materials and treatments

VectoBac tablets (Bti, 2,700 ITU/mg; Lot #64-164-BD-XR-10, Valent BioSciences Corp, Libertyville IL, USA) were used at the rate of 1 tablet per 50 L of water. Average weight of tablet was 0.37 g. Temephos sand granules 1% (BASF Thai Limited, Bangkok, Thailand), at the operational rate of 5 g of the formulation per 50 L of water, yielding 1 ppm temephos was used. A new experimental temephos zeolite granular 1% formulation (Ikari Trading Co., Ltd., Bangkok, Thailand) was also used at the dosage of 5 g per 50 L of water,

yielding 1 ppm temephos. This formulation is made on zeolite green granules (clinoptilolite mineral, a sodium aluminium silicate) commercially available and used for water filtration, animal feed filler and pharmaceutical purposes.

There were 12 treatments in this experiment using 3 larvicides and control in 3 water regimens. Each treatment consisted of 4 jars, set in a row from east to west. The larvae were added to the jars longitudinally, to spread larval variability over treatments. The treatments were: VectoBac tablets, temephos SG 1%, temephos zeolite granules 1%, and control. For each material 3 water regimens were used: full jars, full jars 1/2 water removed and refilled weekly, and 1/2 full jars. Treatments were arranged in block design and made on February 8, 2002. The larvae were placed in the containers and then the required amount of the formulation applied. All applied materials sank to the bottom of the jars.

Reduction and inhibition of emergence

The magnitude of reduction (%) and inhibition of emergence (IE%) were calculated on the basis of larval mortality (48 hr post addition and based on total number added) and on the number of pupal skins (indicating adult emergence, one week after addition) as compared to the initial number of larvae added. Mortality in the checks was not considered in the calculations, as in general it was low. Although treatments were challenged weekly with new cohorts of larvae, we report here part of the data. From the 26 cohorts used during this experiment over a period of 6 months, we present here data on 13 cohorts only. Presentation of all the data will be voluminous and not necessary for elucidating the longevity of the treatments and drawing meaningful inferences.

Results and discussion

Water-storage containers

Artificial containers holding or storing water constitute major habitats for the development of *Ae. aegypti*. These containers include water jars, concrete tanks, pails, barrels, drums (plastic and metal), uncapped empty beverage bottles, tires, ant guards, potted plant saucers and etc. Among these water containers, water-storage jars and tanks constitute by far the largest proportion of water volume producing *Ae. aegypti* (Kittayapong and Strickman 1993,

Thavara et al. 2004). Our observations in rural and semi-urban areas in Thailand in 2003 (Thavara et al. 2004) revealed that in terms of water volume, water-storage jars inside and outside dwellings accounted for more than 90-95% of the water volume stored. We categorized the water jars into two groups, mini jars with capacities of 50 to 150 L and mega jars with volumes ranging from 200 to 2,500 L. The smaller size jars (50-200 L) are generally made from clay and glazed on the outside, while the larger jars are molded from cement mix, having either gray cement color or a red earthen coat on the outside. The mini jars used for daily consumption of water are cleaned frequently (Thavara et al. 2004) while the mega jars used for long-term storage are cleaned occasionally. This practice obviously influences efficacy of formulations. Observations in several villages on the size of jars showed that the 1,000-2,000-L cement jars were more common for long-term water-storage, while for short-term storage and daily use of water the 150-200-L clay jars were common. It should be pointed out that even with the availability of domestic water supply or well water, rural and sub-urban people in Thailand prefer to store and consume rain water collected from roof tops and stored in mega jars for future use (Thavara et al. 2004). Rainwater is considered to have better taste and more cleansing power. Moreover, domestic water supplies in many communities especially rural areas are not secure and sometimes the water lines could be dry for days.

Sampling methods

For determining the comparative efficacy and longevity of larvicidal formulations over time, we found that visual counting of live larvae, pupae and pupal skins was accurate having little or no variation among samplers in jars (200 L) painted with white paint. The paint coat lasted for the entire duration of the test period (over 6 months) and the paint had no adverse effects on larval and pupal survival. It should be pointed out that painting of jars is practical for use in experimental research units and not in operational research or evaluation of area-wide *Ae. aegypti* control programs. In the latter situations, estimates of larval or pupal populations or the simple assignment of positivity - negativity scores (Bang and Tonn 1969a, Bang et al. 1972) are deemed adequate. We also employed small fish nets or syringes, removing pupal skins floating on surface of water in non-painted jars. The pupal skins were easily netted or syringed out and counted by inverting the nets containing pupal skins into water in

plastic trays. Two or 3 netting removed all the pupal skins from water surface. This sampling technique provides a sensitive and least disruptive means for assessing efficacy in both mini and mega jars. Both visual counting and removal of pupal skins in white painted jars yielded essentially the same numbers. Visual counting and syringing of pupal skins in unpainted jars is difficult in mega water-storage jars, but netting of pupal skins provides rapid and accurate sampling for determining the magnitude of emergence of adults which reflects the extent of overall control. Netting of live larvae and pupae was not deemed desirable and practical in mega jars. Pupal skin counts by netting from unpainted jars over a 16-day period, where 50 pupae were stocked per jar yielded data on their persistence. The number of countable pupal skins either visually or by netting decreased over the 16-day period, the counts being essentially the same 2 days (94% by each method with 6% mortality in the pupae) and after 7 days (72 and 78% by the two methods) post addition of pupae. The pupal skins began to disintegrate or sink after 16 days at which time only 46% (visually) and 32% (netting) were noted and those netted were broken and hard to count. Persistence of pupal skins for up to one week is adequate for assessment purposes. We noted that netting of pupal skins changed their floating ability, the number counted or netted after putting the netted pupal skins back into the jars became smaller as compared to the first visual or netting count. Once the pupal skins are netted out, they should not be put back into the containers, and for assessing the efficacy of larvicides or determining the extent of adult emergence this procedure is not necessary.

Larvicidal efficacy

In order to assess the efficacy and longevity of the 3 formulations under conditions of 3 water practices, we challenged the treatments with weekly placement of third-instar larvae from a laboratory colony of *Ae. aegypti*. In total, 26 cohorts were used during the course of the experiment for 6 months, but we here report the data from half of the cohorts (13). The larval mortality and % control were determined 48 hrs after addition of larvae. The surviving larvae and pupae were followed further for a week after addition of each cohort, by which time almost all surviving larvae had pupated and emerged as adults. As the prevailing temperatures in the water jars were high and relatively constant (max 31-34 °C, min 27-30 °C during the 6-month period), larval and pupal

development went fast. Precise counting of pupal skins and calculating the inhibition of emergence (IE%) are used as measures of the overall effectiveness of treatments. The % IE was always equal to or higher than the % reduction calculated from the larval mortality. The reason for the increase in IE% is due to delayed mortality in larvae or pupae. It is for the first time that pupal skin counts and IE% have been used for assessing the overall yield of adults of *Ae. aegypti* emerging from water-storage containers.

The assessment of the 1st cohort (added on the day of treatment) and the 2nd cohort added 7 days after treatment, showed that all treatments yielded 100% mortality of larvae, resulting in no pupae or adult emergence as indicated by the absence of pupal skins (data omitted for both cohorts). The 3rd cohort larvae showed some larval survivorship in VectoBac tablet treatments, but further mortality beyond the 48-hour assessment resulted in high level of inhibition of emergence (91-100%) at the one week assessment (Table 1). There was slight emergence of 7 and 3% (93 and 97 IE%) in two temephos ZG treatments too, the full jar treatment, however, showing IE of 100%, despite the fact that there was some survivorship of larvae (see Table 1). For the 4th cohort larvae (data omitted), slight emergence occurred in the full jars treated with VectoBac tablets and temephos ZG. The 5th cohort larvae were added to the jars 28 days post-treatment where in the VectoBac tablets in full with half removed and refilled jars, the inhibition of emergence was 100% while in the other two VectoBac water regimen treatments, the IE was lower but still satisfactory (see Table 1). Temephos SG, on the other hand, yielded close to 100% IE, while the ZG yielding 2-6% emergence in cohort 5. In cohort 6 (added 35 days post-treatment), the 3 water regimens of VectoBac tablets yielded larval mortality and adult emergence (Table 2) similar to those in cohort 5, and both temephos granules produced EI 100% in cohort 6. Assessment of cohorts 7 and 8 yielded results (data omitted) similar to the previous 2 cohorts. The results obtained with cohort 9 (added 56 days post-treatment), showed 100% IE in all temephos granule treatments (see Table 2). The level of inhibition of emergence was from 79 to 89% in the various VectoBac tablet treatments.

In cohort 10 (added 63 days post-treatment) and 13th (added 84 days post-treatment), there was about 100% EI in all temephos granules treatments (Table 3). In VectoBac tablet treatments, however, there was some emergence (2-17%), the results being similar to cohort 6 and 9. In cohort 11 and 12 (70

and 77 days post-treatment, data omitted) temephos granules yielded about 100% IE, while VectoBac tablets yielded 87 to 98% IE. Assessment of cohort 13 produced results (see Table 3) which showed similar trends as the previous 2 cohorts (11 and 12). The IE in temephos granules was 100% in this cohort and slight emergence (2-11%) was noted in VectoBac tablet treatments (see Table 3). In cohort 14, all treatments of VectoBac tablets and temephos granules and regimens, except VectoBac tablets in 1/2 full jars, yielded high level of inhibition of emergence of 95 to 100% (Table 4). The IE in VectoBac tablets in 1/2 full jars was 75%, showing declining efficacy. In both 15th and 16th cohorts (data omitted), the inhibition of emergence, especially in 1/2 full jars was mediocre in VectoBac, but it was almost 100% in all temephos treatments. In cohort 17 (added 112 days post-treatment), some of the VectoBac treatments began to decline by showing some emergence. In cohort 17, the full jar tablet treatment still yielded 96% IE, while the other two jar treatments yielded 67 and 74% IE (Table 4). This level of decline in efficacy in these two treatments was also noted in cohorts 15 and 16 (data not presented). All the temephos treatments provided 100% IE in cohort 17.

To confirm the decline in efficacy of VectoBac tablets, 3 additional cohorts 18 (added 119 days post-treatment), 19 (added 126 days post-treatment) and 20 (added 133 days post-treatment) were used. VectoBac treatments showed low levels of inhibition of emergence in all of these 3 cohorts (Table 5, data for cohort 19 omitted). In all 3 cohorts the IE in VectoBac treatments amounted to 33-71%, further confirming the breakdown of all VectoBac treatments after 112 days. From the data of cohort 17, it can be concluded that VectoBac tablets (full jars) showed excellent efficacy for 112 days, the full 1/2 removed and 1/2 full were efficacious for 91 days (cohort 14). It is further concluded that VectoBac tablets give satisfactory control for about 100 days (in full jars) at the very low dosage of 1 tablet (0.37 g) per 50 L of water. VectoBac tablets will, therefore, avail another option for achieving long-lasting control of *Ae. aegypti* larvae in water-storage jars. After 133 days of treatment, the temephos SG provided 100% IE in cohort 20 in all treatments, while the ZG provided high level of IE (92-98%) (see Table 5). After assessment of the 19th and 20th cohorts, we discontinued assessing VectoBac tablets but continued assessing the efficacy of temephos treatments by challenging them with successive cohorts of larvae.

Table 1. Assessment of the efficacy of controlled release formulations of VectoBac tablets, temephos SG, and temephos ZG, in water storage jars (200 L) against 3rd and 5th cohorts of larvae (3rd instars) of *Ae. aegypti****. Treated Feb. 8, 2002.***

Materials	Row	Water level in jars	Larvae (48 hrs)*		Emergence (1 week)	
			Larvae	Reduction (%)	Pupal skins	EI (%)
			3rd cohort added 14 days post-treatment (Feb. 22, 02)			
VectoBac tablets	A	Full	16	84	9	91
	B	Full, 1/2 removed	3	97	0	100
	C	1/2 Full	6	94	1	99
Temephos SG 1%	E	Full	0	100	0	100
	F	Full, 1/2 removed	0	100	0	100
	G	1/2 Full	0	100	0	100
Temephos ZG 1%	H	Full	9	91	3	100
	I	Full, 1/2 removed	39	61	7	93
	J	1/2 Full	19	81	3	97
Control	D	Full	100	099	1	
	K	Full, 1/2 removed	96	4	98	2
	M	1/2 Full	100	0	98	2
			5th cohort added 28 days post-treatment (Mar. 8, 02)			
VectoBac tablets	A	Full	33	67	24	76
	B	Full, 1/2 removed	0	100	0	100
	C	1/2 Full	23	77	18	82
Temephos SG 1%	E	Full	2	98	0	100
	F	Full, 1/2 removed	0	100	0	100
	G	1/2 Full	0	100	0	100
Temephos ZG 1%	H	Full	12	88	2	98
	I	Full, 1/2 removed	8	92	3	97
	J	1/2 Full	21	79	6	94
Control	D	Full	96	4	98	2
	K	Full, 1/2 removed	92	8	92	8
	M	1/2 Full	99	1	991	

* In cohorts 1 and 2 there was 100% mortality of larvae 48 hrs post-exposure.

In cohort 22 (added 153 days post-treatment), both temephos granules yielded almost 100% IE (Table 6). However, in cohort 23 (added 160 days post-treatment data omitted), the IE was still high in temephos SG, but in ZG the IE declined to 81% in full jars, but still high in the other two regimens (data

omitted). In cohorts 24 (167 days), 25 (175 days, data omitted) and 26 (185 days) the IE% was 99 to 100% indicating both temephos granules are highly efficacious. The last reading of assessing inhibition of emergence was made on August 19, 2002, some 197 days post-treatment. At this point both temephos granules yielded 100% IE, most mortality occurring in the larvae.

Table 2. Assessment of the efficacy of controlled release formulations of VectoBac tablets, temephos SG, and temephos ZG, in water storage jars (200 L) against 6th and 9th cohorts of larvae (3rd instars) of *Ae. aegypti****. Treated Feb. 8, 2002.***

Materials	Row	Water level in jars	Larvae (48 hrs)		Emergence (1 week)	
			Larvae	Reduction (%)	Pupal skins	EI (%)
			6th cohort added 35 days post-treatment (Mar. 15, 02)			
VectoBac tablets	A	Full	27	73	19	81
	B	Full, 1/2 removed	7	93	3	96
	C	1/2 Full	24	76	14	86
Temephos SG 1%	E	Full	0	100	0	100
	F	Full, 1/2 removed	0	100	0	100
	G	1/2 Full	0	100	0	100
Temephos ZG 1%	H	Full	6	94	0	100
	I	Full, 1/2 removed	3	97	0	100
	J	1/2 Full	14	86	0	100
Control	D	Full	2	98	99	1
	K	Full, 1/2 removed	7	93	94	6
	M	1/2 Full	3	97	98	2
			9th cohort added 56 days post-treatment (Apr. 5, 02)			
VectoBac tablets	A	Full	21	79	11	89
	B	Full, 1/2 removed	21	79	18	82
	C	1/2 Full	37	63	21	79
Temephos SG 1%	E	Full	0	100	0	100
	F	Full, 1/2 removed	0	100	0	100
	G	1/2 Full	0	100	0	100
Temephos ZG 1%	H	Full	16	84	0	100
	I	Full, 1/2 removed	4	96	0	100
	J	1/2 Full	14	86	0	100
Control	D	Full	98	2	98	2
	K	Full, 1/2 removed	98	2	98	2
	M	1/2 Full	94	6	95	5

In conclusion, these long-term studies show that the three larvicidal formulations tested provide long-lasting control of *Ae. aegypti* in water-storage jars under conditions of the experiment. Vactobac tablets at the low dosage of one tablet (0.37 g) per 50 L water, yielded satisfactory inhibition of emergence (> 90%) for 112 days in full jars. IE% was lower in the other two water regimens, but satisfactory control obtained for about 91 days in both.

Both temephos granular formulations at the dosage of 5g/50 L consistently yielded high level of IE (95-100%) for more than 190 days post-treatment. In some of the cohorts, the level of IE may have reached 80%, but subsequent cohorts exhibited high level of IE, indicating that low IE reading on occasions may have been due to experimental errors. Where IE in any two or three consecutive cohorts reached below 80%, the assessment of efficacy was terminated. It should be noted that under controlled experimental conditions it is possible to get this kind of residual activity. Under real world conditions, such a long-term activity is not to be expected (Bang and Tonn 1969ab). As a sequel to this study, we investigated temephos ZG (1%) at 5g/50L, in village trials and found the residual activity to last for only 3 months (Thavara et al. 2004). The longevity of temephos SG (1%) in early field trials was also reported to be for 3 months (Bang and Ton 1969ab). On the basis of these field studies, and those of Thavara et al. (2004), longevity of control for 2-3 months in water-use containers is all that can be expected under normal water-use practices. Many factors especially water use practices (adding, removing, draining, cleaning and etc) influence the longevity of control, and in practice, even a long-lasting formulation will be lost due to removal or dilution. In mega jars, however, where water is removed through a faucet and added at the top and are infrequently washed and cleaned out, the applied materials will persist for longer periods providing control for 6 months or longer.

From these studies it is clear that all 3 larvicidal formulations tested have a good potential for the control of *Ae. aegypti* in water-storage containers which constitute a major habitat for this mosquito in the tropical regions. With the likelihood of appearance of resistance to temephos (used in control programs for many years) as it has been reported from other areas of the world, Bti tablets offer a good and acceptable substitute. Temephos SG are known to have two drawbacks, having objectionable odors and turning water turbid, two factors discouraging its use by homeowners. Temephos ZG do not have these

drawbacks. We noted that water in jars treated with temephos zeolite granules was consistently clear. Bti tablets also possess these advantages as it has no odor problem and it has been shown to reduce water turbidity and algae growth (Su and Mulla 1999).

Table 3. Assessment of the efficacy of controlled release formulations of VectoBac tablets, temephos SG, and temephos ZG, in water storage jars (200 L) against 10th and 13th cohorts of larvae (3rd instars) of *Ae. aegypti****. Treated Feb. 8, 2002.***

Materials	Row	Water level in jars	Larvae (48 hrs)		Emergence (1 week)	
			Larvae	Reduction (%)	Pupal skins	EI (%)
			10th cohort added 63 days post-treatment (Apr. 12, 02)			
VectoBac tablets	A	Full	23	77	17	83
	B	Full, 1/2 removed	23	77	16	84
	C	1/2 Full	38	62	12	88
Temephos SG 1%	E	Full	0	100	0	100
	F	Full, 1/2 removed	0	100	0	100
	G	1/2 Full	0	100	0	100
Temephos ZG 1%	H	Full	1	99	0	100
	I	Full, 1/2 removed	1	99	0	100
	J	1/2 Full	9	91	1	99
Control	D	Full	95	5	98	2
	K	Full, 1/2 removed	96	4	97	3
	M	1/2 Full	98	2	98	2
			13th cohort added 84 days post-treatment (May 3, 02)			
VectoBac tablets	A	Full	-	-	2	98
	B	Full, 1/2 removed	-	-	3	97
	C	1/2 Full	-	-	11	89
Temephos SG 1%	E	Full	-	-	0	100
	F	Full, 1/2 removed	-	-	0	100
	G	1/2 Full	-	-	0	100
Temephos ZG 1%	H	Full	-	-	0	100
	I	Full, 1/2 removed	-	-	0	100
	J	1/2 Full	-	-	0	100
Control	D	Full	-	-	90	10
	K	Full, 1/2 removed	-	-	97	3
	M	1/2 Full	-	-	87	13

Table 4. Assessment of the efficacy of controlled release formulations of VectoBac tablets, temephos SG, and temephos ZG, in water storage jars (200 L) against 14th and 17th cohorts of larvae (3rd instars) of* Ae. aegypti*. Treated Feb. 8, 2002.

Materials	Row	Water level in jars	Larvae (48 hrs)		Emergence (1 week)	
			Larvae	Reduction (%)	Pupal skins	EI (%)
			14th cohort added 91 days post-treatment (May.10, 02)			
VectoBac tablets	A	Full	2	98	2	98
	B	Full, 1/2 removed	21	79	4	96
	C	1/2 Full	31	69	25	75
Temephos SG 1%	E	Full	0	100	0	100
	F	Full, 1/2 removed	0	100	0	100
	G	1/2 Full	0	100	0	100
Temephos ZG 1%	H	Full	0	100	1	99
	I	Full, 1/2 removed	2	98	2	98
	J	1/2 Full	13	87	5	95
Control	D	Full	91	9	92	8
	K	Full, 1/2 removed	94	6	95	5
	M	1/2 Full	76	24	93	7
			17th cohort added 112 days post-treatment (May.31,02)			
VectoBac tablets	A	Full	12	88	4	96
	B	Full, 1/2 removed	56	44	33	67
	C	1/2 Full	47	53	26	74
Temephos SG 1%	E	Full	0	100	0	100
	F	Full, 1/2 removed	0	100	0	100
	G	1/2 Full	0	100	0	100
Temephos ZG 1%	H	Full	0	100	0	100
	I	Full, 1/2 removed	0	100	0	100
	J	1/2 Full	98	2	0	100
Control	D	Full	93	7	98	2
	K	Full, 1/2 removed	98	2	98	2
	M	1/2 Full	97	3	97	3

It is important to note that the assessment techniques developed here, constitute quantitative sampling methods for larvae, pupae and pupal skins of mosquitoes in water containers. Pupal skin sampling either by counting them visually in white painted jars or netting them out from the surface of water in small to large water containers (painted or unpainted) was appropriate for

estimating adult yield. Pupal skins assessment either visually or by netting out provide critical data on the ultimate efficacy of larvicides, and avails useful information on the magnitude of adult mosquitoes emerging from many types of water-storage containers which constitute major sources of *Ae. aegypti*.

Table 5. Assessment of the efficacy of controlled release formulations of VectoBac tablets, temephos SG, and temephos ZG, in water storage jars (200 L) against 18th and 20th cohorts of larvae (3rd instars) of *Ae. aegypti****. Treated Feb. 8, 2002.***

Materials	Row	Water level in jars	Larvae (48 hrs)		Emergence (1 week)	
			Larvae	Reduction (%)	Pupal skins	EI (%)
			18th cohort added 119 days post-treatment (Jun. 7, 02)			
VectoBac tablets	A	Full	46	54	29	71
	B	Full, 1/2 removed	72	28	57	33
	C	1/2 Full	65	35	38	62
Temephos SG 1%	E	Full	0	100	0	100
	F	Full, 1/2 removed	0	100	0	100
	G	1/2 Full	0	100	0	100
Temephos ZG 1%	H	Full	0	100	0	100
	I	Full, 1/2 removed	2	98	0	100
	J	1/2 Full	19	81	3	97
Control	D	Full	94	6	96	4
	K	Full, 1/2 removed	95	5	96	4
	M	1/2 Full	98	2	98	2
			20th cohort added 133 days post-treatment (Jun. 21, 02)			
VectoBac tablets	A	Full	62	38	46	54
	B	Full, 1/2 removed	72	28	47	53
	C	1/2 Full	50	50	50	50
Temephos SG 1%	E	Full	0	100	0	100
	F	Full, 1/2 removed	90	10	0	100
	G	1/2 Full	96	4	0	100
Temephos ZG 1%	H	Full	23	77	2	98
	I	Full, 1/2 removed	22	78	8	92
	J	1/2 Full	23	77	3	97
Control	D	Full	96	4	97	3
	K	Full, 1/2 removed	98	2	99	1
	M	1/2 Full	97	3	97	3

Table 6. Assessment of the efficacy of controlled release formulations of VectoBac tablets, temephos SG, and temephos ZG, in water storage jars (200 L) against 22nd, 24th and 26th cohorts of larvae (3rd instars) of *Ae. aegypti****. Treated Feb. 8, 2002.***

Materials	Row	Water level in jars	Larvae (48 hrs)		Emergence (1 week)	
			Larvae	Reduction (%)	Pupal skins	EI (%)
			22nd cohort added 153 days post-treatment (Jul.12, 02)			
Temephos SG 1%	E	Full	4	96	0	100
	F	Full, 1/2 removed	16	84	0	100
	G	1/2 Full	21	79	0	100
Temephos ZG 1%	H	Full	18	82	0	100
	I	Full, 1/2 removed	14	86	1	99
	J	1/2 Full	34	66	1	99
Control	D	Full	99	1	99	1
	K	Full, 1/2 removed	97	3	97	3
	M	1/2 Full	98	2	98	2
			24th cohort added 167 days post-treatment (Jul. 26, 02)			
Temephos SG 1%	E	Full	1	99	0	100
	F	Full, 1/2 removed	3	97	0	100
	G	1/2 Full	11	89	1	99
Temephos ZG 1%	H	Full	19	81	1	99
	I	Full, 1/2 removed	6	94	1	99
	J	1/2 Full	12	88	0	100
Control	D	Full	98	2	99	1
	K	Full, 1/2 removed	97	3	97	3
	M	1/2 Full	98	2	99	1
			26th cohort added 185 days post-treatment (Aug.13,02)*			
Temephos SG 1%	E	Full	0	100	0	100
	F	Full, 1/2 removed	0	100	0	100
	G	1/2 Full	0	100	0	100
Temephos ZG 1%	H	Full	0	100	0	100
	I	Full, 1/2 removed	0	100	0	100
	J	1/2 Full	1	99	0	100
Control	D	Full	95	5	95	5
	K	Full, 1/2 removed	93	7	93	7
	M	1/2 Full	96	4	96	4

*Experiment terminated on Aug. 19, 2002, 197 days post-treatment

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Efficacy and Longevity of a New Formulation of Temephos Larvicide Tested in Village-Scale Trials against *Aedes aegypti* Larvae in Water-Storage Containers

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Abstract

Field trials on the initial and long-term efficacy of a new formulation of temephos granules (1% on zeolite) applied at 1 ppm AI were conducted in water-storage containers against *Aedes aegypti* in 3 villages in the Kanchanaburi Province in Thailand. A total of 316 water-storage containers of various types and sizes were included in the study. In the initial survey, we found that some containers were positive for *Ae. aegypti* larvae, while others were devoid of larvae prior to the initiation of treatments. The containers were all numbered with paint and divided into 4 groups: with larvae and treated, without larvae and treated, with larvae untreated, and without larvae and untreated. Assessment of larval abundance was made 48 hrs post-treatment and monthly thereafter for 5 months. Containers with larvae and treated, exhibited almost complete absence of *Ae. aegypti* larvae for 2 months, but a small proportion became positive after 3 months. Most of these positive containers were devoid of zeolite granules which are visible in the containers. The number of positive containers increased in month 4 and 5, despite the fact that residues of temephos granules were present in some of the positive containers. The containers group without larvae (initially) and temephos treated, essentially were devoid of larvae for 2 months. After 3, 4 and 5 months about 6-23% of the containers became positive despite the fact that some had visible amounts of temephos granules. In the two control groups with larvae and no larvae initially, there was sustained and consistent production of larvae. Even in the group without initial larval population, the containers became positive for larvae one month after start of the experiment. The positivity rate increased as the trial proceeded. From these studies it can be concluded that a single application of temephos zeolite granules

at 1 ppm AI can provide highly satisfactory control of *Aedes aegypti* larvae in water-storage containers for period of at least 3 months in the field under normal water use practices.

Keywords

Aedes aegypti, mosquito control, village trials, temephos, zeolite granules

Introduction

Dengue haemorrhagic fever (DHF) has been reported in Thailand since the late 1950s (Hammon et al. 1960, Halstead 1966, Ungchusak and Kunasol 1988) and since then it has become one of the major public health problems of the country. The incidence of the disease has been increasing with cyclic outbreaks occurring every 2-3 years. Although the incidence of DHF has been increasing over the past decades, case fatality rate (CFR) has decreased from 10% in the late 1950s to about 0.7% in the late 1980s (Ungchusak and Kunasol 1988). The most severe outbreak of DHF occurred in 1987 with 174,285 reported cases and 1007 deaths (Gratz 1993) in Thailand. Since the turn of the 21st century, incidence of DHF has been high, estimated at 100,000 reported cases each year with CFR of about 1% or less. DHF is caused by dengue viruses (Hammon et al. 1960), which were isolated from *Aedes* mosquitoes in Thailand: *Aedes aegypti* (L.) and *Ae. albopictus* Skuse (Thavara et al. 1996). Scanlon (1965) reported that the first record of *Ae. aegypti* in Thailand was published by Theobald in 1907, and thereafter the mosquito was found in various places of the country as reported by several researchers. It is believed that *Ae. aegypti* now exists in almost every village of Thailand. It occurs in urban, suburban and rural areas of the country where ample developmental sites are present. At the present time there is no effective vaccine available for DHF, and the disease control therefore relies mainly on the control of mosquito vectors. The two main approaches used against *Ae. aegypti* control in Thailand are larviciding and adulticiding. Larval control by larvicide applications and source reduction of mosquito breeding sites is primarily measures relied upon and used routinely, whereas the adult control by space spraying of adulticides is usually carried out as emergency measures for suppressing vector populations during epidemic outbreaks of DHF. Abate (temephos SG 1%) was tested as a larvicide for the control of *Ae. aegypti* in Thailand in water-storage containers in early 1970s (Bang and Pant 1972, Bang et al. 1972), and since then temephos

sand granules have been used in DHF vector control program. The temephos sand granules showed good initial and residual larvicidal efficacy against *Ae. aegypti* larvae in water-storage containers; however, this formulation due to its unpleasant odor has faced major obstacles from the villagers for use in potable and daily-used water (Phanthumachinda et al. 1985, Thavara et al. 2001). The development of a temephos granular larvicide formulation lacking the unpleasant odor poses a challenge to researchers who deal with *Ae. aegypti* control. Mulla et al. (2004) evaluated efficacy of a newly developed temephos zeolite granules, against *Ae aegypti* larvae in Thailand, and found that the formulation possessed high initial and residual efficacy against the larvae for over 6 months under the experimental conditions. This formulation lacks the unpleasant odor when added to water-storage containers breeding *Ae. aegypti* and has the additional advantage of rendering water less turbid. The present study was carried out to evaluate the field efficacy of this new formulation of temephos larvicide in village trials against *Ae. aegypti* larvae in water-storage containers. A large number of water-storage containers were treated for the purpose of determining its efficacy under normal water-use conditions. It is hoped that this formulation will provide a new acceptable alternative for use of temephos by villagers. The acceptance of this larvicide for use in water-storage containers by villagers was then investigated.

Materials and methods

Test material

The new temephos zeolite formulation AZAI-SS (provided by Ikari Trading Co., Ltd. Bangkok, Thailand) was evaluated for larvicidal efficacy and longevity under normal village conditions in this study. This product contains temephos 1% (w/w) and the inert mineral clinoptilolite (zeolite) 99% (w/w). This product was used at the dosage of 10 g per 100 L of water, yielding 1 ppm of temephos AI in each container, a concentration that is currently used in national *Aedes aegypti* control program in Thailand.

Study sites

Field evaluation studies were carried out in 3 villages namely Sahakornnikom, Ongthi and Hindad of the Thong Pha Phum District of Kanchanaburi Province, Thailand. This district lies in western region of Thailand

and is about 240 Km from Bangkok, and each village is approximately 5-10 Km away from each other. The 3 villages were chosen as the study sites due to the historical background of absence of any chemical larvicides used in water-storage containers for at least 1 year. A total of 103 houses (30, 31 and 42 from each village, respectively) in the district were selected for this evaluation which was based on prevalence of *Ae. aegypti* larvae in water-storage containers. Most houses are single-storey residences, each having several water-storage containers of various types and capacities placed both inside and outside the houses. The containers included glazed clay jars (50-200 L), mega cement jars (1,500-2,000 L), plastic pails and metal drums (100-200 L) and concrete tanks in bathrooms (50-1,000 L), all of which support *Ae. aegypti* production.

Field evaluation procedures

To gather baseline data, visual larval surveys were carried out in the study sites to estimate prevalence of *Ae. aegypti* larvae in water-storage containers prior to the initiation of the study. Visual assessments were made by experienced entomologists and survey team. The number of larvae in each container was estimated roughly and scored in the following categories: 0, 1+, 2+, 3+ and 4+, where the number of larvae estimated in each container were 0, 1-10, 11-30, 31-100 and >100, respectively. Based on larval presence or absence, the water-storage containers in this study were categorized into 4 groups: T-1 containers with larvae and treated, T-2 containers without larvae and treated, C-1 containers with larvae and not treated, and C-2 containers without larvae and not treated. T1 and T2 constituted treatments with temephos zeolite granules while C-1 and C-2 were controls. Each container was estimated for water capacity in order to administer the product at the designated concentration of 1 ppm of temephos. For example, glazed clay jars (200 L) and mega concrete jars (1,500 L) were treated with 20 g and 150 g of the product, respectively. All of the 4 groups (T-1, T-2, C-1 and C-2) of water-storage containers were assigned randomly to the various treatments in most houses. It was noted that most houses in this study contained all 4 groups of containers in the same house, whereas the rest had at least 2 groups on the same premises. Prior to treatment, each water-storage container was inspected for larvae, scored and larval presence and abundance recorded and treated (T-1 and T-2 groups only). In addition, each container was numbered and marked with permanent-color

paint spray on its side for subsequent follow-up surveys. This method of marking guaranteed that the same containers are inspected each time in the future assessment of larval absence or presence. Treatments were made only once in T-1 and T-2 groups of containers after the first survey. Assessments were carried out by larval inspection, scoring, and recording results at 48 hours post-treatment and at monthly intervals thereafter. Assessment of efficacy and control was carried out by inspecting the containers and categorizing the larval abundance for 5 months. Attitude of residents for larvicide application was investigated by interviewing one member of each treated house.

Results

At the outset, a total of 357 containers were inspected on the first visit when the study was initiated. The number of containers assigned to T-1, T-2, C-1 and C-2 groups were 147, 70, 70 and 70, respectively. However, the numbers of inspected containers decreased during the course of this study because in some containers all water was used up and were dried up, turned upside down, some were broken and some had disappeared or used for other purposes. As a result of this attrition, only 316 containers (i.e. 129, 61, 62 and 64 for T-1, T-2, C-1 and C-2, respectively) were thoroughly and repeatedly inspected and included in the study for larval absence or presence, and data gathering during the 5 months period post-treatment. The containers included in this study were of different types and sizes, but the majority of them were glazed clay jars, mega cement jars (Figure 1), concrete tanks and metal drums (Table 1). Overall the plastic pails represented the smallest proportion of the containers in the villages included in this study. It was noted that the glazed clay jars, especially 200-L in capacity with and without larval infestations were the most commonly used water-storage containers in this study. Several researchers in Thailand also reported similar results relating to the type of water-storage containers used and infested with Aedes larvae (Kittayapong and Strickman 1993, Jamulitrat et al. 1998, Thavara et al. 2001). In many areas of Thailand, we have noted that the glazed clay jars are commonly used as water-storage containers for drinking as well as daily-use water. The glazed clay and mega cement jars are therefore important target containers, which should be focused on for treatment when larval control programs against DHF vectors are carried out.

Table 1. Types and proportion of water-storage containers in the 4 designated groups (total 316 containers) that were inspected for data gathering in 3 villages, Kanchanaburi, Thailand.

Group and larval occurence	No. of containers	Type of container (%)				
		Glazed clay jars	Mega cement jars	Concrete tanks	Metal drums	Plastic pails
T-1 +ve treated	129	56.5	15.5	16.3	7.8	3.9
T-2 -ve treated	61	63.9	1.6	11.5	19.7	3.3
C-1 +ve no treatment	62	50.0	14.5	4.9	29.0	1.6
C-2 -ve no treatment	64	62.5	17.2	10.9	7.8	1.6

Post-treatment larval prevalence in the containers that were positive for larvae at the start and treated with temephos zeolite granules (T-1 group) is shown in Table 2. All of the treated containers had no larvae at 48 hours, 1 month and 2 months post-treatment with the exception of 1 container (plastic pail) becoming positive with few larvae 2 months post-treatment. The one treated container that became positive for larvae two months after treatment had no visible traces of temephos zeolite granules, apparently the container had been drained, cleaned and refilled. The temephos zeolite granules leave bright green residues at the bottom and if present were easily visible in each container. At the 3-month observation the numbers of larva-free containers were 78% but declined some more during the experimental period. By the end of the 4 and 5-month period larval free containers dropped to 71% with 29% positive for larvae. Heavy larval infestation (category 2, 3, 4) was noted in 18 to 24% of the containers 3 to 5 months after treatment. The proportion of treated jars becoming positive for larvae (in all categories 1, 2, 3, 4) ranged from 22 to 29% after 3, 4 and 5 months post-treatment. It was noted that no granular material was visible in most of the containers that had become positive for larvae during the 3 months post-treatment. We noted that these containers were emptied, cleaned and refilled with new water, and the treatment materials were entirely washed out during the cleaning process. These results imply that the treatment could be effective for larval control for longer periods had the material not been washed out. In the last two assessments (4 and 5 months post-treatment), some containers that had visible amounts of granules were also positive for larvae. This indicates that the formulation even though not washed out in those containers had lost activity by the 4^{th} and 5^{th} months

probably due to heavy water use and refilling resulting in dilution. This scenario implies that temephos zeolite granules are highly efficacious under normal water use conditions for about 3 months. One other important thing we noted was that all of the containers having material and becoming positive for larvae were always those on the outside and exposed to sunlight most of the day, or they had received windborne organic debris, dry grass or plant roofing materials. It is possible that temperature, organic debris and UV light from sunlight degraded the active compound rather rapidly. Coupled with these factors, rapid water use, draining and refilling are practices which get rid of the granules and thus shorten the residual efficacy of larvicidal formulations. It is likely that larvicidal formulations will last longer in mega jars (Figure 1) which store water for longer periods. These jars are drained and washed infrequently, water is drained off through a faucet set 10-15 cm above the bottom and water is added to these jars from the top.

Figure 1. Typical cement and earthen jars used in the study area for water use and storage. Two of the jars to the right are large (2000 L), while 3 jars to the left are small (150 L). The mega jars used for long-term water storage, while the small ones are refilled from the large ones for daily water use. Note the trough for carrying rain water from the roof gutter into the mega jars.

The larval prevalence in the containers that were negative for larvae at the start and were treated with temephos zeolite granules (T-2 group) is presented in Table 2. The purpose of this treatment was to see as to how long temephos zeolite granules at operational dose would prevent larval appearance in the larval-negative containers. The results reveal that no container became positive for larvae at 48 hours post-treatment and only 2 containers were found with low number of larvae 1 month post-treatment. At two months after treatment the number of positive containers amounted to only 6%, increasing to 23% at month 4 and going down to 8% positive 5 months post-treatment. No applied granules were visually seen in the positive containers 3 months post-treatment, but the granules were found in some larval-positive containers on 4- and 5-months post-treatment. It was also noted that some of the containers that had visible amounts of granules became positive for larvae during the 4- and 5-month post-treatment period. This study shows that temephos zeolite formulation is effective in preventing larval occurrence for at least 3 months after application in most of the containers where the material still remained. This experiment lends support to the longevity of temephos zeolite granules tested in T-1 group, which lasted for about 3 months.

In contrast to the treated containers (see Tables 2), containers with larvae at the start and not treated supported sustained and constant presence of larvae. The larval prevalence in these containers (C-1 group) is shown in Table 2. This group of containers was used as the control group to see how larval positivity will progress over time. These containers, 62 in total, experienced natural fluctuations in larval prevalence during the study period. It was found that the numbers of containers positive for larvae at any evaluation inspection fluctuated between 60 and 71% positivity for larvae. This experiment documented that larval populations of *Ae. aegypti* without intervention are always prevailing in most of these containers. The abundance and constant presence of larvae in productive containers provide a sound basis for comparison of larval populations with the treated containers.

Larval prevalence in the containers that were negative for larvae at the start and also were not treated with temephos (C-2 group) is presented in Table 2. The data shows that some of the larval negative containers do become positive for larvae in due time. The data show that the number of containers becoming positive for larvae increased dramatically from 0% at the first 48-hr evaluation to 22% (one month) and to 37% (2 months), reaching the peak of 55% positive

at the fourth evaluation (3 months post-treatment), and then declining to 41% (4 month) and to 36% at the last evaluation (5 months post-treatment). These trends reveal that larval population of *Ae. aegypti* appeared naturally in the initially negative containers and prevailed at relatively high frequency over the course of this study.

Table 2. Larval prevalence (% positive) in 4 groups of containers (with or without larvae initially) treated or not treated with temephos zeolite (1%) granules at 1 mg/L AI, kanchanaburi, Thailand.

Prevalence score*	Larval presence and abundance in containers positive (%) post-treatment (month)						
	Pre-treatment	48 hrs	1 month	2 month	3 month	4 month	5 month
	T-1 (120 containers), positive for larvae initially and treated						
0	0	100	100	99.2	77.5	70.5	70.5
1+	6.2	0	0	0.8	4.7	5.4	9.3
2+	19.4	0	0	0	3.1	7.8	3.9
3+	48.8	0	0	0	3.9	2.3	10.1
4+	25.6	0	0	0	10.8	14.0	6.2
	T-2 (61 containers), negative for larvae initially and treated						
0	100	100	96.7	93.5	83.6	77	91.8
1+	0	0	3.3	1.6	4.9	3.3	3.3
2+	0	0	0	3.3	3.3	8.2	0
3+	0	0	0	0	4.9	3.3	1.6
4+	0	0	0	1.6	3.3	8.2	3.3
	C-1 (62 containers), positive for larvae initially and not treated						
0	0	0	37.1	29	29.1	32.3	40.4
1+	6.4	6.4	12.9	19.4	17.7	17.7	14.5
2+	22.6	22.6	11.3	9.7	12.9	12.9	17.7
3+	35.5	35.5	8.1	12.9	12.9	14.5	16.1
4+	35.5	35.5	30.6	29	27.4	22.6	11.3
	C-2 (64 containers), negative for larvae initially and not treated						
0	100	100	78	62.5	45.3	59.3	64.1
1+	0	0	9.4	7.8	20.3	17.2	10.9
2+	0	0	4.7	17.2	14.1	9.4	7.8
3+	0	0	4.7	4.7	7.8	4.7	17.2
4+	0	0	3.2	7.8	12.5	9.4	0

* 0 without larvae, + with larvae at different densities: 0 = 0; 1 = 1-10; 2 = 11-30; 3 = 31-100; and 4 = >100 larvae/container

Acceptability

In order to gauge the level of resistance or acceptability to the use of temephos granules in domestic water-storage containers by the residents, we interviewed 96 residents in the test area in Kanchanaburi. Of these, 89% did not use any larvicide in their water containers, even though, it was supplied by the local health authorities. These individuals objected to the use of currently used temephos sand granules (1%) in water containers, due to unpleasant odor of the formulation and secondly because of increase in water turbidity which they associated with the formulation. A small percentage (11%), however, used temephos granules but only occasionally, again being reluctant to use the treatment due to odor, water turbidity and safety considerations.When questioned regarding the use of temephos zeolite granules with lack of odor and water turbidity, the respondents indicated their willingness to employ safe and odorless formulations in their water supplies. Such objections to the use of temephos sand granules which has odor were also reported by Phanthumachinda et al. (1985) and Thavara et al. (2001). During the current experiment, the residents acknowledged marked reduction in the abundance and biting activity of adult mosquitoes and they showed eagerness to start treatments if a safe and odorless product is made available to them.

Discussion

At present, there are 3 major problems regarding the use of larvicides for the control of *Ae. aegypti* in water-storage containers in Thailand and other dengue-endemic areas. These are larvicide formulation characteristics, water-consumption styles of the dwellers and insufficient provision of larvicide formulation by government agencies. Firstly, the current larvicide used for *Ae. aegypti* larval control in Thailand is temephos sand granules (1%) which provides high degree of control, but it possesses unpleasant odor when applied to water and renders water more turbid. These drawbacks are not accepted by many dwellers and they usually refuse to use the larvicide as reported by Phanthumachinda et al. (1985) and Thavara et al. (2001). Secondly, with regard to water-consumption styles of villagers, it is found that the people always keep water for drinking and daily use in various kinds of containers, such as jars, tanks, drums, pails, etc., with capacity ranging from 50 to 2,000 L. The main sources of water are from rain, wells, canals, rivers and piped-water supply.

In most rural areas of Thailand people stock water because of drought, especially in dry season but in many urbanized areas having piped-water supply, people still keep water in their water-storage containers for 2 reasons: irregular water supply and traditional styles of water usage, most people preferring rain water over other supplies. The vast numbers of water-storage containers in use constitute major breeding sources of *Ae. aegypti*. From our observations, it was noted that many containers (mostly < 200 L in capacity) were frequently washed and cleaned up and refilled with new water, losing the granular materials applied. As shown in Table 2 some containers treated with larvicidal granules later were noted to be devoid of applied material and became positive for mosquito larvae. This practice of washing, cleaning and refilling reduces the residual efficacy of treatments. Under controlled experimental conditions, Mulla et al. (2004) demonstrated that 2 temephos formulations (sand and zeolite granules) at the rate of 1 ppm AI with 3 water regimens, full, full 1/2 removed and refilled and 1/2 full were equal in efficacy yielding almost 100% control of *Ae. aegypti* for over 6 months. In this experiment the applied materials remained in jars for the duration of the experiment. Moreover, the water-storage jars in that experiment were covered and located in shade under a roof, keeping light out. Apparently normal water-use practices as noted in the trial villages culminate in decreased residual activity. A number of environmental factors also influence residual activity in the containers.

Finally, we have noted that governmental agencies responsible for distributing larvicidal materials to the public for *Ae. aegypti* control, deliver quantities insufficient for treatment of all larval sources. As a rule a family receives only 20-40 grams of larvicide once or twice a year from the local health station. This amount is grossly insufficient to treat all water-storage containers, as there are at least 4-5 containers having various capacities (50-2,000 L) in each house. Not having sufficient quantities of larvicide, we noted that some families declined from using the larvicide provided in sachets. Due to the large number of water-storage containers in Thailand, requiring a large amount of larvicide, financial resources are inadequate to supply the needed quantities. Because of the limited government budget to provide larvicides to most of the country each year, it is desirable to supervise larval control program and to identify key infested containers to be treated routinely in a community based vector control program. At the present time, application of larvicides to

control *Ae. aegypti* larvae in water-storage containers is the most appropriate and effective measure. Implementing integrated control technology employing larvicides, larvivorous fish (Wu et al. 1987, Wang et al. 2000) and mosquito proof covers will yield sustainable control especially in mega jars used for long-term water-storage.

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Field Trial of *Bacillus thuringiensis* H-14 (Larvitab®) against *Aedes aegypti* Larvae in Amphoe Khlung, Chanthaburi Province, Thailand

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Abstract

Bacillus thuringiensis (H-14), an entomopathogenic bacteria, was mostly used for the control of mosquito larvae. The product named Larvitab® was formulated into a l-gm tablet which contained 600 ITU of the potency against *Aedes aegypti* larvae in laboratory and was then evaluated for its efficacy in village scale.

The study was carried out in 2 rural villages of Chanthaburi province during April 8-November 12, 1992, by visual larval survey and adult collection. A tablet of *Bti* was applied individually for 200 litres jar and less proportional dose to capacity of the water containers. Repeated treatments were whenever the mosquito larva was found. Four pre-treatment and eight post-treatment surveys were achieved and we found that more than 80% reduction of Breteau Index (BI) could be as long as 17 weeks (mean = 13.8 ± 2.5 weeks). Moreover other *Aedes* indices including House Index (HI), Container Index (CI), landing and biting rate also decreased more than 70%, particularly for drinking containers. Not only good cooperation of the people but also no complaint in using of this *Bti* formulation was found in this study.

It could be concluded that this *Bti* formulation was very effective and practical for the control of *Aedes aegypti* larva in Thai communities and it will overcome the insecticides by its safety to human beings.

Keywords

Bacillus thuringiensis, *Aedes aegypti*, vector control, dengue

Introduction

Aedes aegypti Linnaeus is the well-known principal vector of Dengue Haemorrhagic Fever (DHF) in the Southeast Asia, including Thailand (Halstead, 1990). To control this mosquito-borne disease, eradication of the mosquito larva by reducing their breeding sites is well recognized (WHO 1983). Abate

1% sand granule, an organophosphate insecticides, has been widely used for water containers in which *Aedes* larva was found. The target dose at 1 ppm of Abate® sand granule was recommended for controlling the larvae in various types of water containers in Bangkok Metropolitan, Thailand (Bang and Pant 1972). Nevertheless, a lot of people rejected this insecticides because of its oily and the awareness of chemical hazard (Phanthumchinda et al. 1985). Recently, *Bacillus thuringiensis* H-14, an entomopathogenic bacteria, was developed into biological products for mosquito control. Bactemos®, a bacteria granule, was found to be effective in Bangkok community (Laojana et al. 1987). During the period of 33 weeks and triple consecutive treatment of 20 mg and 60 mg, the efficacy was persisted for 7-10 days after each treatment. In 1990, we investigated the using of *Bti* H-14 sand granule (400 ITU/mg) in a village of Nonthaburi province (near Chao Phraya river) and found that 65% of Breteau Index (BI) decreasesd below that of check area (BI = 170) for the period of 10 months and there was no complaint neither on the detrimental effects nor on the physical change of water was found.

This study was aimed to evaluate the *Bti* H-14 formulated tablet (≥ 600 ITU/mg) at 20 times of LC_{95} dosage for *Aedes* larvae in a rural village of Chanthaburi province. Visual larval surveys and adult collections were obseved as the efficiency indicators.

Materials and methods

Study area

Amphoe Khlung, one of the districts in Chanthaburi province was selected as the study area as shown in Figure1. It located about 300 kms eastern of Bangkok Metropolitan. Most vicinities are mountainous with clumping soil, orchards and rubber trees. Durian, rambutan and mangosteen are the main trees planted together with the woodenhouses in the gardens. There was no water supply available besides wells and water containers which keep the rain. Cement jar with capacity of 150-200 litres is the predominent water container in most houses, including cement bath and ant-guard. Average precipitation for one year is 300 mm. Village No. 3 and Village No. 5 of Tambon Taporn were selected as treated and untreated areas because of their similarities in general conditions such as type of houses, water storages and living style.

Aedes surveys

A survey team consist of 1 scientist (as a suprevisor) and 5 mosquito scouts (2 officers and 3 volunteers) will look over each individual house in both study areas. Visual larval surveys and adult collections were done by following the standard procedure of WHO (1983). Pre-treatment survey was carried out once a month during April 8th-July 8th and then became twice a month after the application of *Bti*.

Bti H-14

(Larvitab®), a 1-gram formulated tablet with 600 ITU/mg potency was applied for a 200 litres water. More or less dosage was in proportion to the capacity of each container, only 1/4 (0.25 gram) was applied for an ant-guard. Repeated treatment would be done as necessary as mosquito larva was found in the containers.

Data analysis

Each *Aedes* index was analyzed by statistically test in order to find the homogeneity of the population (*F* test) and the significance of *Bti* efficacy between treated and untreated areas (*t*-test). Percentage reduction of *Aedes* larval indices in both areas were also compared together.

The larva free period for each kind of container was calculated by following formula:

$$\text{Average larva free period} = \frac{\text{Total average duration for individual container}}{\text{Total number of container}}$$

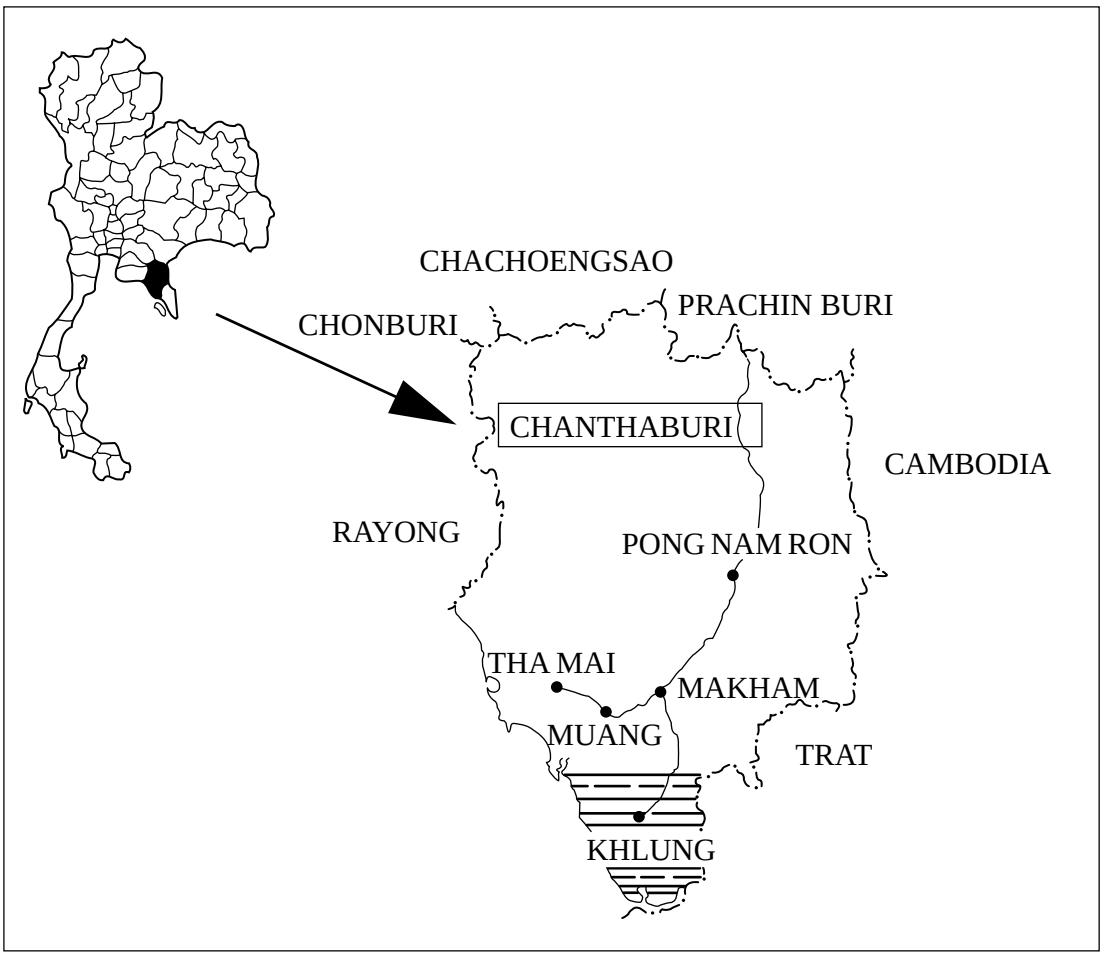

Figure 1. Amphoe Khlung, the study area in Chanthaburi province of the Easthern region of Thailand.

Results

Average *Aedes* larvae indices in treated and unteated villages before *Bti* application were listed in Table 1. It showed that most houses in both areas were highly positive in presenting of *Aedes* mosquito larvae even only 50% of the containers was positive. Although the number of jar was highest among other contatiners but ant-guard represented the highest positive one in both areas. The average value of House Index (HI) was 85.0, Container Index (CI) was 51.8 and Breteau Index (BI) was 429.8 in treated village whereas those in untreated area were 86.5, 44.8 and 344.5 respectively. Statistical analysis (*F* test) showed good homogeneity of the data in both areas.

Since 2nd week after *Bti* application, mosquito indices in treated area decreased gradually as seen in Figure 2. Almost 90% reduction was achieved up to 17 weeks with the average reduction of 69.8% for HI, 84.1% for CI, 84.4% for BI, 73.9% for landing and 73.6% for biting rate whereas there was no such trend found in untreated area (Table 2). Moreover, statistical significances of all indices were found ($p<0.05$).

Table 3 showed the average larval free period for each type of container grouping as drinking, washing bathing and ant-guard. Drinking containers showed longest mean larval free duration (16.4 ± 2.5 Weeks) followed by ant-guard (13.8 ± 2.5 weeks), washing containers (12.9 ± 2.5 weeks) and bathing containers (12.3± 2.5 weeks).

Table 1. Number of houses and** Aedes **mosquito breeding sites in treated and untreated villages in Amphoe Khlung, Chanthaburi (April 8-9, 1992).

Items	Treated Village	Untreated Village	*F*-test
No. of house serveyed	61	92	
No. of positive house	53	76	
House Index (HI)	86.9	82.6	N.S.
No. of water jar	337	506	
No. of positive	108	179	
% positive for water jar	32.0	35.3	N.S.
No. of cement bath	125	143	
No. of positive	44	66	
% positive for cement bath	35.2	46.1	N.S.
No. of ant-guard	88	92	
No. of positive	41	52	
% positive for ant - guard	46.6	55.4	N.S.
Total No. of containers	550	741	
No. of positive containers	193	296	
Container Index (CI)	35.0	39.9	N.S.
Breteau Index (BI)	316.4	321.7	N.S.

N.S. = not significant difference at p = 0.05

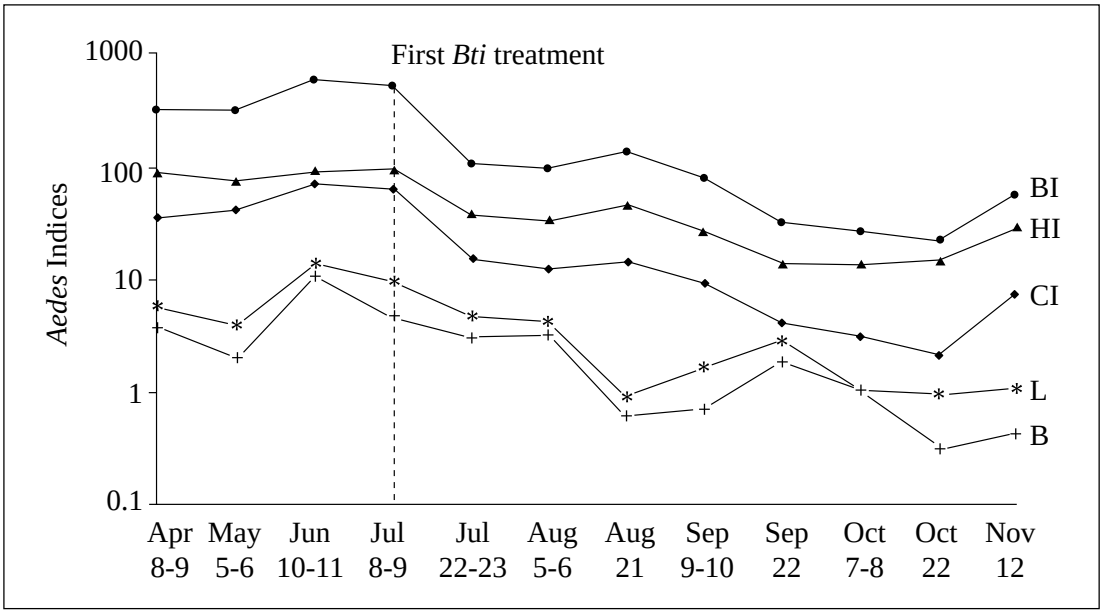

Fig. 2. *Aedes* ***survey indices in treated village along the study period (April 8-Nov 12, 1992). 1st treatment is shown by dotted line. (BI = Breteau Index, HI = House Index. CI = Container Index, L= landing rate and B = biting rate).***

Table 2. Percent reduction of *Aedes* ***indices after*** *Bti* ***H-14 application in treated village comparing with untreated villge during April 8-Nov 12, 1992. (HI = House Index, BI = Breteau Index, CI = Container Index).***

Time	Treated Village					Untreated Village				
	HI	CI	BI	Landing	Biting	HI	CI	BI	Landing	Biting
Before	85	51.8	429.8	8.1	5.2	86.5	44.8	344.5	8.2	5.8
% recduction after treatment										
2nd week	56.5	71.0	76.0	43.2	42.3	Data not obtained				
4th week	62.4	76.8	77.9	48.2	38.5	-0.6	10.7	16.1	35.4	36.2
6th week	48.2	73.0	69.3	88.9	88.5	Data not obtained				
8th week	69.4	82.6	81.8	80.2	86.5	2.9	28.6	30.0	24.4	22.4
10th week	84.7	92.3	82.8	66.7	65.4	Data not obtained				
12th week	84.7	94.2	94.0	87.6	80.8	20.2	37.5	44.8	51.2	48.3
14th week	83.5	96.1	95.1	88.9	94.2	Data not obtained				
17th week	69.4	86.5	87.9	87.6	92.3	22.5	44.2	51.5	54.9	65.5
Average*	69.8	84.1	84.4	73.9	73.6	11.2	35.6	43.1	30.2	41.5

*Statistical Signicicance ($p < 0.05$)

Table 3. Percent reduction and average** Aedes **larva free period in containers (grouped by using purpose) after** Bti **application.

Use of container	*Total number*	*Larva free period (weeks)*	
		x ± S.D.	range
Drinking	37	16.4 ± 2.5	4-17
Washing	176	12.9 ± 2.5	2-17
Bathing	68	12.3 ± 2.5	2-17
Ant-guard	40	13.8 ± 2.5	2-17
Total	321	13.8 ± 2.5	2-17

Discussion

Although water jar was a numerous water containers found in this study, but only 1/3 of them contained mosquito larvae. In contrast with the ant-guards which were highly positive (~ 50%) because such containers were usually uncovered and were occasionally attentive. However high density of *Aedes* harvae was noticeably found in the cement baths, but we missed to count its total number.

Breteau Index (BI) commonly found as high as 350-400 in rural areas of Thailand decreased by 80% in the first two weeks of *Bti* application. The reason was that some drinking containers were free from treatments due to misunderstanding of the local people. However, they became sincerely and gave more cooperation in the following surveys, then larvae indices decreased grasually more than 90% till the end of the study. Nevertheless, it still remained few untreated containers which we asked the owners to protect by either covering or dipping as they can.

According to larval free period in various types of container in Table 3, *Bti* product could stand longest in drinking water jar (16.4 weeks) with ranging from 4-17 weeks. The minimum larval free period was only 2 weeks after the application in other type of containers. It could be caused by frequent use and filling of water, then *Bti* organisms in the containers were too much diluted to kill the mosquito larvae. However, the repeating of *Bti* in positive containers could maintain quite a long duration of larval free.

The efficacy of *Bti* in this study was rather practical because we formulated 1-gram tablet to be used for a 200 litres jar, so that the dose was 20 times as much as LC_{95} in laboratory. The formulation was quite convenient for health

workers and people in using by their owns. Moreover, no complaint about smell, turbidity, taste of water of illness after drinking water has been found in our study. It revealed that this formulation of *B. thuringiensis* H-14 could be applied for the control of *Ae. aegypti* larvae in water containers without any opposition, particularly in case of the safety comparing with the chemical insecticides.

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Development of Bti-Formulated Products and Efficacy Tests against *Aedes aegypti* Populations

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Abstract

Several *Bacillus thuringiensis israelensis* (Bti)-formulated products (i.e, sand granules, paper strips and tablets) were developed in order to improve its potency and efficiency in reducing *Aedes aegypti* larvae. A 1-g tablet, with potency of 500 ITU/mg, demonstrated the best performance among other Bti-formulated products produced in this study. Results from efficacy tests of this product revealed that the tablet was highly toxic to *Ae. aegypti*, followed by *Culex* and *Anopheles* larvae. Observations on the efficacy of Bti-formulated tablets in the 160-1 water containers showed that the tablets are effective against *Ae. aegypti* larvae for as long as 3 mo without changing the water by refilling and removing and about 2 mo in water containers with daily removal/refill of 20% water in volume. In addition to additives of Bti-formulated products, another ingredient, the grounded powder developed from cadavers of mosquito larvae (which were fed on Bti-pure powder) was tested for its efficacy against *Ae. aegypti* larvae. Results from the bioassay of such powder demonstrated that there was an adequate amount of toxin retained in the mosquito cadavers, and this can be used as an addition ingredient.

Keywords

Bacillus thuringiensis israelensis, formulated products, field trials, *Aedes aegypti*, mosquitoes

In recent years, there have been a growing number of insects becoming resistant to chemical pesticides in many parts of the world. In addition, the inherent problem of acute and chronic toxicity of many pesticides to non-target

organisms, especially mammals, and impact on the environment has created the need for more efficient and environmentally sound alternatives. Research on alternative control agents has come up with a number of biological control agents, especially the entomopathogenic organisms, e.g., microbial insecticides-bacteria, viruses and fungi etc. However, *Bacillus thuringiensis* (Bt) has generated more interest than others. Because it is generally accepted that Bt, commonly found in natural soil, is a harmless organism and does not pose any threat to the environment and/or consumers. Despite its safety, Bt is commercially attractive. It has low development cost compared with chemical insecticides and a wide range of the pesticidal activity spectrum.

Bacillus thuringiensis israelensis (Bti) serotype H-14, an aerobic, gram positive, spore and crystal forming bacterium, was designated by de Barjac (1978a,b). It is one of the entomopathogenic bacteria that has been used as an effective agent for controlling insect vectors, especially mosquito larvae, i.e., *Culex pipiens* and *Aedes aegypti* (Goldberg & Margalit 1977). The larvicidal activity is mainly associated with its sporulating cells that produce a high molecular weight of the crystalline, proteinaceous material known as delta-endotoxin. This delta-endotoxin is highly toxic and lethal with its specificity to mosquito larvae.

Therefore, with its specificity, efficacy and safety, Bti is considered one of the safer alternatives to the currently used, highly hazardous and environmentally unsafe chemical insectidal products.

Several attempts have been made to develop and produce Bt-based products for use against agricultural pests and insect vectors. Therefore, it was our main objective in this study to develop a locally produced formulation of Bti-based products for use in controlling *Ae. aegypti* populations in Thailand.

Materials and methods

Strain Selection.

Standard strain of *B. thuringiensis israelensis* H-14 (IPS.82 Lot. 91509) (potency of 15,000 ITU/mg) from Pasteur Institute, France, was chosen for this study.

Isolation of Pure Culture Bti & Selective Media.

Isolation of pure culture Bti H-14 without having bacteriophage is an important step and had to be accomplished. Selection of media is also crucial, especially when we decided to mass produce this pure Bti on a solid medium.

Bacteria will produce spores only when the condition is unfavorable for their normal/routine growth. Several recipes for media have been tested; however, the Nutrient agar supplement with salt (NAS) (Nutrient broth = 0.8%, $CaCl_2.2H_2O$ = 0.008%, $MnCl_2.4H_2O$ = 0.005%, $ZnSO_4.7H_2O$ = 0.005%, $CuSO_4.H_2O$ = 0.005% and Agar = 2.00%) appeared to be the best medium for harvesting the Bti.

After incubation at 28±2 °C for 72 hr, the maximum growth of Bti with about 10^8-10^{10} spore/ml could be expected. The bacterial colonies were removed with 0.85% NaCl solution, then centrifuged, and later the precipitant was separated from supernatant. The precipitant was stored at -77 °C for 24 hr, then lyophilized. The end product was a wettable powder.

Mosquito Populations.

Efficacy tests of the Bti pure powder and Bti-formulated products were performed on the fourth-instar *Ae. aegypti* larvae as well as other mosquitoes: *Ae. albopictus, Culex quinquefasciatus, Culex tritaeniorhynchus, An. dirus* A. and *Toxorhynchites rutilus*.

Results

Efficacy Test of Bti as Active Ingredient.

Potency of Bti wettable powder was examined. Efficacy tests on the fourth-instar *Ae. aegypti* larvae as well as the other mosquito larvae followed the Method for Titration of Bti Preparation by World Health Organization (WHO), collaborating Centre for Entomopathogenic Bacilli Pasteur Institued, France. The potency of products defined as the "International Toxicity Unit (ITU/mg)" was calculated for LC_{50} using Probit Analysis of Novo.

Death rates of mosquito larvae on different concentrations of Bti were recorded and compared with the standard (Table 1). Our Bti-formulated products were calculated and found to have potency of 17,462 ITU/mg *Ae. aegypti*.

Activity of Bti Powder in Different Types of Water.

Pure Bti powder with potency of 17,462 ITU/mg was adjusted to the level of 12,000 ITU/mg with silicon dioxide. The main objective of this experiment was to test the performance of Bti-based products in different types of water. Because different kinds of mosquito larvae have different types of

breeding habitats in different types of water, i.e., clear water (e.g., distilled, rain and tap water) or polluted (canal) water with different chemical and physical characteristics, i.e., the pH of water, chemical/mineral contents (nitrate, sulfate and chlorine), and the amount of dissolved oxygen (DO).

Table 1. Activity of Bti H-14 powder cultured on NAS medium against *Ae. aegypti* ***larvae.***[a]

Conc. of Bti (mg/l)	standard IPS 82	%mortality Bti H-14 (powder)
0.005	30.8	37.2
0.008	58.8	57.2
0.01	64.0	77.2
0.02	82.8	98.8
0.03	94.8	100.0
0.04	98.8	100.0
LC_{50} (mg/l)	0.0065	
LC_{90} (mg/l)	0.0134	

[a] 25 larvae used per cup. Mortality assessed 48 hr after exposure.

The test was performed using four different concentrations of Bti at the Lethal Concentration of 100 (LC_{100}): (1 x LC_{100}) = 0.07 mg/l, (5 x LC_{100}) = 0.35 mg/l, (10 x LC_{100}) = 0.70 mg/l and (20 x LC_{100}) = 1.40 mg/l, respectively. Results are shown in Table 2. The same experiment was also performed with the 5% Bti tablet. Results are shown in Table 3.

Bti-Formulated Products.

The adjusted potency of Bti (with 12,000 ITU/mg *Ae. aegypti*) was used in developing different kinds of formulated products. Three different kinds of formulated products-sand granules, paper strips, and 1-g tablets-were developed in this study. Comparison of potency of different types of Bti-formulated is presented in Table 4.

1. Sand Granule. Active ingredient: 3 or 5% Bti (12,000 ITU/mg); other ingredients: molasses and fine sand granule; mixing ratio-sand granule:-molasses: Bti; 3% Bti sand granule 175.90 ITU mg (cost = 1,000.00 Baht/kg); 5% Bti sand granule 348.30 ITU mg (cost = 1,650.00 Baht/kg)

2. Paper Strip. Active ingredient: 3 or 5% Bti (12,000 ITU/mg); other ingredients: flour, molasses, postlip paper (1.5 x 10 cm); mixing ratio-flour:-Bti: molasses; 3% Bti paper strip 526 ITU/mg (cost = 1.01 Baht/strip); 5% Bti paper strip 1,088 ITU/mg (cost = 1.67 Baht/strip)

3. 1-g Tablet. Active ingredient: 3-15% Bti (12,000 ITU/mg); other ingredients: sodium bicarbonate, magnesium stearate, citric acid, lactose, floguard, cellulose etc.; 3% Bti H-14 tablet 442.10 ITU mg *Ae. ae.* (cost = 1.08 Baht/1 tablet); 5% Bti H-14 tablet 535.32 ITU mg *Ae. ae.*; 8% Bti H-14 tablet 936.71 ITU mg *Ae. ae.*; 10% Bti H-14 tablet 1,005.90 ITU mg *Ae. ae.*; 15% Bti H-14 tablet 1,293.87 ITU mg *Ae. ae.*

Table 2. Activity of Bti (H-14) wettable powder (12,000 ITU/mg) in different types of water.

	Physical analysis				Chemical analysis			% mortality (hr)			
	pH	turb.	Temp.	DO	NO_3	SO_4	Cl_2	1	3	12	24
1 x LC_{100}(0.07 mg/l)											
Distil. water	5.1	001	25.9	6.0	0.1	2.0	0.0	83	100	100	100
Rain water	6.8	001	25.9	7.4	0.6	2.5	1.0	76	96	100	100
Tap water	7.0	001	26.2	7.3	0.5	5.5	32.0	71	97	100	100
Canal water	7.4	006	26.5	2.0	16.1	2.5	85.5	3	69	87	100
5 x LC_{100}(0.35 mg/l)											
Distil. water	5.7	001	26.1	6.5	0.2	1.5	0.0	99	100	100	100
Rain water	7.1	001	26.1	7.5	1.0	2.5	0.0	92	100	100	100
Tap water	7.0	001	26.4	7.4	1.3	4.5	49.0	93	100	100	100
Canal water	7.5	005	26.5	2.0	16.4	2.5	93.0	1	93	100	100
10 x LC_{100}(0.70 mg/l)											
Distil. water	4.7	001	25.0	6.3	-	-	-	99	100	100	100
Rain water	6.8	001	25.1	6.1	-	-	-	96	100	100	100
Tap water	7.4	001	25.1	6.4	-	-	-	96	100	100	100
Canal water	7.9	007	25.2	4.5	-	-	-	95	99	100	100
20 x LC_{100}(1.40 mg/l)											
Distil. water	4.7	001	25.1	6.3	-	-	-	100	100	100	100
Rain water	6.0	001	25.2	6.1	-	-	-	100	100	100	100
Tap water	7.4	001	25.1	6.4	-	-	-	100	100	100	100
Canal water	7.9	001	25.2	4.5	-	-	-	92	96	100	100

Tablet Formulations.

Different concentrations of Bti-formulated products were also tested in order to compare the potency against *Ae. aegypti* larvae (Lethal concentration LC_{50} & LC_{100}). Results from Table 5 show that Bti-formulated tablets with the concentration of pure Bti-powder higher than 8% have the potency close to 1,000 ITU/mg *Ae. ae.*, (936.71 for 8% tablet, 1,005.90 for 10% tablet and 1,293.87 for 15% tablet). However, for the efficacy test of these tablet-products, under laboratory conditions, results revealed that a 1-g 5% Bti-formulated tablet with potency of 500 ITU/mg *Ae. ae.*, had an adequate amount of toxin to kill *Ae. aegypti* larvae, and this tablet was highly toxic to *Ae. aegypti* larvae.

Table 3. Activity of formulated Bti H-14 products (5% tablet) (12,000 ITU/mg) in different types of water .

	Physical analysis				Chemical analysis			% mortality (hr)			
	pH	turb.	Temp.	DO	NO_3	SO_4	Cl_2	1	3	12	24
1 x LC_{100}(0.07 mg/l)											
Distil. water	6.1	003	26.3	6.1	0.1	3.0	1.0	63	99	100	100
Rain water	6.1	008	26.3	6.6	0.5	2.5	7.0	19	87	95	96
Tap water	6.6	009	26.2	6.3	0.3	4.0	27.0	43	87	99	100
Canal water	7.0	016	26.1	4.1	0.9	4.0	95.0	0	0	23	32
5 x LC_{100}(0.35 mg/l)											
Distil. water	5.8	008	22.8	5.0	-	-	-	95	100	100	100
Rain water	6.5	009	22.8	5.0	-	-	-	91	99	100	100
Tap water	6.8	009	22.8	5.6	-	-	-	84	97	99	100
Canal water	6.9	009	22.6	3.4	-	-	-	0	0	22	51
10 x LC_{100}(0.70 mg/l)											
Distil. water	5.8	008	24.1	6.0	0.3	3.0	0.0	95	97	100	100
Rain water	6.1	009	23.8	5.5	0.95	1.7	0.0	96	100	100	100
Tap water	6.5	009	24.0	5.6	0.95	5.7	22.0	56	97	100	100
Canal water	6.9	009	24.0	4.6	1.1	4.5	43.0	7	76	97	97
20 x LC_{100}(1.40 mg/l)											
Distil. water	5.8	009	22.8	5.0	-	-	-	99	100	100	100
Rain water	6.1	009	22.8	4.8	-	-	-	100	100	100	100
Tap water	7.1	009	23.0	4.0	-	-	-	88	100	100	100
Canal water	6.8	015	23.0	4.2	-	-	-	0	44	97	97

Table 4. Comparison of potency of formulated Bti (H-14) products.

Formulated products (%Bti)	Lethal concentration		Potency (ITU/mg)[a]
	LC_{50}(mg/l)	LC_{90}(mg/l)	
IPS-82(standard)	0.0077	0.0186	15,000
3% sand granule	0.6599	1.5818	176
5% sand granule	0.3332	0.5801	348
3% paper strip	0.2204	0.5528	527
5% paper strip	0.1066	0.2832	1,089
3% tablet	0.2853	0.5377	407
5% tablet	0.1949	0.4784	595

[a] Potency = $\frac{\text{Potency of standard}}{LC_{50}\text{ test}}$ x LC_{50} of standard

Table 5. Comparison of potency of different Bti (H-14) formulated tablets against *Ae. aegypti* ***larvae.***

Tablet formulations (%Bti)	Lethal concentration		Potency ITU/mg. *Ae. aegypti*
	LC_{50}(mg/l)	LC_{90}(mg/l)	
3	0.302	0.886	442
5	0.250	0.711	535
8	0.143	0.530	937
10	0.133	0.525	1006
15	0.103	0.448	1294

Efficacy Tests of Bti-Formulated Tablets in the 200-l Water Container.

Efficacy tests of 1-g Bti-formulated tablet of different Bti pure-powder concentrations (3, 5, 8, 10 and 15% respectively) were used in the 200-l water jars, with 40-, 80- and 160-l volumes of water. Table 6 shows that all products exhibited 100% mortality using 25 *Ae. aegypti* larvae in the three different volumes of water in the 200-l water containers. This demonstrated that a l-g tablet would be suitable for application in this kind of water container, which are widely used in the village.

Efficacy Tests of Different Types of Bti products on Mosquito Larvae.

Table 7 shows the efficacy of different types of Bti-formulated products produced in this study in comparison with the Bti H-14 Standard Powder of 15,000 ITU/mg and local commercial product (Larvicos Pong) of 6,000 ITU/mg,

on six different species of mosquito larvae: *Ae. aegypti, Ae. albopictus, Cx. quinquefasciatus, Cx. tritaeniorhynchus, An. dirus* A., and *T. rutilus*. Most of products demonstrated higher toxicity to *Ae. aegypti* over *Culex* and *Anopheles* larvae but had no effect on *Tritaeniorhynchus* larvae.

Table 6. Comparison of potency of different Bti-formulated tablets against** Ae. aegypti **larvae in water containers of various capacities.

Formulated tablets (%Bti)	%mortality		
	40 l water	80 l water	160 l water
3	100	100	100
5	100	100	100
8	100	100	100
10	100	100	100
15	100	100	100

Table 7. Efficacy of different types of Bti-formulated products against different species of mosquito larvae.

Formulated	*Ae. ae.*		*Ae. albo.*		*Cx. quin.*		*Cx. tri.*		*An. dirus*	
Bti products	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
IPS-82 (Standard ITU)	0.012	0.287	0.014	0.028	0.013	0.030	0.014	0.029	0.043	0.136
Larvicos Pong (6,000 ITU)	0.039	0.092	0.042	0.121	0.034	0.100	0.046	0.136	0.062	0.643
3% Bti H-14 (Sand Granule)	0.660	1.582	0.790	3.163	1.035	4.098	-	-	-	-
5% Bti H-14 (Sand Granule)	0.333	0.058	0.759	3.751	1.485	4.723	2.846	5.571	5.660	13.94
3% Bti H-14 (Paper Strip)	0.220	0.553	1.243	3.427	5.564	17.56	-	-	-	-
5% Bti H-14 (Paper Strip)	0.107	0.283	1.397	3.028	2.363	4.857	5.519	14.68	6.261	13.29
3% Bti H-14 (Tablet)	0.285	0.538	1.691	3.154	1.799	4.098	-	-	-	-
5% Bti H-14 (Tablet)	0.195	0.478	0.858	2.983	0.848	2.351	0.252	0.526	1.537	3.409

Table 8. Longevity of different Bti-formulated tablets against** Ae. aegypti **larvae in 160-l water containers in shaded area.[a]

Days after treatment		% mortality									
		20% water change (in&out) every day, % Bti tablet					No water change (in or out), % Bti tablet				
	Control	3	5	8	10	15	3	5	8	10	15
1	0	100	100	100	100	100	100	100	100	100	100
20	0	100	100	100	100	100	100	100	100	100	100
60	0	96	100	100	100	100	100	100	100	100	100
100	0	80	96	96	100	100	100	100	100	100	100
150	0	80	88	92	100	100	92	96	100	100	100

[a] Although assessment was made every 10 d, for brevity some of the readings are omitted.

Efficacy Tests of Bti-Formulated Tablets in Artificial Water Containers.

An experiment was designed to simulate the real situation of using water containers, the main breeding habitats of *Ae. aegypti*, by the villagers, because the villagers normally used up water daily from all kinds of water containers, which are located around the house. Therefore, the volume of water in each container varied, with or without the routine of using or refilling water in or out of the jar, as well as the location of jar being in shaded areas with or without sunshine (morning or afternoon sunlight). These studies were carried out to determine the effect of sunlight on the performance of Bti-tablets. A 1-g Bti tablet was used in each 200-l water jar, with the starting volume of water being 160-l. Twenty-five fourth-instar *Ae. aegypti* larvae were released into the water-jar every 5 d for 5 mo. Observations on the efficacy of these formulated tablets in the 160-l water containers revealed that the Bti-formulated tablet was effective against *Ae. aegypti* larvae for as long as 3 mo in containers with no change, refilling or removal of water and about 2 mo in the containers with daily removal/refill of 20% water in volume. Results of these tests are presented in Tables 8-10, and 5% Bti-formulated tablet exhibited the best products.

Preparation of Powder Developed from Mosquito Larval Cadavers.

Another attempt was made to improve the performance and persistence of Bti-formulated products, since there are some indications that this bacterium has the ability to recycle it-self, especially in nature. An experiment was designed to use the powder ground up from cadavers of mosquito larvae, that

were fed on Bti-powder, as part of the ingredient of Bti-formulated products. Results of bioassay tests of such powder on *Ae. aegypti* larvae are in Tables 11 and 12, which indicate that such powder has its efficacy against mosquito larvae. Thus, there is adequate amount of toxin protein remaining in mosquito larval cadavers.

Table 9. Comparison of potency of different Bti-formulated tablets against *Ae. aegypti* ***larvae in 160-l water containers in shaded area with morning sun (8:00-12:00).***[a]

Days after treatment		% mortality									
		20% water change (in&out) every day, % Bti tablet					No water change (in or out), % Bti tablet				
	Control	3	5	8	10	15	3	5	8	10	15
1	0	100	100	100	100	100	100	100	100	100	100
40	0	100	100	100	100	100	100	100	100	100	100
80	0	92	92	96	100	100	100	100	100	100	100
120	0	76	80	80	84	88	100	100	100	100	100
150	0	64	68	80	80	80	88	88	100	100	100

[a] Although assessment was made every 10 d, for brevity some of the readings are omitted.

Table 10. Longevity of different Bti-formulated tablets against *Ae. aegypti* ***larvae in 160-l water containers in shaded area with afternoon sun (12:00-17:00).***[a]

Days after treatment		% mortality									
		20% water change (in&out) every day, % Bti tablet					No water change (in or out), % Bti tablet				
	Control	3	5	8	10	15	3	5	8	10	15
1	0	100	100	100	100	100	100	100	100	100	100
40	0	88	100	100	100	100	100	100	100	100	100
80	0	72	88	100	100	100	100	100	100	100	100
100	0	64	80	96	100	100	96	100	100	100	100
120	0	60	72	76	76	88	92	92	96	100	100
150	0	44	60	60	64	72	76	88	88	100	100

[a] Although assessment was made every 10 d, for brevity some of the readings are omitted.

Table 11. Bioassay of powder products developed from mosquito larvae fed on different concentrations of Bti expressed as number of dead larvae in dry weight (mg/250 larvae).[a]

Timing (hr)	Powder from mosquito larva fed on					
	$1xLC_{100}$=0.075 mg/l Bti		$5xLC_{100}$=0.375 mg/l Bti		$10xLC_{100}$=0.75 mg/l Bti	
	No. dead larvae	Dry weight (mg/250 larvae)	No. dead larvae	Dry weight (mg/250 larvae)	No. dead larvae	Dry weight (mg/250 larvae)
0.5	0	50	25	54	50	50
1.0	0	54	192	56	215	50
1.5	3	50	225	55	232	50
2.0	3	50	243	54	243	46
2.5	3	50	245	55	250	50
3.0	6	54	247	53	250	50
3.5	6	50	247	50	250	50
4.0	9	56	249	54	250	50

[a] 250 larvae used.

Table 12. Bioassay of powder products developed from mosquito larvae fed on different concentrations of Bti against fourth-instar *Ae. aegypti* ***larvae.***

Powder from mosquito larvae fed on Bti powder (hr)	Bioassay test no. dead larvae[a] concentration in µl/(mg/l)				Lethal concentration			Potency (ITU/mg)
	1000 /33.3	1500 /50.0	2000 /66.7	2500 /83.3	3000 /100.0	LC_{50}	LC_{90}	
1 x LC_{100} Bti -.075 mg/l								
0.5	0	0	0	0	0	-	-	-
1.0	0	0	0	0	0	-	-	-
1.5	0	0	0	0	0	-	-	-
2.0	0	0	0	0	0	-	-	-
2.5	0	0	0	0	0	-	-	-
3.0	0	0	0	0	0	-	-	-
3.5	0	0	0	0	0	-	-	-
4.0	0	0	0	0	0	-	-	-

Table 12. Bioassay of powder products developed from mosquito larvae fed on different concentrations of Bti against fourth-instar *Ae. aegypti* ***larvae. (cont.)***

Powder from mosquito larvae fed on Bti powder (hr)	Bioassay test no. dead larvae[a] concentration in µl/(mg/l) 1000 /33.3	1500 /50.0	2000 /66.7	2500 /83.3	3000 /100.0	Lethal concentration LC_{50}	LC_{90}	Potency (ITU/mg)
5 x LC_{100} Bti -0.375 g/l								
0.5	8	17	22	24	25	40.77	68.06	5.35
1.0	18	22	24	24	25	22.56	53.43	9.66
1.5	14	17	20	23	24	34.40	77.30	6.34
2.0	5	8	13	14	17	68.43	194.06	3.19
2.5	16	24	24	25	25	28.87	47.97	7.55
3.0	23	24	24	25	25	3.40	27.05	64.22
3.5	23	24	24	25	25	3.40	27.05	64.22
4.0	23	24	24	25	25	3.40	27.05	64.22
10 x LC_{100} Bti -0.75 mg/l								
0.5	7	16	22	23	24	42.45	75.88	4.72
1.0	19	23	24	25	25	20.65	47.80	9.70
1.5	14	21	23	24	25	30.13	61.27	6.65
2.0	19	23	24	25	25	16.60	38.26	12.08
2.5	20	23	24	25	25	17.49	46.22	11.46
3.0	16	19	24	25	25	18.93	33.61	10.59
3.5	19	22	24	25	25	21.52	50.29	9.31
4.0	15	20	23	24	25	17.51	37.74	11.45

[a] No. dead larvae using 25 larvae in each bioassay. There was no mortality in the controls.

Discussion

The standard strain of *B. thuringiensis israelensis* H-14 with potency of 15,000 ITU/mg was chosen as an active ingredient for the development of Bti-formulated products in this study. Data and information gathered from the standard strain would be used to establish the criteria for the future development of local strain for both Bti and *B. sphaericus* (Bs)-formulated products in controlling mosquito larval populations.

However, the method of harvesting bacteria on hard medium has both advantages and disadvantages. For instance, a high number of bacterial spores could be expected with this method. But the cost of harvesting bacteria would

be more expensive [on average, about 33.00 Baht (US $1.25)/g] as well as time consuming (to mass produce 500 g of pure wettable powder Bti would take at least 2 weeks), were examples of some disadvantages.

Therefore, for the future development of active ingredient (Bti pure powder), especially in the scale-up procedure and/or process of mass production, the technique of fermentation has to be the method of choice. With the fermentation technique, the cost of producing Bti pure culture will be lower.

Formulation technology of Bti-formulated products is not going to be emphasized only on the active ingredient but also other ingredients or additives as well. The performance and quality of Bti-based products, i.e., its efficacy, dispersion and persistence etc., depend upon both factors. Some additives play a major role in the development of a good and effective formulation. Too much or too little of some additives definitely would influence its performance. For example: the amount of sodium bicarbonate and citric acid will affect the pH level or some adhesive substances, i.e., cellulose, would affect the dispersion of the product. Bti-formulated products must not have any color, odor or create any turbidity in the water containers.

Results from the efficacy test of powder developed from cadavers of mosquito larvae, which were fed on different concentrations of Bti pure powder, was promising. It revealed that there is enough toxin protein contained in the mosquito cadavers. The mosquito larval tissue could become a good source of nutrition for bacterium to grow and be able to produce more spores. This leads to more toxin production. Therefore, such powder obtained from ground-up mosquito larval cadavers could be used as another ingredient in the future Bti-based products and provide for good persistence of Bti-based products.

Several field trials of Bti-based products have been conducted by many investigators, especially in the United States (Lacey 1985). However, such field study/trial in Thailand has not been conducted scientifically. The implementation of Bti-formulated products developed in this study is now being done in Amphoe Mae Sot (Country), Tak province. The application of Bti-based products is being integrated along with the normal/routine vector control programs, i.e., Abate-sand granule ULV spraying, at the community level. One of the main reasons this village had been chosen for the study sites was because this area is one of the Dengue Haemorrhagic Fever (DHF) endemic areas for the past several years. Two treatment sites (Mae Kasa and Mae Kud Leung villages) and a placebo/control site (Mae Kud Sam Tha village) were designated.

Bti-formulated tablets of 1 g 5% are being used in these field trials. Results and data received from this field study will be analyzed and compared. Entomological, ecological and geographical surveys (GIS-Geographical Information System) will also be conducted to determine whether or not the use of Bti-based products can reduce *Ae. aegypti* populations and subsequently reduce the number of DHF cases.

Improvement of the formulated products of bacterial larvicidal agents, especially using the newly discovered local strains of both Bti and Bs, in conjunction with ecological aspects will provide us with better vector control strategies yielding better results.

Acknowledgments

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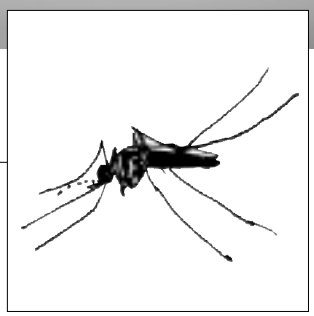
Filariasis Vector



Field Trials with *Bacillus sphaericus* Formulations against Polluted Water Mosquitoes in a Suburban Area of Bangkok, Thailand

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Abstract

Two newly developed *Bacillus sphaericus* larvicidal formulations, VectoLex CG® (corncob granules) and VectoLex WDG® (water dispersible granules), were tested against *Culex quinquefasciatus* larvae in 4 highly polluted breeding sites in Thailand. VectoLex CG, applies at rates of 0.5-2 g/m^2, gave satisfactory to complete control of late-instar larvae and pupae for up to 4 wk after treatment. The VectoLex WDG, which had higher potency and was applied at rates of 0.1-0.5 g/m^2, gave satisfactory control for 1-4 wk after treatment. Among the factors influencing longevity of control were dosage of a given formulation, precipitation, and flooding of the treated sites; the latter had the greatest impact. Presence of larvivorous fish did not seem to influence larval populations because there were heavy populations of mosquito larvae present in the test sites in the presence of moderate numbers of fish before the application of *B. sphaericus* treatment.

Keywords

Bacillus sphaericus, mosquitoes, biopesticides, vector control

Introduction

Bacillus sphaericus Neide is a spore-forming becterium found commonly in soil, water, and other substrates in nature. Most strains of this bacterium are nonpathogenic to insects. However, in recent years, a number of strains have been discovered and isolated that produce parasporal toxins that are toxic to larvae of several genera of mosquitoes. The binary crystalline toxins produced by *B. sphaericus* strain 2362 have high activity against larvae of *Culex* mosquitoes (Mulla 1991). A corncob granular formulation of this strain with the

trade name of VectoLex CG® (Abbott Laboratories, North Chicago, IL) was registered for mosquito control by the United States Environmental Protection Agency in 1991. This formulation and others are just now becoming available for experimental use and for the control of mosquitoes around the world.

Bacillus sphaericus formulations have been tested and evaluated in a variety of habitats, especially the breeding sites of *Culex* mosquitoes. Karch et al. (1991) evaluated a granular formulation (VectoLex) of this microbial larvicide against *Anopheles gambiae* Giles in clear water and *Culex quinquefasciatus* Say in pulluted water in Zaire. The granular formulation was found to be highly effective against the polluted water mosquito. Similarly, *B. sphaericus* formulations were found to show good activity against *Anopheles stephensi* Liston (Kumar et al. 1994).

The most important and desirable attributes of a microbial larvicide formulation are persistence and recycling, as well as suspension of the particulate toxins in the feeding zone of mosquito larvae. Unfortunately, most evidence to date points out that the toxin particles in currently available formulations settle out from the feeding zone of larvae and sink to the bottom of the water (Davidson et al. 1984, Mulla et al. 1988, Matanmi et al. 1990). Recycling of *B. sphaericus* at least under laboratory conditions has been suggested by numerous workers. Mosquito larval cadavers have been reported to serve as substrates for spore porduction (Becker et al. 1995, Correa and Yousten 1995). Nicolas et al. (1987) reported persistence and recycling of *B. sphaericus* in a field test in West Africa. The studies of Skovmand and Bauduin (1997) clearly showed the recycling of this agent where larvae were added to treated regimens, the dead larvae acting as a medium for the production of spores.

Because field studies on the persistence and longevity of larval control with *B. sphaericus* formulations, especially in polluted waters, are few, the present studies were initiated to determine the efficacy of 2 newly developed formulations of *B. sphaericus* against *Culex* larvae in highly polluted water habitats in the Nonthaburi Province in the suburb of Bangkok. To find the minimum effective dosage, various rates of the experimental formulations were evaluated for initial efficacy as well as long-term control of mosquito larvae. The studies were initiated in August 1996 and continued into January 1997.

Materials and methods

Four small breeding sites, of various surface areas, where heavy populations of *Culex* larvae prevailed were selected for these studies. Mosquito larvae breeding in these habitats were primarily *Cx. quinquefasciatus*. The formulations evaluated and the site descriptions are as follows.

Formulations

VectoLex CG corncob granules with 50 ITU/mg (lot no. 16-723-NB containing 7.5% of a technical powder of *B. sphaericus*) and VectoLex WDG® water dispersible granules with 350 ITU/mg (designated as ABG-6461, lot no. 21-105-BR) were provided by Abbott Laboratories. It should be noted that the potency and characteristics of the VectoLex corncob granules have changed over time, and the new formulation evaluated here was different from those studied earlier. The VectoLex WDG formulation tested here was also a new product that has not been field tested previousely.

Sites

The four mosquito breeding sites used in these experiments were located in a low income area of Nonthaburi Province in the suburbs of Bangkok, Thailand. All sites received wastewater from dwellings that were raised on posts of pillars. Wastewater accumulated in depressions both under the houses and in open areas around the houses. There was a great amount of solid waste in the water as well as on the ground above the water line. Plastic containers and bags as well as plastic and glass bottles and decaying plant wastes constituted the bulk of the solid waste mass. Solid waste accumulation created discontunuous water pools, some of them very small and holding a few hundred ml of water. The accumulated water came from washing clothes, kitchen utensils, and household wares, from bathing and showers, and, in some cases, from leaky or broken sewers and septic tanks. Frequent rains during the early part of the experimental period also added water. At times, there was substantial precipitaton that flooded the catwalks, the only access to the communities and dwellings from paved city streets. Because of the continuous addition of domestic wastewater and rainwater, there was somewater flow into and out of the treated plots, but in the absence of rain, the flow rate was minimal.

Thanausi community: This site, which consisted of a ditch and pool extended under and around a house, received wastewater from domestic sources.

The area terated with VectoLex CG and WDG granules was about 50 m^2. This site was treated 4 times, the first 2 treatments with VectoLex CG, and the last 2 with the WDG formulation. VectoLex CG was applied at the rate of 2 g/m^2 on September 3 and October 17, 1996. The WDG applications were made on November 14 and December 11, 1996 at the rate of 0.25g/m^2, a lower dosage than that of VectoLex CG because the WDG formulation possessed high potency.

Raevadee community: This site consisted of a pool and depression under and around houses that received wastewater from domestic uses. The area treated with VectoLex CG and WDG granules was about 100 m^2. The VectoLex CG formulation was used at the rate of 2 g/m^2 in the first 2 treatments made on September 3 and October 17, 1996. This same area was then treated twice with the WDG formulation at the rate of 0.1 g/m^2 and 0.25 g/m^2 (lower dosages than that of VectoLex CG because of the high potency of the WDG) on November 14 and December 25, 1996, respectively.

Wat Lannaboon community: This site consisted of a small ditch that opened into a larger area under and beside houses. Again, wastewater from domestic uses accumulated in this area. The total area of the plot initially treated (only the ditch) was 50 m^2; it was treated twice at the rate of 0.5 g/m^2 of VectoLex CG on September 5 and October 17, 1996. For the WDG treatments, the area was increased to 100 m^2 and treated on November 14 at the rate of 0.25 g/m^2 and again on January 4, 1997 at the rate of 0.5 g/m^2 of the WDG formulation.

Wat Tinnakornnimit: This site consisted of a small pool of highly polluted water under and beside houses that received wastewater from the dwellings and also contained a large amount of solid waste and floating debris. An area of about 16 m^2 was administered 2 treatments of VectoLex CG at the rate of 2 g/m^2 on September 3 and 20, 1996 and a 3rd treatment of 1.6 g/m^2 on October 17, 1996. The WDG formulation was applied twice (November 14 and December 11, 1996) at 0.1 g/m^2.

Applications

The required amounts of the VectoLex CG corncob granules were broadcast by hand as evenly as possible. With this method of application, it was not possible to treat water under the houses; only the open accessible areas were

covered. For assessment of larval populations before and after treatment, only the accessible areas were sampled.

The WDG granules were easily suspended in a small amount of water in a hand-operated compression spray tank. The tank was filled with 2-5 l of water (depending on area of the plot) and manually shaken. This action was sufficient to completely suspend the formulation in water with no need for further agitation. The tank was pressurized and the water suspension sprayed out through a T-jet nozzle producing coarse spray. The spray stream could be directed under the houses and could reach 1-2 m from the edge inward.

Sampling

Larval populations were sampled in all sites by the standard dipping technique using a 400-ml dipper and taking 5-10 dip samples at each site and at each interval depending on plot size. Samples were taken in a biased manner, sampling those spots where mosquito larvae were noted in large numbers. For counting, the contents of the dipper were transferred to white plastic trays (15 x 30 x 4 cm deep), and the larvae were counted and categorized as 1st and 2nd instars and 3rd and 4th instars and pupae. The sites were samples before and at intervals (as shown in the figures) after treatment. When the counts of 3rd and 4th instars and pupae resurged, reaching or exceeding the pretreatment counts, the sites were retreated with the same or different dosage or a different formulation. The numbers of 1st and 2nd instars, although counted, are omitted from the figures because they are not a good indicator of the level of control. *Bacillus sphaericus* acts slowly on mosquito larvae, and 1st and 2nd instars can survive for 24-48 h before they suffer mortality. This is especially ture of 1st instars, which could have hatched a few hours before assessment and would not have had a sufficient exposure period.

Results

Thanausi community: The 1st treatment at the rate of 2 g/m^2 of VectoLex CG formulation produced complete control of late-instar larvae and pupae for almost 28 days (Figure 1). Five weeks after treatment, there was reappearance of 3rd and 4th instar larvae, as well as pupae. This resurgence was preceded by rains, which possible flushed the treated site. The populations further increased, and the site was then retreated with VectoLex CG at the same rate.

The 2nd treatment of VectoLex CG produced complete control of all immatures for 7 days. On the 7th day, heavy rains occurred, and the flushing out of the treated site resulted in slight resurgence of larvae 14 and 21 days after treatment, but the pupal population was still low (Figure 1). One month after this 2nd treatment, the larval and pupal populations increased markedly, reaching or exceeding the pretreatment populations, at which time the site was treated with the WDG Formulation.

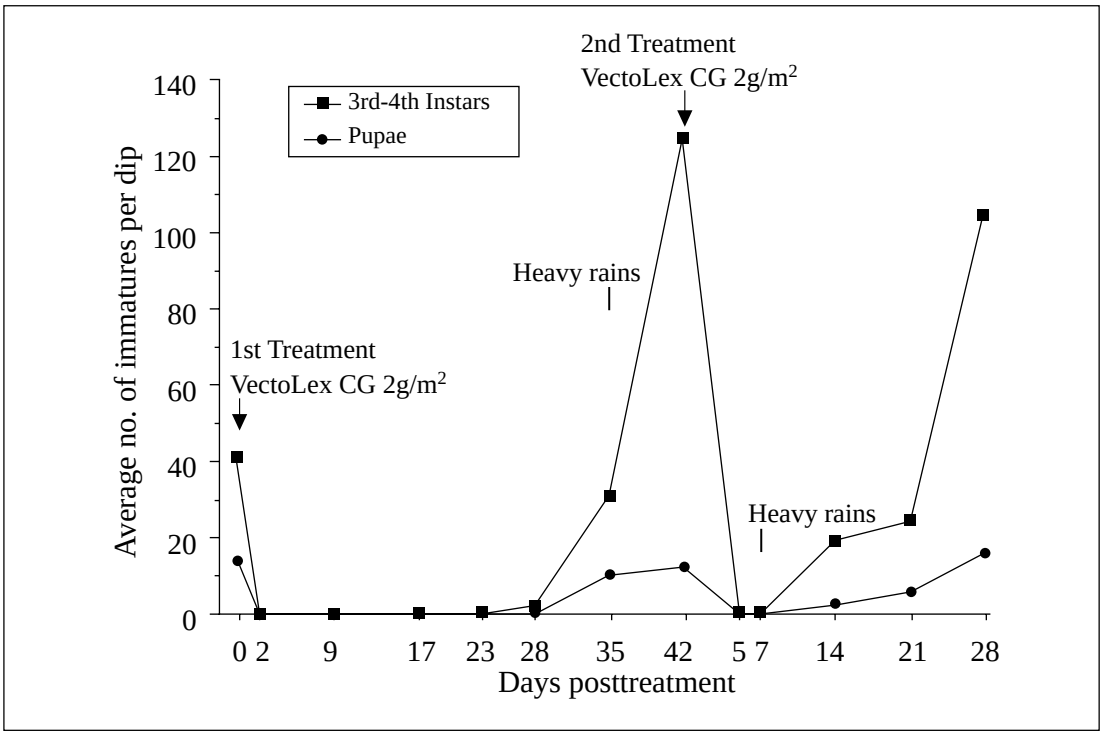

Figure 1. Evaluation of VectoLex CG® of *Bacillus sphaericus* ***(strain 2362) against larvae of*** *Culex quinquefasciatus* ***in domestic polluted waters in Thanausi community, Nonthaburi, Bangkok (1996).***

The 3rd and 4th treatments were made with the VectoLex WDG formulation at 0.25 g/m^2 (Figure 2). After the 3rd treatment, the immatures almost reached zero 4 and 7 days posttreatment, but the numbers of immatures increased slightly 14 days posttreatment. Population recovery, especially 3rd and 4th instars, was substantial 21 and 28 days posttreatment, after which time the site was retreated with the 4th application of WDG. This treatment markedly suppressed all immatures for 14 days, after which time there was

some recovery of larvae. Recovery was complete in 1 month, but the populations declined 6 wk posttreatment. At this site, a good number of larvivorous fish (either *Gambusia* or *Poecilia*, species not determined) prevailed in the open water. Even with fish present, there were heavy populations of mosquito larvae prevailing in protected areas.

Raevadee community: This site supported heavy populations of immature mosquitoes prior to the 1st treatment. VectoLex CG treatment at the rate of 2 g/m^2 suppressed the larval populations markedly, and the pupal population reached zero 2 days after treatment (Figure 3). Nine days posttreatment, the larval populations increased slightly but were still considered low for this sites, and the pupal population remained negligible. By 15 days posttreatment, the larval and pupal populations increased substantially. This increase could have been a result of heavy rains (on the 9th day posttreatment) flushing out the site. From 15 days to 6 wk posttreatment, populations of all immature stages, including the pupae, prevailed in high numbers. At 6 wk posttreatment, the site was retreated at the same rate of VectoLex CG.

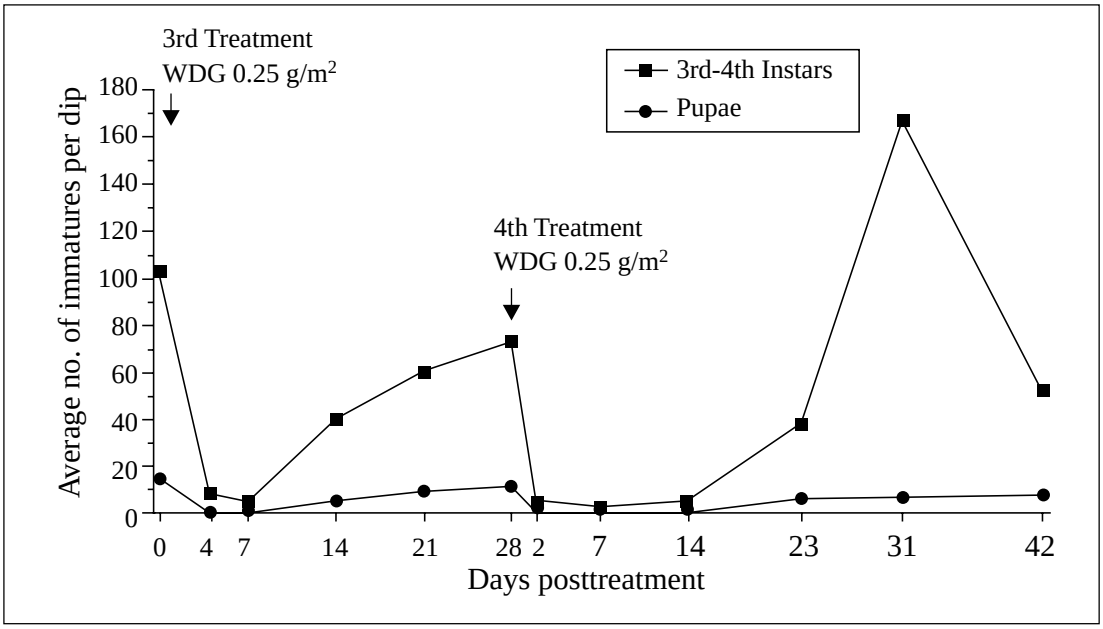

Figure 2. Evaluation of WDG formulation of *Bacillus sphaericus* ***(strain 2362) against larvae of*** *Culex quinquefasciatus* ***in domestic polluted waters in Thanausi community, Nonthaburi, Bangkok (1996).***

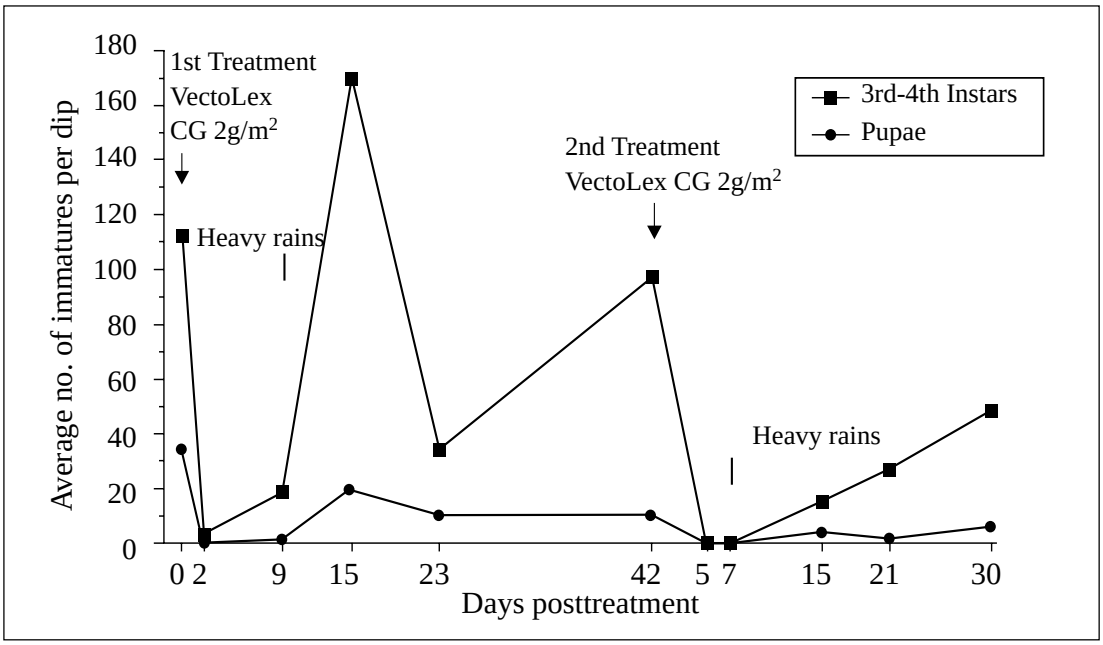

Figure 3. Evaluation of VectoLex CG of *Bacillus sphaericus* ***(strain 2362) against larvae of*** *Culex quinquefasciatus* ***in domestic polluted waters in Raevadee community, Nonthaburi, Bangkok (1996).***

The 2nd treatment with VectoLex CG produced complete control of immatures for 7 days posttreatment. Soon after assessment, heavy rains occurred in the area. Because of flooding and flushing of the treated site, there was slight recovery of all immature stages 15 days posttreatment, but the density of immatures was considered low for 15 and 21 days posttreatment. Thirty days after this treatment, populations of immatures increased further. This area as well as additional contiguous area were treated with the WDG formulation at 0.1 g/m^2. This 3rd treatment with WDG yielded excellent control of the immatures up to 28 days posttreatment (Figure 4). Because of the lack of precipitation, even this low dosage of WDG yielded excellent control of larvae and pupae for 28 days. There was a marked recovery of the immatures (especially 3rd and 4th instars) 33 and 40 days posttreatment, when the site was administered the 4th treatment using the WDG formulation at 0.25 g/m^2. This treatment produced a high level of control 7 days posttreatment. Recovery of larval and pupal populations ensued 15 days posttreatment because of the water flow from an adjacent canal with possible movement of larvae and pupae into the plot and dilution of the *B. sphaericus* toxins.

This site also supported populations of larvivorous fish that were active in the deeper, open parts of the habitat. Despite their presence, this site harbored heavy populations of larvae prior to treatment with *B. sphaericus*.

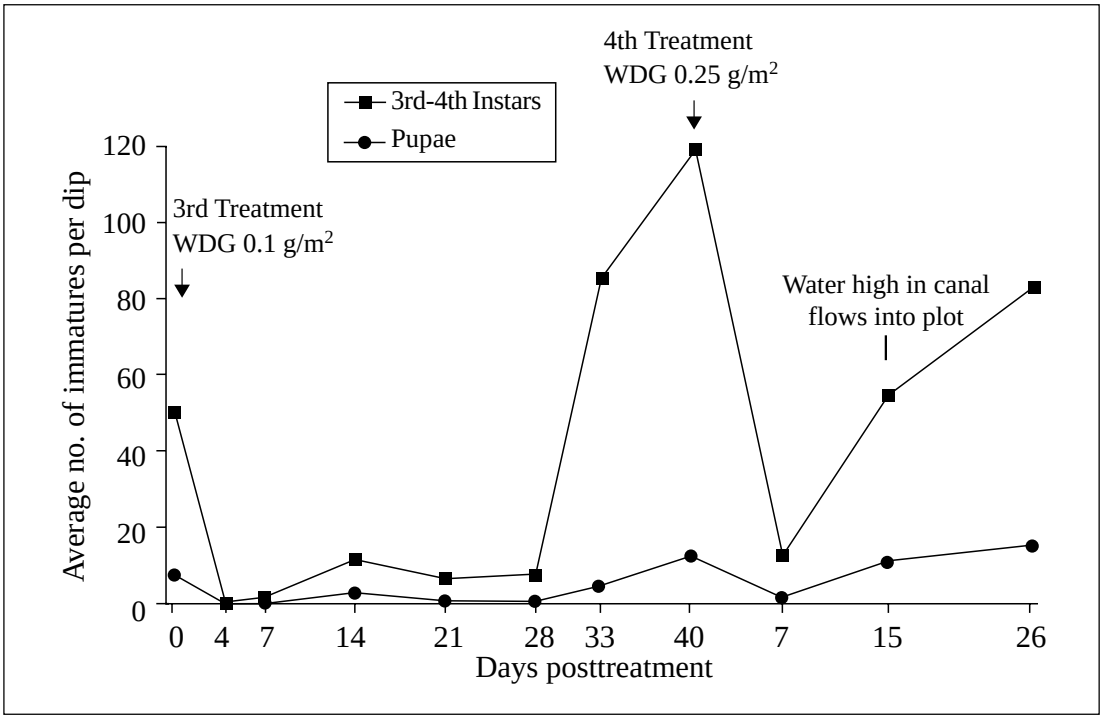

Figure 4. Evaluation of WDG formulation of *Bacillus sphaericus* ***(strain 2362) against larvae of*** *Culex quinquefasciatus* ***in domestic polluted waters in Raevadee community, Nonthaburi, Bangkok (1996).***

Wat Lannaboon community: This small site, which consisted of a ditch that flowed continuously, received water from upstream from a pool that accumulated water under and beside a house and could not be treated. The 1st treatment with VectoLex CG and 0.5 g/m^2 yielded almost complete control of all immature stages 4 days after treatment (Figure 5). Seven days after treatment, there was slight recovery in the larvae, but the pupal population remained zero. At 13 days posttreatment, there was substantial recovery of all immature stages, but they declined markedly 21 and 28 days posttreatment. At 5 wk posttreatment, there was heavy rainfall and the population recovered, and after 6 wk, the site was retreated with the same rate of VectoLex CG formulation.

The 2nd treatment at 0.5 g/m^2 of VectoLex CG produced almost complete control of all immatures for up to 7 days, after which heavy rains occurred (Figure 5). No samples were taken at 14 days posttreatment because flooding made the area inaccessible. The sampling at 21 days posttreatment still showed low populations of larvae and pupae.The last samples at 30 days posttreatment showed good recovery of populations, which equaled the pretreatment level. This area as well as additional water accumulations were then treated with sprays using the WDG formulation.

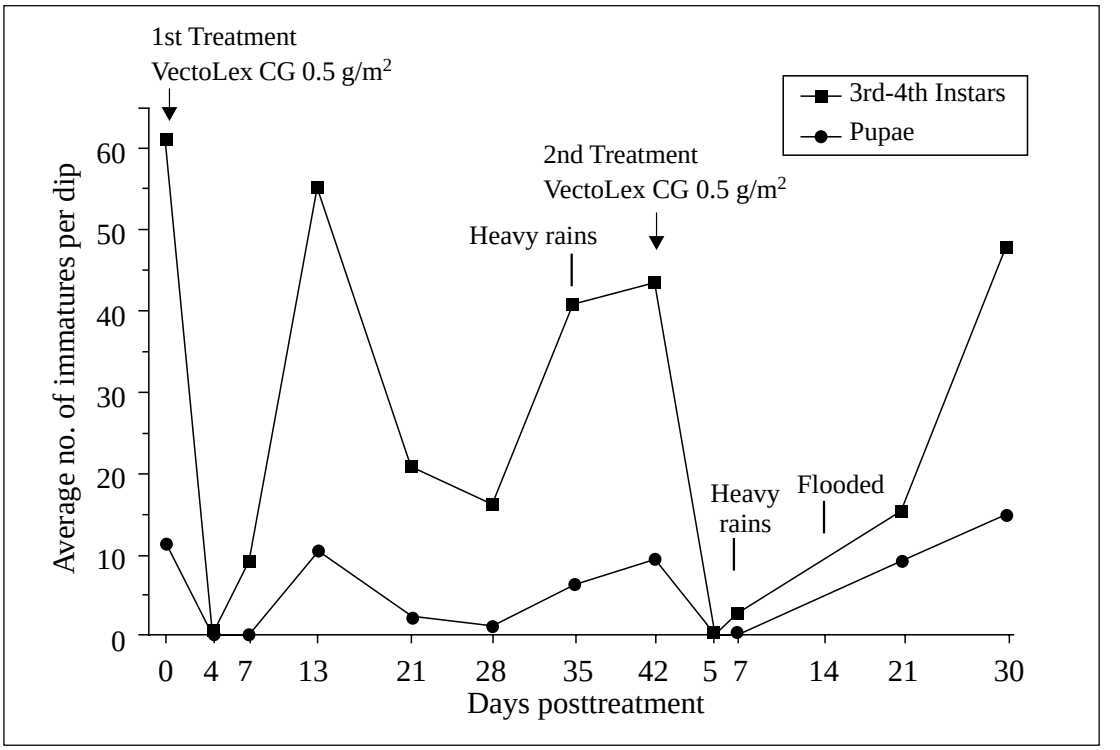

Figure 5. Evaluation of VectoLex CG of *Bacillus sphaericus* ***(strain 2362) against larvae of*** *Culex quinquefasciatus* ***in domestic polluted waters in Wat Lannaboon community, Nonthaburi, Bangkok (1996).***

The 3rd treatment using the WDG formulation at 0.25 g/m^2 provided good control for 4 days posttreatment, but mediocre control was noted 7 days posttreatment because the site was heavily flooded by rains (Figure 6). The populations increased further at 14 and 21 days posttreatment, and the site was retreated with WDG at 0.5 g/m^2. This treatment yielded almost complete control of immatures for 37 days posttreatment. Immature populations remained very

low for up to 48 days posttreatment because there was little of no rain during this period at this site.

This breeding source had a low level of larvivorous fish population. The fish were seen to swim in the open parts of the habitat, leaving the larvae to occupy protected and discontinuous niches.

Wat Tinnakornnimit community: This area consisted of a small pool of highly polluted wastewater accumulated under houses. Prior to treatment with VectoLex CG (2 g/m^2), the pool supported heavy populations of mosquito larvae and pupae. Up to 1 wk posttreatment, all immatures were reduced to very low levels. Slight resurgence occurred 13 and 15 days after treatment, and the site was retreated with VectoLex CG at the same rate (Figure 7).

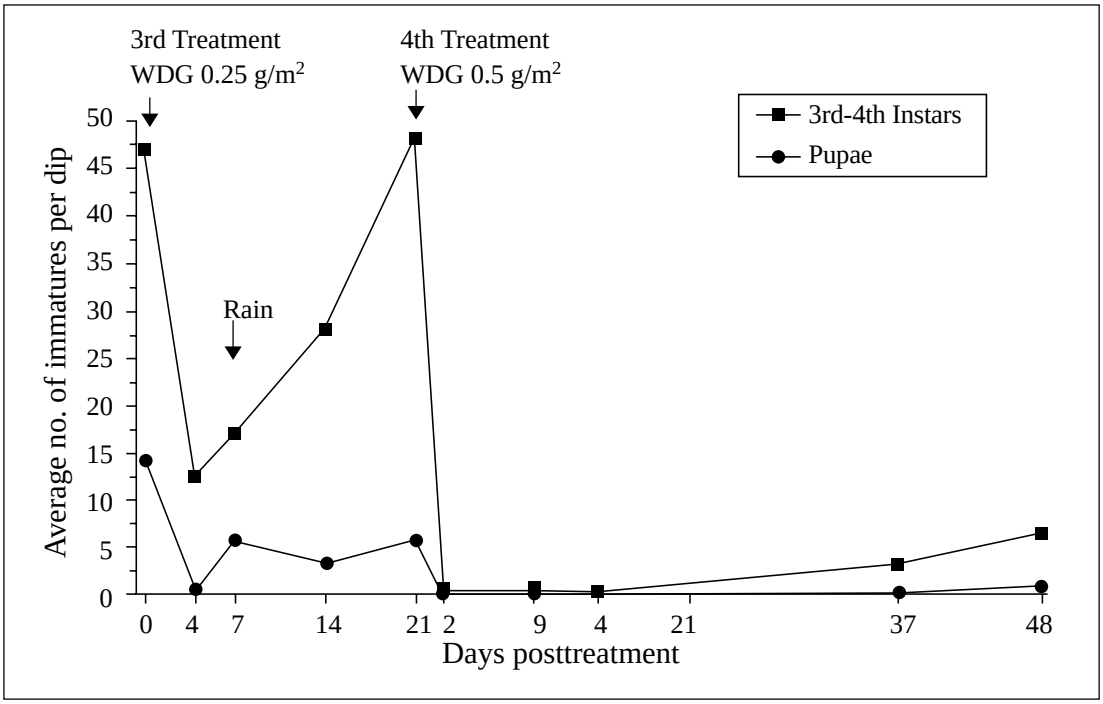

Figure 6. Evaluation of WDG granular formulation of** Bacillus sphaericus **(strain 2362) against larvae of** Culex quinquefasciatus **in domestic polluted waters in Wat Lannaboon community, Nonthaburi, Bangkok (1996).

The second treatment suppressed immatures markedly although not completely for about 6 days (Figure 7). There was some recovery 2 and 3 wk posttreatment but notable recovery, of the immatures especially, after the rains occurred 4 wk posttreatment, at which time the site was administered a 3rd

treatment at 1.6 g/m^2 of VectoLex CG, which produced almost complete control of immatures for up to 7 days and a high level of control of pupae for up to 29 days (Figure 7). Twenty-nine days after treatment, there was slight recovery of the larvae, and the area was sprayed with WDG formulation.

The 4th treatment with WDG formulation (0.1 g/m^2) suppressed larval populations for 7 days (Figure 8). Thereafter, the larval populations increased, and the 5th treatment at the same rate of WDG was made 28 days after the 4th treatment. This 5th treatment suppressed late-instar larvae significantly, but the pupal populaition was suppressed markedly for 15 days, after which period all immatures increased in numbers.

This very small treated site was adjacent to larger water accumulations under houses that could be treated 2 m inward at the most under the houses. Encroachment of immatures from the adjacent untreated area and flooding of the small treated area by rains up to the end of the 3rd treatment are possible reasons for lower efficacy of the treatments at this site.

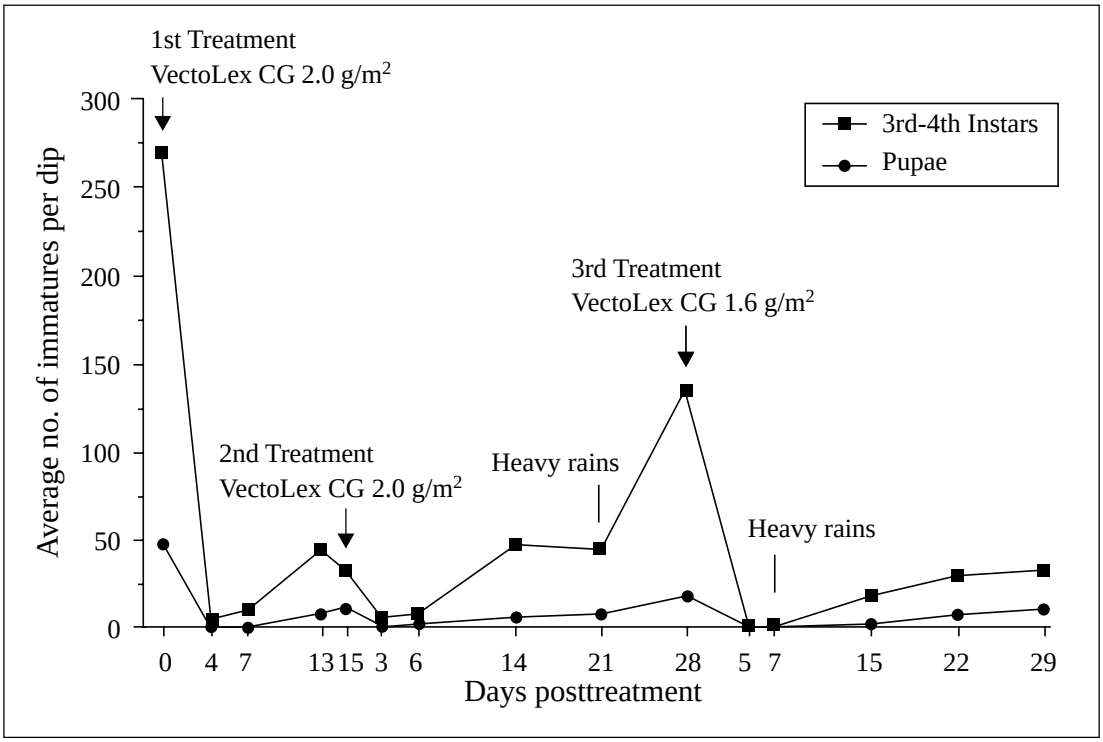

Figure 7. Evaluation of VectoLex CG of *Bacillus sphaericus* ***(strain 2362) against larvae of*** *Culex quinquefasciatus* ***in domestic polluted waters in Wat Tinnakornnimit community, Nonthaburi, Bangkok (1996).***

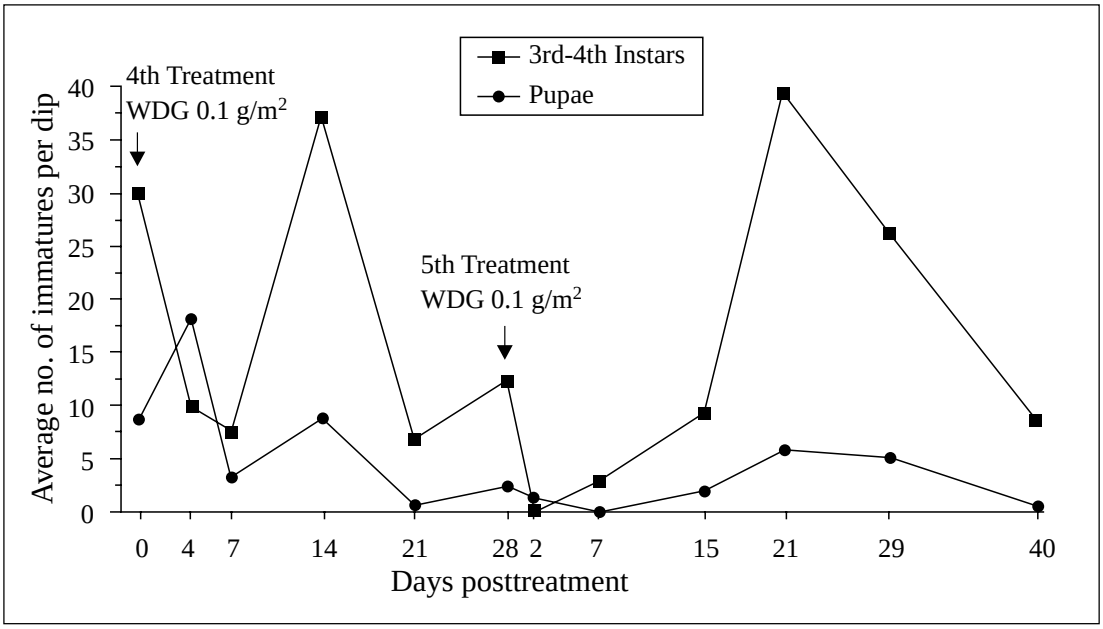

Figure 8. Evaluation of WDG formulation of *Bacillus sphaericus* ***(strain 2362) against larvae of*** *Culex quinquefasciatus* ***in domestic polluted waters in Wat Tinnakornnimit community, Nonthaburi, Bangkok (1996).***

Discussion

It is evident from these field experiments that *B. sphaericus* formulations can provide excellent control of mosquitoes in polluted waters. The persistence and longevity of efficacy to a large extent depended on formulations, rate of application, and extent of rains and flooding. Although some of the sites supported low populations of larvivorous fish, the role of the fish in control of larvae seemed to be minor. Even with fish present, these sites supported heavy populations of mosquito larvae prior to treatment. The 1st treatment with VectoLex CG at the rate of 2 g/m^2 of the formulation provided excellent suppression of mosquito larvae and pupae for 28 days at Thanausi, after which period there was a significant population resurgence following heavy rains. The 2nd treatment at the same rate at this site provided good control for only 14 days because of heavy rains (7 days posttreatment) flushing out the site (Figure1). Similarly, the 1st treatment of VectoLex CG (2 g/m^2) at Raevadee provided excellent control but for only 9 days. On the 9th day, the site was flooded by heavy rains, and the population fully recovered on the 15th day

(Figure 3). The 2nd treatment of VectoLex CG at the same rate provided control for 15 days because heavy rains 7 days posttreatment again flooded this site (Figure 3). Another treatment with VectoLex CG (2 g/m^2) of a very small site (Wat Tinnakornnimit) yielded excellent control for 13 days; the 2nd treatment (the same rate) at this site produced excellent control for 3 wk, after which heavy rains caused decline in efficacy. A 3rd treatment with VectoLex CG (1.6 g/m^2) yielded excellent control for 15 days, after which the efficacy declined somewhat as a result of rains 7 days posttreatment (Figure 7) but still kept the immature population at a low level.

At one site (Wat Lannaboon) receiving a low dosage treatment of VectoLex CG (at 0.5 g/m^2), the efficacy of this treatment lasted for only 7 days despite the fact that there was no heavy rain (Figure 5). The 2nd treatment at the same rate also yielded a high level of control for 7 days, and the efficacy declined when the area was flooded and could not be sampled 14 days post-treatment (Figure 5). This treated area was very small and was surrounded by larger mosquito breeding areas that could not be treated. It is likely that larvae and pupae moved into the treated plot.

Various dosages of VectoLex CG can provide various degrees of control depending on the site and prevailing environmental conditions. In the real-world situation, extent of control will vary because of these uncontrollable factors. It is evident, nevertheless, that dosages in the range 0.1-2 g/m^2 can provide effective control. During the rainy season, lower dosages will be the method of choice because no long-lasting control of larvae will be expected. In the dry season when the habitat is more stable, higher dosages for lasting control (for a month or longer) should be employed.

The water dispersible granules (WDG) tested in all 4 sites yielded variable results that were influenced by dosage, characteristics of the sites, and precipitation. Two WDG treatments (0.25 g/m^2) at Thanausi produced excellent control for 7-14 days (Figure 2). At Raevadee site, the 1st WDG treatment (0.1g/m^2) yielded excellent control for 28 days, whereas the 2nd treatment at the same rate gave good control for 7 days only (Figure 4). At 15 days posttreatment, immatures increased because of flooding from a canal. Even low dosages of the WDG can provide control for 1 month or so where there is little or no precipitaion .

It should be pointed out that heavy rains, flooding, and flushing of the treated sites reduce longevity of control. It should also be noted that some of the sites, such as Wat Lannaboon and Wat Tinnakornnimit, were small and were receiving water from untreated areas upstream. It is possible that immatures from the untreated areas encroached onto the treated plots.

It is also important to note that in using *B. sphaericus* one should not attempt to get 100% control because the existence of larvae aids in recycling; the larvae seem to serve as manufacturers of *B. sphaericus* spores. Once the larvae are completely annihilated, there will be little or no propagation of this bacterial agent, and resurgence of immature mosquitoes will follow. It is therefore advisable to employ moderate dosages rather than high dosages of *B. sphaericus* formulations and achieve moderate to high levels (80-95%) of control rather than to eliminate the total population. But it is equally important to note that higher dosages are apt to give longer control than lower dosages under certain conditons.

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Efficacy of New Formulations of the Microbial Larvicide *Bacillus sphaericus* against Polluted Water Mosquitoes in Thailand

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Abstract

Two new water dispersible granular (WDG) formulations of the microbial control agent *Bacillus sphaericus* (strain 2362) were extensively evaluated in polluted waters against *Culex quinquefasciatus* in Thailand. The studies were carried out in stagnant as well as flowing waters during August 1997 to July 1998. The trail period covered both rainy and dry seasons. The two WDG formulations of *B. sphaericus* tested were low potency (350 ITU/mg) and high potency (630 ITU/mg) products. Both formulations were used at various rates to determine initial efficacy and longevity. The high potency formulations provided excellent control (80 to 90%) of immature mosquitoes at the rates of 50 to 100 mg/m^2, while the less potent formulation yielded similar control at the rates of 89 to 250 mg/m^2. Longevity of control was anywhere from one week to four weeks or longer depending on the dosage, habitat, and environmental conditions. Two treatments with low dosages of *Bacillus thuringiensis* ssp. *israelensis* WDG provided short-term control lasting for one week. Frequent episodes of heavy rains facilitated longterm suppression of immature mosquitoes in klongs by flushing out the larvae. In the absence of rain, the longevity of treatments in flowing waters was shorter than in the presence of rain. Rain, however, had some but not pronuounced effect on longevity in the stagnant water habitats. Operation of floodgates controlling water flow from stagnant water habitats into the Chao Phraya River had greater influence on the abundance of mosquito immatures in the ponded polluted water under dwellings. Precipitation, adding large amounts of water, was probably responsible for diluting the control agents and also resulted in lower counts of immatures per unit volume of water. These variables could influence the efficacy (especially longevity) of treatments employing formulations of

microbial agents in operational control programs. The WDG formulations are preferred over other formulations, such as granules, because the former contain larger quantities of toxins per unit mass than the latter and are easily applied as aqueous sparys. Potent formulations with greater content of active ingredients are less costly to ship and transport to distant areas.

Keywords

Mosquitoes, *Bacillus thuringiensis israelensis, Bacillus sphaericus,* control polluted water, formulations

Introduction

In recent years, two mosquitocidal microbial control agents, *Bacillus sphaericus* Neide (*B. sphaericus*) and *Bacillus thuringiensis* ssp. *israelensis* (B.t.i.) de Barjac have been developed for the control of mosquitoes and other insects of public health importance (Mulla 1990, 1991). Both agents are spore-forming bacteria, which produce parasporal proteinaceous crystal toxins showing high activity against larvae of certain dipterans. The latter agent (B.t.i.) was developed and labeled for the control of mosquitoes and blackflies around 1980 and has been used since then, while the former (*B. sphaericus*) was registered in the United States in 1991 and found its use in mosquito control programs in 1996. *Bacillus sphaericus* is highly specific and shows maximum activity against *Culex* and a few other groups of mosquitoes (Barbazan et al. 1998, de Barjac and Sutherland 1990, Mulla 1991, Mulla et al. 1988, 1997, Skovmand and Bauduin 1997). Some of the desirable attributes of *B. sphaericus* are its persistence in the habitat after treatment and its recycling potential in the habitat or in larval cadavers (Becker et al. 1995, Corea and Yousten 1995, Lacey 1990, Mulla et al. 1988, Nicolas et al. 1987, Skovmand and Bauduin 1997).

The first formulation of *B. sphaericus* that received EPA registration for mosquito control in the United States was VectoLex CG (Abbott Laboratories N. Chicago, IL), a corn grit formulation with 7.5% active ingredients and a potency of 50 ITU/mg. This formulation is now a commercial product and is the only product currently used in the USA. Since mosquitoes are a diverse group of insects breeding in a variety of developmental sites, the manufacturer (Abbott Laboratories, N. Chicago, IL) embarked upon the development and production of new formulations with high potency for the purpose of developing

and facilitating additional use patterns. There is a great need for formulations that can be applied with water to a variety of habitats where VectoLex CG is not the material of choice. Among these new products, two new water dispersible granular (WDG) formulations of *B. sphaericus* (strain 2362) were produced and made available for laboratory and field evaluation in 1997. Field evaluation of the less potent early WDG formulation was initiated in Thailand in late 1996 and the results reported elsewhere (Mulla et al. 1997), and efficacy data on WDG formulations of both *B. sphaericus* and B.t.i. were generated in mesocosms (Su and Mulla 1999) under controlled conditions.

We here report on extensive field evaluation of the two new WDG formulations of *B. sphaericus* carried out in 1997-1998 against polluted water mosquiotes (*Culex quinquefasciatus* Say) in Thailand. The WDG formulations evaluated had potencies much higher than the currently used VectoLex CG (corn grit) formulation. They were extensively evaluated in stagnant water habitats as well as flowing water canals that received polluted water and supported heavy population of *Cx. quinquefasciatus* larvae.

Materials and methods

These studies were carried out in ponded domestic wastewater accumulations and two polluted water klongs (canals) in the Nonthaburi Province (adjacent to Bangkok) in Thailand. The studies were initiated in August 1997 and terminated in July 1998. This period encompassed both rainy (May-October) and dry (November-April) seasons. During the dry season there were episodes of rain, but the frequency and intensity were less than that in the rainy season. Several treatments (with the exception of Klong Keha-Chumchon) were made to each site to determine longevity and optimum dosage of the formulations to control mosquito larvae and pupae. The general mothods and procedures employed in the treatments and assessments are those used in our earlier studies in Thailand (Mulla et al. 1997). In brief, these methods are presented below.

Experimental Sites

Soi Jumpa (ponded water).

The community located in the Pak Kret District of the Nonthaburi Province in Thailand has a cluster of low income housing without and adequate wastewater disposal system. The dwellings are all raised on posts, and domestic wastewater accumulates over the entire area under the houses as well as between houses. The water depth ranged from 10-25 cm and water level fluctuated with rains and opening or closing of flood control gates in the klongs which drain into the Chao Phraya River. At the outset, two plots of 300 m^2 and 600 m^2 at Soi Jumpa were selected, Each plot was treated several times with different dosages; the dosages are shown in figures for each treatment. In subsequent experiments, most of Soi Jumpa (3,600 m^2) water accumulation was used as one plot and treated several times with various dosages of *B. sphaericus* WDG formulation and two treatments of B.t.i. WDG formulation. The latter formulation was used for comparison with the *B. sphaericus* formulation. Concomitantly with this large test, and area (Raevadee) similar to Soi Jumpa was designated as a control, which supported heavy production of mosquitoes as did the Soi Jumpa habitat.

Klongs (canals).

Two klongs were subjected to treatments of the WDG formulations. A portion (200 m long, 4 m wide, 800 m^2) of Klong Keha Chumchon located in the Pak Kret District of Nonthaburi, was treated only once on August 22, 1997 with the older WDG formulation (350 ITU). Due to frequent flushing caused by rains, clean out , and dredging three weeks after treatment, mosquito density did not increase enough to require another treatment until June 1998. Water in this portion of the klong was 10-50 cm deep, and water flow was sluggish (2m/min.) in the dry season. Water velocity increased during the rainfalls. Most of the larval aggregations were noted along the margins in vegetation or in accumulations of solid wastes in the water; and, therefore, only the margins were sampled for larvae and samples (frequency of samples shown in figures) were taken only in spots where mosquito larvae were abundant.

The second klong (Bangkraso) is located in the Muang District of Nonthaburi Province. A small, accessible, L-shaped section of this klong was selected where heavy populations of mosquitoes occurred. The treated section

measured 170 m long by 4.7 m wide. Water depth was 10-50 cm and water flow was 6 m/min. in the dry season. Mosquito larvae prevailed along the margins in vegetation or in accumulations of solid wastes and the samples (for frequency and number see sampling below) were taken in these areas.

This klong was treated four times, the first treatment on September 4, 1997 and the last treatment on March 4, 1998. Although this klong was flushed out several times during the rainy season, it was not dredged or cleaned out during the course of this study, and mosquito larval resurgence was noted after the effectiveness of the treatment had ceased.

Formulations

Two water dispersible granular formulations (WDG) of *B. sphaericus* and one WDG formulation of B.t.i. were field tested. These formulations were produced by Abbott Laboratories, N. Chicago, Illinois. The first WDG formulation of *B. sphaericus* (ABG 6491, lot no. 30-073-BR, 350 ITU/mg) became available in mid-1997, while the second WDG formulation (ABG-6491, lot no. 32-094BR, 630 ITU/mg) was received in October 1997. A third formulation of B.t.i. WDG (lot no. 31-078-BR, 4000 ITU/mg) was also tested at very low dosages to see if it will provide satisfactory (short-term) control of polluted water mosquitoes. All three formulations readily dispersed in water on shaking in the spray tank, no further mechanical agitation was needed during spraying. The suspension was easily applied through a cone nozzle that shot the spray stream to a distance of 4-5 m onto the target area. Areas farther away could not be reached by the spary streams.

Applications

The required amount of the WDG formulations was placed in an 8-liter compression spray tank and the required amount of tap water added. During addition of water, the mixture was stirred with a stick and then shaken in the tank. The tank was then sealed and pumped to pressurize the spray mixture. The WDG suspension was sprayed through a cone nozzle that facilitated the spray stream to travel and hit target areas up to 4 to 5 m away.

The plots in Klong Keha Chumchon (200 m x 4 m) and in Klong Bangkraso (170 m x 4.7 m) were also treated with 7 liters of suspension, spraying both sides and the middle of the klongs. The samll plot (300 m^2) in

Soi Jumpa was sprayed with 4 liters of the suspension, while the larger plot (600 m^2) was sprayed with 7 liters of the spray. The whole stagnant water area (3600 m^2) in Soi Jumpa was treated with three 7-liter tanks (21 liters) of the spray. In treating Soi Jumpa from walkways and catwalks, the spray stream could only reach 4-5 m away, and in some cases all the water underneath the houses could not be reached by the spray. The dosages, however, were based on the total area of the habitat and are noted in the figures.

Sampling

All sampling for larvae and pupae was done by dipping with a standard 400 ml dipper. Samples were taken in a biased manner, taking samples only in those spots where heavy aggregations of mosquito larvae occurred. The contents of each dipper were transferred into white plactic trays (30 x 15 x 4 cm deep), and the immatures were counted and categorized into 1st - 2nd instars, 3rd - 4th instars, and pupae. At Soi Jumpa, 10 dip samples were taken in each of the small plots, but 20 dips were taken when the total breeding habitat was treated. Similarly, 20 dip samples were taken each time in the Raevadee control plot. The two klongs were sampled by taking 20 samples at each interval as shown in the figures.

Efficacy Assessment

In order to determine the magnitude of reduction in immatures, the densities of posttreatment intervals were compared with those of the pretreatment. It was not possible to establish untreated plots in the klongs, as most of the klongs upstream or downstream from the treated area were not readily accessible. Similarly, it was not possible to establish untreated plots in the Soi Jumpa area in the small tests, as comparable mosquito breeding areas were not available in the vicinity. However, when all the Soi Jumpa mosquito developmental sites were treated, we selected a comparable area (Raevadee) as a control. The two areas were noted to be similar in their potential of producing mosquitoes. Retreatments were administered when immature mosquitoes (larvae and pupae) resurged, reaching approximately the 40-50% mark or higher of the pretreatment populations.

Standard error (SE) values of the mean for each sampling date were detemined by using Stat View SE+Graphics (Abacus Concepts, Inc., Berkeley, CA), and these values are inserted in the figures.

Results and Discussion

Klong Keha Chumchon

Klong Keha Chumchon received a single treatment of the WDG formulation of *B. sphaericus*. (350 ITU/mg) at the rate of 250/m^2 on August 21, 1997. This single treatment yielded excellent control of all immatures especially the pupae (*B. sphaecicus* does not kill pupae, pupal control is a result of the larval control) for four days with moderate resurgence of 3rd and 4th instars, 7 and 12 days posttreatment (Figure 1) and declining 14 days posttreatment. This slight to moderate resurgence is characteristic of *B. sphaericus* treatments, where the populations rise slightly and then decline subsequently. This secondary decline is probably due to the recycling of *B. sphaericus* in larval cadavers (Becker et al. 1995, Corea and Houston 1995, Skovmand and Bauduin 1997). A drastic reduction was noted on days 14 and 21 posttreatment before the dredging and clean-out operation. On day 21, the klong was cleaned out and dredged and experienced frequent flooding due to rains up to 53 days posttreatmen. Thereafter the immature populations, especially the pupae, remained zero to very low with the exception of a slight resurgence on day 97 posttreatmen. It seems that a treatment with *B. sphaericus* WDG formulation combined with clean-out and/or flooding action can provide long-lasting control of *Cx. quinquefasciatus* in polluted klongs. The immatures (3rd and 4th instars and pupae) did not reach the pretreatment level over the entire period of 10 months or so (not shown in Figure 1). From this experiment it seems that the WDG formulation of *B. sphaericus* at 250 mg/m^2 provided almost complete control of pupae for 21 days or so, and thereafter immature poputations remained very low or nil due to heavy rains or combination of rains and the treatment. After cessation of rain (53 days posttreatment), the poputation of larvae increased moderately on days 84 and 97 and then declined naturally thereafter.

Klong Bangkraso

This klong, with higher water velocity than the previous one, supported high populations of *Cx. quinquefasciatus* along the margins. It was treated twice with the less potent WDG formulation (350 ITU/mg). The first treatment was made on September 4, 1997 at the rate of 100 mg/m^2, while the second treatment at the same rate was made on November 19, 1997. The first

treatment appears to have yielded excellent suppression of all immature stages up to 43 days or longer posttreatment (Figure 2), a period which also experienced frequent rains. On day 86 (dry season) after the first treatment, there was a moderate resurgence of immatures, when the klong was retreated with 100 mg/m^2 of the *B. sphaericus* WDG formulation (350 ITU/mg). This treatment provided good control of larvae for about 28 to 35 days, larval populations increasing on day 40 and beyond this period, reaching extemely high numbers on February 19, 1998 (82 days posttreatment), when it was retreated with the more potent WDG formulation (see Figure 3).

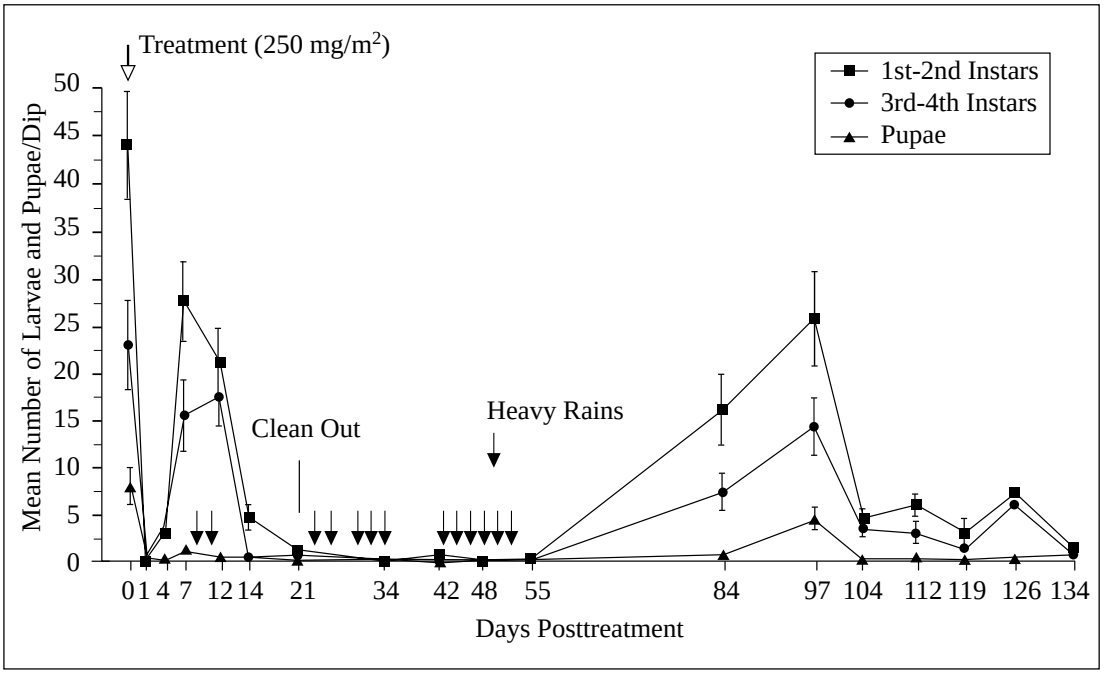

Figure 1. Evaluation of *Bacillus sphaericus* ***WDG formulation (350 ITU/mg) against*** *Culex quinquefasciatus* ***in Klong Keha Chumchon, Pak Kret District, Nonthaburi Province, Thailand (Starting August 21, 1997).***

It seems that precipitation influenced the efficacy and longevity of the first treatment with the low potency WDG formulation. Eight days after the first treatment (September 13, 1997), heavy rains came practically every two to three days up to 43 days posttreatment. As can be seen from the chart (Fig. 2), immature populations remained very low during the heavy rainy period, with moderate resurgence occuring 86 days posttreatment, during a period when no heavy rains were encountered. This dry period continued up to

March 10, 1998 (82 days post second treatment) when the immatures recovered markedly. The results of the second treatment are in contrast to the first treatment suggesting that heavy rains and flooding flushed out immatures of mosquitoes, resulting in apparent long-term suppression of immatures during the first treatment period. This trend was similar to that observed in Klong Keha Chumchon.

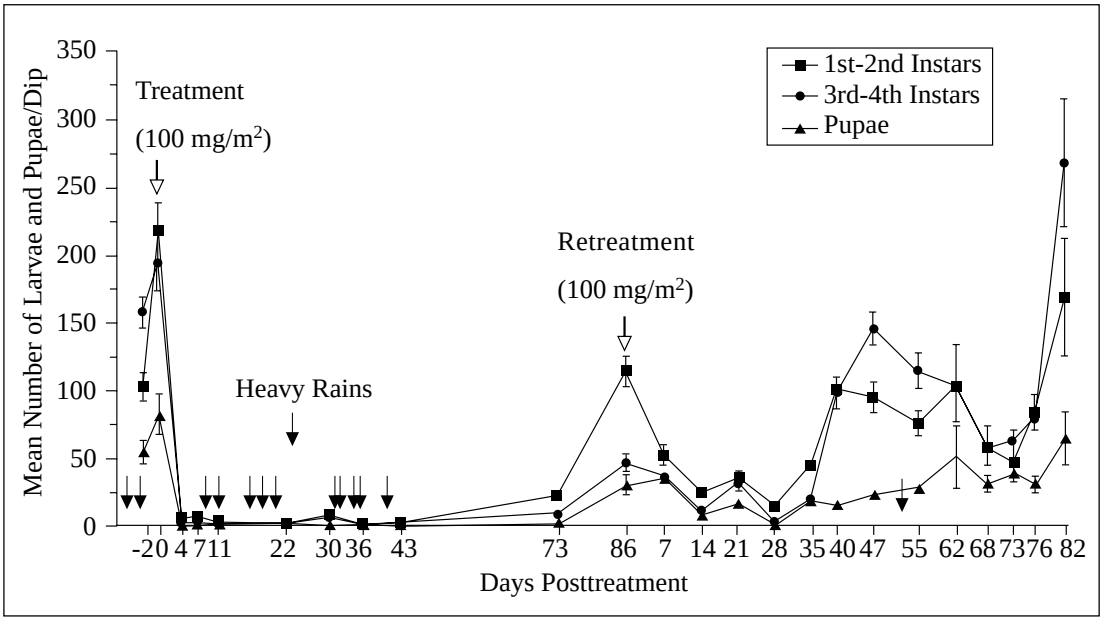

Figure 2. Evaluation of *Bacillus sphaericus* ***WDG formulation (350 ITU/mg) against*** *Culex quinquefasciatus* ***in Klong Bangkraso, Muang District, Nonthaburi Province, Thailand (starting September 4, 1997).***

After recovery of the immatures on day 82 post second treatment, Klong Bangkraso was administered a third treatment on March 10, 1998 (dry season), using the more potent WDG formulation (630 ITU/mg) at the rate of 106 mg/m^2. This treatment yielded excellent control of immatures for up to 26 days posttreatment (Figure 3) despite the fact that there was no significant precipitation during this period. It is likely that the immature suppression was solely due to *B. sphaericus* treatment. On day 33 (posttreatment), the immature population increased somewhat (although not to the pretreatment level) and the klong was administered a fourth treatment at the rate of 125 mg/m^2 of the more potent WDG formulation (630 ITU/mg).This fourth treatment, as the third treatment, yielded excellent control of immatures for up to 28 days or

longer posttreatment. During these 28 days of the dry season, no heavy rains were encounterd, but the rainy season began soon thereafter. During this ensuing rainy period, no resurgence of immatures was noted up to 56 days post this treatment, at which time the fifth treatment at the low dosage of 50 mg/m^2 of the *B. sphaericus* WDG (630 ITU/mg) was made. This low dosage treatment yielded good control for seven days posttreatment (Figure 3). The decline in immatures continued up to 14 days posttreatment when the experiment was terninated.

From the previous five treatments (Figure 2, 3) in Klong Bangkraso, it is apparent that under dry season conditions the more potent WDG formulation provided longer-lasting control of immatures than the less potent WDG formulation at approximately similar dosages. A dosage of 100 mg/m^2 of this potent formulation seems to be adequate to provide larval and pupal reduction for three to four weeks or longer.

Soi Jumpa (Plots 1 and 2)

An area of shallow, stagnant wastewater totaling 3,600 m^2 was selected in this community. In the first series of tests, a subarea was divided into plot 1 (300 m^2) and 2 (600 m^2). They were treated on August 21, 1997 at the rate of 250 mg/m^2 and 100 mg/m^2 of the less potent WDG formulation (350 ITU/mg), respectively. In plot 1, a high level of control was achieved up to 14 days posttreatment (Figure 4). A slight resurgence was noted 21 days posttreatment; but the immature populations declined on the 25th day posttreatment, increasing again 34 days posttreatment, when the plot was retreated at the very low rate of 10 mg/m^2 of the WDG (350 ITU/mg). This low rate of application as expected yielded excellent suppression for two days only, the population rebounding eight days posttreatment (Figure 4). The population reached a high level of density 50 and 54 days post second treatment when this plot and plot 2 were made a part of a large plot, which was treated with the same WDG formulation on November 18, 1997 (see Figure 6). The purpose of this test was to treat the whole mosquito infested water and to include another similar area as a control for comparison.

The second plot in Soi Jumpa (supporting extremely high densities of immatues than plot 1) treated on the same date as plot 1 with the *B. sphaericus* WDG formulation (350 ITU/mg) at the rate of 100 mg/m^2, experienced almost

complete control for 12 days posttreatment and better than 84% control (based on 3rd and 4th instars and pupae) for 14 days posttreatment (Figure 5). The level of control achieved was about 45% or better up to 34 days posttreatment. Immature populations prevailed at low levels, but the plot was retreated with a low dosages (25 mg/m^2) of the WDG formulation (350 ITU/mg) on September 24, 1997 (34 days post first treatment). This second treatment administered at the low rate of 25 mg/m^2 yielded excellent control of immatures on day 2 of sampling, populations resurging moderately 8 to 14 days posttreatment and reached maximum numbers 50 and 54 days post second treatment (see Figure 5). This plot was then combined with plot 1 (see above) and additional breeding sites were also added to make a larger plot that was teated on November 18, 1997 (see Figure 6).

During this test period in Soi Jumpa (34 days) there were seven episodes of heavy rains. Immature populations fluctuated and remained low in both plots. The second treatment at the very low rates of 10 and 25 mg/m^2 to plot 1 and 2, respectively, yielded short-term control, indicating that these rates of this WDG (low petency) formulation are not sufficient to provide long term control, but could suppress populations markedly for a very short period.

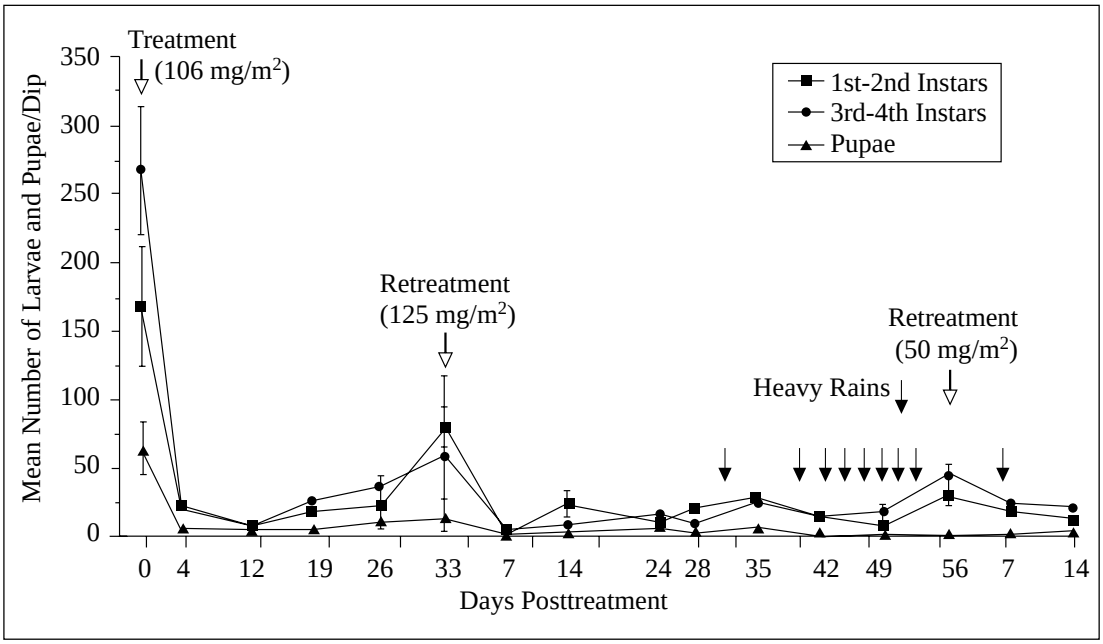

Figure 3. Evaluation of *Bacillus sphaericus* ***new WDG formulation (630 ITU/mg) against the polluted water mosquito*** *Culex quinquefasciatus* ***in dry season in Klong Bangkraso, Muang District, Nonthaburi Province, Thailand (starting March 10, 1998).***

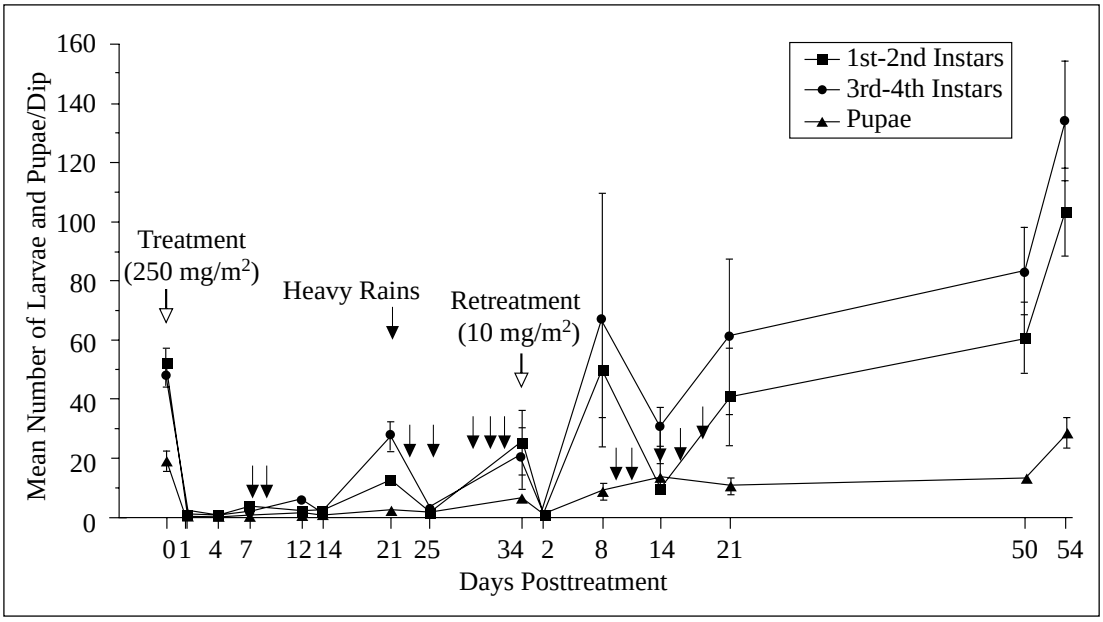

Figure 4. Evaluation of *Bacillus sphaericus* ***WDG formulation (350 ITU/mg) against*** *Culex quinqiefasciatus* ***in domestic waste water accumulation, Soi Jumpa (Plot 1), Pak Kret District, Nonthaburi Province, Thailand (starting August 21, 1997).***

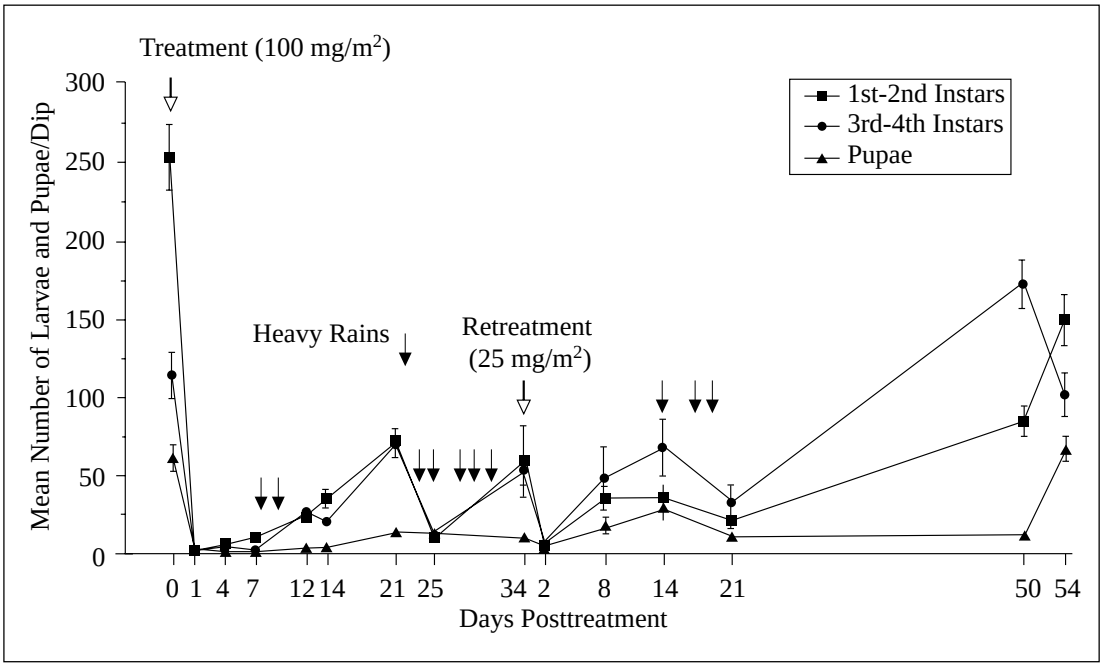

Figure 5. Evaluation of *Bacillus sphaericus* ***WDG formulation (350 ITU/mg) against*** *Culex quinqiefasciatus* ***in domestic waste water accumulation, Soi Jumpa (Plot 2), Pak Kret District, Nonthaburi Province, Thailand (starting August 21, 1997).***

Soi Jumpa (large plot)

After termination of the experiments in plots 1 and 2 in Soi Jumpa on Nomvember 18, 1997, the two plots as well as additional areas of breeding sources were combined into one large plot amounting to 3,600 m^2. At the same time an equivalent and similar area in the Revadee community close to Soi Jumpa was established as a control and sampled for larvae along with the treated area after treatment (intervals shown in figures). The large plot (Soi Jumpa) was treated with the less potent WDG formulation (350 ITU/mg) of *B. sphaericus* at the rate of 88.9 mg/m^2. Very high levels of control were achieved up to nine days posttreatment (Figure 6), and the extent of control was still appreciable (based on 3rd and 4th instars and pupae) up to 15 days posttreatment. The immature populations in the control plot in contrast incrased tremendously at this time. The immatures in the treatment further increased on day 24, when the plot was treated the second time. The second treatment with the same formulation was administered at 100 mg/m^2 on December 12, 1997. This treatment yielded excellent control (based on 3rd and 4th instars and pupae) for 14 days (Figure 6). Thereafter, the immature populations fluctuated remaining low to moderate for up to 54 days posttreatment. At this time, the plot was reteated for the third time with the same formulation. This third treatment with the less potent WDG formulation was made at 100 mg/m^2 on February 5, 1998. This treatment yielded good control (80% plus) of 3rd and 4th instars and pupae for up to 30 days. After 30 days the level of control dropped to about 75% and the plot was treated with B.t.i. WDG on March 10, 1998 (30 days post third treatment) (Figure 7). It should be noted that during these treatments (Figure 6) of Soi Jumpa, the population of immatures in the control plot, Raevadee, remained very high. The treated and control plots had similar populations at the time of the first treatment. The controls remainded very high (except for one dip) most of the time.

Due to the potential for resistance development to *B. sphaericus* (Rodcharoen and Mulla 1994) it was deemed desirable to determine the initial short-term efficacy of B.t.i. WDG (4000 ITU/mg) by making two treatments. The first treatment of B.t.i. WDG at the very low rate of 28 mg/m^2 was made on March 10, and the second treatment at the rate of 42 mg/m^2 was made on April 1, 1998. The first B.t.i. treatment yielded about 89% reduction as measured on day 7 posttreatment, but the immatures resurged reaching high densities 14

and 21 days posttreatment (Figure 7). The second treatment with B.t.i. yielded 76% control up to seven days posttreatment, but immature populations increased reaching close to the pretreatment level and the plot was retreated with the more potent WDG formulation (630 ITU/mg) of *B. sphaericus* at 50 mg/m^2 on April 17, 1998. This third treatment of the large plot produced excellent control of the immatures up to 20 days or longer posttreatment (Figure 7). It gave 92% control 20 days posttreatment. The rainy season started and there were frequent episodes of heavy rains during the period 14 to 27 days posttreatment.

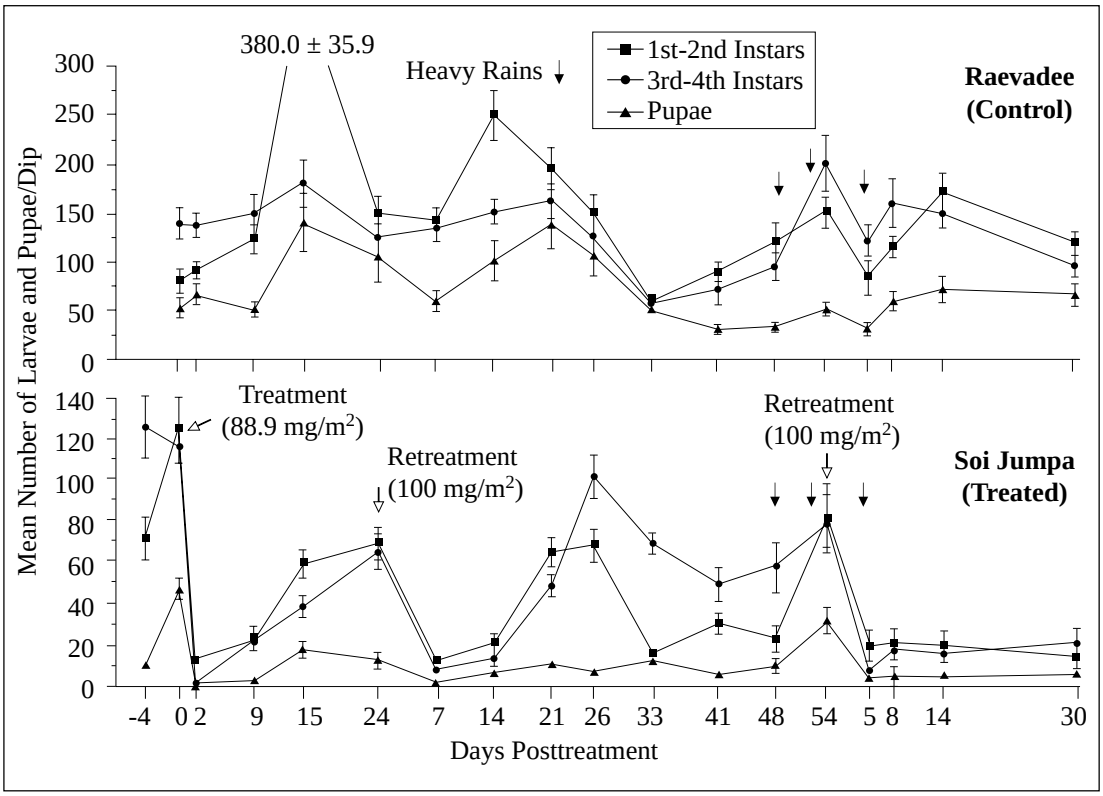

Figure 6. Evaluation of** Bacillus sphaericus **WDG formulation (350 ITU/mg) against the polluted water mosquito** Culex quinqiefasciatus **in dry season in Raevadee (as control area) and Soi Jumpa (treated total area), Pak Kret District, Nonthaburi Province, Thailand (starting November, 1997).

During the course of these three treatments of the large plot at Soi Jumpa (two teatments with B.t.i. and one treatment with *B. sphaericus*), the population of immatures, with the exception of one dip, remained quite high in the control. Larval cohorts prevailed at more than 80/dip of each cohort during this test

period. It is thus apparent that B.t.i. and *B. sphaericus* treatments effectively suppressed immatures.

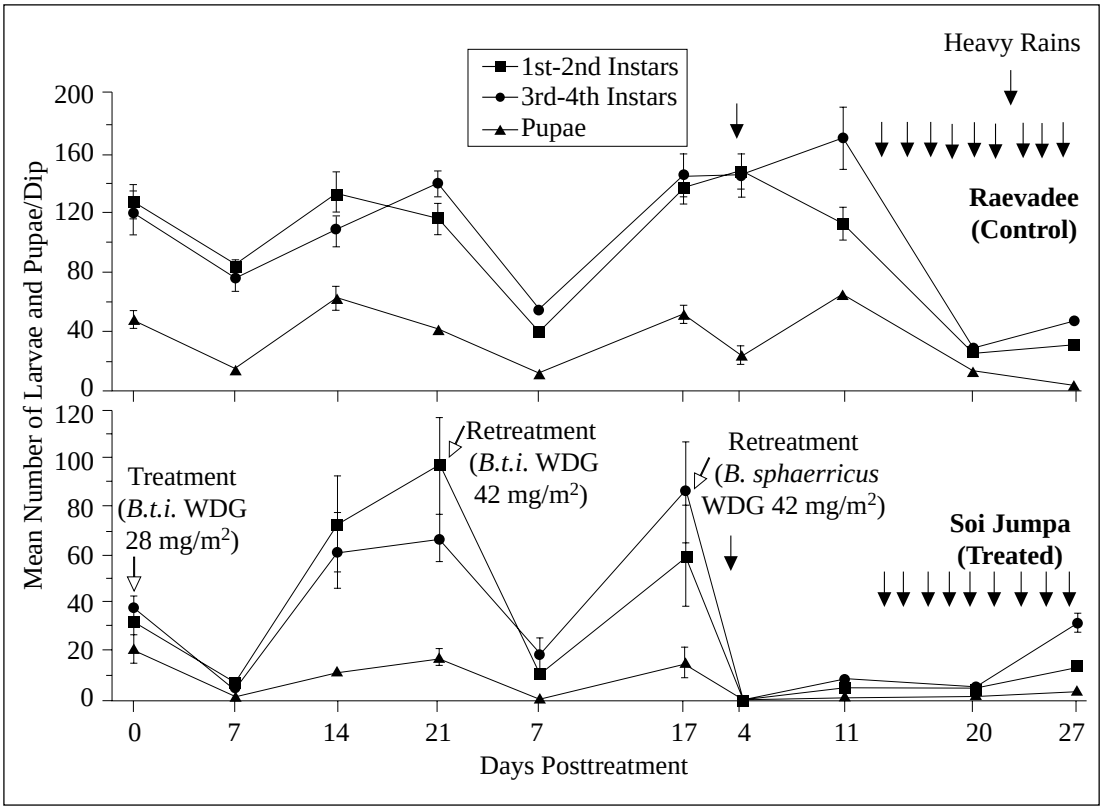

Figure 7. Evaluation of B.t.i. WDG (4,000 ITU /mg) and *Bacillus sphaericus* ***(630 ITU/mg) WDG against the polluted water mosquito*** *Culex quinquefasciatus* ***in dry season in Raevadee (as control area) and Soi Jumpa (treated total area), Pak Kret District, Nonthaburi Province, Thailand (starting March 10, 1998).***

On day 27 (May 14, 1998) post third treatmnet to July 23, 1998, this large plot was treated three times (May 14, June 4, and July 2, 1998) with the potent *B. sphaericus* WDG formulation (630 ITU/mg), each time at the low rate of 50 mg/m^2. The data for these low rate treatments are presented in Figure 8. Briefly, during the nine-week period of the three treatments (May 14 to July 23), populations of immature mosquitoes fluctuated at low levels, each treatment suppressing the immature populations for a week or ten days and then resurging moderately. The immature populations in general exceeded the densities observed on the day of the first treatment of this series of tests (May 14) indicating the 50 mg/m^2 rate was not too effective. During the course of

observation during these and previous tests, we have noted that once a habitat is treated with *B. sphaericus*, mosquito populations in general even after resurgence prevail at a low equilibrium level. The population trends in the control remained stable with the exception of one dip. They prevailed in much higher numbers in the control plot than in the treated plot even though the two areas had similar potential in supporting larvae.

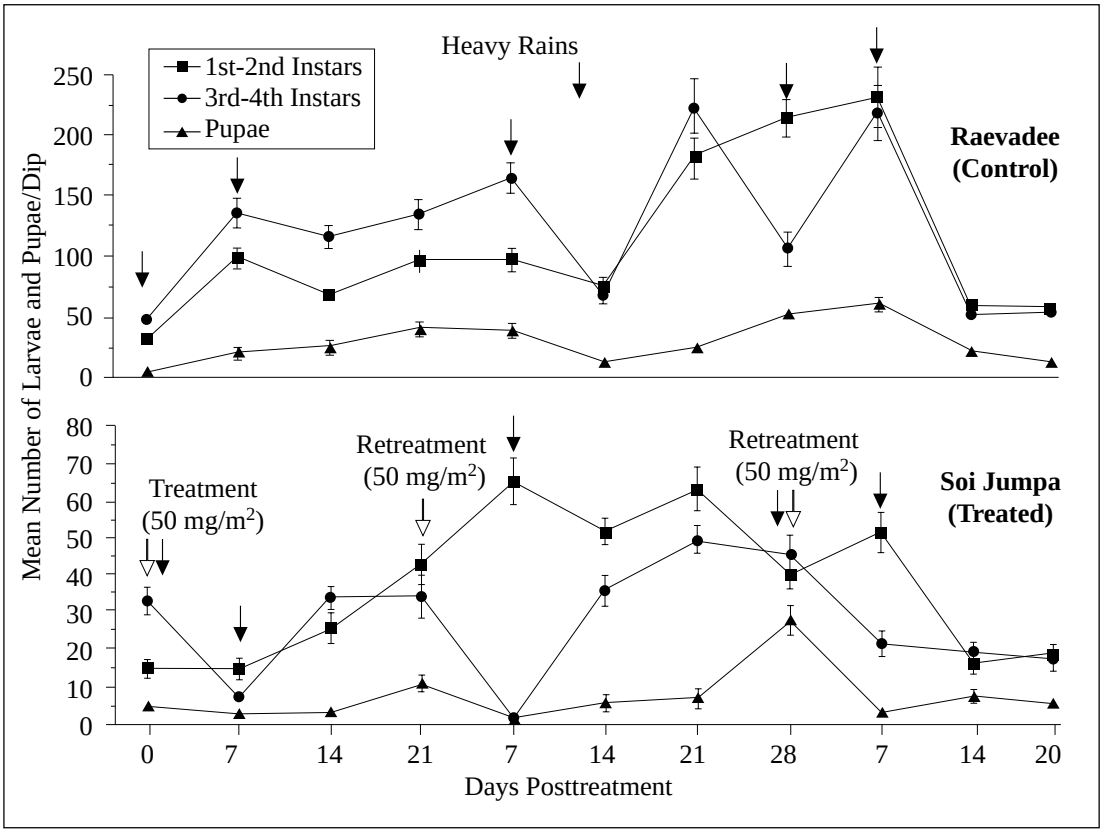

Figure 8. Evaluation of *Bacillus sphaericus* ***WDG formulation (630 ITU/mg) against the polluted water mosquito*** *Culex quinquefasciatus* ***in Raevadee (as control area) and Soi Jumpa (treated total area), Pak Kret District, Nonthaburi Province, Thailand (starting May 14, 1998).***

Summary

In summary, it can be concluded that *B. sphaericus* (strain 2362) WDG formulations yielded excellent control of immature mosquitoes in stagnant as well as flowing water situations. The range of effective dosages was 50-100 mg/m^2. The more potent WDG formulation was effective at lower dosages

than the less potent formulation. This relationship was also neted in microcosm studies (Su and Mulla 1999) on these formulations. Due to uncontrollable field conditions (similar to those under operational paradigms) the results as expected were quite variable and typical of what might be expected in operational control programs. Thiery et al. (1997) in outdoor small scale experiments on *B. sphaericus* reported even greater variability among tests in different locations and habitats and different times. Such variability in population trends of mosquitoes is to be expected especially when one subjects large areas to treatments.

In our trials, rains, especailly episodes of heavy precipitation, caused flooding and flushing of the treated habitats (to a greater extent in the klongs), washing away immature stages of mosquitoes. Experimental evidence showed that *B. sphaericus* treatments administered during the rainy season could provide long-lasting suppression of immature mosquitoes. In addition to rains, water level changes due to closure or opening of floodgates to let water flow into the Chao Phraya River could also influence abundance of immature mosquitoes. Light rains did not seem to have marked efffects on the efficacy of treaments especially in the relatively stagnant water habitats. One other impact of rain could be due to the rise in water level, leading to reduced larval density per unit volume of water resulting in lower density counts. Increase in the volume of water could also cause a dilution of the toxins. These uncontrollable elements have to be dealt with in the administration of treatments in operational control programs. Appropriate surveillance programs have to be employed to determine the need for treatment of a given habitat for mosquito control in operational control programs.

Acknowledgments

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Mosquito Larval Control with *Bacillus sphaericus:* Reduction in Adult Populations in Low-Income Communities in Nonthaburi Province, Thailand

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Abstract

During 1999 and 2000 several larvicidal treatments of *Bacillus sphaericus* strain 2362 water dispersible granular (WDG) formulations were made at 50 to 200 mg/m^2 in mosquito developmental sites in low-income communities in Nonthaburi Province, Thailand to determine whether larviciding dense populations would result in a noticeable reduction of adults mosquitoes in small treated areas. In the treated area in 1999 (Soi Jumpa), immature populations were suppressed to extremely low levels for extended periods, especially at the higher dosages. This decline in immature populations was followed by a substantial decline in adult mosquitoes. There was a lag of 7 to 14 days post-larval treatments before maximum decline in adults was noted. Adults that emerged prior to treatments survived for 7-14 days or longer, thus no drastic reduction was noted soon after treatments. Despite a slight resurgence in adult mosquitoes during the middle of the experimental period, adult female mosquitoes (over 98% *Cx. quinquefasciatus*), remained low during the 5-month period of trials. During the last 2 weeks (17 days post last treatment) of the experimental period, female populations reached the pre-treatment level. During the 2000 tests at Wat Pikul reduction in larvae was 87-98% for 7 weeks after first treatment at 200 mg/m^2, resulting in a reduction of 24 to 73% (2 and 7 days post-treatment respectively) and 87 to 98 (2-6 weeks) in the adults. In the second and third treatments at 50 mg/m^2, larval control and subsequent adult reduction were lower and shorter-lived than at the high dosage, and the fourth treatment at 100 mg/m^2 did not yield a high level of reduction in the larvae (18 to 33%), but reduction of adults was still 80%. The final fifth treatment at 200 mg/m^2 yielded only 18% control of larvae, suggesting tolerance to *B. sphaericus*

at this site. It was shown that at both treated sites repeated treatments with a larvicide such as *B. sphaericus* could result in substantial reduction in adult mosquitoes. Vigilance for detection of resistance development should be practiced, as resistance could emerge in certain populations following a few treatments.

Keywords

Culex quinquefasciatus, Bacillus sphaericus, larval control, adult control, Thailand

Introduction

Larval control of mosquitoes either by source reduction, use of larvicides or a combination of these, is a preferred method for reducing adult mosquitoes in many areas of the world. In arid regions where the extent of mosquito developmental sites is limited, larval control through the use of chemical and microbial larvicides has been the method of choice. In urbanized areas, larval control is also practiced extensively in both arid and tropical regions, supplemented by other measures used against adult mosquitoes.

In general, it is believed that area-wide control programs of mosquito larvae also result in the control adult mosquito populations that are responsible for annoyance and the transmission of pathogens. The extent and magnitude of such suppressions are not adequately documented, especially in the tropics. Two recent studies allude to the relationship of larvicidal interventions to reductions in abundance or biting rates of adult mosquitoes and the subsequent impact on disease incidence. Barbazan et al. (1998) in studies carried out in Maroua (north Cameroon), showed effective control of anophelines using *Bacillus sphaericus* strain 2362 larvicidal treatments at the rate of 10 g/m^2. In this program, the onset of malaria was delayed by 2 months and a subsequent reduction in the number of malaria cases was noted. In Zanzibar towns, Maxwell et al. (1999), showed that larval control using polystyrene beads in pit latrines and cesspits (supporting *Culex quinquefasciatus* Say) in combination with mass drug therapy (diethyl carbamazine) reduced the incidence of bancroftian filariasis significantly. In another town, treatment of pit latrines and cesspits with polystyrene beads reduced the adult mosquitoes by about 65%. This level of reduction in adult mosquitoes was deemed sufficient to result in a decline in the incidence of filariasis.

For the past 4 years we have been evaluating formulations of the microbial control agent *Bacillus sphaericus* strain 2362 against *Cx. quinquefasciatus* larvae in polluted water sites in several low income communities in Nonthaburi Province, Thailand. Results of these treatments applied to canals and standing water around and under dwellings were reported earlier (Mulla et al. 1997, 1999). In these studies we only reported the impact of treatments on larval populations without reference to the trends of adult mosquito populations, although the residents reported a marked reduction of mosquito adults and their biting activity. The present studies were initiated to elucidate the relationship between larviciding and population trends of adult mosquitoes, where all accessible mosquito developmental sites in small squatter communities were treated with a larvicide. The larvicide used was a new formulation of water dispersible granules (WDG) of *B. sphaericus* strain 2362 applied to mosquito developmental sites in 3 squatter communities (1 control and 2 treated) in Nonthaburi Province near Bangkok, Thailand. In addition, to assessing the magnitude of larval control, we obtained quantitative information on the abundance and trends of adult mosquitoes on a weekly basis during the test periods using blacklight traps placed either inside or just outside dwellings in 2 test areas, a treated site and a control.

Materials and methods

These studies were initiated in the beginning of May, 1999 and terminated in December 2000. The tests were carried out during the rainy seasons in the central part of Thailand in both 1999 and 2000 when frequent heavy showers occurred. Two sites, Soi Jumpa and Soi Raevadee in Pak Kret District (Mulla et al. 1997, 1999) and one new site, Wat Pikul in Bang Yai District in Nonthaburi Province, were selected for these studies. All 3 sites supported heavy *Culex* (mostly *Cx. quinquefasciatus*) mosquito development. In the first series of tests from May-October 1999, Soi Raevadee (control) and Soi Jumpa (treated) were selected for the studies. In the second series of tests form September-December 2000, Wat Pikul was subjected to treatments while Soi Jumpa was used as the control.

Soi Raevadee

This site was established as a control in the 1999 studies, with no treatments during the test period. Mosquito developmental sites consisted of wastewater accumulations under, around, and between dwellings. A large canal in this community supported heavy populations of mosquitoes. All stagnant water accumulations and the canal received a great deal of solid wastes (glass, plastics, metal cans, plant waste materials, etc.), which provided protective niches and rich sources of nutrients for the propagation of *Cx. quinquefasciatus*. Despite the presence of guppies in these mosquito developmental sites, larval populations were quite heavy most of the time. At the outset, this site had lower productivity in terms of mosquito larvae as compared to the treated area (Soi Jumpa) in 1999 (Figure 1). Raevadee community, like most low-income squatter communities, is densely populated (Mulla et al. 2001). The housing was substandard with many homes having no windows, opening on the sides, and floors and doors with large cracks that allowed the ingress and egress of adult mosquitoes. In this area *Cx. quinquefasciatus* larvae constituted 98-99% of the larvae sampled. The total area of water supporting mosquito production was about 3000 m^2.

Soi Jumpa

This community consisted of some 100 dwellings located about 3 km from Soi Raevadee. There were ample polluted water accumulations from rain and domestic waste water with sewage seepage under, between, and around the dwellings. Large amounts of solid wastes were present in the water. This habitat provided ideal conditions for the propagation of *Cx. quinquefasciatus*, the dominant mosquito (98-99%). This site was found to support heavier populations of this species than Soi Raevadee (Figure 1). Therefore, the Soi Jumpa site was assigned the treatment regiment under more demanding conditions. Like in Raevadee, the construction of houses was substandard, allowing easy ingress and egress of mosquitoes. The total area of water subjected to treatments was 3600 m^2.

Wat Pikul

In September, 2000 this site with an extremely high density of immature mosquitoes was selected for treatment while the site at Soi Jumpa with a

relatively lower larval density was used as a control. The two sites were similar in terms of type of housing and density and the level of organic and solid wastes pollution. Wat Pikul, however, had developmental sites for *Cx. gelidus* Theobald and *Mansonia* species in the adjacent fruit garden areas (not treated), which were absent in Soi Jumpa. There were about 200 dwellings at Wat Pikul and in some dwellings as many as nine families (one family per room) occupied the units. The nature and type of mosquito developmental sites were essentially the same as in Soi Jumpa, except as noted above. Wat Pikul supported slightly heavier populations of *Cx. quinquefasciatus* at the start of the experiment than the control site Soi Jumpa (Figure 2). The treated area in Wat Pikul was divided into 7 sites for the purpose of spraying, and the total area of this site subjected to treatments amounted to 4100 m^2.

Sampling

Mosquito larvae and pupae were sampled by the dipper method. Each site in the tests was sampled before treatment and then 2 days and weekly after treatment for determining the trends of larval and pupal populations. Every site was sampled using 20 dips in spots considered to support heavy aggregations of larvae. It was predetermined that some spots regularly supported heavy larval densities and these spots were sampled repeatedly before and after treatments. The contents of the dipper were transferred to a white enamel pan (15x30x4 cm deep) and the larvae and pupae were counted on the sites and categorized by instars and if they were pupae. After each treatment, as the larval counts resurged in the treated area, the site was re-treated, provided it was accessible or when no heavy rains were falling.

Adult mosquitoes were sampled for one night approximately every week by placing 3 blacklight traps (6 watt black light tube, 220 volt) in each of the treated and untreated sites. The traps were hung 1.3 m above the floor or ground. The traps were set and activated at 1800 h turned off at 0600 h and collected at 0800 h the next day. To preclude ant predation on mosquitoes, a band of petroleum jelly was applied to the cord hanging the trap. The mosquito collections were brought into the laboratory and counted. Species identification was made under a stereomicroscope. The data are presented in the figures giving the average number (with standard error) of mosquitoes per trap/night at each interval at each site.

Materials and Treatments

In 1999 the treatments were made with experimental preparations of a water dispersible granular (WDG) formulation of *B. sphaericus* strain 2362 provided by Abbott Laboratories, N. Chicago IL. (ABG-6491, lot no. 31-077BR 600 ITU/mg and lot no. 32-094 BR 630 ITU/mg). In the September, 2000 tests a commercial VectoLex^{TM} formulation (lot no. 56-809 PG with 650 ITU/mg) was used. This latter product was manufactured in 1999 by Abbott Laboratories and provided by Valent Biosciences Corp. (Libertyville, IL.). Additional quantities of VectoLex^{TM} were obtained from the NW Mosquito and Vector Control District (Corona, California). The products were applied by spraying most of the accessible larval infested waters, which amounted to about 3600 m^2 in Soi Jumpa treated in 1999 and 4100 m^2 in Wat Pikul treated in 2000. The required amount of the WDG formulation for each spray tank was mixed with water in a plastic bucket and then poured into a 7-liter compression sprayer filled to capacity. The formulations readily went into a homogeneous suspension made in plastic buckets and poured into spray tanks. The spray tank was pressurized and the bacterial suspension was sprayed over the water surface through a cone nozzle. The nozzle generated a coarse jet stream of spray that could reach over water surface 4-5 m away. Since there was standing water under rows of houses, not all of the larval infested areas could be reached with the spray jet. In total, 6 spray tanks (42 liter spray) were used in each treatment to cover the target area in Soi Jumpa in each of the 1999 tests, while 7 tanks (49 liter) of spray were used to treat Wat Pikul in each of the 2000 tests.

In total, 4 treatments were made in Soi Jumpa during the 5 months of the rainy season (May to October 1999). At this treated site, the dosages applied were somewhat on the low side and varied from 72 mg/m^2 to 115 mg/m^2 (0.72-1.15 kg/ha). In previous studies we noted that the WDG formulation gave good control of mosquito larvae at the dosage of 100 + mg/m^2 (Mulla et al. 1999). We employed dosages lower than 100 mg/m^2 to assess the initial and persistant control of larvae. In the 2000 tests, Soi Jumpa was established as a control site while a new site Wat Pikul (not used before in our studies) was selected as a treated area, because the latter had somewhat heavier larval densities (Figure 2) before *B. sphaeric*us treatments were initiated. Adjacent to the study area there were substantial developmental sites for *Cx. gelidus* and *Mansonia* species which added to the adult counts in light traps. In Soi

Jumpa vicinity developmental sites for these species were lacking. The Wat Pikul site receiving a total of 5 treatments was treated initially with a high dosage (200 mg/m^2 or 2.0 kg/ha), to see if higher dosages (at the upper end of label dosage) would provide long-lasting control. This first treatment at Wat Pikul was made on September 14, 2000, and the subsequent four treatments, two at the low dosage of 50 mg/m^2 (0.5 kg/ha), one at 100 mg/m^2 (1.0 kg/ha) and the last at the high dosage of 200 mg/m^2 were made on October 26, November 16, November 30, and December 8, 2000 respectively. In Wat Pikul 2000 tests, the percent reductions in immatures and adult mosquitoes were calculated by comparing mean post-treatment counts vs. the original pre-treatment counts. In this calculation, we did not consider the population trends in the control area, as in general the mosquito populations increased during the post-treatment period. We believe comparison of post-treatment populations with the pre-treatment in the treated area provide a better indication of the extent of suppression.

Results and Discussion

Control of Larval Mosquitoes

Soi Jumpa Tests (1999)

Prior to the initiation of the treatments, the total number of larvae (all instars) and pupae per sample was much higher in the Soi Jumpa community (treated) than Soi Raevadee (control). The immature mosquito populations remained at a relatively stable level in Soi Raevadee for about a month during the post-treatment monitoring, then experienced a slight decline for 5 weeks, but began to increase substantially in numbers thereafter. On the other hand, the first treatment with VectoLex^{TM} WDG formulation at 115 mg/m^2 in Soi Jumpa on May 10, 1999 suppressed the immatures to a very low level from 165 (pre-treatment) to 2/dip (Figure 1). This very low level of immatures prevailed for about 4-5 days post-treatment with a slight resurgence on day 8 (37/dip) post-treatment. On day 11, the site was given a second treatment at the reduced dosage of 93 mg/m^2. The second treatment suppressed the immature populations to extremely low levels initially (<1 to 9/dip) with moderate levels (below 25/dip) occurring for up to 28 days post second treatment. From day 35-49 post second treatment there was a slight but sustained resurgence of the immatures, the immatures density reaching 43-60/dip. The site was then

retreated at the low dosage of 72 mg/m^2 (0.77 kg/ha). This treatment depressed the immatures to a mean number of 4/dip but then increased on day 14 to about 50/dip. From day 21-49 post third treatment (at 72 mg/m^2), high populations of larvae and pupae were present ranging from 88 to 136 total/dip. Then, the fourth treatment at 114 mg/m^2 (1.14 kg/ha) was applied. This, treatment in this series suppressed populations drastically to 8/dip on day 3 post-treatment, remaining below 40/dip up to 17 days post-treatment, then resurging to high levels (92-113/dip) on days 24-32 post-treatment. During the 5 month test period, treated larval populations in Soi Jumpa never returned to the pre-treatment levels.

The total immature mosquito population trends at the Soi Raevadee control site, however, remained at the pre-treatment level or higher except during the concomitant period of post-second treatment. Soi Raevadee population declined from a total of 63/dip pre-treatment to about 10/dip during days 28-42 corresponding post-second treatment. The populations began to resurge to the pre-treatment level or higher during the twelve weeks corresponding to the third and fourth treatment periods. For this extended period the densities of immature populations ranged from 67-243/dip.

Wat Pikul Tests (2000)

Prior to treatment, Wat Pikul had a high density of immature mosquitoes, (275+ per dip). The first treatment at the relatively high dosage of 200 mg/m^2 of VectoLex^{TM} WDG caused a high level of reduction (>98%) in immatures. This low density of immatures lasted for 35 days post-treatment (Figure 2). On day 41 post-treatment the immatures resurged to 60/dip and the level of control dropped to 83%. At this time (day 41 post-treatment), the site was retreated at a lower dosage to see if adequate larval suppression could be sustained.

The second treatment at the low dosage of 50 mg/m^2, suppressed the immature *Cx. quinquefasciatus* populations to a very low level (5/dip 2 days and 8/dip 6 days post-treatment). The reduction was 89-98% for a period of 2 weeks. The immature populations then increased (145/dip), and the level of control dropped to 47% at 20 days post-second treatment (Figure 2). The site was given a third treatment (20 days post-second treatment) at the low dosage of 50 mg/m^2. This treatment gave an initial control of 88% of immatures, but

the level of control dropped to 64 and 47% on days 6 and 13 post-treatment respectively (Figure 2). The fourth treatment was then applied at the moderate dosage of 100 mg/m^2 of VectoLex^{TM} from NWMVCD. Surprisingly, this treatment did not cause much reduction of immatures; the level of reduction was only 35% on day 2 and 13% on day 6 post-treatment. We suspected that the population of *Cx. quinquefasciatus* at Wat Pikul became somewhat tolerant to *B. sphaericus*. To test this hypothesis, we made the fifth and last treatment at the original high dosage of 200 mg/m^2.

The fifth and last treatment (at the high rate of 200 mg/m^2) yielded only 18% reduction of immature mosquitoes three days after treatment. The density of immatures per dip by instars and stage were 40 (first-second), 153 (third-fourth) and 6.7 (pupae). This high density of immatures, especially that of third and fourth instars, suggests the emergence of tolerance to *B. sphaericus* in mosquitoes at Wat Pikul after 5 treatments. The nature and magnitude of resistance of this population are not understood, but the evidence signals a warning that in certain locations, specific populations can acquire tolerance to this microbial control agent rather quickly. Further studies on the nature and scope of this tolerance are in progress.

The first treatment at Wat Pikul at the high dosage (200 mg/m^2) yielded a high level of immature control (95%+) for extended periods of up to 5 weeks post-treatment. The two subsequent treatments, made at the low dosage of 50 mg/m^2, each provided lower levels of control, lasting for only 6 to 13 days. The level of control further dropped following the 4th treatment at 100 mg/m^2. The last treatment at 200 mg/m^2 (a dosage that gave over 95% control in the first treatment) yielded little or no control. It should be noted that pupal populations were reduced by almost 100% within 2 days after each of the first two treatments rendering 95-98% larval control, however, pupal reduction was not complete in the next three treatments. *B. sphaericus* toxins do not affect pupae as they do not feed or ingest the toxins. Pupal disappearance is the result of emergence of adults or absence of larvae from becoming pupae. Heavy larval mortality led to the reduction in the pupal population. It appears that the rates of 100-200 mg/m^2 would be necessary to achieve satisfactory initial and extended control of immatures under conditions as in this study area, but vigilance should be exercised in detecting tolerance after a few treatments in certain developmental sites.

Reduction in Adult Mosquitoes

Soi Jumpa Tests (1999) Adult Mosquitoes

Prior to the initiation of treatments at Soi Jumpa, the total of male and female mosquitoes collected in light traps was about 6 times higher in the Soi Jumpa treated area (2531 to 3739 total/trap/night) than in the Soi Raevadee control site (291-544/trap/night). Four days after the first *B. sphaericus* larvicidal treatment, the adult mosquitoes were suppressed to a very low level (619/trap/night) in the Soi Jumpa treatment area and their populations further declined to 394/trap/night on day 11 post-treatment. The adult population decline continued, with the total number reaching 243, 242 and 177 per trap/night on day 2, 7, and 14 post-second treatment respectively (Figure 3). This low level of adult mosquitoes prevailed for about 2 weeks post-second treatment, but experienced a moderate resurgence (944-1419/trap/night) on days 21-49 post-second treatment. The level of resurgence was again suppressed, although, not as precipitously as before by the third treatment of the larvae at the very low dosage of 72 mg/m^2. Overall, the total adult mosquito populations prevailed at moderate levels (973-2085/trap/night) for 49 days post third treatment. The adult populations remained at high levels after the 4th larvicidal treatment at 114 mg/m^2 of VectoLex^{TM} WDG. The adult collections fluctuated between 1345 and 4117 adults/trap/night.

The adult populations in the Raevadee control site, in contrast to the treated Soi Jumpa site, increased during the 3-4 week after the first treatment period (571-660/trap/night) but declined about a month later. The population remained low for 8 weeks (161-708/trap/night), then resurged to higher levels (ranging from 305 to 4269/trap/night) during the remaining 2 month period of the test (Figure 3). During most of this period of the test, the adult mosquitoes in the control area prevailed at higher numbers than their pre-treatment populations. Toward the end of the test period corresponding to 31-38 days post 4th treatment, the total adult capture was 2-13 times more than the pre-treatment counts in the control site, while no such resurgence was noted in the treated area. There was a substantial increase in adults in the treated area following the fourth treatment. This increase corresponds to the increase in larval density during this period (Figure 1). The adult mosquitoes, especially females, in the treated Soi Jumpa area, reached pre-treatment levels or higher about 31 to 38 days after the fourth treatment when the test was terminated.

During the course of the 5-month studies, *Cx. quinquefasciatus* constituted over 98-99% of the adult mosquitoes trapped at both sites, Soi Jumpa and Raevadee. A few adults, mostly females, of *Armigeres subalbatus* Coquillet and *Cx. (Lutzia) fuscanus* Wiedemann and *Mansonia* spp. were also trapped occasionally. To gain information on the collection of blood-fed female *Cx. quinquefasciatus* for future studies we assessed the numbers of blooded females. In this species the number of blood-fed varied from 0.3% to 36% (36% only in one collection) in the 50 collections made in the treated and untreated sites. Most of the collections contained an average of only 3-5% blooded mosquitoes. There were only 4 collections in the treated and 3 collections in the untreated area, which had no blood-fed females.

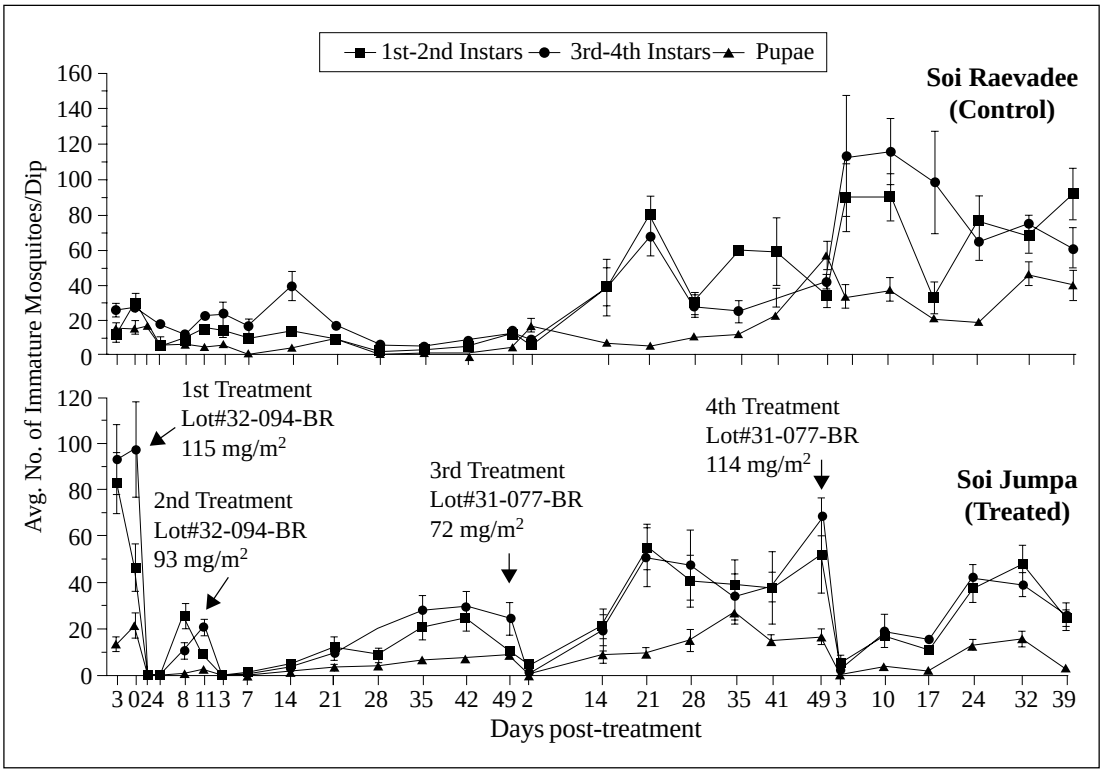

Figure 1. Population trends of mosquito larvae and pupae (predominantly *Cx. quinquefasciatus****) in treated (with*** *B. sphaericus* ***WDG formulations) and untreated low-income communities, Nonthaburi Province, Thailand. During the 5-month testing period (May-October, 1999), there were 30 episodes of rain amounting to 5-15 mm each.***

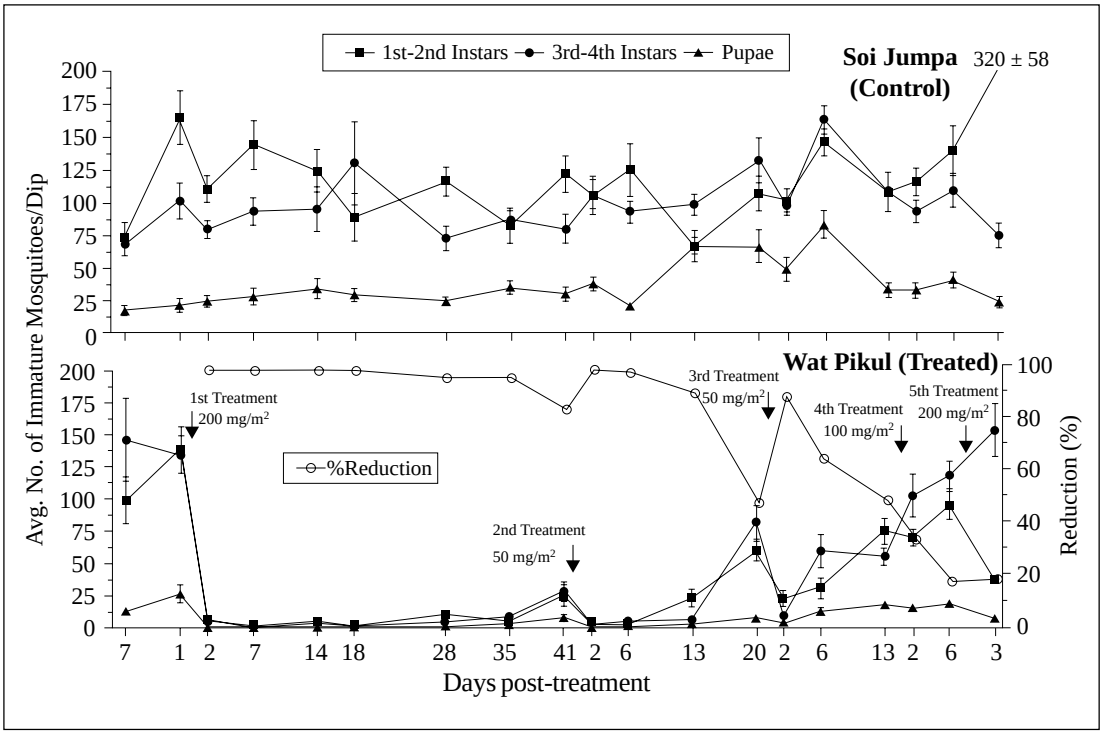

Figure 2. Population trends of mosquito larvae and pupae (predominantly *Cx. quinquefasciatus****) in treated (with VectoLex™ WDG formulation) and untreated low-income communities, Nonthaburi Province, Thailand. During the 2.5-month testing period (September-December, 2000), there were 13 episodes of rain amounting to 5-15 mm each.***

Wat Pikul Tests (2000) Adult Mosquitoes

Following the first larval treatment with VectoLex™ WDG formulation (200 mg/m^2) there was a marked reduction in adult mosquitoes collected by blacklight traps. The reduction adults was 24 and 73% compared to the pre-treatment counts after 2 and 7 days of the treatment respectively. The decline in adult populations increased and reached 91 and 95%, on days 14 and 28 post-treatment. The reductions in adult collections were 89 and 87% on days 35 and 42 post-treatment respectively (Figure 4). A lag time was noted of about 1-2 weeks after larval treatment before the adult mosquito populations were markedly suppressed following larvicidal treatment. Adult mosquitoes that emerged prior to larval treatment can survive for one to two weeks or longer. Thus, this lag time in adult suppression was expected. Following the second treatment at the low dosage of 50 mg/m^2, the reduction in adults was 83% one week and 74% two weeks post-treatment.

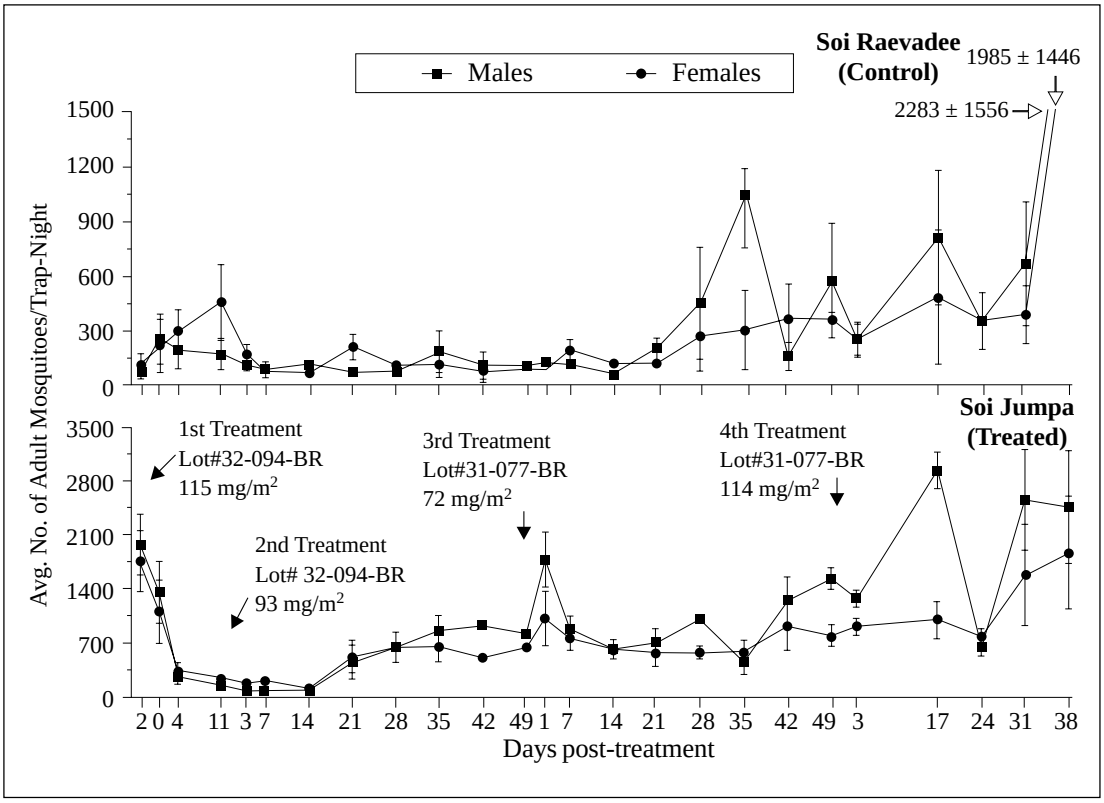

Figure 3. Impact of larval control using** B. sphaericus **WDG formulation on the abundance of adult mosquitoes (**Cx. quinquefasciatus**) as measured by blacklight traps in low-income communities, Nonthaburi Province, Thailand. During the 5-month testing period (May-October, 1999), there were 30 episodes of rain amounting to 5-15 mm each.

The reduction in adult populations was somewhat lower following the third treatment (50 mg/m^2) when compared to the second treatment. The reduction after the third treatment was 60-80%. The reduction in adults after the fourth treatment (100 mg/m^2) was 79%, despite the fact that there was a low level of larval control (18-33%) with this treatment (Figure 2). It seems that there is a lag time of 1-2 weeks for resurgence of adult populations following failure or termination of larvicidal treatments. The last larvicidal treatment in this series of tests was made at the high rate of 200 mg/m^2. This treatment yielded only 18% control of the immatures (Figure 2). Because of this failure of control, adult populations were not trapped after this last treatment. At this point we were convinced that the decreasing efficacy of larviciding was due to the appearance of tolerance to *B. sphaericus* at this site. Further studies on the rapid emergence of resistance at this site are continuing.

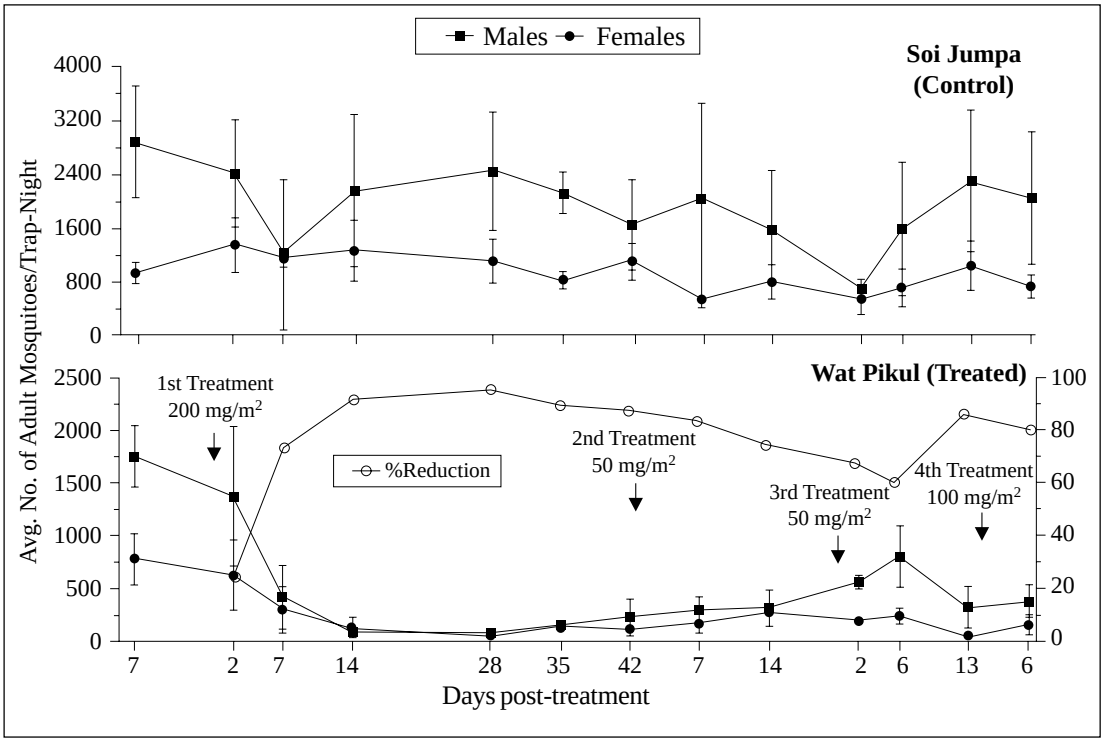

Figure 4. Impact of larval control with VectoLex™ WDG formulation on the abundance of adult mosquitoes (*Cx.quinquefasciatus*) as measured by blacklight traps in low-income communities, Nonthaburi Province, Thailand. During the 2.5-month testing period (September-December, 2000), there were 13 episodes of rain amounting to 5-15 mm each.

Concomitantly, the adult populations in the untreated Soi Jumpa community reamined relatively stable over the entire test period, never declining more than 36% of the original populations, and for the most of the study period increased over the pre-treatment level (Figure 4). In Soi Jumpa, the mosquitoes were for all purposes *Cx. quinquefasciatus* (over 98%) with few *Cx. gelidus* trapped during October and November. The proportion of *Cx. gelidus* in the collections increased as adults of *Cx. quinquefasciatus* declined due to treatments. In Wat Pikul, however, we collected *Cx. gelidus* that made up to 7-27% of the collections. This species probably developed in standing water in fruit gardens across the road from the Wat Pikul study site. We collected *Cx. gelidus* females, mostly without blood, and very few males, in every trap collection, ranging from 28 to 404 per trap/night. Since males were absent, this mosquito is believed to be not propagating in the treated polluted water

site. It is hypothesized that the females were flying into the treated site, as we found no *Cx. gelidus* larvae breeding in the polluted water at Wat Pikul.

These studies illustrate that control of larvae of *Cx. quinquefasciatus* can result in a marked and sustained reduction in the adult populations within a short period of one to two weeks after the first treatment. This decline in adult mosquitoes was noted by the residents, who experienced reduced host-seeking and biting activity of mosquitoes during most of the treatment period. Residents at Soi Raevadee (control) expressed no such perceptions, except during the 6-week period when the larval and adult populations declined naturally. It should, however, be noted that the very high level of larval control did not result in a correspondingly high level of control of adults. This discrepancy was expected because not all developmental sites of larvae were accessible and thus could not be treated. Likewise the areas treated were not large enough to preclude the movement of adult mosquitoes from outlying untreated areas. Lastly, adults that emerged prior to larval treatments survived for 2 weeks or longer, contributing to the slow decline of adults for a few days following larval treatments. Regardless, 80-98% reductions in female mosquitoes was achieved in both test areas with the application of a larvicide.

We conclude that treatments with larvicides even in limited areas can lead to a substantial reduction in the number of active host-seeking adult *Cx. quinquefasciatus.* Similar larvicidal treatments and larval control efforts reduced anopheline adults (Barbazan et al. 1998) and *Cx. quinquefasciatus* adults (Maxwell et al. 1999), which resulted in a decline of malaria and bancroftian filariasis respectively. Studies of this nature on other mosquitoes in a variety of habitats are warranted to document the effectiveness of larvicidal programs in suppressing adult mosquitoes.

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Emergence of Resistance and Resistance Management in Field Populations of Tropical *Culex quinquefasciatus* to the Microbial Control Agent *Bacillus sphaericus*

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Abstract

In recent years, highly potent mosquitocidal strains of the microbial agent *Bacillus sphaericus (Bsph)* have been isolated and developed for the control of mosquito larvae around the world. Laboratory selection experiments with the most active strains and their use in large-scale operational mosquito control programs resulted in the emergence of resistance in larvae of the *Culex pipiens* complex. This generated great concern among vector control agencies around the world, who feared reduced efficacy of this highly active larvicidal agent. To address this issue, the current studies were started to find practical strategies for controlling resistant mosquitoes and more importantly to develop resistance management strategies that would prevent or delay development of resistance. We initiated field studies in 3 low-income communities in Nonthaburi Province, Thailand. In 1 of the communities, larvae of *Culex quinquefasciatus* that were highly resistant (>125,000-fold) to *Bsph* strain 2362 were successfully controlled with applications of *Bacillus thuringiensis* var. *israelensis (Bti)* alone or in combination with *Bsph*. To prevent or delay resistance to *Bsph*, 2 other sites were selected, 1 treated with *Bsph* 2362 alone and the other treated with a mixture of *Bsph* 2362 and *Bti*. Mosquitoes treated with *Bsph* 2362 alone showed some resistance by the 9th treatment and almost complete failure of control occurred by the 17th treatment. After 9 treatments with the mixture over a 9-month period at another site, no noticeable change in susceptibility to *Bsph* was detected. During this period, the site treated with *Bsph* alone required 19 treatments, whereas the site treated with mixtures took only 9 treatments because of slower resurgence of larvae at the site treated with the mixture than at the site treated with *Bsph* alone. This is the 1st field evidence for delay or prevention of resistance to microbial agents in larval *Cx. quinquefasciatus* by

using mixtures of *Bti* and *Bsph*. Further studies on the use of mixtures for the management of field resistance are warranted.

Keywords

Culex quinquefasciatus, Bacillus sphaericus, resistance, resistance management, prevention

Introduction

In the past 25 years, several isolates and strains of 2 species of spore-forming bacteria have been found that produce parasporal proteins that show high toxicity against mosquito larvae (WHO 1985, de Barjac and Sutherland 1990). *Bacillus thuringiensis* var. *israelensis* de Barjac *(Bti)* was discovered in 1976 (Goldberg and Margalit 1977) and was developed for use in mosquito and black fly control programs (de Barjac 1990a, Mulla 1990). During this same period, research on isolating and developing mosquitocidal strains of *Bacillus sphaericus (Bsph)* Neide was intensified. Of the approximately 300 strains isolated, 17 were highly toxic to mosquito larvae (de Barjac and Sutherland 1990). Of these, strains 2297 from Sri Lanka (Wickremesinghe and Mendis 1980), 1593M from Indonesia (de Barjac 1990b, Singer 1990), 2362 from Nigeria (Weiser 1984), and C3-41 from China (Liu et al. 1989) were studied extensively and are now available commercially as mosquito larvicides. Strain 2362, which is the most commonly used material, has been used in Europe and elsewhere since 1989 and in the USA since 1996.

In 1990, we initiated studies on the potential development of resistance to *Bsph* 2362 in larvae of *Culex quinquefasciatus* Say in the laboratory. A moderate to high level of resistance was obtained on selection with this strain (Rodcharoen and Mulla 1994, Wirth et al. 2000, Zahiri et al. 2002). Moderate to high levels of resistance in the *Cx. pipiens* L. complex were reported in the field after 2-3 years of use of strains 2362 (Sinègre et al. 1994, Silva-Filha et al. 1995), 1593M (Adak et al. 1995, Rao et al. 1995), and C3-41 (Yuan et al. 2000). In the evaluation and development process, *Bsph* strains were tested once or twice in field trials for initial activity in several countries (Liu et al 1989; Zhang et al. 1989; Karch et al. 1992; Mulla et al. 1997, 1999; Ali et al. 2000). Once implemented in field control programs, little or no monitoring of susceptibility levels of mosquito species was conducted and resistance was only detected when gross failure in field control programs was noted.

During the course of several years of field evaluation of *Bsph* formulations against *Cx. quinquefasciatus* in polluted water in squatter communities in Thailand (Mulla et al. 1997, 1999, 2001), almost 100% initial and 35 days of residual control of larvae was achieved with the 1st few treatments of *Bsph* 2362. However, with further treatments, the level of control declined and longevity of control also progressively decreased (Mulla et al. 2001). Rapid failure of control occurred with the VectoLex water dispersible granules (WDG) formulation at Wat Pikul Community in Nonthaburi Province in 2000. Control of larvae was reduced after the 4th treatment, and no control was achieved in the 5th treatment at the maximum label dosage of 200 mg/m^2 (Mulla et al. 2001). A high level of resistance in *Cx. quinquefasciatus* evidently had developed after 4-5 repeated applications of *Bsph* at this site. This rapid development of resistance caused concern in vector control programs.

To respond to these concerns of rapid development of resistance, the current studies were initiated to document the magnitude of resistance in *Cx. quinquefasciatus* at Wat Pikul and to develop practical strategies for the control of *Bsph*-resistant mosquitoes. Laboratory assays provided evidence for lack of cross resistance to *Bti* in *Bsph*-resistant mosquitoes (Rao et al. 1995; Rodcharoen and Mulla 1996; Zahiri et al. 2002; Su and Mulla, unpublished data) and this information led to the hypothesis that *Bsph*-resistant populations at Wat Pikul could be controlled with *Bti* alone or with a mixture of *Bti* and *Bsph*. *Bacillus sphaericus* produces higher mortality and longer residual activity than does *Bti* in polluted water. The belief was held that a mixture of *Bsph* and *Bti* could yield initial and persistent control. Therefore, field studies were initiated to ascertain control of *Bsph*-resistant mosquitoes by mixtures of *Bsph* and *Bti*, to investigate prevention of resistance by use of *Bsph*-*Bti* mixtures, and to study the emergence of resistance as a result of treatments with *Bsph* 2362 alone. Results are presented in figures.

Materials and methods

Control of** Bsph-**resistant mosquitoes at Wat Pikul: To control *Bsph*-resistant *Cx. quinquefasciatus*, treatments with *Bti* or *Bsph* 2362 alone or in a mixture were initiated. Wat Pikul (Bang Yai District, Nonthaburi Province) is a low-income community with high-density housing and a dense human population (Mulla et al. 2001). In some dwelling units, as many as 9 families live

together, in some cases with 1 family per room. As in other low-income communities in this area, wastewater and solid organic and inorganic wastes are thrown outside. These accumulate under structures built above ground on posts and pylons. These conditions (solidwaste mixed with polluted water) create ideal habitats for the propagation of mosquitoes, especially *Cx. quinquefasciatus.* The breeding area for mosquitoes at this location was about 4,000 m^2, a relatively large mosquito-producing area of this type. After detecting resistance to *Bsph* in this area in December 2000, the total area was subjected to treatments of WDG formulations of *Bti* or *Bsph* 2362 alone or in various mixtures from January to late October 2001.

Another community, Soi Jumpa (Pak Kret District, Nonthaburi Province), was used as an untreated control. Mosquitoes at this site were not resistant to *Bsph*, because a treatment with 100 mg/m^2 of the WDG formulation yielded almost 100% initial control. Detailed descriptions of this site were published by Mulla et al. (1999, 2001). This community is similar in size to Wat Pikul and has similar potential for supporting polluted-water mosquitoes. However, monitoring of this site was terminated at the time of the 10th treatment at Wat Pikul on May 23, 2001. The trends of immature mosquitoes were rather stable during the 1st 4 months of the experiment and we saw no point in further following these populations as a control. We have noted that mosquitoes in these types of developmental sites resurge quickly after cessation of treatment and reach pretreatment populations; a comparison of posttreatment counts versus pretreatment counts provides an accurate estimation of control.

Emergence of resistance to** Bsph **and prevention of resistance with mixtures: These studies were carried out in 2 communities in the Nonthaburi Province, Thailand. The purpose of these studies was to determine if repeated treatments of a small breeding source with *Bsph* 2362 alone would result in the development of resistance, as was observed at Wat Pikul. At the same time, we examined whether mixtures of *Bti* and *Bsph* could prevent or delay emergence of resistance to *Bsph*. The 1st community, Wat Lahan, Bang Bua Thong District, was subjected to repeated treatments of *Bsph* WDG formulation at 100 mg/m^2, a highly effective dosage for susceptible larvae.This site encompassed a total mosquito-producing area of 400 m^2, an area much smaller than that of Wat Pikul and Soi Jumpa. This area also had some inaccessible breeding sources of mosquitoes that could not be treated. The studies in Wat

Lahan community started on May 17, 2001, and terminated on March 15, 2002, after the last (19th) treatment at 200 mg/m^2 failed to provide satisfactory control of larvae.

In previous years, Soi Jumpa community was treated intermittently with WDG formulations of *Bsph* 2362, receiving 7 treatments in 1997, 3 treatmetnts in 1998, and 4 treatmetnts in 1999 (Mulla et al. 1999, 2001). In each period, the last treatment was found to be highly effective, pointing to the absence of resistance in this population. Since October 1999, this site remained untreated. To test the susceptibility of larvae at this site, it was treated in early May 2001 at 100 mg/m^2 of *Bsph* WDG, which yielded almost 100% initial control. Because of the lack of apparent resistance to *Bsph*, this site was selected for the mixture trials.

Sampling: Mosquito larval populations were sampled by 400-ml-capacity dippers. Larvae were sorted into 1st, 2nd, 3rd, and 4th instars and pupae and counted. The sites were sampled before treatment and at various intervals after treatments. When the larval and pupal counts resurged to with-in 60-80% of the pretreatment levels, the sites were treated again.

Larval sampling was biased toward areas where mosquito larvae and pupae were dense because we wished to judge effectiveness of control in areas of dense aggregations of larvae and pupae. The number of dips taken varied depending on the size of the treated area. In Wat Pikul, Wat Lahan, and Soi Jumpa, 20, 10, and 20 dip samples were taken each time, respectively.

Materials and application: Commercial products of *Bti* (VectoBac WDG) and *Bsph* 2362 (VectoLex WDG) formulations were used in these experiments. Both products were provided by Valent BioSciences Corporation (Libertyville, IL). The dosages given in mg/m^2 are for the formulations used. The product containers were kept at room temperature until used. The required amounts of the WDG formulation was mixed with water in a plastic bucket, transferred to a 7-liter stainless steel spray tank, pressurized, and sprayed onto the water surface of the habitat. The aqueous spray was applied through a cone jet nozzle that projected the spray jet up to 4-5 m from the sprayer. Most, if not all, of the habitat was covered by the application.

In Wat Pikul, the treatable area was 4,000 m^2 through the 9th treatment, but drained to 2,000 m^2 after this time. Wat Lahan had only 400 m^2 of mosquito-breeding area. In Soi Jumpa, the control area for the Wat Pikul test, and subsequently an area treated with the mixture to examine prevention of resist-

ance, had a breeding source of 3,000 m^2. At Wat Pikul, the large area was sprayed with 7 tanks (7 liters each), whereas the reduced area was sprayed with 5 tanks. Wat Lahan was sprayed only with one 7-liter tank, whereas Soi Jumpa was sprayed with 5 tanks (7 liters each) each time.

Population reduction: Population reduction in percent was calculated by comparing counts of 3rd and 4th instars and pupae to the pretreatment counts of the same stages. Pretreatment counts used in the calculations were those just before each treatment at a given site. Although *Bti* and *Bsph* do not kill pupae, the pupal numbers quickly declined because of larval kill by the treatments and, therefore, they were included in calculations of percent reduction. Comparison of posttreatment with pretreatment populations in the treated site provide consistent and reliable levels of control.

Results and Discussion

Control of resistant mosquitoes at Wat Pikul

Culex quinquefasciatus, the dominant mosquito, was treated 5 times at this site with *Bsph* 2362 WDG formulation in 2000. After the 4th and 5th treatments, larval control was not satisfactory (Mulla et al. 2001). The magnitude of resistance in this population was further quantified by laboratory bioassays, which showed more than 125,000-fold resistance compared to susceptible mosquitoes from Soi Sirichai, Nonthaburi Province, Thailand, to *Bsph* 2362 technical powder (Su and Mulla, unpublished data). To control this highly resistant mosquito population, treatments were initiated mostly with *Bti-Bsph* 2362 mixtures starting in January 2001 and continued them until late October 2001. In total, 18 treatments with single agents or mixtures of the 2 agents were made (Figure 1).

To further confirm the resistance at this site, the 1st treatment of VectoLex WDG was made on January 22, 2001, at 200 mg/m^2, a highly efficacious dosage against susceptible mosquitoes in polluted water. This rate of application gave no control of 3rd and 4th instars and pupae 48 h after treatment. The larval density increased after this treatment (Figure 1A). In our studies at this site in 2000, before the mosquitoes developed resistance to *Bsph* 2362, we obtained almost 100% control of larvae with the 1st treatment at 200 mg/m^2 of VectoLex WDG for over 35 days (Mulla et al. 2001). After documenting lack of control at a high dosage of VectoLex, the effectiveness of an equal dosage

of VectoBac WDG was tested against this *Bsph* 2362-resistant population. This treatment gave almost complete control of larvae, lasting, as expected, for 14 days. After this treatment, the efficacy of lower dosages of VectoBac WDG was determined by applying this formulation at 50, 25, and 20 mg/m^2 in the next 3 treatments. The 50 mg/m^2 treatment gave 100% control of larvae 2 days after treatment, with the control dropping to 78% 8 days after treatment; the population resurged to the pretreatment level 13 days after treatment. The treatments at 25 and 20 mg/m^2 yielded 98 and 91% initial control of larvae, which dropped to 40 and 47% 7 days after treatment, respectively (Figure 1A). Two more applications of *Bti* alone (13th and 14th treatmetns) were made at the 20 mg/m^2 dosage of VectoBac WDG. These treatments gave 99-100% control of larvae 2 days after treatment. The level of control dropped to 60 and 33% in the 13th and 14th treatments 7 days after treatment, respectively. The 17th treatment in this series was made with VectoBac WDG at 200 mg/m^2. This treatment, as for the 2nd treatment at 200 mg/m^2, yielded 100% control initially, which declined to 92% in 7 days and to 64% 15 days after treatment (Figure 1B). These treatments with *Bti* showed that the *Bsph*-resistant mosquitoes at Wat Pikul are completely susceptible to *Bti*. This was confirmsed by laboratory studies (Su and Mulla, unpublished data). The 7 treatments (2nd through 5th, 13th, 14th, and 17th) made with VectoBac WDG also provided evidence that *Bti* treatments have no residual activity (although this increased slightly at higher dosages) beyond 7 days and that repeat treatments would be necessary every 7-10 days at practical dosages. This study also indicated that the optimum dosages for the control of this *Bsph*-resistant polluted-water mosquito are between 20 and 50 mg/m^2 for initial and short-term control.

After 4 treatments (2nd through 5th) with *Bti* against *Bsph*-resistant mosquitoes, various mixtures of *Bti* and *Bsph* 2362 were used for treatment. The 6th and 7th treatments were made with mixtures of VectoBac WDG and VectoLex WDG at 5 mg/m^2 of each (total 10 mg/m^2) and 10 mg/m^2 of each (total 20 mg/m^2), respectively. The treatment with 5 mg/m^2 of each formulation yielded little or no control of larvae, but treatment with 10 mg/m^2 of each yielded 96% control of larvae 2 days and 52% control 7 days after treatment. The population increased beyond the pretreatment level 14 days after the 7th treatment. For the 8th treatment, the dosages were increased to 20 mg/m^2 each (total 40 mg/m^2). This treatment yielded similar control as the 7th treatment, 98% at 2 days after treatment and 31% 1 week later (Figure 1A).

After 7 treatments with *Bti* alone or with a *Bti-Bsph* mixture, a single treatment with VectoLex WDG at 30 mg/m^2 was administered to determine if a shift in level of *Bsph* resistance occurred. This treatment yielded no control of the larvae, hence showing no decline in *Bsph* resistance (Figure 1B). After noting these results, 20 mg/m^2 of each agent was applied in the 10th treatment. This treatment yielded 98% control initially and only 12% control 9 days after treatment (Figure 1B). The mixture treatment thus far offered no residual activity against *Bsph*-resistant mosquitoes, and the results were similar to those with *Bti* alone.

To determine if a higher ratio of *Bsph* in the mixture would generate residual activity beyond that of *Bti* alone, a mixture of *Bti-Bsph* at 20 and 80 mg/m^2, respectively, was applied in the 11th and 12th treatments. In both treatments, initial control was high (95-99%), but control 7 days after treatment was 69 and 60% in the 11th and 12th treatments, respectively. No residual control was noted in either treatment at 14 days after treatment. These 2 experiments indicated that residual activity against *Bsph*-resistant populations could not be increased substantially by increasing the ratio or dosage of *Bsph* in the mixture. As evidenced in the 13th and 14th treatments with *Bti* WDG alone at 20 mg/m^2, the initial control and longevity were essentially the same as in the mixture with *Bsph* at 80 mg/m^2 and *Bit* at 20 mg/m^2. Addition of *Bsph* to *Bti*, even at 4 times the dosage of *Bti*, did not increase residual activity. Use of *Bti* and *Bsph* at 20 mg/m^2 each (15th treatment) yielded 100% initial control, similar to that with *Bti* alone at 20 mg/m^2. Addition of *Bsph* to *Bti* did not improve the extent or longevity of control. In 1 further treatment (16th), where the quantity of *Bsph* in the mixture was increased further (*Bti* 20 mg/m^2, *Bsph* 100 mg/m^2), the level of control was similar to that with *Bti* alone (20 mg/m^2) or with the mixture of *Bti-Bsph* at 10 mg/m^2 each. For the 17th treatment, *Bti* was again applied at 200 mg/m^2 to test for residual activity. This treatment produced 100% initial control, but the level of control was 92 and 64% at 7 and 15 days after treatment, respectively. No significant level of persistence was noted beyond 7 days (Figure 1B).

For the final treatment (18th) against *Bsph*-resistant mosquitoes at Wat Pikul, the efficacy of the high label dosage of *Bsph* WDG at 200 mg/m^2 was tested. Little or no initial control (31%) was achieved with this treatment (Figure 1B). This clearly indicated that the *Bsph* resistance in this population was stable, with no marked shift noted after 15 treatments of *Bti* or *Bti-Bsph* mixtures,

showing that once a high level of resistance was established, it could not be reversed with 15 treatments of *Bti* or of *Bti-Bsph* mixtures and that use of *Bsph* 2362 against *Bsph*-resistant *Cx. quinquefasciatus* would not be practical. Increasing the ratio of *Bsph* in mixture did not result in persistence against resistant larvae. Thus, development of strategies to prevent or delay development of resistance to mosquitocidal *Bsph* strains is needed. From the foregoing, *Bsph*-resistant *Cx. quinquefasciatus* in the tropics clearly can be controlled easily by the use of *Bti* alone at relatively low rates, with the drawback being that *Bti* would have to be applied at least weekly, which would increase treatment costs.

Development of resistance in sites treated only with *Bsph*

To provide further information on the speed of development of resistance as found in Wat Pikul in previous studies (Mulla et al. 2001), a small polluted water source of mosquitoes (400 m^2) at Wat Lahan was subjected to repeated treatments with VectoLex WDG alone at 100 mg/m^2. In total, 19 treatments were made (Figure 2) between May 2001 and March 2002. The larval threshold for repeat treatments was set low at 30 larvae/dip.

The 1st 6 treatments yielded approximately 99% control for 7 days and 79% control 14 days after treatment (Figure 2). However, the 7th treatment yielded a low level of control on day 2 (85%) and day 7 (18%). The 8th treatment yielded excellent initial control (96%) on day and 76% control on day 8. During the following treatments (9th through 16th), extent of control declined substantially both on day 2 and day 7 after treatment. Likewise, substantial residual activity was not noted in any of these 8 treatments (9th through 16th). The 17th and 18th treatments exhibited complete failure of control, because initial control was only 8% for the 17th treatment. After the 18th treatment, the initial population density increased 30% and then doubled over the pretreatment level 14 days after treatment. For the next treatment (19th), the dosage of VectoLex WDG was increased from 100 mg/m^2 to 200 mg/m^2. At this relatively high dosage, only 60% control on day 2, 55% control on day 9, and 29% control on day 15 was achieved. The conclusion was made that larval *Cx. quinquefasciatus* at Wat Lahan community had developed resistance to *Bsph* 2362, and therefore further treatments were stopped.

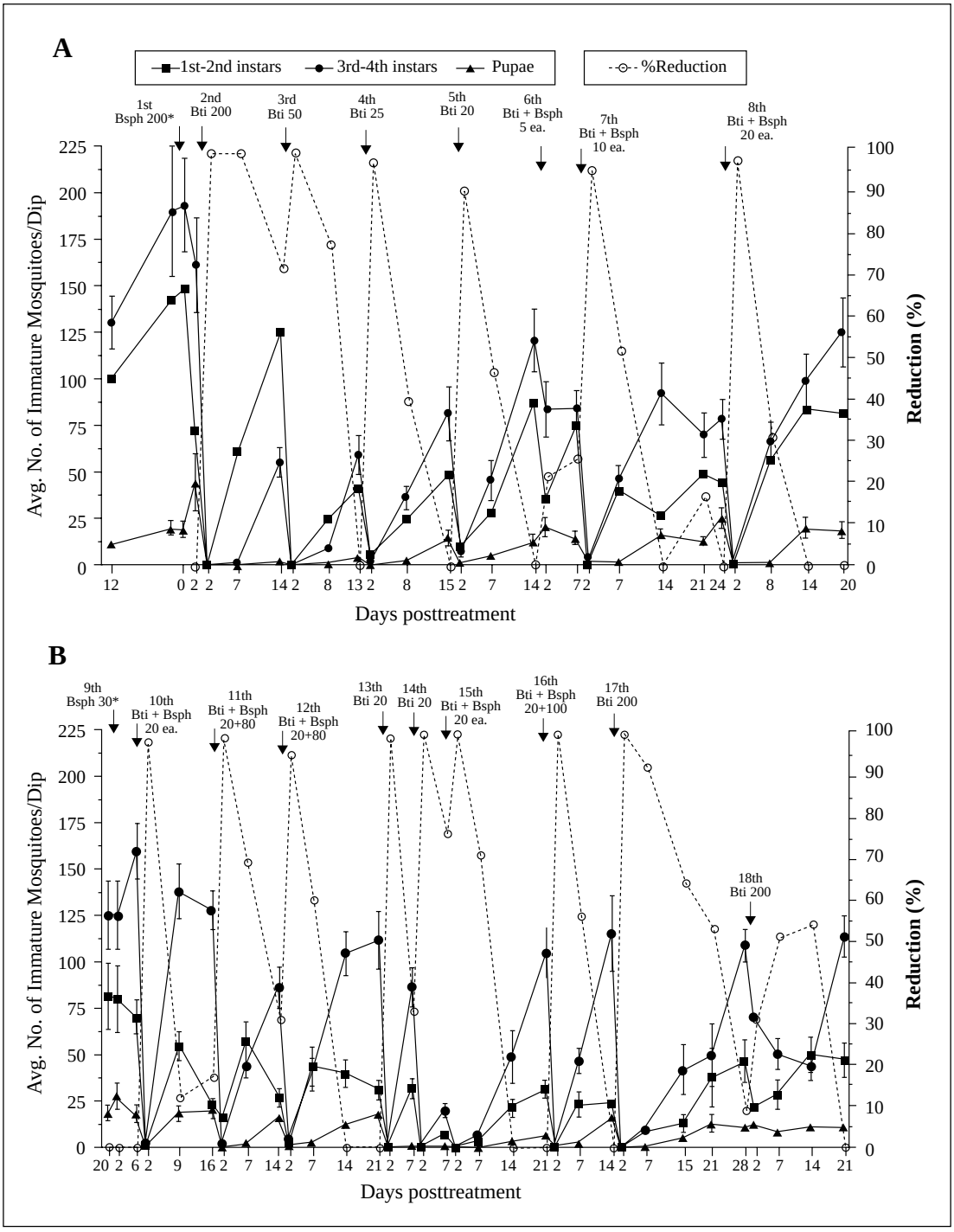

Figure 1. Control of *Culex quinquefasciatus* ***resistant to*** *Bacillus sphaericus (Bsph)* ***2362 (resistance ratio 125,000-fold) with (A) 1st through 8th treatments (January to April 2001) or (B) 9th through 18th treatments (May to October 2001) of*** *Bacillus thuringiensis* ***var.*** *israelensis (Bti)* ***or*** *Bsph* ***alone or*** *Bti-Bsph* ***mixtures at Wat Pikul, Bang Yai District, Nonthaburi Province, Thailand. Dosage is given as mg/m^2.***

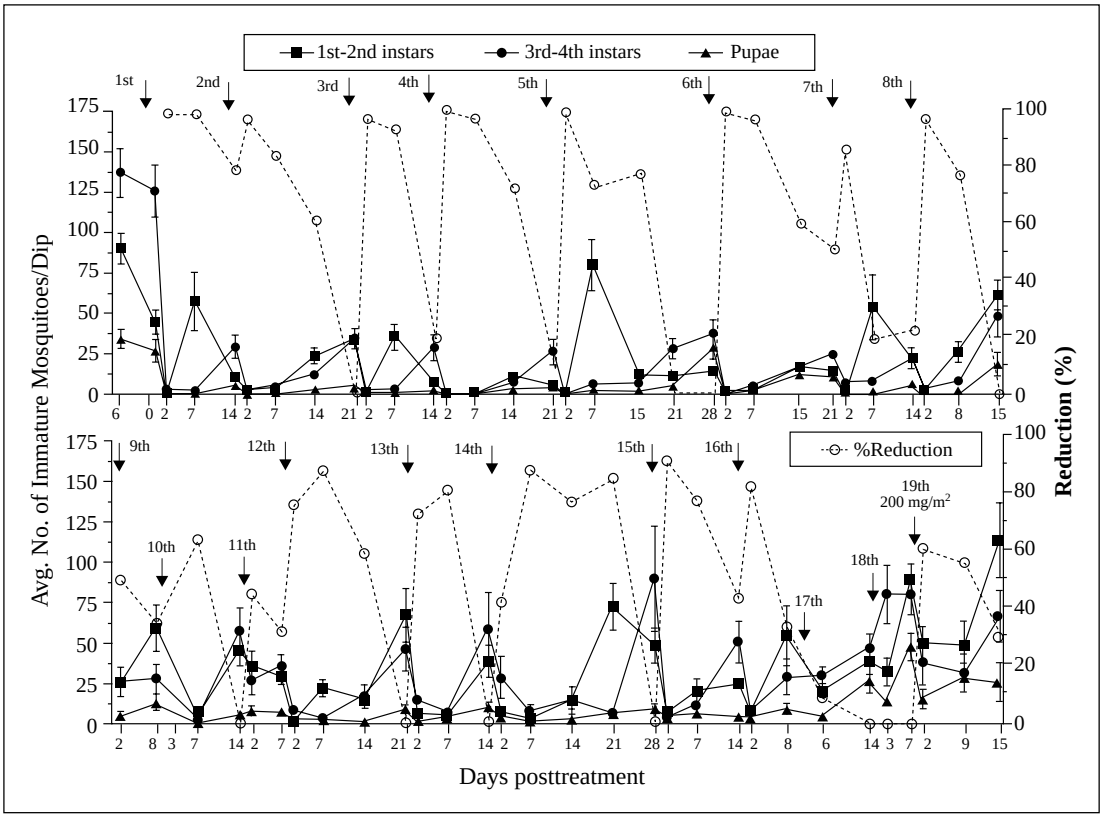

Figure 2. Emergence of resistance in larval** Culex quinquefasciatus **treated 19 times (May 2001 through March 2002) with** Bacillus sphaericus **2362 water dispersible granules formulation at 100 mg/m^2 at Wat Lahan Community, Bang Bua Thong District, Nonthaburi Province, Thailand.

Preventive measures against resistance to *Bsph*

As shown in the previous section, once *Bsph* resistance emerges, use of *Bsph* after treatments with *Bti* or a mixture of *Bti* and *Bsph* is impractical. Establishment of proactive antiresistance measures is more desirable before the widespread use of *Bsph* or other chemical and microbial agents. Based on the previous study and our laboratory studies (Zahiri and Mulla, unpublished data), a mixture of VectoBac and VectoLex WDG at 50 mg/m^2 each (total 100 mg/m^2) was tested in the low-income community of Soi Jumpa, where the total breeding habitat was treated repeatedly.

In Soi Jumpa community, 9 treatments with a mixture of *Bti-Bsph* were made from May 23, 2001, to January 2002 (Figure 3). The 1st 6 treatments provided 98-100% control 2 days after treatment, 98-99% control 7 days after

treatment, and up to 86% control 14 days after treatment, indicating residual activity for up to 2-3 weeks after treatment. Thereafter, moderate levels of control were achieved 21-28 days after treatment (Figure 3). The 7th and 8th treatments produced 96-99% control on days 2 and 7 after treatment; however, the level of control was quite low on day 14 after treatment (54 and 46%, respectively). However, the following treatment (9th) yielded excellent control on day 2 (99%) and day 7 (91%) after treatment; this declined to 84% but was still good on day 14 after treatment.

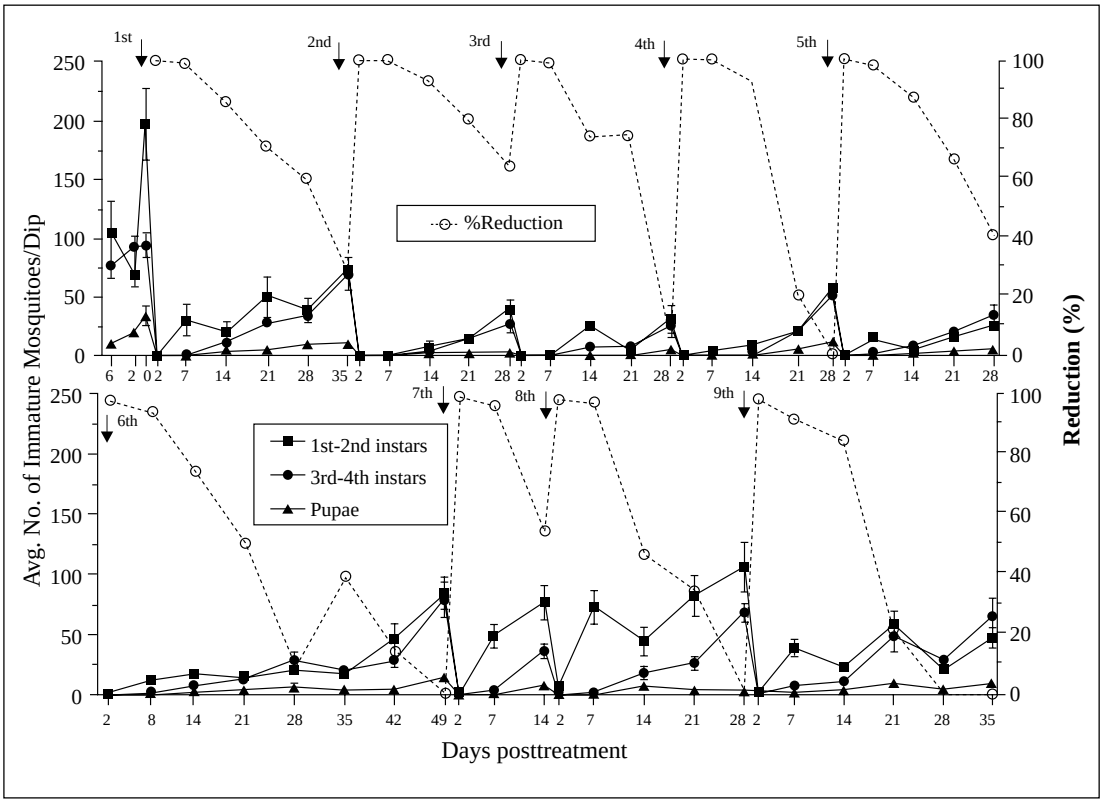

Figure 3. Prevention of resistance development in *Culex quinquefasciatus* ***treated 9 times (May 2001 through January 2002) with mixture of water dispersible granules formulation of*** *Bacillus sphaericus-Bacillus thuringiensis* ***var.*** *israelensis* ***(1:1) at 100 mg/m^2 total in Soi Jumpa Community, Pak Kret District, Nonthaburi Province, Thailand.***

Although the field treatments at Wat Lahan (*Bsph* alone) and Soi Jumpa (with mixture) covered the same period, 19 treatments were made at the former site compared to 9 at the latter site. The reason for fewer treatments at Soi Jumpa was that resurgence of larvae here was slower than at Wat Lahan.

From these studies, mixtures of *Bti-Bsph* (1:1) used against *Bsph*-susceptible larvae did not cause any noticeable shift in the susceptibility of larval *Cx. quinquefasciatus* at Soi Jumpa. All treatments were equally effective, with good residual activity up to 14 or 21 days after treatment. This is in contrast to the *Bsph*-resistant populations at Wat Pikul, where mixtures readily controlled the resistant larvae but provided no residual control. One other aspect that was observed was that over the 8-month period of our study at Soi Jumpa, the immature populations subjected to treatments with *Bti-Bsph* mixtures occurred at lower numbers, and never recovered to the level prior to the 1st treatment. On the basis of these few treatments, the conclusion was made that development of resistance will be materially delayed or possibly prevented by administering a mixture of *Bti-Bsph* to susceptible larval populations. However, additional field tests and possibly more treatments with mixtures are warranted to document prevention of resistance. At the present time, adequate information is not available on the optimum ratios of *Bti-Bsph* that will result in desired level of initial and persistent control of susceptible larvae.

Conclusions

In previous and present studies, evidence was provided that tropical *Cx. quinquefasciatus* can develop resistance rapidly to *Bsph* 2362. Emergence of a high level of resistance (>125,000-fold) at Wat Pikul (Su and Mulla, unpublished data) was noted after 5 treatments. This study documents the reemergence of resistance at Wat Lahan after 17 treatments. Possible reasons that more treatments at Wat Lahan than at Wat Pikul were needed for development of resistance to *Bsph* are that only 400 m^2 of possible mosquito-breeding source was treated at Wat Lahan and that sources that could not be treated were nearby. Factors such as refugia, size of area treated, and migration of susceptible genes into the target area can influence the speed, nature, and magnitude of emergence of resistance in mosquitoes. Note that resistance to *Bsph* is a feature of *Cx. pipiens* complex and no reports of development of resistance to *Bsph* in other species of mosquitoes have surfaced to date.

As a result of these studies, the conclusion is made that *Bsph*-resistant mosquitoes can be controlled with *Bti* alone or mixtures of *Bti* and *Bsph*. The conclusion also is made that use of a mixture of *Bti* and *Bsph* against susceptible *Cx. quinquefasciatus* can delay or prevent emergence of resistance, although

additional studies are needed. The inclusion of *Bsph* in the mixture is justified, because it provides longer residual control of susceptible, but not resistant, mosquito larvae. Prolonging the efficacy of control for 1 or 2 more weeks has advantages in operational vector control programs in terms of costly surveillance, monitoring, and application of treatments.

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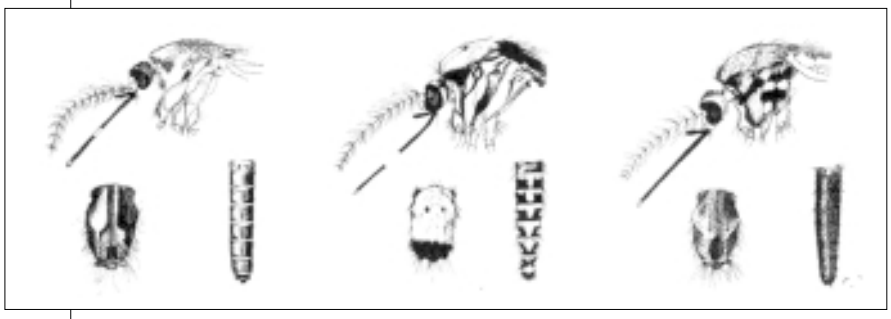
JE Vectors



Estimation of Gonotrophic Cycle Lengths and Survival Rates for Vector Mosquitoes of Japanese Encephalitis in the Suburbs of Bangkok, Thailand

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Abstract

Frequencies of blood meals and survival rates of vector mosquitoes are important parameters influencing transmission efficiency of pathogens. We applied the time series analysis proposed by Holmes and Birley (1987) for estimation of gonotrophic cycle lengths and survival rates of *Culex tritaeniorhynchus* and *Cx. gelidus*, Japanese encephalitis (JE) vectors in Thailand. Females of these species were collected at a buffalo shed and a pigsty in a suburban area near Bangkok during 30 and 24 consecutive nights, respectively. Specimens were dissected daily to determine the parity status by tracheation of ovaries. In total, 17,482 *Cx. tritaeniorhynchus* and 13,011 *Cx. gelidus* females were caught, including 15 and 19% of parous individuals, respectively. The time series analysis for the data set collected at the buffalo shed yielded a gonotrophic cycle length of 5 days and a survival rate per cycle of 20% for *Cx. tritaeniorhynchus* and 8 days and 16% for *Cx. gelidus*. From these estimates, daily survival rates were calculated as 72% and 80% for *Cx. tritaeniorhynchus* and *Cx. gelidus*, respectively. However, the data set collected at the pigsty did not yield significant results. Holmes and Birley's (1987) method could be an effective tool in epidemiological studies for Japanese encephalitis vectors, but its applicability is influenced by conditions at collection sites.

Keywords

Culex tritaeniorhynchus, *Culex gelidus*, Japanese encephalitis vector, gonotrophic cycle length, survival rate, Thailand

Introduction

Vector survival rate is a key parameter determining the transmission efficiency of pathogens by arthropods. Various methods have been proposed for survival rate estimation of female mosquitoes (Service 1993). Among them, the method based on parous rate (Davidson 1954) has widely been used due to its simplicity. However, the method assumes that the target population has stable age structure, which is rarely found in the field. Furthermore, for estimation of daily survival rate, it requires gonotropic cycle length (an interval between two successive blood meals) determined separately.

Birley and Rajagopalan (1981) introduced an innovative method based on time series analysis using daily numbers of total and parous females. An advantage of this method is that both gonotrophic cycle lengths and survival rates can be estimated from field data without assuming stable age structure. Holmes and Birley (1987) improved the method so as to remove spurious but significant correlation due to external factors such as weather. These methods or their modified versions were applied for survival rate estimation of malaria and other disease vectors in various geographical regions generally with successful results (Service 1993).

Japanese encephalitis (JE), one of the important mosquito-borne diseases in Thailand, is now expanding its prevalence up to a thousand cases per year. The disease regularly occurs during the rainy season, corresponding with high densities of vector populations. Several studies on JE vectors in Thailand (Gould et al. 1974; Mori et al. 1983; Leake et al. 1986; Somboon et al. 1989; Ginglich et al. 1992) revealed that, besides *Culex tritaeniorhynchus* Giles, *Cx. gelidus* Theobald, *Cx. fuscocephala* Theobald, and *Cx. vishnui* Theobald may be involved in the transmission of JE virus. Mori et al. (1983) and Somboon et al. (1989) had used Davidson's method to estimate daily survival rates of JE vectors by assuming the gonotrophic cycle duration of 3-4 days.

This paper presents the result of a trial study of applying Holmes and Birley's (1987) method to estimate survival rate of *Cx. tritaeniorhynchus* and *Cx. gelidus* in Pathum Thani, a suburban area near Bangkok. One feature in this area was that main blood source animals were protected by mosquito nets at night.

Materials and methods

Mosquito collection

The study was done in Amphoe Lat Lum kaeo, Pathum Thani Province, west of Bangkok. The area was mostly consisted of rice fields, including human houses with 962 residents and animal sheds with 50 cattle, 30 buffaloes, 20 goats and 17 pigs. Humans used mosquito nets when they slept at night. Most animal sheds were covered with mosquito nets after sunset. The other blood sources available in the area were dogs, chickens, ducks and wild birds such as egrets. During the study, most rice fields had been filled with water due to frequent rain.

Mosquitoes were collected at two animal sheds, one with 12 buffaloes and the other with 5 pigs. The distances between thesesheds was about 1.5 km. Traps used for mosquito sampling were modified from the Fujihira super light trap (Malainual 1988) by using 1 kg dry ice as an attractant instead of light. This sampling method was adopted to keep the sampling efficiency constant and to exclude non-biting insects such as moths and beetles. Traps were hanged at 1.5 m above the ground under the eave of the animal sheds and were operated during 18 : 30-19 : 30 for 30 consecutive nights (October 4th-November 3rd, 1988) at the buffalo shed and 24 nights (October 10th-November 3rd) at the pigsty. Mean temperatures during the collection ranged from 24 to 28 °C. There were often weak or moderate winds but they did not disturb the flight activities of mosquitoes.

Trapped mosquitoes were stored in an ice box and brought back to the laboratory for examination on the following day. Only unfed females were processed. After identification and count, *Cx. tritaeniorhynchus* and *Cx. gelidus* were dissected and the tracheation of ovaries was examined to distinguish between nulliparous and parous (Detinova 1962). The maximum of 100 females for each species were dissected. When more than 100 females were collected, numbers of parous females in total catches were interpolated the with parous rate of 100 females.

Estimation of gonotrophic cycle and survival rate

Gonotrophic cycle lengths and survival rates were estimated by the method of Holmes and Birley (1987). The key assumption of this method is that females having blood meals on day *t* are parous when they take the next meal on day *t+d*. Hence, it is expected that there is a positive correlation between

the number of total females on day $t(T_t)$ and that of parous females on day $t+d(M_{t+d})$. The actual process is to find a time lag (d integer days) which yields significant cross-correlation coefficients (CCs) between T_t and M_{t+d}. To remove spurious significant correlations, T_t and M_t filtered by the following formula were used for calculation of CCs;

$$z_t = x_t - \alpha \times x_t - 1$$

Where z_t = time series filtered, x_t = time series to be filtered, and α = autoregression parameter calculated as

$$\alpha = \Sigma\{(x_t - \bar{x}) \times (x_t - \bar{x})\} / \Sigma(x_{t-1} - \bar{x})^2$$

Calculation of CCs followed a standard correlation coefficient formula. When CCs were larger than $2\sqrt{\text{number of samples in time series}}$, the departure from zero was regarded as significance at $P<0.05$. Then, d was adopted as a gonotrophic cycle length with a corresponding estimate of a survival rate per gonotrophic cycle (p) as $p = \Sigma M_{t+d} / \Sigma T_t$. Daily survival rates were calculated as the d root of p. All the calculation was performed by using a computer program written in BASIC.

Results

Species composition and density

Nearly a thousand of female mosquitoes were collected each night, most of them were *Cx. tritaeniorhynchus* and *Cx. gelidus* (Table 1). The trap operated at the buffalo shed collected mosquitoes 8 times of the collection at the pigsty. Of females captured at the buffalo shed, 54% were *Cx. tritaeniorhynchus*, whereas, this species occupied 70% at the pigsty. *Cx. gelidus* represented 43% and 24% at each shed, respectively. Other mosquito species of *Culex*, *Mansonia*, *Anopheles* and *Aedes* were collected less than 10% of the totals at both collection sites.

Numbers of *Cx. triaeniorhynchus* and *Cx. gelidus* females fluctuated widely from day to day at both sites (Table 2), but there were no tendencies for the population levels either to increase or to decrease. Correlations between numbers of the two species was significant at the buffalo shed (r = 0.89, t = 10.50, d.f. = 28, $P<0.001$) and the pigsty (r = 0.69, t = 4.49, d.f. = 22, $P<0.001$). Number of the female mosquitoes at both collection sites did not correlate to each other for *Cx. tritaeniorhynchus* (r = 0.06, t = 0.296, d.f. = 22, $P>0.05$) or *Cx. gelidus* (r = -0.12, t = -0.567, d.f. = 22, $P>0.05$).

Age structure

Parous rates fluctuated daily within the range of 0-56%, but were generally low (Table 2). Parous rates for totals at each site were 22% and less irrespective of species. When both collection sites were pooled, parous rates were 15 and 19% for *Cx. tritaeniorhynchus* and *Cx. gelidus*, respectively.

Survival rate

Significant peaks of CCs between total and parous females were detected at a lag of 5 days for *Cx. tritaeniorhynchus* and at a lag of 8 days for *Cx. gelidus* at the buffalo shed (Figure 1). Estimated survival rates per gonotrophic cycle were larger than the mean parous rate for *Cx. tritaeniorhynchus* but smaller for *Cx. gelidus* (Table 2 and 3). Daily survival rates assuming constancy during the cycle were within the range of 70-80% (Table 3).

CCs were not significant for both species at the pigsty, therefore gonotrophic cycle lengths and survival rates could not be estimated by the method of Holmes and Birley (1987).

Table 1. Species and numbers of female mosquitoes collected at animal sheds in Pathum Thani during the period of October 4th-November 2nd, 1988.

Species	Buffalo			Pig		
	Total	No./night*	%	Total	No./night*	%
Cx. tritaeniorhynchus	15,535	517.8	53.86	1,947	81.2	69.26
Cx. gelidus	12,342	411.4	42.79	669	27.9	23.80
Culex spp.	771	257.0	2.67	169	7.0	6.01
Mansonia spp.	144	48.0	0.50	20	0.8	0.71
Anopheles spp.	16	5.3	0.06	5	0.2	0.18
Aedes spp.	2	0.7	0.01	0	0.0	0.00
Unidentified specimens	33	11.0	0.11	1	0.04	0.04
Total	28,843	961.4	100.00	2,811	117.1	100.00

* 30 nights at the buffalo shed and 24 nights at the pigsty.

Table 2. Number and parity of** Cx. tritaeniorhynchus **and** Cx. gelidus **collected at animal sheds in Pathum Thani during the period of October 4th-November 2nd, 1988.

Day	*Cx. tritaeniorhynchus*						*Cx. gelidus*					
	Buffalo			Pig			Buffalo			Pig		
	Total	Parous	P%*	Total	Parous	P%*	Total	Parous	P%*	Total	Parous	P%*
1	168	17	10	** —	—	—	73	8	11	—	—	—
2	67	14	21	—	—	—	24	2	8	—	—	—
3	43	3	7	—	—	—	5	0	0	—	—	—
4	164	11	7	—	—	—	83	7	8	—	—	—
5	237	18	8	—	—	—	151	25	16	—	—	—
6	3,232	216	7	—	—	—	3,578	541	15	—	—	—
7	1,577	64	4	71	9	13	832	177	21	8	1	13
8	557	68	12	86	2	2	92	12	13	86	2	2
9	604	63	10	192	36	19	341	53	16	64	12	19
10	511	57	11	45	7	16	463	74	16	12	2	17
11	782	238	30	43	3	7	630	179	28	32	4	13
12	384	61	16	57	14	25	314	72	33	15	2	13
13	238	46	19	198	40	20	30	8	27	57	15	26
14	652	110	17	45	8	18	1,644	419	26	38	10	26
15	491	76	16	201	28	14	412	49	12	76	10	13
16	443	110	25	308	67	22	263	76	29	63	15	24
17	487	119	24	192	58	30	161	49	30	58	17	29
18	178	72	40	50	28	56	63	28	44	33	14	42
19	601	239	40	70	33	47	166	74	45	20	11	55
20	99	37	38	32	12	38	35	14	40	21	7	33
21	450	129	29	20	7	35	76	21	28	4	1	25
22	349	74	21	59	18	31	84	30	36	8	2	25
23	861	159	19	44	5	11	279	52	19	10	2	20
24	251	14	6	14	3	21	293	44	15	5	1	20
25	568	11	2	77	10	13	450	39	9	24	1	4
26	408	28	7	21	0	0	593	33	6	5	0	0
27	207	41	20	10	0	0	104	25	24	55	7	13
28	282	34	12	35	5	14	417	83	20	21	5	24
29	371	60	16	66	19	29	233	20	9	13	1	8
30	268	61	23	11	6	55	453	73	16	7	0	0
Total	15,535	2,252	15	1,947	419	22	12,342	2,292	19	669	142	21

* Percentage parous.

** Not collected.

Table 3. Population parameters estimated for** Cx. tritaeniorhynchus **and** Cx. gelidus **collected at animal sheds in Pathum Thani during the period of October 4th- November 2nd, 1988.

Parameter	*Cx. tritaeniorhynchus*		*Cx. gelidus*	
	Buffalo	Pig	Buffalo	Pig
Gonotrophic cycle length (day)	5	—	8	—
Survival rate per cycle (%)	20	—	16	—
Daily survival rate (%)	72	—	80	—

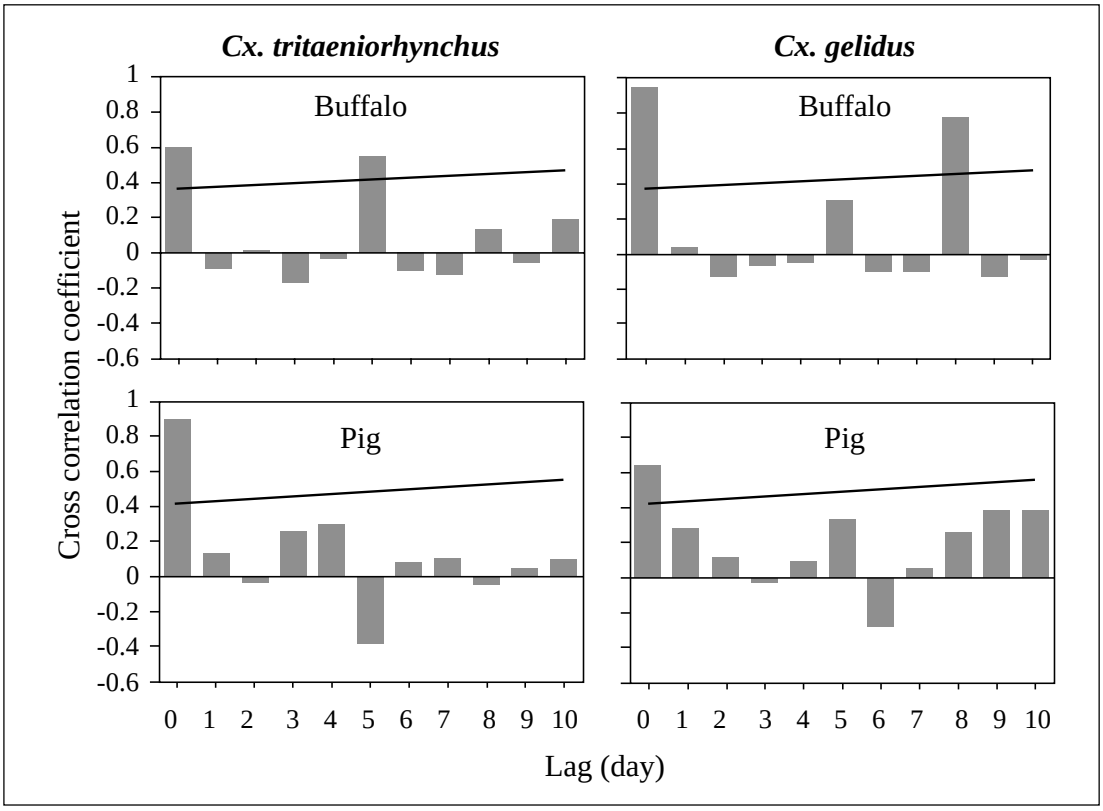

Figure 1. Cross-correlation coefficients (CCs) at specific time lag intervals between numbers of total and parous females for** Cx. tritaeniorhynchus **and** Cx. gelidus **collected at the buffalo shed and the pigsty. Solid lines on each panel indicates 95% confidence limits; CC Values larger than this limit significantly depart from zero.

Discussion

The obtained results clearly indicated both advantages and limitations of the time series analysis of Holmes and Birley (1987). Data at the buffalo shed yielded significant CCs, which enabled estimation of gonotrophic cycle lengths and survival rates. In contrast, data at the pigsty failed to yield significant CCs. The two collection sites were in the same village, 1.5 km apart from each other. However, there was no correlation between mosquito numbers at both sites. This means that the fluctuation pattern of mosquito densities at each collection site reflected the mosquito abundance in the study area and the micro condition at each site. The latters includes such factors as the direction and strength of wind, and the density and size of trees and architecture around each site. They would influence the spatial distribution of flying mosquitoes (Bidlingmayer 1975).

The difference in attractiveness between the two collection sites might also have contributed to the different fluctuation pattern in mosquito numbers. Buffaloes in the shed were more abundant and individually much larger than pigs in the pigsty. The buffaloes were mature, while the pigs were of 3 months after birth. Attraction ranges of baits for mosquitoes depend at least partly, on the number and size of baits (Gillies and Wilkes 1972; McIver and McElligott 1989). Therefore, the buffaloes was certainly much more attractive for host-seeking mosquitoes than the pigs. In fact, 8 times more mosquitoes were collected at the buffalo shed. Probably, samples at the buffalo shed better represented the mosquito population in the study area.

The gonotrophic cycle length of 5 days detected at the buffalo shed for *Cx. tritaeniorhynchus* was longer than 3 days determined in Japan by the mark-recapture method (Buei et al. 1980). The 3-day cycle corresponded to the shortest period observed in the laboratory (Kawai 1969). The man interval between two successive blood meals was about 4 days at 27 °C (Kawai 1969), therefore the 5-day cycle is a reasonable estimation. By the mark-recapture study for *Cx. tritaeniorhynchus* in Pakistan, 5-8 days were detected as the period from emergence to the first oviposition (Reisen et al. 1978). Birley and Rajagopalan's (1981) method used for *Cx. tarsalis* Coquillett in California also detected a cycle of 5-7 days (McHugh 1990).

For *Cx. gelidus*, the cycles of 8 days was detected. Because little is known about bionomics of this species (Malainual 1992), it is difficult to discuss

further about adequacy of this estimation. It was provisionally treated as a gonotrophic cycle length. However, it is noted that, in the time series analysis, factors other than gonotrophic cycle lengths may yield significant CCs (Holmes and Birley 1987).

Parous rates observed in this study were lower than 30-70% reported for these mosquito species (Harada et al. 1967, 1968; Aslam et al. 1977; Reisen et al. 1986; Somboon et al. 1989), but were similar to 15-22% in some reports (Buei et al. 1982; Mori et al. 1983). Sampling in this study was limited to 1 hour after sunset. Parous rates of *Cx. tritaeniorhynchus* might increase towards later part of night (Aslam et al. 1977), but Reisen et al. (1986) found that, on average, a half of the females attracted to cattle during 30 minutes after sunset were parous. Therefore, a short sampling time limited to evening was not a reason of relatively low parous rates. During the study, breeding habitats of the vectors were abundant due to wet rice fields. These habitats probably produced high numbers of new adults, lowering parous rates.

Protection of main blood source animals with mosquito nets may also had an influence on the low parous rate. In addition, it may have made the detection of significant CC peaks more difficult as exemplified for the data obtained at the pigsty. Increased difficulty in access to blood sources would make feeding success less synchronized, which could render feeding peaks indistinct. Impact of animal protection on mosquito populations and JE epidemiology, either desirable or undesirable for humans, deserves further research.

Daily survival rates calculated by Buei et al. (1982) and Mori et al. (1983) were 60% *odd*, because they combined low parous rates with a gonotrophic cycle length of 3 days. Our estimates with longer cycle lengths directly obtained for the target population are comparable to 70-90% in other reports.

Summarizing, Holmes and Birley's (1987) method could be an effective tool in studies of Japanese encephalitis vectors. However, it is noted that this method is not robust but rather sensitive to conditions at collection sites. Establishment of a standard sampling technique, which can increase the efficiency of this method, is desirable.

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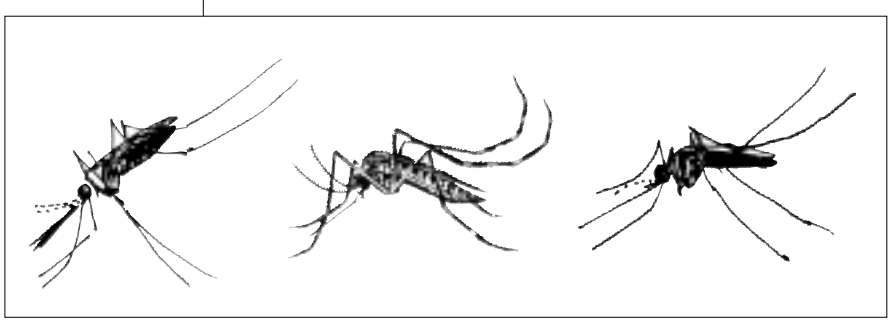
Mosquito Vectors & Personal Protections



Laboratory and Field Evaluations of the Insect Repellent 3535 (Ethyl Butylacetylaminopropionate) and Deet against Mosquito Vectors in Thailand

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Abstract

The insect repellents 3535 (ethyl butylacetylaminopropionate or IR3535) and deet (N,N-diethyl-3-methylbenzamide) were prepared as 20% solutions in absolute ethanol and evaluated for repellency against many mosquito species in Thailand under laboratory and field conditions using human subjects. In the laboratory, 0.1 ml was applied per 30-cm^2 of exposed area on a volunteer's forearm (0.66-0.67 mg active ingredient [AI]/cm^2), whereas in the field, volunteers' legs (from knee to ankle, with a surface area of about 712-782 cm^2) were treated with 3 ml per exposed area (0.76-0.84 mg AI/cm^2). In the laboratory, both IR3535 and deet showed equal repellency ($P>0.05$) for 9.8 and 9.7 h against *Aedes aegypti*, for 13.7 and 12.7 h against *Culex quinquefasciatus*, and for 14.8 and 14.5 h against *Cx. tritaeniorhynchus*, respectively. *Anopheles dirus* was significantly less sensitive to IR3535 than to deet ($P<0.05$), with a mean protection time of 3.8 and 5.8 h, respectively. Under field conditions, both IR3535 and deet provided a high degree of protection against various mosquito vectors ranging from 94 to 100% during the test periods. Both repellents provided a high level of protection for at least 8 h against *Ae. albopictus* and for at least 5 h against *Cx. gelidus*, *Cx. tritaeniorhynchus*, *Cx. quinquefasciatus*, *Mansonia dives*, *Ma. uniformis*, *Ma. annulata*, *Ma. annulifera*, *Anopheles minimus*, and *An. maculatus*. This study clearly documents the potential of IR3535 for use as a topical treatment against a wide range of mosquito species belonging to several genera.

Keywords

Repellents, IR3535, deet, mosquitoes, Thailand

Introduction

Mosquito-borne diseases, such as malaria, filariasis, dengue fever, dengue hemorrhagic fever, yellow fever, and encephalitis are still some of the major public health problems for people in tropical countries (Service 1993). Up to the present time, no effective vaccine has been available for protection from these diseases, except yellow fever and Japanese encephalitis. Therefore, protection from mosquito bites is 1 of the best strategies to prevent these diseases or reduce their incidence. Since the late 1950s, deet *(N,N*-diethyl-3-methylbenzamide) has been 1 of the most commonly used repellents against a broad range of mosquitoes and other biting insects (Smith 1957, Thavara et al. 1990, Coleman et al. 1993). However, several workers have reported occasional risks resulting from the topical use of deet. Contact urticaria syndrome due to application of deet was reported by Maibach and Johnson (1975). Zadikoff (1979) reported 2 cases and Edwards and Johnson (1987) reported 1 case of toxic encephalopathy in children. Reuveni and Yagupsky (1982) reported skin eruptions in 10 soldiers after application of 50% deet. Recently, Qiu et al. (1998) reviewed the pharmacokinetics, formulations, and safety of deet, and concluded that deet exhibits a good margin of safety, but does manifest some adverse effects in humans. To find safer and more acceptable repellents for topical use, many workers have searched for other chemicals providing repellency equal to or better than that obtained from deet (Schreck and McGovern 1989, Coleman et al. 1993, Frances et al. 1996, Walker et al. 1996, Yap et al. 1998, Debboun et al. 1999). Insect repellent 3535 (ethyl butylacetylaminopropionate or IR3535) is considered to have a high margin of safety to humans, including infants, and lack of toxic effects when recommended usage is followed (U.S. EPA 1999).

This study was designed to evaluate the repellency of IR3535 against mosquito vectors under both laboratory and field conditions. In the laboratory, the testes were conducted against 4 mosquito species. Field evaluations were carried out in 5 provinces of Thailand to cover a broad range of mosquito vectors. The most commonly used repellent, deet, was used as the standard against which the efficacy of IR3535 was evaluated.

Materials and methods

Test materials: Two repellents, IR3535 (purity 99.8%; provided by Merck KgaA, Darmstadt, Germany) and deet (purity 99.3%; purchased on the market), were evaluated. The repellents were prepared as 20% (w/w) solutions in absolute ethanol.

Test mosquitoes in the laboratory: The mosquitoes used in this study were laboratory-reared female *Aedes aegypti* (L.), *Culex quinquefasciatus* Say, *Culex tritaeniorhynchus* Giles, and *Anopheles dirus* Peyton and Harrison. These mosquitoes were reared according to the standard protocol of the Biology and Ecology Section, National Institute of Health, Ministry of Public Health, Thailand, and maintained in the insectary of the institute. Sugar-fed, 3- to 5- day-old females of these mosquitoes were used in laboratory repellent tests. Before testing, the mosquitoes were starved for 24 h. The tests against *Ae. aegypti* were carried out from 0600 to 1800 h, whereas those against *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus*, and *An. dirus* were conducted between 1800 and 0600 h. However, because our preliminary study found that both repellents could protect against biting of *Cx. quinquefasciatus* and *Cx. tritaeniorhynchus* for more than 12 h, the treatments were then applied at 1400 h, 4 h before the beginning of each test against the 2 species.

Laboratory repellent test procedure: The tests were conducted at the National Institute of Health, Thailand, in a room maintained at 27 ± 2 °C and relative humidity 70 ± 10%. The light intensity was regulated at 300-500 lux for the testing of day-biting mosquitoes and at about 10-50 lux for the night biters. The evaluation method used was similar to that described by Tawatsin et al. (2001). For testing, 0.1 ml of the 20% solution of IR3535 (0.67 mg active ingredient [AI]/cm^2) was applied onto a 3x10 cm marked area of 1 forearm of each of 3 human volunteers (25-37 years old) and a similar dose of deet (0.66 mg AI/cm^2) was applied to the other forearm. Each arm was covered by a paper sleeve with a 3x10 cm exposed area corresponding to the marked and treated site. After treatment, every 30 min, each volunteer put the arm into a mosquito cage (30x30x30 cm) containing 250 female mosquitoes and left the arm there for 3 min. Before the start of each exposure period, mosquitoes were tested for their readiness to bite by placing an untreated bare hand of each volunteer into a test mosquito cage for up to 15 sec for *Ae. aegypti*, and for up to 30 sec for *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus*, and *An. dirus*.

The mosquitoes were blown from the hand before any blood was taken. If at least 2 mosquitoes landed or bit (generally many more mosquitoes bit during this period) the hand, the repellency test was carried out, otherwise the test was not conducted. For the actual test, the number of biting mosquitoes on the marked area was recorded at each interval until either 2 bites occurred in a single 3-min exposure period, or 1 bite occurred in each of 2 consecutive exposure periods. At this point the test was terminated. The duration between the application of repellent and the first 2 bites or 2 bites in successive observations was recorded as the protection time.

Field test sites: The field evaluations were carried out in various areas of Thailand during both day and night to include a wide range of mosquito species. First, Surat Thani, a province in southern Thailand, was selected to conduct the tests against day-biting mosquitoes. *Aedes albopictus* (Skuse) was the dominant daytime biter here. Several provinces in other regions of Thailand (Mae Hong Son, Nonthaburi, Nakhon Si Thammarat, Surat Thani, and Satun) were chosen to run the tests against night-biting mosquitoes. Test sites are shown in Figure 1.

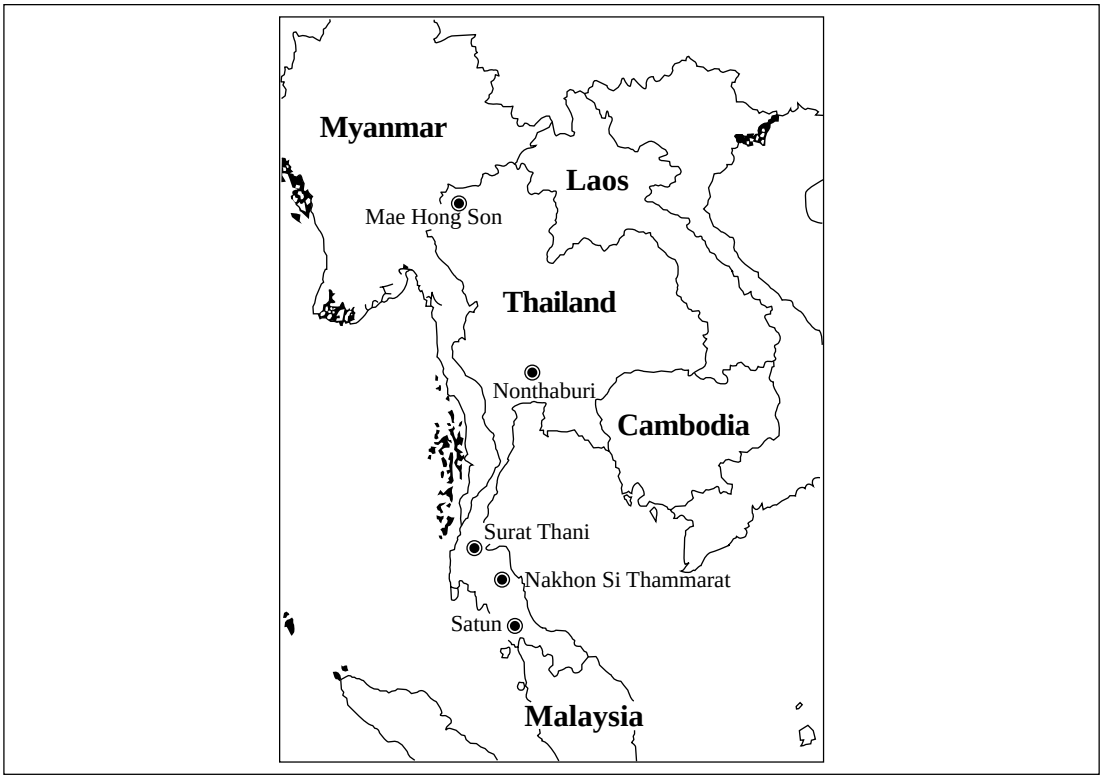

Figure 1. Map of Thailand showing the study sites.

Field evaluation procedure: The human-bait method was used to evaluate the efficacy of the test repellents (WHO 1996). In the treated group of 6 adult volunteers (18-42 years old), each person was treated with IR3535 on 1 leg and deet on the other leg. The volunteers rolled their pants up to their knees. These 2 repellents were directly applied to lower part of their legs, from the knee to the ankle. Three milliliters of the 20% repellent solutions were applied to each leg (surface area of about 712-782 cm^2), providing dosages of about 0.77-0.84 mg AI/cm^2 for IR3535 and 0.76-0.84 mg AI/cm^2 for deet. Nothing was applied to the legs of 6 other adult volunteers (18-42 years old) assigned as controls. Assessments of the efficacy of the tests were conducted by comparisons between control (untreated) and treated volunteers. The volunteers were seated in pairs, each pair consisting of 1 control and 1 treated volunteer sitting about 1 m apart from each other. The pairs were located at least 5 m away from any other pair. The tests were run in protected locations with minimal wind disturbance where mosquito landing or biting activity was high. The pairs of volunteers sat on chairs and collected all of the mosquitoes landing on or biting their legs in the specified area for a 10-min period. Each exposure period was followed by a 10-min break before the next mosquito collection was conducted. Each hour of the test included 3 mosquito collections and 3 breaks. The tests were conducted for 8 h (0900-1700 h) against day-biting mosquitoes, whereas tests against night-biting mosquitoes were carried out for 5 h (1900-2400 h). The captured mosquitoes were brought to the laboratory and identified to species under a stereomicroscope. The percentage reduction in landing and bites during every hour of test was calculated according to Mani et al. (1991) and Yap et al. (1998):

$$\text{Percentage reduction} = \frac{C - T}{C} \times 100$$

Where *C* is the number of mosquitoes collected by the control volunteers and *T* is the number collected by the treated volunteers.

Statistical analysis: The repellency comparisons of IR3535 and deet under laboratory conditions against each mosquito species were analyzed as mean protection time comparisons using Student's *t*-test. For field evaluations, percentage reduction for each hour was transformed to log (x + 1) for analysis of variance (Yap et al. 1998). The transformed data were analyzed for analysis of variance and mean comparisons using the SPSS program (version 9.0) (SPSS Inc., Chicago, IL).

Results and Discussion

Laboratory tests

Relative repellency (mean protection time) under laboratory conditions provided by IR3535 and deet against the 4 mosquito species is shown in Figure 2. Both IR3535 and deet demonstrated equal repellency ($P>0.05$) for 9.8 and 9.7 h against *Ae. aegypti*, for 13.7 and 12.7 h against *Cx. quinquefasciatus*, and for 14.8 and 14.5 h against *Cx. tritaeniorhynchus*, respectively. Mean (± SE) biting on the control areas (the untreated bare hands) for *Ae. aegypti*, *Cx. quinquefasciatus*, and *Cx. tritaeniorhynchus* was 4.7 ± 0.2, 4.8 ± 0.3, and 3.7 ± 0.3 bites, respectively. On the other hand, *An. dirus* was significantly less sensitive to IR3535 than to deet ($P<0.05$), with mean protection time of 3.8 and 5.8 h, respectively. Mean (± SE) biting on the control areas for *An. dirus* was 2.6 ± 0.3 bites. With regard to deet, Frances et al. (1996) found that 20% deet provided protection from *An. dirus* (6-7 days old) bites for an average of 105 min in a test cage containing 200 mosqutioes. This protection time is shorter than that found in our studies using 4- to 5-day-old mosquitoes. This discrepancy between the 2 studies can be explained in terms of different evaluation procedures and different responses in different species or populations of the same species. Such differences in response to chemical repellents have been reported by Rutledge et al. (1978) and Robert et al. (1991), where variable responses in time and location have been noted.

Field tests

The relative efficacies of IR3535 and deet against day-biting mosquitoes in the field at Surat Thani, Thailand, studied in April and July 1999, are presented in Table 1. In the April test, IR3535 and deet provided an everage reduction of field mosquito bites of 98 and 97%, respectively, during the 8 h of exposure period. In the April test, only 9 and 14 mosquitoes, all *Armigeres subalbatus* (Coquillett) were caught on the volunteers treated with IR3535 and deet, respectively (mosquito collections on the untreated volunteers are presented in Table 3), during the entire 8 h of testing. In the July test, the 2 repellents provided complete repellency against mosquitoes during the test period of 8 h.

The relative repellencies of IR3535 and deet against night-biting mosquitoes at various study sites are shown in Table 2. The 2 repellents yielded equally excellent repellency with almost complete prevention of mosquito

landing and biting in the 4 study sites. Note that no significant difference was found in efficacy of both repellents among the test sites and the test months ($P>0.05$). At Nakhon Si Thammarat (July), deet provided complete reduction of mosquito bites, whereas IR3535 gave an average of 99% protection over the 5 h exposure period. In fact, only 1 *Culex sitiens* Wiedemann bit 1 of the 6 volunteers treated with IR3535. At Mae Hong Son, IR3535 showed an average of 99% biting reduction in July and August, whereas deet gave an average of 98 and 99% reduction, respectively. In the July test, only 3 and 6 mosquitoes were captured on the 6 volunteers treated with IR3535 and deet, whereas in August, 2 and 1 mosquitoes were captured by the treated group, respectively. These very few mosquitoes caught belonged to 2 species, *Anopheles hyrcanus* (Pallas) and *Anopheles minimus* Theobald.

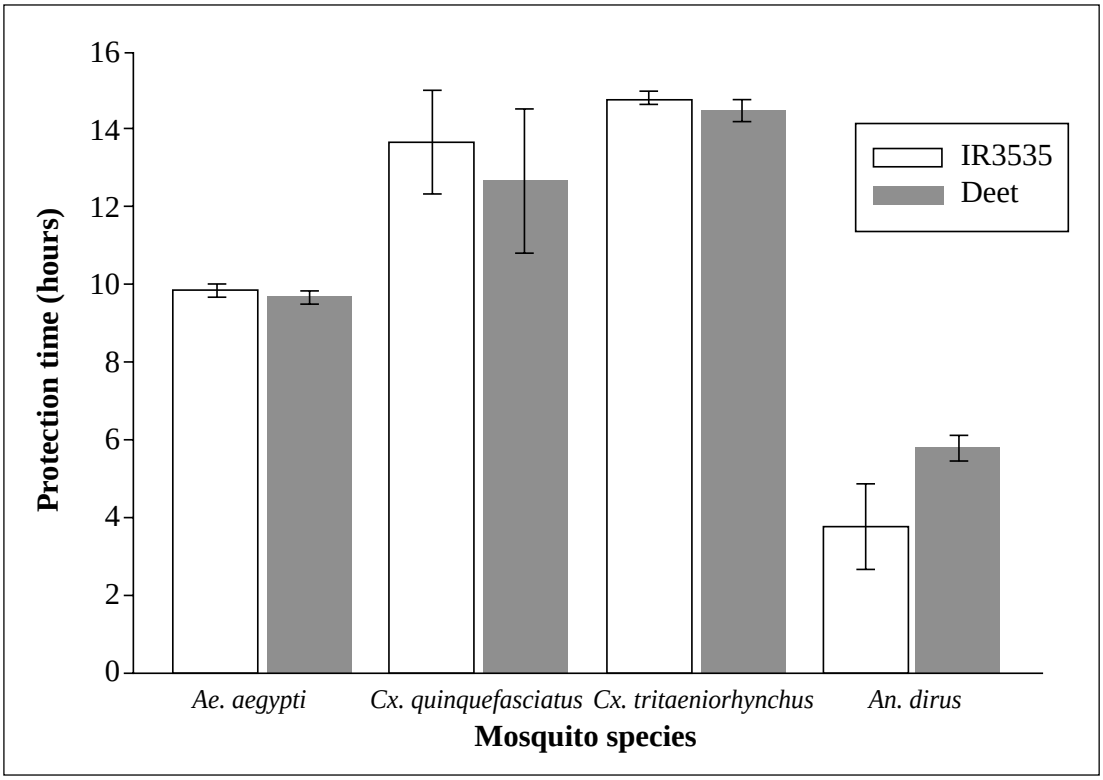

Figure 2. Relative repellency (mean ± SE) of IR3535 and deet against 4 mosquito species under laboratory conditions.

The total number of mosquitoes caught by the volunteers of the control group and predominant species are presented in Table 3. For repellency tests conducted against day-biting mosquitoes in Surat Thani, the mosquitoes captured on controls belonged to 3 species: *Ae. albopictus*, *Ar. subalbatus*, and *Coquillettidia crassipes* (Van der Wulp). In the April test, both *Ae. albopictus* and *Ar. subalbatus* mosquitoes were caught on the untreated volunteers, whereas in July, *Ar. subalbatus* was replaced by *Cq. crassipes*. Therefore, we conclude that both IR3535 and deet provide complete repellency of *Ae. albopictus* and *Cq. crassipes* for at least 8 h under field conditions. For the tests conducted against night-biting mosquitoes in Nakhon Si Thammarat, Nonthaburi, Satun, and Mae Hong Son, the mosquitoes caught by the control groups included 13 species belonging to 3 genera. These were *Anopheles maculatus* Theobald, *An. hyrcanus*, *An. minimus*, *Anopheles pseudowillmori* Theobald, *Anopheles sawadwongporni* Rattanarithikul and Green, *Culex gelidus* Theobald, *Cx. quinquefasciatus*, *Cx. sitiens*, *Cx. tritaeniorhynchus*, *Mansonia annulata* Leicester, *Mansonia annulifera* (Theobald), *Mansonia dives* (Schiner), and *Mansonia uniformis* (Theobald).

It is quite clear that in Nakhon Si Thammarat both in April and July, significant number of *Culex* species and 3 *Mansonia* species were landing on and biting the control groups (Table 3). The 2 test repellents provided almost complete protection from the *Culex* species (see Table 3). In Satun, the mosquitoes biting during the test were *Ma. dives* and *Ma. uniformis*, with the repellents again providing complete protection during the test period. In the Mae Hong Son area in July and August, 5 species of *Anopheles* were actively landing and biting the control groups during the test periods (see Table 3). Treatment with IR3535 and deet provided 94-100% protection from landing and biting of these *Anopheles* mosquitoes. No rash, skin irritation, or hot sensation was observed on arms and legs of the test volunteers treated with IR3535 and deet during and after application.

In summary, IR3535 demonstrated excellent repellency (100% protection in most tests) against both day- and night-biting mosquitoes under laboratory and field conditions. A high degree of protection averaging 94-100% was observed under a variety of field conditions for the various biting mosquitoes. Therefore, this study clearly indicates the potential of IR3535 for use as an effective topical repellent against a wide range of mosquito species belonging to various genera.

Table 1. The relative efficacy of IR3535 and deet against day-biting mosquitoes (*Aedes albopictus****) over an 8-h exposure period (0900-1700 h) in April and July 1999, at Surat Thani, Thailand.***

Month	Repellent	Reduction (%) of mosquito bites during 8 h of exposure								
		1	2	3	4	5	6	7	8	Mean ± SE [1]
April	IR3535	98.6	98.9	97.5	100	94	97.8	100	100	98.4±0.7a
	Deet	97.3	98.9	97.5	95.9	94	95.7	100	100	97.4±0.8a
July	IR3535	100	100	100	100	100	100	100	100	100±0 b
	Deet	100	100	100	100	100	100	100	100	100±0 b

[1] Means in this column followed by different letters are significantly different from each other (P<0.05).

Table 2. The relative efficacy of IR3535 and deet against night-biting mosquitoes over a 5-h exposure period (1900-2400 h) in tests conducted from April to August 1999, at various locations in Thailand.

Study site (province)	Month	Repellent	Reduction of mosquito bites (%) during 5 h of exposure					
			1	2	3	4	5	Mean ± SE [1]
Nakhon Si Thammarat	April	IR3535	100	100	100	100	100	100 ± 0
		Deet	100	100	100	100	100	100 ± 0
Nakhon Si Thammarat	July	IR3535	100	100	100	94.1	100	98.8 ± 1.2
		Deet	100	100	100	100	100	100 ± 0
Nonthaburi	May	IR3535	100	100	100	100	100	100 ± 0
		Deet	100	100	100	100	100	100 ± 0
Satun	July	IR3535	100	100	100	100	100	100 ± 0
		Deet	100	100	100	100	100	100 ± 0
Mae Hong Son	July	IR3535	100	100	100	97.1	98	99.0 ± 0.6
		Deet	100	97.4	97.4	94.2	100	97.8 ± 1.1
Mae Hong Son	August	IR3535	100	100	100	100	93.8	98.8 ± 1.2
		Deet	100	100	96.4	100	100	99.3 ± 0.7

[1] Means of all treatments at all locations are not significantly different from each other (P>0.05).

Table 3. Total mosquitoes captured, biting rate, and predominant species of mosquitoes collected at various study sites in Thailand, April-August 1999.[1]

	Surat Thani		Nakhon Si thammarat		Nonthaburi	Satun	Mae Hong Son	
	April	July	April	July	May	July	July	August
Total mosquitoes	541	542	93	55	544	66	230	137
Biting rate (no./ person-hour)	22.5 [2]	22.6[2]	6.2 [3]	3.7[3]	36.3[3]	4.4[3]	15.3[3]	9.1[3]
Predominant species (%)	*Ae. albopictus* (83) *Ar. subalbatus* (16) Other species (1)	*Ae. albopictus* (69) *Ar. subalbatus* (11) *Cq. crassipes* (18) Other species (2)	*Cx. sitiens* (42) *Cx. tritaenio-rhynchus* (15) *Ma. annulata* (19) *Ma. annulifera* (10) Other species (5)	*Cx. sitiens* (17) *Cx. tritaenio-rhynchus* (55) *Ma. annulifera* (19) *Ma. dives* (6) Other species (3)	*Cx. gelidus* (64) *Cx. quinque-fasciatus* (27) *Cx. tritaenio-rhynchus* (6) Other species (3)	*Ma. dives* (79) *Cq. crassipes* (17) *Ma. uniformis* (4)	*An. hyrcanus* (38) *An. maculatus* (6) *An. minimus* (45) *An. sawad-wongporni* (6) Other species (5)	*An. hyrcanus* (62) *An. minimus* (24) *An. pseudowill-*mori (5) *An. sawad-wongporni* (4) Other species (5)

[1] *Ae., Aedes; Cx., Culex; Ma., Mansonia; An., Anopheles; Ar., Armigeres; Cq., Coquillettidia*

[2] Biting rates were computed according to mosquitoes captured between 0900 and 1700 h

[3] Biting rates were computed according to mosquitoes captured between 1900 and 2300 h

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Repellency of Volatile Oils from Plants against Three Mosquito Vectors

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Abstract

Volatile oils extracted by steam distillation from four plant species turmeric (*Curcuma longa*), kaffir lime (*Citrus hystrix*), citronella grass (*Cymbopogon winterianus*) and hairy basil (*Ocimum americanum*)), were evaluated in mosquito cages and in a large room for their repellency effects against three mosquito vectors, *Aedes aegypti*, *Anopheles dirus* and *Culex quinquefasciatus*. The oils from turmeric, citronella grass and hairy basil, especially with the addition of 5% vanillin, repelled the three species under cage conditions for up to eight hours. The oil from kaffir lime alone, as well as with 5% vanillin added, was effective for up to three hours. With regard to the standard repellent, deet alone provided protection for at least eight hours against *Ae. aegypti* and *Cx. quinquefasciatus*, but for six hours against *An. dirus*. However, deet with the addition of 5% vanillin gave protection against the three mosquito species for at least eight hours. The results of large room evaluations confirmed the responses for each repellent treatment obtained under cage conditions. This study demonstrates the potential of volatile oils extracted from turmeric, citronella grass and hairy basil as topical repellents against both day-and night-biting mosquitoes. The three volatile oils can be formulated with vanillin as mosquito repellents in various forms to replace deet (*N,N*-diethyl-3methylbenzamide), the most common chemical repellent currently available.

Keywords

Repellents, plant volatile oils, deet, mosquitoes

Introduction

Over two billion people, primarily in tropical countries, are at risk from mosquito-borne diseases, such as dengue hemorrhagic fever, malaria and filariasis (Service 1993). The search for effective vaccines against these diseases is still in progress. Mosquito control and personal protection from mosquito bites are currently the most important measures to control these diseases. The use of repellents is an obvious practical and economical means of preventing the transmission of these diseases to humans. The most common mosquito repellent formulations available on the market contain deet (*N*,*N*-diethyl-3-methylbenzamide), which has shown excellent repellency against mosquitoes and other biting insects (Yap 1986, Coleman et al. 1993, Walker et al. 1996). However, human toxicity reactions after the applications of deet vary from mild to severe (e.g., Zadikoff 1979, Robbins and Cherniack 1986, Edwards and Johnson 1987, Qiu et al. 1998). To avoid these adverse effects, research on repellents that are derived from plant extracts to replace deet has been conducted in many laboratories. Recently, extracts of several plants, including neem (*Azadirachta indica* A. Juss), basil (*Ocimum basilicum* L., *O. basilicum* L. fa. *citratum* Bach, *O. gratissimum* L., *O. americanum* L., *O. tenuiflorum* L.) citronella grass (*Cymbopogon nardus* Rendle), galingale (*Alpinia galanga* L.), clove (*Syzyaium aromaticum* L.) and thyme (*Thymus vulgaris* L.), have been studied as possible mosquito repellents (Sharma et al. 1993, Chokechaijaroenporn et al. 1994, Suwonderd and Tantrarongroj 1994, Boonyabancha et al. 1997, Barnard 1999). These natural repellents have demonstrated good efficacy against some mosquito species but some were evaluated only by olfactometry or by using laboratory mice as hosts of *Aedes aegypti* (L.) under laboratory conditions. However, the evaluation of repellency should preferably be carried out using human subjects because laboratory animals may inadequately simulate the condition of human skin to which repellents will be eventually applied (WHO 1996).

This study investigates the repellency of volatile oils derived from four plant species against three mosquito vectors using human bait methods in mosquito cage and large room conditions. Also, the usefulness of the additive vanillin to increase the protection time of the oils was studied.

Materials and methods

Volatile oils

At least 20 kg of turmeric (*Curcuma longa* L.) rhizomes, kafir lime (*Citrus hystrix* DC.) leaves, citronella grass (*Cymbopogon winterianus* Jowitt) leaves, and hairy basil (*O. americanum*) leaves were extracted for volatile oils by steam distillation. One or two kg of fresh plant material at a time was 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 and allowed to boil until the distillation was completed. The distillate was collected in a separating funnel in which the aqueous portion was separated from the volatile oil. The water (lower) layer was slowly drawn off until only the oil layer remained. This procedure was repeated until at least 20 ml of oil had been recovered. The volatile oil was collected and kept in a stoppered cylinder at 4 °C until it was tested for mosquito repellency. For efficacy evaluation, each oil as well as deet was prepared in two formulations: 25% (v/v) in absolute ethanol with and without 5% vanillin.

Test mosquitoes

The mosquitoes used in this study were laboratory-reared female *Ae. aegypti* (dengue hemorrhagic fever vector), *Anopheles dirus* Peyton & Harison (malaria vector) and *Culex quinquefasciatus* Say (filariasis vector). These were reared according to the standard protocol of the Biology & Ecology Section, National Institute of Health, Thailand, and maintained in the insectary of the institute. Three to five-day-old females of these species were used for repellency tests.

Repellent test procedure

The repellency of the volatile oils was evaluated using the human-bait technique (Schreck and McGovern 1989, WHO 1996). The testing period lasted up to eight hours, depending on the efficacy. The timing of the tests depended on whether the target mosquitoes were day-or night-biters; *Ae. aegypti* was tested from 0800 h to 1600 h while *An. dirus* and *Cx. quinquefasciatus* were tested between 1800 h and 0200 h. Evaluations were carried out in a 6x6x3 m room, at 25-29 °C and relative humidity of 60-80%. An area 3x10 cm on each forearm of three human volunteers was marked out with a permanent marker.

Approximately 0.1 ml of test repellent was applied to the marked area of one forearm of each volunteer while the other forearm was treated with the same repellent with 5% vanillin added. As a blank control, a solution of 5% vanillin in ethanol was placed on one forearm of the some volunteer with the same process as the test repellents, whereas the other forearm was untreated. During the test, the forearm was covered by a paper sleeve with a hole corresponding to the marked area. Each volunteer put the test forearm in a mosquito cage (40x40x40 cm), containing 250 female mosquitoes (3-5 days old), for the first three minutes of every half-hour exposure. However, before the start of each exposure, the bare hand, used as control area of each volunteer, was exposed for up to 30 seconds. If at least two mosquitoes landed on or bit the hand, the repellency test was then continued. The test continued until as least two bites occurred in a three-minute period, or until a bite occurred and was followed by a confirmatory bite (second bite) in the following exposure period. The time between application of the repellents and the second successive bite was recorded as the protection time. Since two hours is the minimum protection time specified for mosquito repellents allowed to be sold in Thailand, repellents providing at least four hours of protection under mosquito cage conditions were then tested for efficacy under large room conditions.

Large room evaluations

The evaluations were conducted in a 6x6x3 m room that had a door and six glass-windows that were always closed during the tests. The room was lit with fluorescent lamps. Ten minutes before the start of each test, 250 avid female mosquitoes (3-5 days old) were released into the test room. To compare the data with the results from the mosquito cage, the same three volunteers were assigned to evaluate the volatile oils under the large room conditions. Assessment areas comprised each leg from knee to ankle, covering surface area of about 782-826 cm^2. Approximately 3 ml of the volatile oil were applied to the test area of one leg of each volunteer. The other leg was the control area. Immediately after oil application, the volunteers went into the test room and sat on chairs in a triangle 1.5 m from each other. Evaluation of the repellency was done by catching the mosquitoes that landed on or bit the assessment areas of the volunteers' legs. For the six hours of each repellent test, volunteers entered the room for 10 minutes each half-hour. Therefore, each test was based on 13 mosquito collections and 12 breaks. Volunterrs' positions were rotated

on each collection occasion to allow for any variation among the positions. All mosquitoes caught during each collection were released again into the room to maintain the same number as at the start. The tests for each volatile oil against each mosquito species were conducted on separate days. Each test was carried out for 6 hours and the timing of the test depended on the target mosquitoes, i.e., 1000-1600 h for *Ae. aegypti* and 1800-2400 h for *An. dirus* and *Cx. quinquefasciatus*.

Data analysis

The median protection time was used as a standard measure of the repellency of the volatile oils and deet against the three mosquito species in the laboratory. The repellency among the oils was compared using the Kruskal-Wallis one-way ANOVA. The effects of vanillin in prolonging the protection time of the repellents were analyzed using the Mann-Whitney U Test. Percentage repellency in the semi-field trial was calculated as follows (Sharma and Ansari 1994, Yap et al. 1998):

$$\% \text{ Repellency} = \frac{C - T}{C} \text{x } 100$$

Where *C* is the number of mosquitoes collected from control areas and *T* is the number collected from the treated areas of volunteers. The total numbers of mosquitoes caught during each exposure at all seat positions for the semi-field evaluation of each mosquito species was compared using a Kruskal-Wallis one-way ANOVA.

Results

The relative repellency of the four volatile oils and deet with and without vanillin against *Ae. aegypti*, *An. dirus* and *Cx. quinaquefasciatus* is shown in Figure 1. There were significant overall differences in repellency among the repellents against each of t he mosquito species ($P<0.01$). There was no repellency against the three mosquito species of the blank control (5% vanillin in ethanol); i.e., biting frequency did not differ from that on the control (untreated) arm. Among the four oils without vanillin, citronella and hairy basil provided repellency against *Ae. aegypti* for three hours while turmeric and kaffir lime gave only one hour (Figure 1A). However, vanillin significantly increased the repellency of these oils against *Ae. aegypti* ($P<0.05$). As a result, citronella and hairy basil with vanillin could repel *Ae. aegypti* for up to 6.5 h,

whereas turmeric and kaffir lime with vanillin had extended repellency to 4.5 and 3 hours respectively. Deet, with and without vanillin, provided repellency against *Ae. aegypti* for at least eight h.

For *An. dirus* (Figure 1B), turmeric oil alone showed outstanding efficacy among the test repellents without vanillin. In fact, turmeric oil provided protection for at least eight hours, whereas deet gave only six hours for this species. The other three oils all had a repellency of less than four hours. However, vanillin prolonged the repellency of citronella, hairy basil and deet to at least eight hours. Vanillin extended the repellency of kaffir lime from 0.5 to 1.5 hours. Again, vanillin significantly increased the repellency of the extracts against *An. dirus* ($P<0.05$).

In contrast with *An. dirus*, citronella and hairy basil oils without vanillin provided as good a repellency as did deet against *Cx. quinquefasciatus* of at least eight hours, whereas turmeric oil alone gave repellency for five hours and kaffir lime only 0.5 h (Figure 1C). With vanillin added, turmeric, citronella and hairy basil oils repelled *Cx. quinquefasciatus* for at least eight hours, but kaffir lime was extended to only 2.5 h. There was no significant difference in the effect of vanillin in prolonging the protection by the four oils against *Cx. quinquefasciatus* ($P>0.05$).

The results of repellency against the mosquitoes under large room conditions are given in Table 1. There were no bites by the mosquitoes for at least four hours after the application of all extracts. Citronella + vanillin demonstrated a repellency equivalent to the standard repellents, deet and deet + vanillin, with at least six hours complete protection against *Ae. aegypti*. Turmeric + vanillin and hairy basil + vanillin were less effective than the deet standard, with repellencies of about 60% and 85.7% six hours after application. In contrast, the four volatile oil formulations, turmeric, turmeric + vanillin, citronella + vanillin and hairy basil + vanillin, showed greater protection against *An.dirus* than did deet. In fact, the four formulations as well as deet + vanillin could completely repel the anopheline mosquito for at least six hours, whereas six hours after application deet alone gave repellency of about 58.3% (Table 1). All repellents demonstrated equally good repellency against *Cx. quinquefasciatus* that lasted for at least six hours after application.

The numbers of *Ae. aegypti*, *An. dirus* and *Cx. quinquefasciatus* biting on the control and treated areas are shown in Table 1. There were no significant

differences in the number of mosquitoes caught among the control groups for each mosquito species (P>0.05).

No skin irritation, hot sensations or rashes were observed on the arms and legs of the test volunteers treated with the volatile oils during five months of the study period or in the following three months, after which time observations ceased.

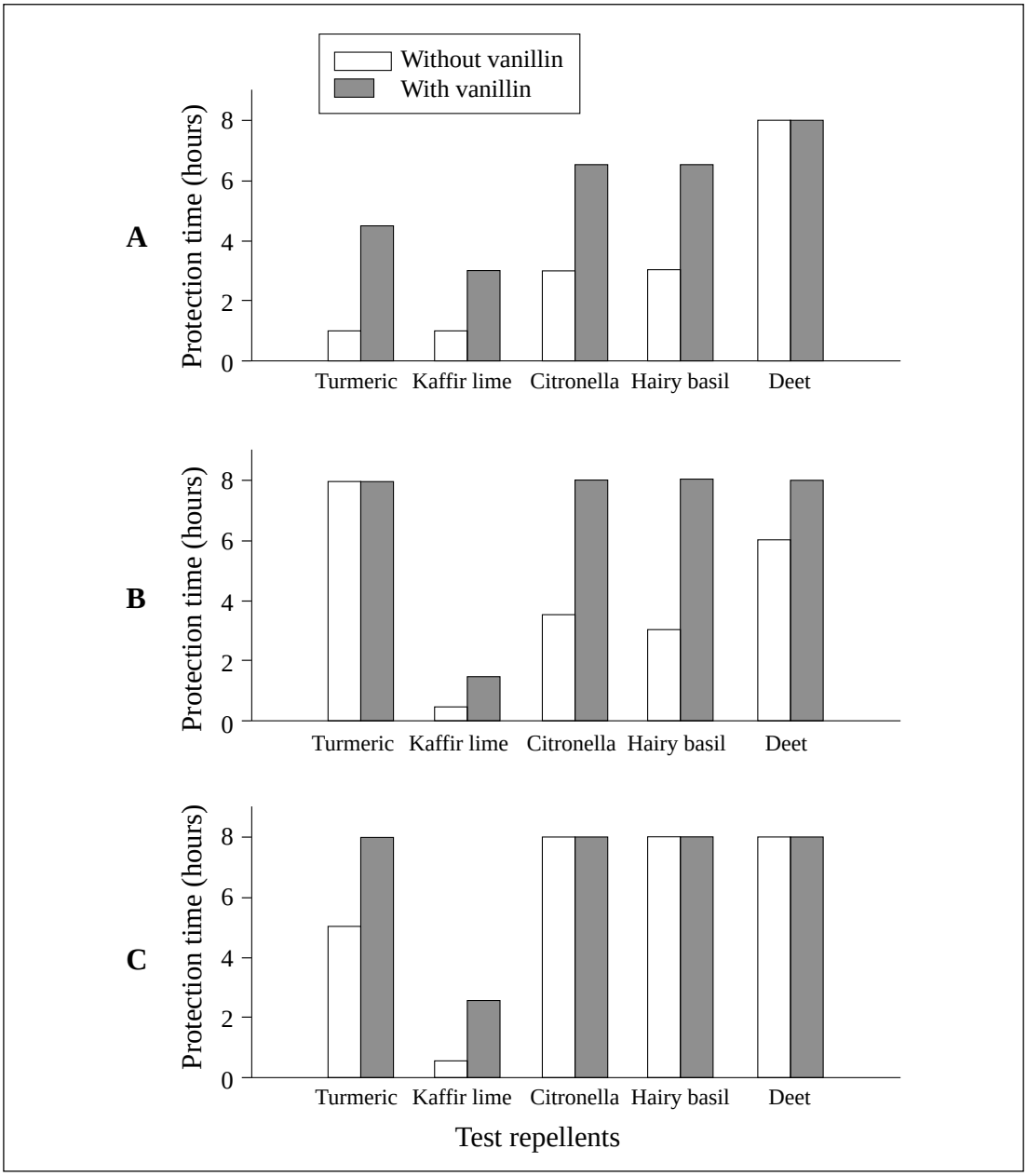

Figure 1. Relative repellency (median protection time) of volatile oils and deet against (A) *Ae. aegypti****, (B)*** *An. dirus****, and (C)*** *Cx. quinquefasciatus* ***under laboratory conditions.***

Table 1. Relative repellency of volatile oils and deet against three mosquito vectors under large room conditions.

Mosquito species	Test repellents	No. of mosquito bites (mean ± S.E.)		% Repellency after application				
		Control	Treated	4.0 h	4.5 h	5.0 h	5.5 h	6.0 h
Ae. aegypti	Turmeric + Vanillin	45.3 ± 2.7	1.2 ± 0.6	100	94.4	76.9	62.5	60
	Citronella + Vanillin	44.8 ± 4.2	0.0 ± 0.0	100	100	100	100	100
	Hairy basil + Vanillin	48.4 ± 2.8	0.3 ± 0.2	100	100	92.3	90.9	85.7
	Deet	38.2 ± 3.5	0.0 ± 0.0	100	100	100	100	100
	Deet + Vanillin	46.1 ± 5.0	0.0 ± 0.0	100	100	100	100	100
An. dirus	Turmeric	29.1 ± 1.7	0.0 ± 0.0	100	100	100	100	100
	Turmeric + Vanillin	35.5 ± 2.3	0.0 ± 0.0	100	100	100	100	100
	Citronella + Vanillin	36.1 ± 3.0	0.0 ± 0.0	100	100	100	100	100
	Hairy basil + Vanillin	33.0 ± 2.2	0.0 ± 0.0	100	100	100	100	100
	Deet	30.6 ± 2.5	0.6 ± 0.4	100	100	88.9	75	58.3
	Deet + Vanillin	34.1 ± 2.2	0.0 ± 0.0	100	100	100	100	100
Cx. quinquefasciatus	Turmeric	33.0 ± 1.9	0.0 ± 0.0	100	100	100	100	100
	Turmeric + Vanillin	26.8 ± 1.8	0.0 ± 0.0	100	100	100	100	100
	Citronella	27.4 ± 2.2	0.0 ± 0.0	100	100	100	100	100
	Citronella + Vanillin	28.6 ± 2.6	0.0 ± 0.0	100	100	100	100	100
	Hairy basil	30.3 ± 1.8	0.0 ± 0.0	100	100	100	100	100
	Hairy basil + Vanillin	31.1 ± 2.3	0.0 ± 0.0	100	100	100	100	100
	Deet	27.2 ± 1.8	0.0 ± 0.0	100	100	100	100	100
	Deet + Vanillin	33.2 ± 1.9	0.0 ± 0.0	100	100	100	100	100

Discussion

The volatile oils derived from turmeric, citronella grass and hairy basil, especially with 5% vanillin added, were very effective against the three mosquito species, and that from kaffir lime alone or with 5% vanillin added showed the least repellency. The results of large room evaluations clearly confirmed these results. The protection time of the four oils was significantly increased by the incorporation of 5% vanillin. These results agree with those of Khan et al. (1975) that vanillin could prolong protection time against *Ae. aegypti* by more than 100% in most cases. However, the volatile oil of kaffir lime is not suitable as a mosquito repellent because of its low repellency; only those of turmeric, citronella and hairy basil should be considered further as topical mosquito repellents. The results of this study are similar to those of

Jaruwichitratana et al. (1988), Wasuwat et al. (1990), Chokechaijaroenporn et al. (1994), Suwonderd and Tantrarongroj (1994) and Boonyabancha et al. (1997), but are potentially more useful in many respects, such as the method used (see below), length of assessmetn and a wider range of mosquito species tested. It is important to note that all those other studies were with citronella oil obtained from a different species of citronella grass (*Cy. nardus*). In fact, Jaruwichitratana et al. (1988) conducted an efficacy test on 14% citronella cream against *Culex* mosquitoes under field conditions for only one hour and showed that the cream could prevent at least 90% of mosquito attacks in thirteen out of twenty volunteers who applied enough cream (1.2 g or more per whole forearm). Wasuwat et al. (1990) demonstrated under laboratory conditions that repellency of a cream containing 14% citronella oil was about two hours against *Ae. aegypti*. On the other hand, Suwonkerd and Tantrarongfoj (1994) showed that a repellent cream containing less than 10% citronella oil provided only two hours protection against *An. minimus* Theobald mosquitoes under laboratory conditions while a 10% formulation could repel this species for at least four hours. Additionally, cream containing a combination of 2.5% citronella oil, 5% galingale oil and 0.5% vanillin cluld prevent biting by *An. minimus* for at least six hours. Unfortunately, this laboratory study was conducted during the day whereas *An. minimus* is a night-biter. In the field, the repellency of a cream containing a combination of 2.5% citronella oil, 5% galingale oil and 0.5% vanillin against *Cx. quinquefasciatus* was six hours from 1800 h to 2400 h.

In contrast to these previous studies, Boonyabancha et al. (1997) used a modified olfactometer incorporating laboratory mice and demonstrated that the EC95 concentrations against *Ae. aegypti* for citronella oil and hairy basil oil were approximately 5.3% and 1.5%. They also showed that at fifteen minutes post-application, a 1% concentration of those two oils provided about 75% and 90% protection against Ae.aegypti biting human arms during a single three-minute exposure under laboratory conditions. In contrast, again using mice, Chokechaijaroenporn et al. (1994) showed that the volatile oil obtained from hairy basil exhibited the least repellency among the oils from five *Ocimum* spp., being only fifteen minutes against *Ae. aegypti*, but those of *O. gratissimum*, *O. basilicum*, *O. basillicum* L. fa. *citratum* and *O. tenuiflorum*, were 135, 75, 75 and 105 minutes respectively. On the other hand, in a study in Guinea Bissau,

West Africa, fresh *O. canum* Sims (syn. *O. americanum*) could reduce biting by anopheline mosquitoes by about 63.6%, mostly *An. gambiae* Giles and *An. pharoensis* Theobald, under field conditions between 2000 h and 2200 h (Palsson and Jaenson 1999). There is clearly inconsistent repellency of volatile oils derived from *O. americanum* among the methods used for evaluation. It would therefore be very valuable to compare the repellency, by evaluations based on human skin, among the different plant species, such as citronella grass (*Cy. nardus* and *Cy. winterianus*), basil (*O. americanum, O. gratissimum*, *O. basilicum*, *O. bsilicum* fa. *citratum* and *O. tenuiflorum*).

This study could not describe the rearing details, e.g., soil, water, and nutritional conditions of the plant materials used for oil extraction because the study plants were purchased from the local market. However, it is important to obtain the most appropriate conditions for growing each plant in order to obtain the best yield, and further studies should emphasize this point. The quality of volatile oils depends on many factors, e.g., plant species, rearing conditions, maturation of harvested plants, plant storage, plant preparation and methods of extraction. Thus, these factors should be carefully considered and standardized when the extraction of volatile oils is being planned.

In conclusion, this study clearly demonstrated the potential of volatile oils derived from turmeric, citronella and hairy basil, for use as topical repellents against both diurnal and nocturnal mosquitoes. To improve their repellent efficacy, these three oils should be formulated with vanillin and could replace deet, currently the most common chemical repellent available. However, testing in the field will be necessary.

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Phytochemicals as Repellents against Mosquitoes in Thailand

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Abstract

Repellents are commonly used for personal protection against mosquitoes worldwide. They are one of the most effective products used in prevention and control of mosquito-borne tropical diseases. Although there are a number of effective repellents containing chemical active ingredients, such as deet, KBR 3023 and IR3535 there is in concern with regard to chemical toxicity. To overcome this concern phytochemicals extracted from various plants have been formulated as mosquito repellents to be sold in Thailand in recent years. Since the year 2000, 44 formulations of mosquito repellents containing plant extracts as active ingredients were evaluated for repellency against *Ae. aegypti* under laboratory conditions at the National Institute of Health (NIH), Thailand. These extracts included citronella oil, eucalyptus oil, tea tree oil, turmeric oil, bergamot oil, lavender extract, tobacco-leaves extract, clove extract and neem-leaves extract. The protection offered by these products was up to 6.3 hours. However, only 12 products were qualified for registration to be sold in the market since minimum protection time of 2 hours is the minimum in requirement. These qualified repellent products were formulated as lotion, spray, cream and balm, where citronella oil, eucalyptus oil and tea tree oil were the main active ingredients. On the other hand, the NIH also formulated a mosquito repellent containing turmeric oil and eucalyptus oil as active ingredients. It was found that this repellent provided protection time for 7 hours against *Ae. aegypti*, and at least 8 hours against *Culex quinquefasciatus* and *Anopheles dirus* under laboratory conditions. This study demonstrated and encouraged the development of alternative active ingredients derived from plants to be formulated as effective mosquito repellents.

Keywords

Repellents, mosquitoes, phytochemicals, biopesticides

Introduction

Repellents are commonly used for personal protection against mosquitoes. They are one of the most effective devices used in prevention and control of mosquito-borne diseases or for protection of mosquito bites. Although there are a number of effective mosquito repellents containing synthetic chemicals, such as deet (Smith 1953, Coleman et al. 1993), KBR 3023 (Yap et al. 1998) and IR 3535 (Thavara et al. 2001) which are currently available in the market, there is increasing concern with regard to their toxicity. Several researchers have reported adverse effects after uses of repellents containing deet, such as contact urticaria (Maibach and Johnson 1975), skin eruption (Yagupsky 1982) or toxic encephalopathy in children (Zadikoff 1979, Edwards and Johnson 1987). Although there is no report of any adverse effects of KBR 3023 and IR 3535 until now, the widespread use of these chemical repellents has been impeded by many people with the concern of possible effects similar to deet. In recent years attempts have then been placed in Thailand on extracting and evaluating extracts of plants for repellent activity against mosquitoes (Jaruwichitratana et al. 1988, Wasuwat et al. 1990, Chokechaijaroenporn et al. 1994, Suwonkerd and Tantrarongroj 1994, Boonyabancha et al. 1997, Tawatsin et al. 2001). Phytochemicals extracted from various plants, thus, have been formulated as mosquito repellent products for sale in Thailand. We initiated detailed studies on the evolution of plant-based products for use as mosquito repellents.

In this study we evaluated the repellency of mosquito repellent products containing plant extracts as active ingredients against mosquitoes in laboratory and under field conditions. The repellent formulation containing turmeric oil and eucalyptus oil was also developed by us and then evaluated under laboratory and field conditions.

Materials and methods

Test mosquitoes in laboratory

The mosquitoes used in this study were laboratory-reared female *Aedes aegypti* (L.), *Anopheles dirus* Peyton & Harrison, and *Culex quinquefasciatus* Say. The mosquitoes were bred and maintained according to the standard operation procedure of the Biology and Ecology Section, National Institute of Health (NIH), Thailand. In each test, 3-5 day-old female mosquitoes had no prior blood meal, were starved for 16 hours prior to testing.

Test repellents

The test repellents were the products proposed to be registered at the Food and Drug Administration (FDA) for sale in Thailand during the period from January 2000 to February 2002. Prior to registration, every product must be evaluated for repellency against *Ae. aegypti* at the NIH. A total of 44 repellent products containing plant extracts as active ingredients only were selected for this study. These extracts included citronella oil, eucalyptus oil, tea tree oil, turmeric oil, bergamot oil, lavender extract, tobacco-leaves extract, clove extract and neem-leaves extract. On the other hand, a repellent containing turmeric oil (5%) and eucalyptus oil (10%) as active ingredients with 5% vanillin added was formulated by us an evaluated for repellency in this study.

Laboratory evaluation

The test repellents were evaluated for repellency under laboratory conditions using human-bait method (Tawatsin et al. 2001, Thavara et al. 2001). The test period was up to 8 hours, depending on duration of repellency of each repellent. Our turmeric and eucalyptus repellent and the 44 other repellent products were tested for repellency against day biting mosquitoes, *Ae. aegypti*, from 0800 h to 1600 h. Moreover, our turmeric and eucalyptus oil repellent was tested against night biting mosquitoes, *Cx. quinquefasciatus* and *An. dirus* during the period between 1800 h and 0200 h. All evaluations were carried out in a 6x6x3 m^3 room, at 25-29 °C with relative humidity of about 60-80%. One of each forearm of three volunteers was marked out for an area of 3x10 cm^2 with a permanent marker. An amount of 0.1 ml of test repellent was applied to the marked area of one forearm of each volunteer. Prior to each exposure, the marked forearm was covered by a paper sleeve, having a rectangular hole corresponding to the marked area. Then, each volunteer inserted the marked forearm into a mosquito cage (40x40x40 cm), containing 250 female mosquitoes for three minutes every half-hour. The test continued until at least two bites occurred in a three-minute exposure period, or until a bite in the previous exposure period followed by the second bite in the following exposure period. Repellency or protection time of each repellent was the time between application of the test repellent and the second successive bite.

Field evaluation

Ten out of 44 repellent products and our repellent (5% turmeric oil and 10% eucalyptus oil with 5% vanillin) were evaluated for repellency against wild population of mosquitoes under field conditions. Among the 10 selected repellent products, half of them were chosen from qualified products (protection time 2.5-5.3 hours) and the rest were from unqualified ones (protection time 0.5-1.8 hours). Field evaluations were conducted by four volunteers (aged 25-57 years) at a research station located in Bang Bua Thong District, Nonthaburi Province. This place was chosen to be the study site as it had developmental sites for a variety of mosquito species. The evaluations were carried out at locations with minimal wind disturbance in an opened-building (6x24 m^2), which had only roof and there were some water jars placed in the building. Mosquitoes therefore could come from surrounding areas to feed on volunteers. Each volunteer was treated with 3 ml of the test repellent on one leg (from knee to ankle) whereas the other leg was left as control. The volunteers were positioned in a row, 5 m apart from each other and they caught all of the mosquitoes landing on or biting both their legs in the desired area (from knee to ankle) within a 10-minute period. Each mosquito collection was followed by a 20-minute break before the next collection was conducted again. As a result, there were two mosquito collections and two breaks during each hour of the test. The tests were carried out for 4 hours from 1800 h to 2200 h. The collected mosquitoes then were identified to species in laboratory. Repellency of each repellent was assessed through comparisons of mosquitoes collected on control (untreated) and treaded legs. The reduction in biting was calculated as follow (Yap et al. 1998):

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

Where *C* denotes the number of mosquitoes collected by the control group and *T* stands for the number collected on the treated ones.

Results and Discussion

Laboratory evaluation

The duration of repellency against *Ae. aegypti* under laboratory conditions obtained from 44 mosquito repellent products ranged from 0 to 6.3 hours, with an average of about 2 hours. However, only 12 products (27.3%) were qualified for registration at the FDA for marketing since minimum repellency of 2 hours

is required for labeling. The repellency of these qualified repellent products ranged from 2.5 hours to 6.3 hours (Table 1). These products were formulated as lotion, spray, cream and balm, whereas citronella oil, eucalyptus oil and tea tree oil were the main active ingredients. The concentrations of the 12 qualified products used were citronella oil 6-13%, whereas those of eucalyptus and tea tree oil were 10-15% and 5%, respectively. The repellency of these qualified products, containing citronella oil, eucalyptus oil and tea tree oil were 2.5-6.3 hours, 5.3-5.8 hours and 3 hours, respectively. However, some products consisting of citronella oil, eucalyptus oil and tea tree oil were also unqualified. It is interesting to note that citronella oil was the most common active ingredient used in both qualified (66.7%) and unqualified (65.6%) products. These imply clearly that repellency of each repellent product depends on not only main active ingredient but also other synergists added in each formulation. Moreover, the same concentration of the same active ingredient obtained from different plant sources could exhibit different repellency. The rest of the products in this study containing turmeric oil, bergamot oil, lavender extract, tobacco-leaves extract, clove extract and neem-leaves extract were found to be unqualified as they provided repellency against *Ae. aegypti* for less than 2 hours (data omitted).

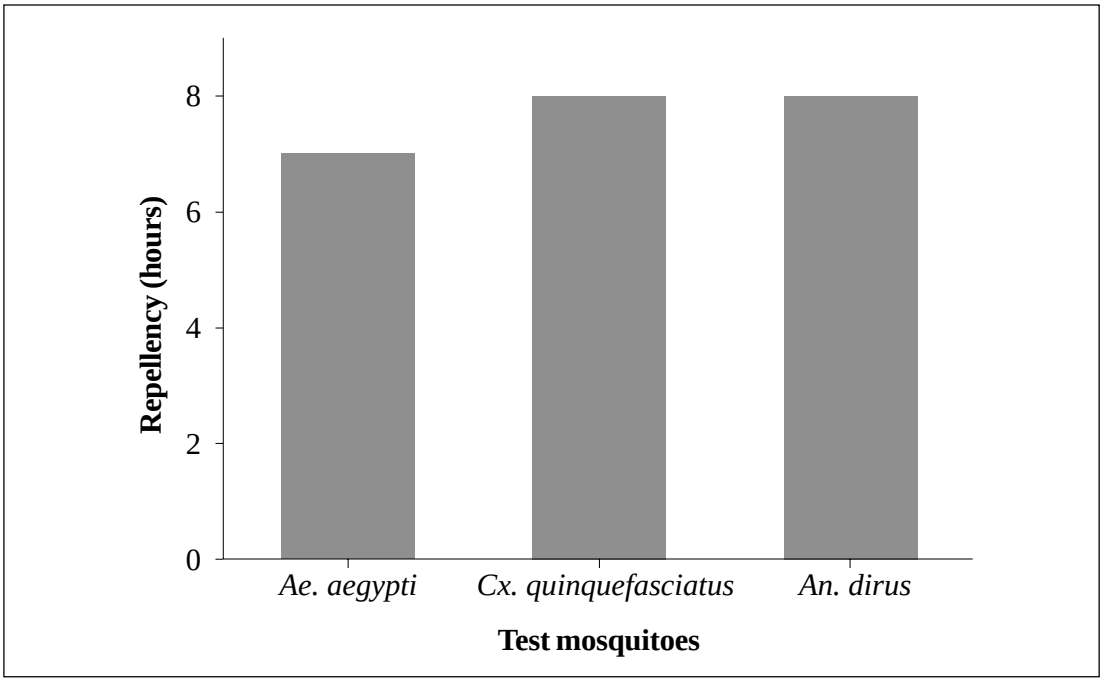

Figure 1. Repellency of repellent (turmeric oil 5% and eucalyptus oil 10%) against *Ae. aegypti, Cx. quinquefasciatus* ***and*** *An. dirus* ***under laboratory conditions.***

Figure 1. shows repellency of our repellent containing turmeric oil 5% and eucalyptus oil 10% with 5% vanillin added, against *Ae. aegypti*, *Cx. quinquefasciatus* and *An. dirus* under laboratory conditions. As can be seen, this repellent showed a high degree of repellency against both day- and night-biting mosquitoes as it provided protection time for 7 hours against *Ae. aegypti*, and at least 8 hours against both *Cx. quinquefasciatus* and *An. dirus*. In comparison with the repellent (20% turmeric oil with 5% vanillin added) as described by Tawatsin et al. (2001), our present repellent composition shows equal repellency against *Cx. quinquefasciatus* and *An. dirus*, but better repellency against *Ae. aegypti*. Previously, we also evaluated the repellency of our repellents consisting of each single oil (i.e. turmeric oil and eucalyptus oil) with different concentrations from 5% to 10% (with 5% vanillin added). However, all of them showed repellency against *Ae. aegypti* for up to 4 hours only. With the same concentrations, turmeric oil provided longer protection time than did eucalyptus oil, but turmeric oil was approximately four times more expensive than eucalyptus oil. To minimize repellent cost, an idea of combinations of both oils with various concentrations then was carried out to study for extended repellency. Finally, the combination of turmeric oil 5% and eucalyptus oil 10% was the most cost-effective formulation, which provided a high degree of repellency against the mosquitoes as mentioned above. This suggests that the eucalyptus oil played an important role as a synergist for the extended repellency.

Field evaluation

The results of 10 selected repellent products and our repellent (turmeric oil 5% and eucalyptus oil 10%) that were evaluated under field conditions are shown in Table 1. The relative repellency is demonstrated in terms of biting reduction. The qualified products substantially provided substantial protection where average biting reduction, ranged from 90.6% to 100%, whereas those of the unqualified products were between 58.2% and 99.4%. It is interesting to note that two qualified repellent products, containing citronella oil 13% and eucalyptus 15% and our repellent (5% turmeric oil and 10% eucalyptus oil with 5% vanillin) completely prevented biting by mosquitoes (Table 1). Actually, these repellents provided protection time against *Ae. aegypti* in laboratory for 4.5, 5.3 and 7 hours, respectively. This indicates that a repellent, which possesses a high degree of repellency against *Ae. aegypti* under laboratory

conditions could also prevent biting of night biting mosquitoes. However, some of the unqualified products also provided a high degree of biting reduction with average ranging from 87.3% to 99.4%, except the product containing eucalyptus 15% provided 58.2% reduction only. In other words, the unqualified repellents that failed to prevent biting of *Ae. aegypti* under laboratory conditions were able to substantially reduce biting of night biting mosquitoes. This may be explained by two possibilities: *Ae. aegypti* is more aggressive than the night biting mosquitoes or *Ae. aegypti* is less sensitive to the phytochemicals than are the night biting mosquitoes.

Table 1. Biting reduction of test repellents that were evaluated under field conditions at Bang Bua Thong District, Nonthaburi Province, during the period between March 30, 2002 and April 18, 2002 (from 1800 h to 2200 h).

Active ingredient (%)	Lab. Repellency* (hours)	Biting reduction field (%)		Total mosquitoes Caught	Predominant species (%)
		Average	Range		
Qualified products					
Citronella oil 6	3.8	98.6	96.3-100	328	*Cx. vishnui* (73), *Cx. gelidus* (12), *Cx. quinquefasciatus* (7), *Ma. uniformis* (4), *Ma. indiana* (3)
Citronella oil 10	2.5	97.3	95.6-100	295	*Cx. vishnui* (42), *Cx. gelidus* (31), *Cx. quinquefasciatus* (14), *Cx. tritaeniorhynchus* (9), *Ma. annulifera* (2)
Citronella oil 13	4.5	100	100-100	237	*Cx. gelidus* (51), *Cx. quinquefasciatus* (19), *Cx. vishnui* (15), *Ma. indiana* (8), *Ma. uniformis* (5)
Eucalyptus oil 15	5.3	100	100-100	272	*Cx. vishnui* (38), *Cx. quinquefasciatus* (29), *Cx. gelidus* (15), *Cx. tritaeniorhynchus* (8), *Ma. uniformis* (4), *Ma. annulifera* (3)
Tea tree oil 5	3.0	93	88.9-100	353	*Cx. quinquefasciatus* (32), *Cx. gelidus* (26), *Cx. vishnui* (25), *Ma. indiana* (9), *Cx. tritaeniorhynchus* (4), *Ma. annulifera* (3)

Table 1. Biting reduction of test repellents that were evaluated under field conditions at Bang Bua Thong District, Nonthaburi Province, during the period between March 30, 2002 and April 18, 2002 (from 1800 h to 2200 h). (Cont.)

Active ingredient (%)	Lab. Repellency* (hours)	Biting reduction field (%)		Total mosquitoes Caught	Predominant species (%)
		Average	Range		
Unqualified products					
Citronella oil 5.6	0.5	96.3	86.7-100	245	*Cx. vishnui* (42), *Cx. gelidus* (31), *Cx. quinquefasciatus* (14), *Cx. tritaeniorhynchus* (9), *Ma. annulifera* (2)
Citronella oil 12	0.5	99.4	97.9-100	325	*Cx. gelidus* (45), *Cx. quinquefasciatus* (18), *Cx. vishnui* (17), *Ma. annulifera* (10), *Ma. indiana* (5), *An. barbirostris* (2)
Citronella oil 15	1.0	87.3	80-100	274	*Cx. vishnui* (38), *Cx. gelidus* (29), *Cx. quinquefasciatus* (15), *Cx. tritaeniorhynchus* (8), *Ma. uniformis* (4), *Ma. annulifera* (3)
Eucalyptus oil 15	1.8	58.2	40-100	259	*Cx. gelidus* (29), *Cx. quinquefasciatus* (25), *Cx. vishnui* (22), *Ma. annulifera* (9), *Ma. indiana* (7), *Ma. uniformis* (6)
Tea tree oil 5	1.5	92.9	71.4-100	314	*Cx. vishnui* (34), *Cx. gelidus* (25), *Cx. quinquefasciatus* (19), *Ma. uniformis* (11), *Ma. indiana* (6), *Cx. tritaeniorhynchus* (3)
Our repellent Turmeric oil 5 and eucalyptus oil 10	7.0	100	100-100	347	*Cx. quinquefasciatus* (41), *Cx. vishnui* (35), *Cx. gelidus* (12), *Ma. annulifera* (5), *Ma. indiana* (4), *Cx. tritaeniorhynchus* (2)

*Against *Ae. aegypti*

The mosquitoes collected from this study in the field evaluation included several species belonging to 3 predominant genera: *Culex, Mansonia* and *Anopheles*. These were *Cx. vishnui* Theobald, *Cx. gelidus* Theobald, *Cx. quinquefasciatus, Cx. tritaeniorhynchus* Giles, *Ma. annulifera* (Theobald), *Ma. indiana* (Edwards), *Ma. uniformis* (Theobald) and *An. barbirostris* Van der Wulp (Table 1.). Among these species, *Cx. vishnui, Cx. gelidus* and

Cx. quinquefasciatus were the most common ones collected. However, some other species, such as *Armigeres subalbatus* (Coquillett), *Ma. bonneae* (Edwards), *An. sundaicus* Rodenwaldt, *An. vagus* Donitz, *Ae. aegypti*, *Ae. albopictus* (Skuse) and *Cx. whitmorei* Giles were also collected in very small numbers. Regarding the mosquitoes captured on the treated areas of volunteers' legs, they were mostly *Ar. subalbatus*, *Cx. gelidus*, *Ma. annulifera*, *Ma. bonneae*, *Ae. aegypti* and *An. vagus*.

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Field Evaluation of Mosquito Coils Derived from Plants against Night-Biting Mosquitoes in Thailand

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Abstract

Nine plants namely greater galangale (*Alpinia galanga*), fingerroot (*Boesenbergia pandurata*), turmeric (*Curcuma longa*), cardamom (*Elettaria cardamomum*), neem (*Azadirachta indica*), Siamese cassia (*Cassia siamea*), citronella grass (*Cymbopogon nardus*), eucalyptus (*Eucalyptus citriodora*) and Siam weed (*Eupatorium odoratum*) were studies for their efficacy in reducing human-mosquito contact when used in mosquito coils. Each plant material was prepared as 25% in each mosquito coil formulation. Repellency of the 9 mosquito coil formulations and the blank coil against night biting mosquitoes were determined under field conditions in an urban area of Nonthaburi Province, Thailand. Evaluations were carried out by capturing landing-biting mosquitoes on human volunteers and were conducted from 1830 h to 2130 h. Results showed that mosquito coils provided protection against mosquitoes with a ranging from 50% to 71% reduction in biting activity while the blank coil consisting of inert materials only reduced mosquito attacks by about 43%. Mosquito coil containing leaves of citronella grass showed highest efficacy whereas that containing rhizomes of turmeric was least effective. Mosquitoes caught in this study included 12 species belonging to 5 genera (*Aedes, Anopheles, Armigeres, Culex and Mansonia*), but *Cx. vishnui, Culex gelidus and Cx. quinquefasciatus* were most predominant species.

Keywords

Mosquito coil, field evaluations, mosquitoes, Thailand, phytochemicals, biopesticides, personal protection

Introduction

Mosquitoes are important vectors of many tropical diseases, such as malaria, dengue haemorrhagic fever and filariasis (WHO 1996). As there is no effective vaccine currently available for these diseases, personal protection from mosquito bites may help to break the transmission and it avails a practical strategy for prevention and control of the diseases. Mosquito coil is one of the most common household insecticide products used for personal protection against mosquitoes in Asian countries (Yap et al. 1996, Mulla et al. 2001), since it is quite cheap to afford in comparison with other products containing insecticides, such as aerosols, liquid sprays, vaporizing mats. Moreover, it is easy to use anywhere without electricity requirement. Actually, mosquito coils are used to repel mosquitoes at night by people in low-income sectors in urban, suburban as well as rural areas. Since many people like to dine out, most of outdoor restaurants in Thailand use mosquito coils to reduce annoyance from mosquitoes for their customers. In Thailand, Mulla et al. (2001) pointed out that a large quantity of aerosols and mosquito are sold on the market, followed by liquid sprays and vaporizers. All of the mosquito coils registered and sold in Thailand contain solely synthetic pyrethroids, for example, d-allethrin, d-transallethrin and transfluthrin as active ingredients. These coils provide a high degree of reduction in numbers of host-seeking mosquitoes (Yap et al. 1990, 1996, Mulla et al. 2001). However, many people still dislike smell of the mosquito coils containing synthetic pyrethroids when they are burned, and these people also feel that the coils may be harmful for their health. Attempts have been made to find out new active ingredients, especially those derived from natural plants to replace the synthetic pyrethroids.

The present studies were carried out to evaluate repellency of mosquito coils derived from plants as well as commercial mosquito coil products containing transfluthrin and d-allethrin against night biting mosquitoes under field conditions in Thailand. The potential plants that expressed a high degree of repellency against mosquitoes were then recommended as new active ingredients for inclusion in mosquito coil formulations.

Materials and methods

Mosquito coil formulations

Five kilograms of each plant part: greater galangale (*Alpinia galanga* Stunz) rhizomes, fingerroot (*Bosenbergia pandurata* Holtt) rhizomes, turmeric (*Curcuma longa* Linn) rhizomes, cardamom (*Elettaria cardamomum* Maton) rhizomes, neem (*Azadirachta indica* A. Juss) leaves, Siamese cassia (*Cassia siamea* Lamk) leaves, citronella grass (*Cymbopogon nardus* Rendle) leaves, eucalyptus (*Eucalyptus citriodora* Hook) leaves and Siam weed (*Eupatorium odoratum* Linn) leaves were cut into small pieces, dried and ground as fine powder. Each plant powder was prepared as 25% (w/w) ingredient in each mosquito coil formulation, whereas the inert materials (75%) in all coil formulations were the same (i.e. wood powder, coconut shell powder, incense powder and starch). As a result, a total of 9 mosquito coil formulations derived from different plant powder as active ingredients were prepared for testing. Blank coils that consisted of inert materials only were also made to use as reference coil in all field evaluations.

On the other hand, three representative commercial mosquito-coil products from among those sold in the market were also evaluated for repellency under field conditions. These coils contained 0.2% (w/w) and 0.3% (w/w) d-allethrin (d 1-3-allyl-2-methyl-4-oxo-2-cyclopentenyl d-cistrans chrysanthemate) and 0.03% (w/w) transfluthrin (1-R-Trans)-(2,3,5,6-tetrafluorophenyl)-methylester3-(2,2-dichloroethenyl)-2,2-dimethyl-cyclopropane-carboxylic acid as active ingredients. The inert ingredients in these coils were wood powder, coconut shell powder, incense powder, malachite green and sodium benzoate.

Field evaluations

The field evaluations were conducted outdoor at a research station in Bang Bua Thong District of Nonthaburi Province, Thailand, at a place that was not too windy and mosquitoes were usually abundant. Meteorological parameters (i.e. temperature, humidity and rainfall) at the evaluation site were also measured and recorded during each evaluation. The 9 mosquito coil formulations were randomly divided into 3 groups and each group consisted of 3 formulations. Each field evaluation included 5 treatments, i.e. a group of 3 formulations, blank coil and control (no coil), and then 5 human volunteers were needed to participate in each trial. Thus, 3 field evaluations were carried

out for all 9 formulations, whereas blank coil and control were presented in every evaluation. The coils were burned for 5 minutes prior to the initiation of each evaluation. For evaluation the 5 volunteers sat in a row positioned 10-m away from each other and approximately 0.5-m away from the burning coils. Assessments were by catches of the mosquitoes that landed or bit the volunteers' legs bared from knee to foot within a 10-min period of collection using plastic vials with screwed caps. In order to compensate for variation in attractiveness for mosquitoes, each catcher was rotated clockwise for the position at every mosquito collection, whereas the test formulations, the blank coil and the control were fixed at the same position throughout the evaluation. Therefore, every catcher collected mosquitoes at each position for 3 times. In each evaluation, a total of 15 collections were made between 1830 h and 2130 h. The 3 commercial mosquito-coil products were also evaluated for field repellency together with a blank coil and control by the same procedure as described previously. Every mosquito coil formulation (i.e. 9 formulations derived from plant powder, and 3 commercial products) was evaluated in the field on 3 different nights. Thus, a total of 12 nights (9 nights for the formulations derived from plants and 3 nights for the commercial products) were spent for field evaluations in this study. The collected mosquitoes were brought back to the laboratory, counted and positively identified.

Data analysis

As the evaluations were carried out in many different nights, the numbers of mosquitoes captured from each formulation, blank coil and control in each 10-minute collection period were then corrected to be the adjusted numbers based on grand mean number of the blank coils obtained from every night. The adjusted numbers were subjected to square-root transformation before computing for mean numbers and analysis of variance. Percent protection from mosquito landing/biting or repellency was computed as compared to control by the following equation (Tawatsin et al. 2001):

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

Where *C* denotes the number of mosquitoes collected by the control group and *T* stands for the number collected on the treated group. All statistical analysis was conducted using a statistical analysis program, the SPSS Version 9.01.

Results and Discussions

The meteorological data recorded at the evaluation site were: temperature 27±2 °C, 84±5 % RH and no rainfall during the trial nights. Results showed that the 9 mosquito coil formulations containing plant powder as active ingredients provided protection against mosquitoes in the field in the range from 50% to 71% reduction in biting/landing, whereas the blank coil consisting of inert materials reduced mosquito attacks by about 43% (Table 1). Mosquito coil containing leaves of citronella grass showed the highest repellency whereas that containing rhizomes of turmeric was the least effective. It is interesting to note that volatile oils extracted from both plant parts (i.e. citronella leaves and turmeric rhizomes) were equally effective against *Anopheles dirus* Peyton & Harrison and *Culex quinquefasciatus* Say (at least 8 hours) and *Aedes aegypti* (L.) (4-6 hours) when formulated with vanillin as topical repellents at the same concentrations (Tawatsin et al. 2001). This implies that some plant parts are suitable for extraction or preparation for particular product formulations only. There was no significant difference in terms of mean numbers of mosquitoes caught per 10 minutes among the coils containing citronella grass, eucalyptus, Siamese cassia, greater galangale and neem, and each one provided more than 61% of repellency. These plants seem to be highly potential plants for mosquito coil formulaions; however, citronella grass and eucalyptus should be the main focus. In Thailand, citronella grass is usually cultivated for volatile oil industry, whereas eucalyptus is primarily grown for paper industry and basement construction and its leaves are usually discarded. This study indicates the usefulness of citronella grass and eucalyptus leaves for mosquito coil formulations. As for the other 3 potential plants, the Siamese cassia leaves are occasionally used as organic fertilizer, greater galangale rhizomes are an essential spice for famous Thai soups (Tom Yum Kung and Tom Kha Kai) whereas neem leaves are usually used as traditional insecticides by some Thai farmers. In addition, it is noteworthy that the mosquito coils containing active ingredients derived from citronella grass, eucalyptus, Siamese cassia, greater galangale and neem provided good smell when they were burned. This may be an advantage of these coils for public acceptance when they are produced as commercial products. According to the results obtained from this study, it is not recommended to use fingerroot, turmeric, cardamom and Siam weed for mosquito coil formulations since their repellency was lower than 60%. Especially, turmeric and cardamom were no more effective than the blank coil

($P > 0.05$). Except the Siam weed found throughout Thailand, the others (i.e. fingerroot, turmeric, cardamom) are common spices for Thai cuisine.

Repellency of commercial mosquito coils containing pyrethroid chemicals as active ingredients and blank coil is shown in Table 2. ANOVA test showed no significant difference in terms of mean number of mosquitoes caught among the 3 formulations that provided more than 84% repellency as compared to the control, whereas the blank coil provided had a repellency of about 40.8% only. There was also no significant difference between the blank coils tested in Table 1 and 2. The results of mosquito reduction obtained with the mosquito coils containing 0.2% and 0.3% d-allethrin in this study were fairly higher than those (0.19% and 0.28% d-allethrin coils) obtained by Yap et al. (1990) that yielded about 71% repellency. In contrast, the result of the coil containing 0.03% transfluthrin of this study was slightly lower than that (0.027% transfluthrin coil) obtained by Yap et al. (1996) who reported 92.5% reduction of mosquitoes landed. There are many factors that might influence these differences of repellency and they might be attributed to the differences of mosquito species and environments among different evaluation sites.

Table 1. Repellency of mosquito coils derived from plants and evaluated under field conditions, at Bang Bua Thong District, Nonthaburi Province, Thailand in March and April 2002.

Active ingredient	No. of mosquitoes caught/10 min (mean ± S.E.)[1]	Repellency (%)
Control	26.2±3.7a	-
Blank coil	14.9±1.7b	43.1
Greater galangale	9.8±1.2cd	62.6
Fingerroot	10.9±2.4c	58.4
Turmeric	13.0±1.7bc	50.4
Cardamom	12.4±2.0bc	52.7
Neem	10.0±2.4cd	61.8
Siamese cassia	9.6±2.5cd	63.4
Citronella grass	7.6±1.2d	71.0
Eucalyptus	8.5±1.5d	67.6
Siam weed	10.8±1.8c	58.8

[1] Mean numbers followed by different letter is significant difference at the 0.05 level based on analysis of variance (ANOVA) and Duncan's Multiple Range Test.

Table 2. Repellency of commercial mosquito coils containing pyrethroids which were evaluated under field conditions, at Bang Bua Thong District, Nonthaburi Province, Thailand in April 2002.

Active ingredient	Mosquitoes caught/10 min (mean + S.E.)[1]	Repellency (%)
Control	24.5+2.9a	-
Blank coil	14.5+2.3b	40.8
Transfluthrin 0.03%	3.8+1.7c	84.5
d-allethrin 0.2%	3.5+1.1c	85.7
d-allethrin 0.3%	3.3+1.3c	86.5

[1] Mean numbers followed by different letter is significant difference at the 0.05 level based on analysis of variance (ANOVA) and Duncan's Multiple Range Test.

Our evaluations were carried out outdoors and several mosquito species, such as *Cx. gelidus* Theobald, *Cx. vishnui* Theobald, *Cx. quinquefasciatus* and *Mansonia indiana* Edwards were found in abundance, whereas those experiments of Yap et al. (1990, 1996) were conducted in living rooms of squatter residences and *Cx. quinquefasciatus* was the only predominant species (84-97%). This explanation may be confirmed by similar results obtained in Thailand by Mulla et al. (2001) who showed average reduction of landing-biting mosquitoes of 82% and 84% with Baygon coil (0.03% transfluthrin) and Swan-2 coil (0.2% d-allethrin), respectively, and the predominant attacking mosquitoes were *Cx. quinquefasciatus*, *Cx. gelidus*, *Ar. subalbatus* and *Cx. tritaeniorhynchus*.

The mosquitoes caught in this study included 12 species belonging to 5 genera: *Aedes*, *Anopheles*, *Armigeres*, *Culex* and *Mansonia*. These were *Ae. aegypti*, *Armigeres subalbatus* (Coquillett), *An. barbirostris* Van der Wulp, *An. sundaicus* Rodenwaldt, *An. vagus* Donitz, *Cx. gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus* Giles, *Cx. vishnui*, *Ma. annulifera* (Theobald), *Ma. indiana* and *Ma. uniformis* (Theobald) (Table 3 and 4). Among these species, *Cx. vishnui*, *Cx. gelidus* and *Cx. quinquefasciatus* were the most predominant ones. These mosquitoes are always abundant at the study site, as the surrounding areas are paddy fields, swamps with aquatic plants and some polluted-water accumulations that are suitable for their breeding. The numbers of *Ar. subalbatus*, *Cx. tritaeniorhynchus*, *Ma. uniformis*, *Ma. annulifera*, *Ma. indiana* and

An. barbirostris constituted small percentages (mostly less than 10%), but those of *Ae. aegypti, An. sundaicus* and *An. vagus* were insignificant. Regarding the field evaluations of commercial mosquito coils as shown in Table 4, it is interesting to note that *Cx. quinquefasciatus* was only the most predominant species among the mosquitoes collected from the coils containing pyrethroids (transfluthrin and d-allethrin). This confirms the results of Yap and Chung (1987) who demonstrated that *Cx. quinquefasciatus* is highly tolerant to pyrethroid-based mosquito coils in comparison with other common mosquito species in Malaysia.

Table 3. Species composition of mosquitoes caught from field evaluations (as shown in Table 1.) at Bang Bua Thong District, Nonthaburi Province, Thailand in March and April 2002.

Treatment	Total mosquitoes caught	Species composition (%)[1]
Control	3,537	*Cx. vishnui* (26), *Cx. gelidus* (24), *Cx. quinquefasciatus* (12), *Ma. uniformis* (11), *Cx. tritaeniorhynchus* (8), *Ma. annulifera* (7), *Ma. indiana* (5), *Ar. subalbatus* (4), *An. barbirostris* (1.8), *An. sundaicus* (0.6), *An. vagus* (0.4), *Ae. aegypti* (0.2)
Blank coil	2,012	*Cx. gelidus* (29), *Cx. vishnui* (23), *Cx. quinquefasciatus* (20), *Ma. uniformis* (9), *Ar. subalbatus* (7), *Cx. tritaeniorhynchus* (6), *Ma. indiana* (3), *An. barbirostris* (2), *Ma. annulifera* (1)
Greater galangale	441	*Cx. vishnui* (34), *Cx. gelidus* (21), *Cx. quinquefasciatus* (21), *Cx. tritaeniorhynchus* (10), *Ar. subalbatus* (9), *An. sundaicus* (3), *Ma. indiana* (1), *Ae. aegypti* (0.5), *An. vagus* (0.5)
(0.5) Fingerroot	491	*Cx. gelidus* (34), *Cx. vishnui* (28), *Cx. quinquefasciatus* (15), *Cx. tritaeniorhynchus* (6), *Ar. subalbatus* (5), *Ma. uniformis* (5), *Ma. annulifera* (4), *An. barbirostris* (2), *An. sundaicus* (1)
Turmeric	585	*Cx. vishnui* (41), *Cx. gelidus* (36), *Cx. quinquefasciatus* (12), *Cx. tritaeniorhynchus* (4), *Ar. subalbatus* (3), *Ma. uniformis* (2), *Ma. indiana* (1), *An. vagus* (1)

Table 3. Species composition of mosquitoes caught from field evaluations (as shown in Table 1.) at Bang Bua Thong District, Nonthaburi Province, Thailand in March and April 2002. (Cont.)

Treatment	Total mosquitoes caught	Species composition (%)[1]
Cardamom	558	*Cx. quinquefasciatus* (31), *Cx. vishnui* (25), *Cx. gelidus* (24), *Cx. tritaeniorhynchus* (10), *Ar. subalbatus* (6), *Ma. indiana* (2), *An. barbirostris* (1), *An. vagus* (1)
Neem	450	*Cx. gelidus* (41), *Cx. vishnui* (24), *Cx. quinquefasciatus* (14), *Cx. tritaeniorhynchus* (11), *Ma. uniformis* (6), *Ma. annulifera* (2), *Ma. indiana* (2)
Siamese cassia	432	*Cx. vishnui* (36), *Cx. quinquefasciatus* (29), *Cx. gelidus* (27), *Cx. tritaeniorhynchus* (3), *Ma. uniformis* (3), *Ar. subalbatus* (0.8), *An. barbirostris* (0.8), *Ae. aegypti* (0.4)
Citronella grass	342	*Cx. vishnui* (39), *Cx. gelidus* (34), *Cx. quinquefasciatus* (18), *Cx. tritaeniorhynchus* (4), *Ar. subalbatus* (2.5), *Ma. indiana* (1), *An. barbirostris* (1), *An. vagus* (0.5)
Eucalyptus	383	*Cx. gelidus* (28), *Cx. vishnui* (28), C*x. quinquefasciatus* (22), *Ar. subalbatus* (10), *Ma. annulifera* (4), *An. barbirostris* (2), *Cx. tritaeniorhynchus* (2), *Ma. indiana* (2), *An. sundaicus* (1), *Ma. uniformis* (1)
Siam weed	486	*Cx. gelidus* (40), *Cx. vishnui* (34), *Ar. subalbatus* (7), *Ma. annulifera* (6), *Cx. quinquefasciatus* (5), *Ma. uniformis* (4), *An. barbirostris* (3), *Cx. tritaeniorhynchus* (1)

[1] Average percentage of mosquitoes caught from 9 different nights (for control and blank coil) and 3 different nights (for the 9 formulations containing plant powder as active ingredients).

Conclusion

In conclusion, citronella grass and eucalyptus leaves are highly active ingredients to be used in mosquito coil formulations whereas Siamese cassia leaves, greater galangale rhizomes and neem leaves also have a good potential. However, it is suggested that these plant parts should be further studied for mosquito coil formulations, such as varying the concentrations used, or combinations of different plant parts with varying proportions. This may lead us to find out new effective active ingredients derived from plants to be used in mosquito coil formulations instead of synthetic pyrethroids.

Table 4. Species composition of mosquitoes caught from field evaluations (as shown in Table 2) at Bang Bua Thong District, Nonthaburi Province, Thailand in April 2002.

Treatment	Total mosquitoes caught	Species composition (%)
Control	1,103	*Cx. quinquefasciatus* (26), *Cx. gelidus* (21), *Cx. vishnui* (19), *Cx. tritaeniorhynchus* (8), *Ma. uniformis* (8), *Ar. subalbatus* (6), *An. barbirostris* (4), *Ma. annulifera* (4), *Ma. indiana* (3), *An. vagus* (1)
Blank coil	653	*Cx. gelidus* (31), *Cx. vishnui* (26), *Cx. quinquefasciatus* (18), *Cx. tritaeniorhynchus* (5), *Ma. indiana* (5), *Ma. uniformis* (5), *Ma. annulifera* (3), *Ar. subalbatus* (4), *An. sundaicus* (1.5), *An. barbirostris* (1), *An. vagus* (0.5)
Transfluthrin 0.03%	171	*Cx. quinquefasciatus* (29), *Cx. gelidus* (22), *Cx. vishnui* (16), *Cx. tritaeniorhynchus* (10), *Ma. uniformis* (10), *Ar. subalbatus* (8), *Ma. indiana* (4), *Ma. annulifera* (1)
d-allethrin 0.2%	158	*Cx. quinquefasciatus* (25), *Cx. vishnui* (22), *Cx. gelidus* (19), *Cx. tritaeniorhynchus* (12), *Ma. uniformis* (9), *Ma. annulifera* (6), *Ar. subalbatus* (3), *Ma. indiana* (2), *An. barbirostris* (1), *An. sundaicus* (0.5), *Ae. aegypti* (0.5)
d-allethrin 0.3%	149	*Cx. quinquefasciatus* (31), *Cx. vishnui* (28), *Cx. gelidus* (14), *Cx. tritaeniorhynchus* (11), *Ar. subalbatus* (7), *An. barbirostris* (3), *An. sundaicus* (2), *Ma. annulifera* (2), *Ma. uniformis* (1.5), *Ma. indiana* (0.5)

[1] Average percentage of mosquitoes caught from 3 different nights for all treatments.

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Mosquito Burden and Impact on the Poor: Measures and Costs for Personal Protection in some Communities in Thailand

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Abstract

To gauge the extent of mosquito problems and their impact on local people in Thailand, a simple questionnaire was designed consisting of 6 questions with multiple choices to be answered in 4 different communities in Thailand in 1998 and 1999. Mosquito biting activity was noted often by respondents. They reported that mosquitoes bit both night and day, and that the insects were abundant both in the dry and the rainy seasons. In all 4 communities, a large proportion of the residents used bed nets, mosquito coils, and aerosol sprays of personal protection: vaporizing mats and repellents were used sparingly. The cost of such measures amounted to $4 to $25 per year per household. For most of the residents, this represented a substantial proportion of their income, and was proportionally greater than the average cost of organized mosquito control in developed countries. This suggests that instituting organized local vector control programs would be cheaper and more effective than the individual use of personal protectants that do not reduce mosquito numbers. An assessment of the available products stocked in neighborhood stores and supermarkets for personal protection was made. A variety of insecticidal aerosols, mosquito coils, liquid sprays, vaporizing mats, and vaporizing liquids was stocked. This ample supply of household insecticides lends support to the preferred methods of protection reported by the respondents. The active ingredients in most of the formulations were synthetic pyrethroids, although a few contained dichlorvos, propoxur, and a few other compounds. Mosquito coils, the most preferred products used by the poor, were evaluated for efficacy, and were found to provide a reduction of 72-96% in landing-biting rates in controled experiments.

Keywords

Mosquitoes, personal protection, aerosols, insecticidal coils, costs of mosquito control, Thailand

Introduction

In the course of studies on the control of mosquito larvae in small communities in Nonthaburi Province of Thailand (Mulla et al. 1997, 1999), we found that all squatter communities (especially Soi Jumpa, Soi Raevadee, and Wat Pikul) harbored extremely heavy densities of mosquito larvae (primarily *Culex quinquefasciatus* Say) in standing water under and around the raised dwellings, as well as in polluted water canals (klongs) draining these water accumulations. Additionally, these communities were infested with *Aedes aegypti* (L.) breeding in water storage jars and tanks in and around the houses. As a result of heavy larval populations of *Cx. quinquefasciatus*, adults of this species were extremely abundant in and around the dwellings. Residents were attacked by hundreds of host-seeking *Cx. quinquefasciatus* at night, and black light traps placed 1 night a week inside or outside houses for about 8 months in 1999 and 2000 collected up to 3,000 or more *Cx. quinquefasciatus* per trap per night. Although larval treatments with microbial insecticides seemed to ameliorate the problem, the currently administered provincial mosquito control program for adult mosquitoes, consisting of monthly thermal fogging, did not seem to help.

In the communities where we conducted our studies monthly incomes were equivalent to $100-$300 per household. To gain relief from mosquitoes, the residents purchased and used a number of insecticidal products for personal protection. Because of the severe mosquito problem, we prepared a simple questionnaire to gain information on public perception of mosquito annoyance and the costs involved in purchase of chemicals and devices for personal protection.

Materials and methods

Questionnaire

The questionnaire was designed to seek information on the impact of adult mosquitoes on the local people and to gauge the spectrum of protective measures employed. The questionnaire was written in the Thai language. The

approach used with these questionnaires was different from that used by Morris and Clanton in Florida (1988, 1989). We sought to determine the type of measures available and routinely employed by the residents for personal protection. In addition to the questionnaire, we surveyed household insecticidal products labeled for personal protection from mosquitoes and stocked in small neighborhood stores as well as large modern supermarkets in several areas of Thailand.

Members of the households were personally interviewed by the entomology staff of the Department of Medical Sciences, National Institute of Health, Ministry of Public Health, in Nonthaburi. The surveyors either visited the residences or secured the information from storeowners or vendors. All information collected was through personal contact. The surveys were carried out in 4 different areas of Thailand, including 2 in Nonthaburi Province (central), 1 in Chiang Mai Province (northern), and 1 in Surat Thani Province (southern).

Questionnaires gained information on the number of residents (related or unrelated) per household, biting frequency and intensity of mosquitoes, mosquito host-biting diel cycles, and mosquito abundance and activity by seasons (dry and rainy). One question addressed measures used for personal protection such as bed nets, mosquito smoke coils, aerosols and sprays, and repellents. Another question was designed to gain information on the annual costs for residents for purchase of insecticidal products from local stores and markets. The questions did not include purchases of insecticides for other household insects.

Insecticidal products for personal protection

In order to determine the kinds of antimosquito products available for sale to the public, small neighborhood stores (results not reported) as well as small convenience stores (results not reported) and modern supermarkets were surveyed. Product surveys were carried out in 5 small or midrange neighborhood stores located in rural or semiurban areas. Additionally, 3 large modern supermarkets (results for 1 market reported) were visited in urban areas. The product surveys covered the central part of Thailand (Bangkok and Nonthaburi provinces) and 2 provinces in northern Thailand (Chiang Mai and Mae Hong Son). The products available at the time of survey were categorized into aerosols, mosquito coils, liquid sprays, and vaporizers.

Efficacy of mosquito coils

Because mosquito coils are the preferred antimosquito products used by low-income communities, we evaluated 3 representative commercial products from among those available to the public. These coils were Baygon (0.03% transfluthrin: Bayer Thai Co., Bangkok, Thailand), Swan 2 (0.2% D-allethrin: Swan Co., Ltd., Thailand), and Elephant 2 (0.15% esbiothrin: Fumakilla/ Technopia, Penang, Malaysia). The coils were tested outdoors in Soi Jumpa, Pak Kret District, Nonthaburi Province for 1 night each in September, October, and November of 1999. The test procedure consisted of capture of landing and biting mosquitoes on legs (bared from knee to ankle) of 4 volunteers. One individual served as a control and was not positioned close to a mosquito coil. The other 3 were seated 5 m away from one another and close (0.5 m) to 1 of 3 burning coils. A total of 12 collections were made, each collection for a 10-min duration each night from 1800 to 2100 h. The volunteers, including the control, were rotated clockwise after each collection time (10 min) and the individual coils were also rotated after every 4th collection. This experiment was designed to equalize positional effects and individual factors of attraction to mosquitoes.

The captured mosquitoes were brought into the laboratory, counted, and segregated into species. Percent protection from mosquito landing and biting was calculated for each treatment as compared to controls.

Results and Discussion

Mosquito abundance, impact, and protective measures

The questionnaire was presented orally to either the head or members of households and the responses to each question or statement were recorded. The same questions were presented to each respondent in the same order. Residents of different communities gave similar responses to the questions.

The residents in the 2 adjacent communities (Soi Raevadee and Soi Jumpa, Nonthaburi Province) in central Thailand provided similar responses with regard to mosquito annoyance and the use of measures for personal protection (Figure 1). In these communities, the number of residents per household was high, ranging from 4 to 12 individuals per dwelling. These communities had extremely high numbers of mosquitoes. The majority of residents stated that mosquitoes bit often or continuously. They also reported

that mosquitoes bit both day and night, a clear indication of the presence of *Ae. aegypti* and *Cx. quinquefasciatus*. The seasonal abundance of mosquitoes in these communities was perceived to be essentially the same in both rainy and dry seasons.

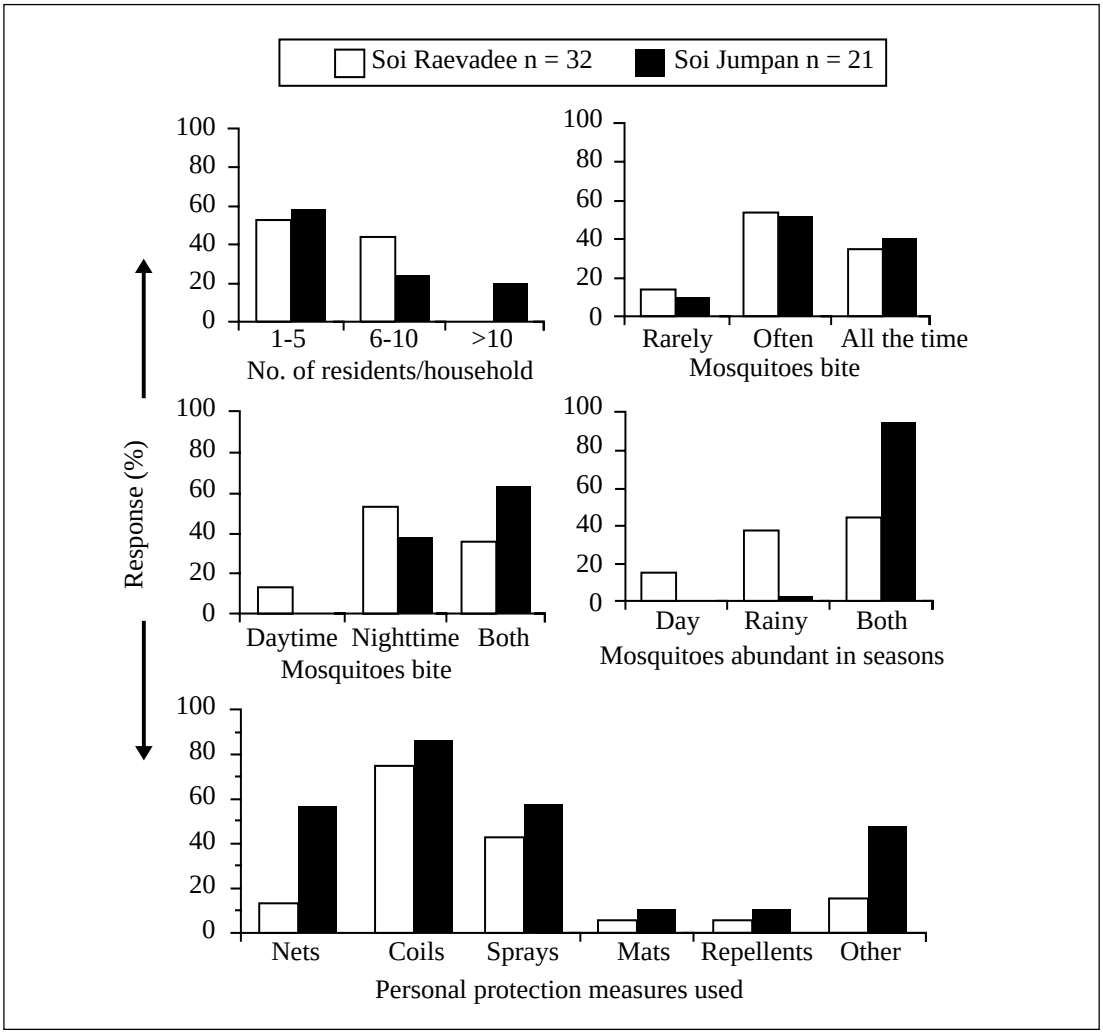

Figure 1. Mosquito abundance and annoyance levels, and protective measures used by residents in 2 low-income communities in Pak Kret District, Nonthaburi Province, in central Thailand based on responses to questionnaires in 1998 and 1999.

We trapped *Cx. quinquefasciatus* mosquitoes at night in 1999 and 2000 by placing black light traps inside or outside the dwellings. It was not uncommon to trap up to 3,000 or more male and female *Cx. quinquefasciatus* per night-trap, with no capture of *Ae. aegypti*, a species not attracted to this type of trap.

Another important aspect of the impact of mosquitoes and their importance as annoying pests to the residents was the widespread use of personal protection measures. Bed nets, aerosol preparations, sprayable liquid formulations, and mosquito coils were the most preferred measures used by the residents (Figure 1). Vaporizing mats (too expensive for low-income residents) and repellents (the types and quantities stocked in stores were limited) were used sparingly. Other measures, such as fans, screened windows, and skin oils, were used by some of the residents. A higher proportion of the residents in Soi Jumpa used bed nets than in Soi Raevadee; the former community is somewhat better off economically but had higher number of mosquitoes.

The same questionnaire and procedures were used in assessing the importance and impact of the biting and nuisance level of mosquitoes in 2 distantly located communities in northern and southern Thailand (Figure 2). In the Mae Tang District (Chiang Mai Province in the north) the proportion of households with 1-5 individuals per household was greater than that in Chaiya District (Surat Thani Province in the south) but the latter indicated more families with 6-10 individuals per household than the former. In both communities, the response to mosquito bites was similar. A majority of the respondents in both communities also reported that mosquitoes bit often and during both night and day. In these communities, mosquitoes were perceived to be more abundant in the rainy season. This may reflect the greater breeding of *Aedes albopictus* (Skuse) in rural areas during the rainy season. This is in contrast to the situation in Soi Raevadee and Soi Jumpa in central Thailand, where mosquito developmental sites are permanent and created by domestic wastewater, including sewage seepage into the canals and other standing water accumulations. These conditions are conducive to heavy production of *Cx. quinquefasciatus*. The rural areas in Mae Tang and Surat Thani are relatively free of organic pollution and therefore have lighter infestations of *Cx. quinquefasciatus*.

The products and measures used for personal protection were essentially the same in the Mae Tang and Chaiya districts (Figure 2) and similar to the use patterns in central Thailand. Bed nets, mosquito coils, and sprays (mostly aerosols) were the most preferred tools for personal protection.

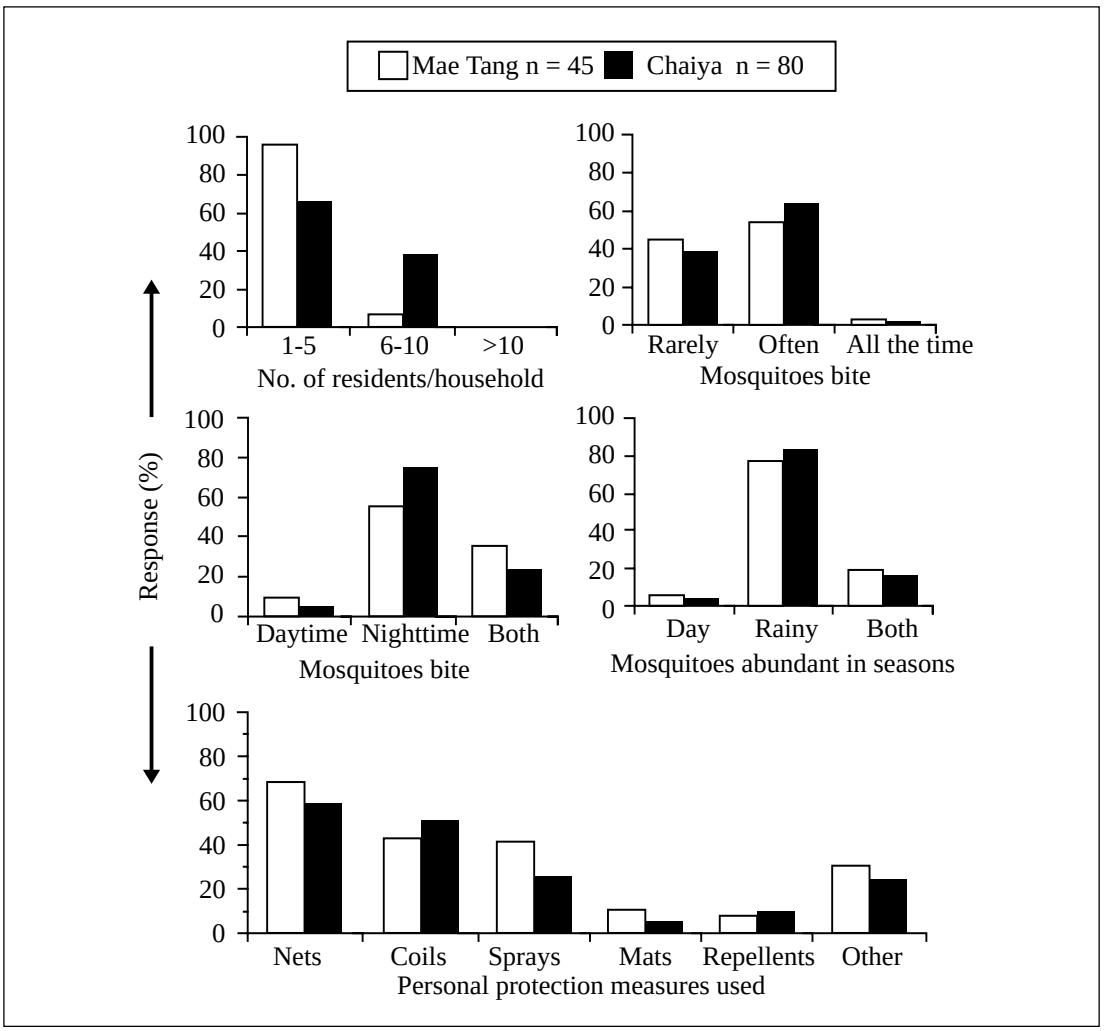

Figure 2. Mosquito abundance and annoyance levels and protective measures used by residents in 2 low-to moderate-income communties in Mae Tang District (Chiang Mai Provinec) and Chaiya District (Surat Thani Province) in northern and southern Thailand, respectively, based on responses to questionnaires in 1999.

Cost of personal protection

The questionnaire provided information on the costs to residents for products used for personal protection against mosquitoes. In Soi Raevadee and Soi Jumpa communities, with heavy populations of mosquitoes, the cost for most of the residents was very high (B500 to > B1,000 per residence per year [$12.50 to > $25]: Figure 3). In the Mae Tang and Chaiya areas, costs were somewhat lower (B100-B1,000 [$2.50-$25]). These expenditures for mosquito protective measures are much higher than the average costs of organized

mosquito control on a per residence basis in industrialized nations. For example, in southern California, where most of the counties are protected by organized mosquito and vector control district programs, the cost of comprehensive vector control is much cheaper per parcel or household than for poor sectors in Thailand. The cost per parcel averages about $2.25 in the Northwest Mosquito and Vector Control District in Riverside County of southern California (Major S. Dhillon, personal communication). In Orange County, the annual cost per parcel is about $5.00 per year (Robert D. Siogren, personal sommunication). The Greater Los Angeles County Vector Control District reported an average cost per parcel of $4.07 per year (Jack Hazelrigg, personal communication).

The cost of organized mosquito control in the Rhine River Valley is about DM2 (=$1.20) per person per annum (Norbert Becker, personal communication). The difference in costs for vector control programs in developed regions vis-à-vis poor communities in Thailand is likely true for most developing countries in the tropics. We conclude that the cost of instituting organized local vector control programs in Thailand would be lower than the aggregate costs borne by individuals, households and provincial programs in mosquito-infested communities.

Insecticidal products for personal protection

We prepared a list of the most commonly used aerosols, sprays, mosquito coils, and vaporizing products available with their chemical composition (active ingredients only). Table 1 lists the aerosol formulations available in Thailand for personal protection against mosquitoes. Six companies were the providers of these products. Most of the formulations we found contained 1 or more synthetic pyrethroids. Dichlorvos, an organophosphate, was the next most frequently encountered chemical in formulations, followed by propoxur, a carbamate insecticide. One formulation contained fenitrothion along with 2 other insecticides.

A similar survey was made for the most commonly available mosquito coils and vaporizers (Table 2). Ten compaines were involved in the manufacturing and distribution of coils. The formulations used in vaporizers (electric heating units) were either liquid or solid, and each type was dispensed with specially made electric vaporizers. The active ingredients in the mosquito coils and in the vaporizing units were solely synthetic pyrethroids.

In terms of ease of application, sustained release over night, and cost, the mosquito coils are the cheapest products available on the market. Most of the residents we talked to reported constant use of mosquito coils. The vaporizers were not used commonly by low-income communities because of the high cost of insecticides as well as the electric vaporizing units.

We also investigated the distribution, availability, and stocking patterns of antimosquito products in neighborhood stores in rural, subruban, and urban areas. Five small neighborhood stores were surveyed for the availability of mosquito protection products in rural and semiurban communities in Thailand (data omitted). The products stocked on the shelves depended on the size of the store. Even small corner stores, such as 1 in Soi Jumpa (of 5 such stores), stocked a wide range of aerosol and mosquito coil products. Midsize stores in rural areas such as in Mae Hong Son Province (data omitted) also stocked a wide variety of products.

We also made a survey of products stocked in 3 supermarkets in urban areas. We only report the data on product availability in 1 of the markets. In the Central Plaza Market in Bangkok, we surveyed only the aerosol products that dominated the supplies stocked. Additionally, we recorded the recommended uses of each product given on the label (Table 3). With the exception of 4 products, which were designated for ant, cockroach, and termite control, all were primarily labeled for mosquitoes. This trend was also noted in the other stores. In 90% of the product labels, mosquitoes were listed as the 1st group of target insects. Most of the products also listed other pests, including cockroaches, ants, flies, spiders, and other household invertebrate pests. In urban areas, the availability of mosquito protection products was determined in 2 of a large chain of supermarkets (Tops Super Market), 1 in Nonthaburi Province (central Thailand), and 1 in Chiang Mai Province in northern Thailand (data omitted). As in other large stores and markets, aerosol products dominated. The range of products stocked in each supermarket was essentially the same, although the number of aerosol products in the market in Chiang Mai was greater. Urban supermarkets apparently stock more aerosols than coils because the demand for coils in urban areas is lower than in rural or low-income communities.

Thailand offers a wide variety of products and formulations designed to reduce the impact of host-seeking mosquitoes. Although we did not assess the

prevalence of other household pests, in discussion with the residents, it became obvious that they rated mosquitoes very high in terms of annoyance and their out of pocket expenditures for purchase of household insecticides.

No published data are available on the use of mosquito protection chemicals and devices in Thailand or most of Southeast Asia. As to the use of commercial household insecticide products, in a study in neighboring Malaysia, Yap and Foo (1984) reported extensive use of aerosol products and mosquito coils. Oil-based liquid sprays and mosquito nets were used by a lower proportion of the households. In a 2nd study, Yap et al. (2000) reported that 70% of the households used some kind of control measure, but the use of household insecticides was the most preferred. The use of aerosol insecticides and mosquito coils was prominent. Vaporizing mats were used only by few of the households, which is also the case in Thailand. In Thailand, from the quantity and kinds of household insecticide products stocked in stores and supermarkets, we conclude that aerosols and mosquito coils are the 2 groups of products used in larger volumes than other products, with the coils primarily by the low-income sector.

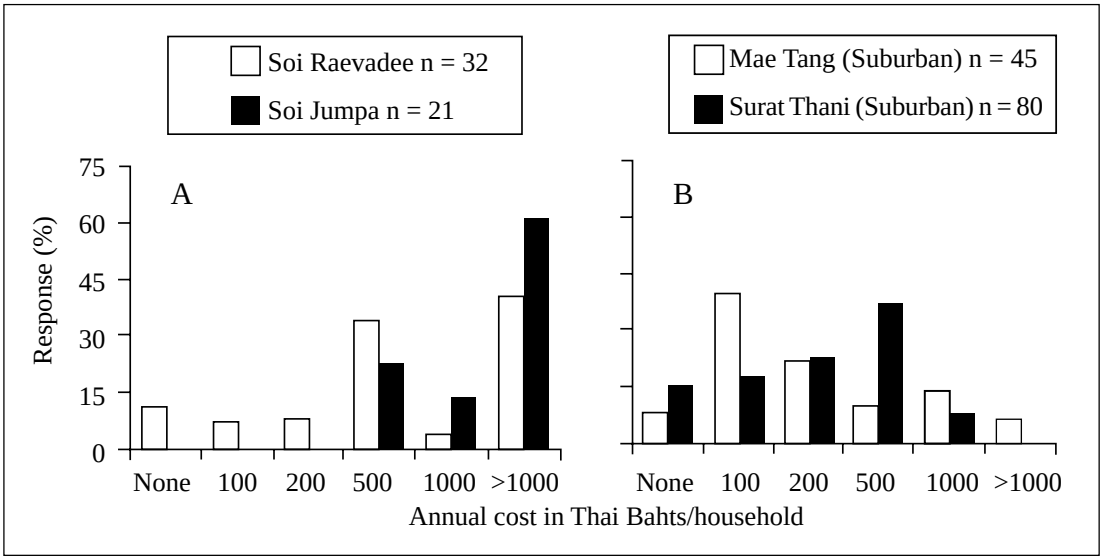

Figure 3. Annual cost for personal protection from mosquitoes based on responses to questionnaires in 1998 and 1999 by residents of Soi Raevadee and Soi Jumpa (A) and Mae Tang District and Surat Thani Province (B).

Table 1. Aerosol formulations available in Thailand for personal protection from mosquitoes (1999).

Manufacturer or distributor	Trad name[1]	Active ingredients %
ARS Chemical Co., Ltd. (Bangkok, Thailand)	ARS Gold OB	Tetramethrin 0.05, dichlorvos 0.50
	ARS 3 Red OB	Tetramethrin 0.20, permethrin 0.10, dichlorvos 0.50
	ARS WB	D-allethrin 0.06, D-tetramethrin 0.06, permethring 0.18
	ARS Mite OB	Cypermethrin 0.12, dichlorvos 0.50
	Household Insecticide	Deltamethrin 0.07, dichlorvos 0.50
Aswin Superman Co., Ltd. (Bangkok, Thailand)	Aswin Gold OB	Dichlorvos 0.50
	Aswin DPP OB	Dichlorvos 0.50, permethrin 0.10, D-allethrin 0.10
Bayer Thai Co., Ltd. (Bangkok, Thailand)	Baygon Blue WB	Cyfluthrin 0.025, transfluthrin 0.04
	Baygon Green 3 OB	Propoxur 0.50, cyfluthrin 0.025, dichlorvos 0.50
	Baygon Yellow 1 OB	Transfluthrin 0.03, dichlorvos 0.50
Cocksec Chemical Industry Co. (Bangkok, Thailand)	Kincho Blue WB	D-Tetramethrin 0.30, permethrin 0.04
	Kincho Green OB	Phthalthrin 0.05, fenitrothion 0.20, dichlorvos 0.50
	Kincho Orange WB	D-Tetramethrin 0.30, cyphenothrin 0.04
	Kincho Red WB	D-Tetramethrin 0.30, chphenothrin 0.09
S.C. Johnson Co., Ltd. (Bangkok, Thailand)	Raid WB	Prallethrin 0.045, permethrin 0.10, pyrethrins 0.05
	Raid Plus WB	Prallethrin 0.06, permethrin 0.20
	Raid Maxx OB	Propoxur 1.00, tetramethrin 0.20, orthophenylphenol 0.15
Reckitt and Colman Co., Ltd. (Slough, Berkshire,United Kingdom)	Shieldtox Blue WB	S-Bioallethrin 0.10, permethrin 0.15
	Shieldtox Green OB	Tetramethrin 0.23, deltamethrin 0.10
	Shieldtox Odourless 1 WB	Bioallethrin 0.241,bioresmethrin 0.046
	Shieldtox Odourless 2 WB	Permethrin 0.279, tetramethrin 0.138
	Shieldtox Odourless 3 OB	Prallethrin 0.0729, D-phenothrin 0.1003
	Shieldtox Yellow OB	S-Bioallethrin 0.10, permethrin 0.20, dichlorvos 0.50

[1] OB, oil based: WB, water based.

Table 2. Coils and vaporizing products available in Thailand for personal protection against mosquitoes (1999).

Manufacturer or distributor	Trade name	Active ingredients %
Mosquito coils		
ARS Chemical Co., Ltd.	ARS	D-Allethrin 0.20
Bayer Thai Co.,Ltd.	Baygon	Transfluthrin 0.03
Cocksec Chemical Industry Co., Ltd.	Kincho	D-Allethrin 0.25
	Elephant	D-Allethrin 0.3
Family Products (Malaysia)	Raid Black Coil 2	NA[1]
Fumakilla/Technopia (Penang Malaysia)	Elephant 1	D-Allethrin 0.30
	Elephant 2	Esbiothrin 0.15
Goose Limited	Product 3	Pynamine Forte 0.2
S.C. Jonhson Co., Ltd.	Raid Smokeless Coil	Esbiothrin 0.10
	Raid	D-Allethrin 0.20
Reckitt and Colman Co., Ltd.	Shieldtox	Esbiothrin 0.10
Sahasamakee Yin Hua Co., Ltd.	Three Goats	D-Allethrin 0.20
	Three Goats (stick)	D-Allethrin 0.125
Swan Co., Ltd. (Thailand)	Swan 1	Esbiothrin (Allethrin) 0.10
	Swan 2	D-Allethrin 0.20
Vaporizing products[2]		
ARS Chemical Co., Ltd.	ARS Mat	D-Allethrin 40 mg/mat
	ARS Liquid	D-Allethrin 2.8
Bayer Thai Co., Ltd.	Baygon Mat 50	D-Allethrin 50 mg/mat
S.C. Johnson Co., Ltd.	Raid 45 (2 in 1)	Esbiothrin 3.0 liquid,
		D-Allethrin 40 mg/mat
Reckitt and Colman Co., Ltd.	Shieldtox Mat	Prallethrin 10 mg/mat

[1] NA, not available.

[2] Electric heating units are available separately for both liquid and solid formulations.

Table 3. Household insecticide formulations (aerosols) available in a large supermarket (Central Plaza) in Bangkok, Thailand, for personal protection against household insects (1999).

Manufacturer or distributor	Aerosol insecticides [1]	
	Formulations [2]	Allowable uses
ARS Chemical Co.	ARS Household Insecticide	Mosquitoes, others
	ARS 3 Household Insecticide	Mosquitoes, others
	ARS Mite Household Insecticide	Ants, termites
	ARS Mosquito Killer WB	Mosquitoes
Bayer Co.	Baygon Blue 1	Mosquitoes, others
	Baygon Foam Spray	Ants, termites
	Baygon Green 3	Mosquitoes, others
	Baygon Yellow 1	Mosquitoes, others
Clearview Co. (Thailand)	Lemonene Insecticide	Mosquitoes
Cocksec Chemical Industry	Kineho Green	Mosquitoes, others
S.C. Johnson	Raid	Mosquitoes
	Raid Plus	Mosquitoes
Johnson Wax (Bangkok, Thailand)	Raid Maxx	Cockroaches, others
Reckitt and Coleman Overseas Ltd.	Shieldtox Odorless 1	Mosquitoes, others
	Shieldtox Water-based	Mosquitoes, others
	Shieldtox Yellow 1	Mosquitoes, others
Sherwood Chemical (Bangkok,Thailand)	Shelldrite 1	Ants, cockroaches, termites

[1] For composition, see Tables 1 and 2.

[2] Mosquito coils and vaporizers and repellents also stocked, but not listed here.

Efficacy of mosquito protectants

The effectiveness and duration of protection afforded by household insecticides is difficult to address. Most of the efficacy data have been gathered by the manufacturers and are not published. Some efficacy studies have been conducted in neighboring Malaysia. Yap and Chung (1987) conducted laboratory studies on the knockdown effects and mortality using mosquito coils containing D-allethrin and D-transallethrin. They showed that *Cx. quinquefasciatus* is less prone to knockdown effects than some other mosquito species, but had 62% mortality after 24 h. In a study inside houses in Malaysia, Yap (1988) evaluated aerosols, mosquito coils, electric vaporizing

mats, and oil-based liquids and determined the reduction in mosquito landing and biting rates over a 4-h period to be 53, 54, 70, and 18%, respectively.

We evaluated 3 representative commercial mosquito coils. The 3 mosquito coils showed similar trends in the reduction of landing-biting mosquitoes. Baygon coils (0.3% transfluthrin) showed a reduction of 77-85%. Swan 2 (0.2% D-allethrin), 72-96%, and Elephant 2 (0.15% esbiothrin), 78-93% (Table 4). In these studies, 6 species of mosquitoes were attracted to human hosts. The composition is given only for the control group, because the numbers of the less dominant species attacking humans under the protection of the coils was very low, ranging from 0 to 4 mosquitoes during the entire test period. In the control group, *Cx. quinquefasciatus* constituted 39-80% of the attacking mosquitoes. The other species attacking were *Culex gelidus* Theobald (11-30%), *Cx. tritaeniorhynchus* Giles (4-8%), *Armigeres subalbatus* (Coquillett) (0-12%), and *Mansonia dives* (Scheiner) (17% in December but 2.5-4.0% in the other 2 tests). Numbers of *Mansonia uniformis* (Theobald) and *Ae. aegypti* were insignificant.

Field studies on the efficacy of mosquito coils containing D-allethrin and D-transallethrin were carried out in living rooms in squatter housing projects in Malaysia (Yap et al. 1990). In this study *Cx. quinquefasciatus* constituted about 85% of the landing-biting mosquitoes. Four mosquito coil formulations tested indoors yielded 29-75% reduction in the landing-biting rate of mosquitoes. In another study using mosquito coils containing either transfluthrin or D-allethrin in houses in a squatter community, all formulations provided good protection (>90%) from biting mosquitoes over a 4-h period (Yap et al. 1996). On the basis of our studies and those in Malaysia, mosquito coils seem to be able to provide good protection from mosquito bites and annoyance if coils are placed relatively close to individuals to be protected.

Published data on the efficacy of aerosols, liquid sprays and vaporizing devices in field situations in Southeast Asia are scanty. However, analysis of our data shows that large quantites of aerosols and mosquito coils are used in Thailand, followed by liquid sprays and vaporizers. Mosquito repellents are used sparingly.

Table 4. Reduction in landing-biting rates of mosquitoes outdoors in the presence of 3 commercial mosquito coils.

Total mosquitoes[1] captured (percent reduction) in presence of coils					
		Type of antimosquito coil			
Date (1999)	Temperature (°C)	None (Control)	Baygon	Swan 2	Elephant 2
September 12	24.3	80 (—)	18 (77)	12 (85)	11 (86)
October 12	24.1	51 (—)	8 (84)	14 (72)	11 (78)
November 12	25.6	122 (—)	18 (85)	5 (96)	8 (93)

[1] In 12 collections (10 min each) from 1800 to 2100 h.

Acknowledgments

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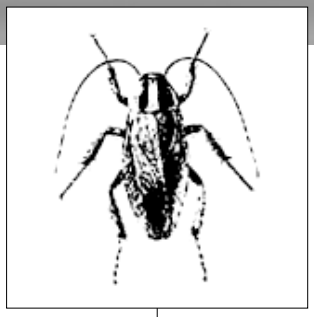
Cockroaches



Cockroach Surveys in 14 Provinces of Thailand

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Abstract

Cockroach surveys using sticky traps were conducted in urban areas of 14 Thailand provinces. At least 30 houses in each province were randomly sampled for cockroaches. Each house was trapped in three areas: kitchen, bedroom and outside. A total of 2,648 cockroaches was caught by 550 out of 1,542 traps (35.7%), from 337 of the 514 houses (65.6%). Overall, relative density ranged from 2.6 to 9.1 with an average of 5.2 cockroaches/house. On the average, 47.7% of the cockroaches were caught in the kitchen, 24.4% and 27.9% were caught in the bedroom and outside of dwellings, respectively. There were 10 species of cockroaches caught from the 14 provinces: *Periplaneta americana* (60.9%), *Periplaneta brunnea* (15.4%), *Neostylpyga rhombiofolia* (9.6%), *Periplaneta australasiae* (9.2%), *Pycnoscelis surinamensis* (3.3%), *Blattella germanica* (0.6%), *Periplaneta fuliginosa* (0.5%), *Supella longipalpa* (0.3%), *Blattella liturricllis* (0.15%) and *Nauphoeta cinerea* (0.05%), belonging to six genera. According to the surveys in this study, *Periplaneta americana* and *Periplaneta brunnea* were the most abundant cockroach species in urban Thailand, whereas the kitchen was the major habitat.

Keywords

Cockroach traps, *Periplaneta*, *Neostylopyga*, *Pycnoscelis*, *Blattella*, *Supella*, *Nauphoeta*.

Introduction

Cockroaches are capable of transmitting many pathogens, including bacteria, viruses, fungi, protozoa and pathogenic helminthes that threaten human health. They act as potential transmitters of agents of bacterial diarrhoea and nosocomial infections in hospitals (Agbodaze and Owusu 1989, Fotedar et al. 1991, Vythilingam et al. 1997). Additionally, there is evidence that substances produced by cockroaches are involved in allergic symptoms (Kongpanichkul

et al. 1997, Pumhirun et al. 1997). Hence, various control strategies should be implemented to suppress cockroach populations. The household agents for cockroach control (mostly aerosol sprays) usually contain chemical insecticides, e.g., pyrethroids (permethrin, tetramethrin, cypermethrin, prallethrin), carbamates (propoxur) and organophosphates (dichlorvos, chlorpyrifos). However, insecticide resistance, especially against pyrethroids, has already become commno among cockroaches (Atkinson et al. 1991, Hemingway et al. 1993). In addition, the use of insecticides has been hindered by a growing concern about possible effects on the environment and non-target organisms. Control strategies should therefore be redirected to emphasize the knowledge of the biology and ecology of the target cockroaches in addition to insecticide use, if any, and should be more selective and less environmentally polluting (WHO 1996).

This study evaluated two commercial cockroach traps commonly sold in Thailand under laboratory and field conditions. The most effective trap was then used in a nationwide cockroach survey. The survey provided information on the cockroach species, their major habitats and the prevalence of cockroaches found in Thailand. The information obtained would, therefore, be valuable for cockroach control and allergy management.

Materials and methods

Candidate Cockroach Traps

CANBIC® is an octagonal plastic box trap (10 cm in diameter), consisting of eight entrances (2.5x2.5 cm^2). Cockroaches enter the trap through a one-way door in front of each entrance. Ground peanuts were used as an attractant and a built-in toxic bait containing 2,2-dichlorovinyl dimethylphosphate (DDVP) (1% w/w) was provided inside each trap.

HOY HOY® is a sticky trap, which does not use any insecticides but uses a simple device that folds into a trapezoid paper-house (10x15x3 cm^3). The sticky area for catching cockroaches is about 9.5x15 cm^2. There are four entrances that cockroaches can use. Cockroaches were lured into the trap by built-in attractants and attached baits that were placed in the middle of the sticky area.

Laboratory Evaluations

Evaluations were carried out in a 70x70x70-cm^3 glass chamber against the American cockroach (*Periplaneta americana*) and the German cockroach (*Blattella germanica*). These cockroaches were colonized in the insectary of the Biology and Ecology Section, the National Institute of Health, Thailand. Adult stages of the American cockroach (8-month-old) and the German cockroach (2-month-old) were used in the study. One day before the test started, 30 adult cockroaches were introduced into the chamber with harborage, feeding source and drinking station. The chamber was covered outside with black paper to darken the inside. One day later, the test cockroach traps, i.e. CANBIC® and HOY HOY®, were placed in the chamber and left for 24 hours; the number of cockroaches caught in each trap were then recorded. The trial against each cockroach species comprised 12 replicates.

Field Evaluations

Evaluations were carried out in an urban area in Pak Kret district, Nonthaburi province of central Thailand. Thirty pairs of candidate cockroach traps, i.e. CANBIC® and HOY HOY®, were placed randomly in 30 houses and left for one night. The cockroaches caught in each trap were identified to species following the handbook of domiciliary cockroach species in Thailand (Asahina 1983) and other relevant references (i.e. Cornwell 1968, Bell 1981, Cochran 1982) and then counted.

Cockroach Surveys

As a result of the preliminary laboratory and field evaluations, HOY HOY® was then used in a nationwide cockroach survey. The survey was conducted in urban areas in 14 Thailand provinces: Chiang Mai, Chiang Rai, Mae Hong Son, Tak, Bangkok, Nonthaburi, Prachuap Khiri Khan, Ranong, Surat Thani, Phangnga, Phuket, Krabi, Trang and Songkhla. At least 30 houses in each province were randomly sampled for cockroaches; each house was trapped in three areas: kitchen, bedroom and outside dwelling. The traps were left for one night. The cockroaches caught in each trap were identified to species following the handbook of domiciliary cockroach species in Thailand (Asahina 1983) and other relevant references (i.e. Cornwell 1968, Bell 1981, Cochran 1982) and then counted.

Data Analysis

The efficacy of candidate cockroach traps was compared by Student's *t*-test according to the number of cockroaches caught under laboratory and field conditions. Number of cockroaches caught in various habitats were compared by a one way ANOVA; if statistical significance was observed, the mean number of cockroaches was then compared by Duncan's test. The number of cockroaches caught in each trap was transformed to $\sqrt{x+0.5}$ data prior to statistical comparisons. The accepted level of significance for all comparisons was $P \leq 0.05$. Analysis was carried out using the SPSS program for Windows version 9.0.

Results

In the laboratory, HOY HOY® caught significantly more American cockroaches (*P. americana*) and German cockroaches (*B. germanica*) than did CANBIC® ($P < 0.001$) (Figure 1). HOY HOY® also caught significantly more German cockroaches than American cockroaches ($P < 0.05$). In contrast, there was no significant difference in trapping the two species by CANBIC® ($P \leq 0.05$).

Under field conditions, HOY HOY® was still significantly more effective than CANBIC® ($P < 0.001$)(Table 1). The cockroaches caught by HOY HOY® from the field belonged to three species: *P. americana* (97.5%), *P. brunnea* (1.7%) and *P. australasiae* (0.8%), but those caught by CANBIC® were *P. americana* only.

Regarding the nationwide cockroach surveys carried out in the 14 Thailand provinces, a total of 2,648 cockroaches were caught by 550 of 1,542 traps (35.7%), from 337 of 514 houses (65.6%) (Table 2). Overall, relative density ranged from 2.6 (in Ranong) to 9.1 (in Prachuap Khiri Khan) with an average of 5.2 cockroaches/house. On the average, 47.7% of the cockroaches were caught in the kitchen, 24.4% and 27.9% were caught in the bedroom and outside dwellings, respectively. Outside dwellings were the major habitats of cockroaches in Bangkok (78.2%), whereas bedrooms were the major habitats of cockroaches in Tak (38.4%) and Ranong (37.9%). There were significant differences between the number of cockroaches caught in kitchens and those obtained from the other two habitats (i.e. bedrooms and outside dwellings) ($P < 0.05$). In contrast, the cockroaches caught in the bedrooms were not

significantly different than those caught from outside the dwellings ($P > 0.05$)

The 10 species of cockroaches caught from the 14 Thailand provinces were: *P. americana* (60.9%), *P. brunnea* (15.4%), *Neostylopyga rhombifolia* (9.6%), *P. australasiae* (9.2%), *Pycnoscelis surinamensis* (3.3%), *B. germanica* (0.6%), *P. fuliginosa* (0.5%), *Supella longipalpa* (0.3%), *B. lituricollis* (0.15%) and *Nauphoeta cinerea* (0.05%) belonging to six genera (Table 3). Seven species were found in Chiang Mai and Phuket, whereas only three species were obtained in Krabi and Ranong. According to the species composition in each province, *P. americana* was captured in all provinces and was the most abundant species found in Chiang Mai (60.2%), Nonthaburi (81.6%), Prachuap Khiri Khan (93.6%), Surat Thani (86.6%), Trang (31.7%), Krabi (83.3%), Phuket (80.4%), Ranong (92.6%) and Phangnga (48.3%). *P. brunnea* (51%) was found slightly more than *P. americana* (47.8%) in Bangkok. *P.australasiae* was most prevailing in Chiang Rai (48.4%), Tak (46.4%) and Songkhla (47.1%), whereas *N. rhombifolia* (56.9%) was most abundant in Mae Hong Son only. In this study, *P. fuliginosa, B. germanica , B. lituricollis, S. longipalpa* and *Na. cinerea* were uncommon species, consisting of less than 1% of the total cockroaches caught.

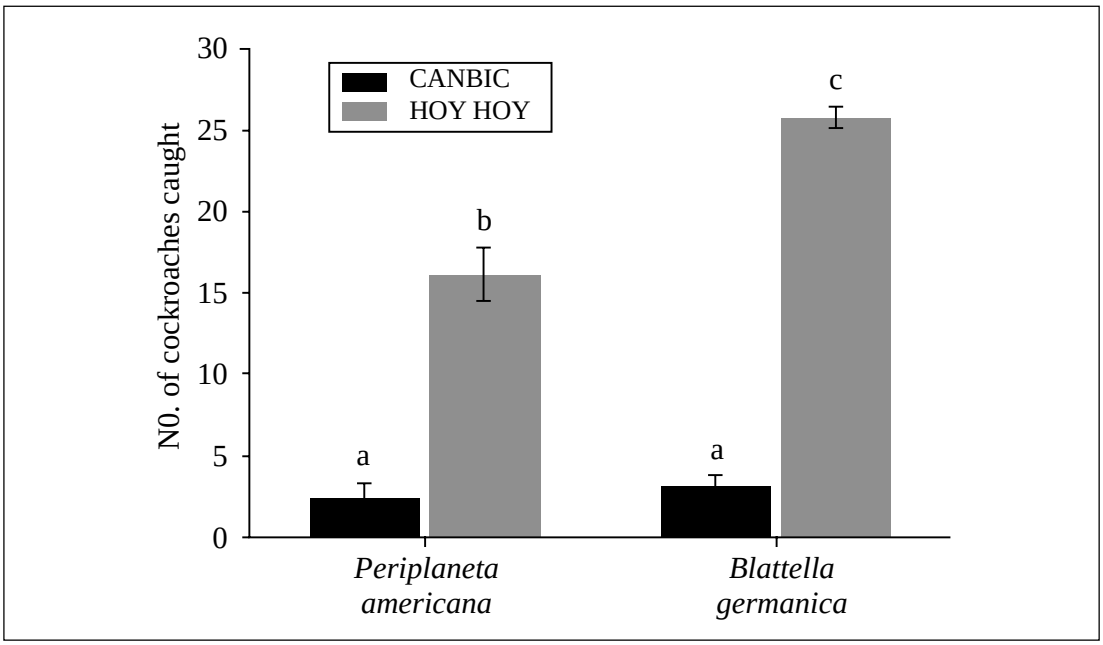

Figure 1. Mean(±S.E.) number of American and German cockroaches caught by CANBIC and HOY HOY traps under laboratory conditions. Different letters above the bars indicate significant differences using a t-test at the 0.001 level.

Table 1. Comparative efficacy of two cockroach traps under field conditions. A mean followed by a different letter is significantly different at the 0.001 level.

Trap	Total cockroaches caught	Mean ± S.E.	Species found
CANBIC®	6	0.2 ± 0.13 a	*P. americana (100%)*
HOY HOY®	351	11.7 ± 3.65 b	*P. americana* (97.5%), *P. brunnea* (1.7%), *P. australasiae* (0.8%)

Discussion

HOY HOY® showed better efficacy than CANBIC® for cockroach trapping under laboratory and field conditions. Its efficacy relies mainly on the built-in attractant and attached bait that effectively lures cockroaches. In the field, HOY HOY® could capture for up to 48 cockroaches/trap/night. Additionally, it never creates any insecticide-related problems, as it is an insecticide-free trap. It could, therefore, be and ideal effective measure for cockroach control. On the other hand, CANBIC® may be and alternative choice for cockroach control. However, it needs improvements on two important points: the attractant and the one-way doors. The ground peanut that was used as an attractant in CANBIC® was not attractive enough to lure cockroaches into the trap, and the small cockroaches (e.g. *B. germanica*, *B. lituricollis*, *S. longipalpa*, or young nymphs of other species) could escape from the trap after they entered and did not nibble at the toxic bait. After improvement of these two points, CANBIC® might be an effective device to control cockroaches.

The data in Table 2 show that cockroaches were common pests infesting homes throughout urban Thailand. The average of 64.1% infested houses with 5.2 cockroaches/house indicated that an important concern about cockroach problems should be raised. Kitchens were predominant habitats for cockroaches in most areas. This confirms the fact that cockroaches flourish where foods are readily available. As stated previously, these cockroaches can transmit diarrhea-causing bacteria (Agbodaze and Owusu 1989, Vythilingam et al. 1997). Bedrooms were the most predominant habitat of cockroaches in Tak and Ranong, but they were slightly more than those caught in the kitchen. Among the remaining provinces, except Bangkok, Mae Hong Son, Nonthaburi and Surat Thani, bedrooms were still second only to kitchens. Only in Bangkok

were cockroaches found in greater abundance outside dwellings than inside. This may be due to a huge number of long sewers throughout Bangkok. The sewer is a suitable environment for cockroaches as it provides plentiful food, darkness, warmth and moisture for them. However, the cockroaches could easily infest dwellings, especially in the kitchen through the sewers connecting to inside. Control measures should, therefore, be considered by homeowners on the major habitat of cockroaches in each particular place.

Table 2. Cockroach surveys carried out in 14 Thailand provinces and occurrence of cockroaches found in each province.

Provinces	Houses		Traps		Total cockroaches caught	Density (No./ house)	Habitats found (%)		
	Total	Positive (%)	Total	Positive (%)			Kitchen	Bedroom	Outside
Chiang Mai	42	78.6	126	42.1	226	5.4	39.8	37.4	22.8
Chiang Rai	40	67.5	120	38.3	122	3.1	48.4	22.2	29.4
Mae Hong Son	30	83.3	90	54.4	167	5.6	38.9	23.4	37.7
Tak	37	73.0	111	36.9	125	3.4	36.8	38.4	24.8
Bangkok	31	64.5	93	25.8	257	8.3	21.4	0.4	78.2
Nonthaburi	39	69.2	117	32.5	179	4.6	64.8	8.9	26.3
Prachuap Khiri Khan	37	64.9	111	46.9	337	9.1	56.1	25.5	18.4
Surat Thani	41	61.0	123	27.6	216	5.3	64.0	12.0	24.0
Songkhla	32	59.4	96	28.1	85	2.7	42.4	32.9	24.7
Trang	40	55.0	120	29.2	183	4.6	54.6	31.7	13.7
Krabi	36	75.0	108	37.0	239	6.6	50.2	31.4	18.4
Phuket	42	64.3	126	38.9	245	5.8	57.6	28.1	14.3
Ranong	36	41.7	108	24.1	95	2.6	33.7	37.9	28.4
Phangnga	31	61.3	93	38.7	172	5.6	44.8	29.7	25.5
Overall*	514	65.6	1,542	35.7	2,648	5.2	47.7[a]	24.4[b]	27.9[b]

*Percentage of overall cockroaches found in each habitat followed by same letter is not significantly different at the 0.05 level.

Ten species of cockroaches, belonging to six genera, were present in this study and *P. americana* and *P. brunnea* were the most common cockroaches in most provinces. In Malaysia, *P. americana* and *P. burnnea* were also reported as the most abundant species found in urban human dwellings (Vythilingam et al. 1997). Due to its prevalence, *P. americana* was the most important cockroach in Thailand. It was also found to be an important source of allergy among the

Table 3. Species composition of cockroaches found in each province.[1]

Provinces	No. of cockroaches found for each species(%)[2]									
	P. americana	*P. brunnea*	*P. australasiae*	*P. fuliginosa*	*N. rhombifolia*	*Py. surinamensis*	*B. germanica*	*B. lituricollis*	*S. longipalpa*	*Na. cinerea*
Chiang Mai	136 (60.2)	15 (6.6)	9 (4)	13 (5.7)	24 (10.6)	25 (11.1)	4 (1.8)	-	-	-
Chiang Rai	13 (10.7)	8 (6.6)	59 (48.4)	-	38 (33.1)	2 (1.6)	2 (1.6)	-	-	-
Mae Hong Son	1 (0.6)	63 (37.7)	-	-	95 (56.9)	-	-	-	8 (4.8)	-
Tak	37 (29.6)	-	58 (46.4)	-	30 (24.0)	-	-	-	-	-
Bangkok	123 (47.8)	131 (51)	-	-	2 (0.8)	-	-	1 (0.4)	-	-
Nonthaburi	146 (81.6)	21 (11.7)	2 (1.1)	-	2 (1.1)	8 (4.5)	-	-	-	-
Prachuap Khiri khan	316 (93.6)	4 (1.2)	1 (0.3)	-	16 (4.7)	-	-	-	-	-
Surat Thani	187 (86.6)	6 (2.8)	19 (8.8)	-	2 (0.9)	-	-	2 (0.9)	-	-
Songkhla	29 (34.1)	9 (10.6)	40 (47.1)	-	7 (8.2)	-	-	-	-	-
Trang	58 (31.7)	44 (24.1)	33 (18.0)	-	5 (2.7)	41 (22.4)	2 (1.1)	-	-	-
Krabi	199 (83.3)	38 (15.9)	-	-	-	2 (0.8)	-	-	-	-
Phuket	197 (80.4)	9 (3.7)	17 (6.9)	-	18 (7.4)	1 (0.4)	2 (0.8)	-	-	1 (0.4)
Ranong	88 (92.6)	6 (6.3)	-	-	-	1 (1.1)	-	-	-	-
Phangnga	83 (48.3)	54 (31.4)	6 (3.5)	-	16 (9.3)	8 (4.6)	5 (2.9)	-	-	-
Overall	1,613 (60.9)	408 (15.4)	244 (9.2)	13 (0.5%)	255 (9.6)	88 (3.3)	15 (0.6)	3 (0.15)	8 (0.3)	1 (0.05)

[1]*P=Periplaneta*; *N=Neostylopyga*; *Py=Pycnoscelis*; *B=Blattella*; *S=Supella*; *Na=Nauphoeta*.

[2]Percentage in each bracket based on total number of cockroaches caught in each province.

asthmatic Thai children and patients with allergic rhinitis (Kongpanichkul et al. 1997, Pumhirun et al. 1997). All of the species found in this study were also identified in the earlier studies in Thailand by Asahina and Hasegawa (1981), Asahina (1983), and Jungwiwattanaporn (1984), but there were differences in numbers of species detected. In fact, Asahina and Hasegawa (1981) found eight species (i.e. *P. americana, P. brunnea, P. australasiae, B. germanica, B. lituricollis, Py. surinamensis, N. rhombifolia* and *Na. cinerea*) of cockroaches surveyed in Chanthaburi province in eastern region of Thailand. However, two years later, Asahina (1983) published a handbook on domiciliary species of cockroaches in Thailand, based on the surveys conducted in Chanthaburi and Bangkok. This included 10 cockroach species and there were two more species (i.e. *S. longipalpa* and *Hebardina concinna*) than those detected in 1981. Jungwiwattanaporn (1984) surveyed domiciliary cockroaches in five provinces (i.e. Khon Kaen, Nong Khai, Nakhon Ratchasima, Ubon Ratchathani and Maha Sarakham) in the northeastern region of Thailand and discovered nine cockroach species similar to the present study, except she found *Py. indicus* instead of *Py. surinamensis*. It is noteworthy that *P. fuliginosa* was first detected and reported in Thailand from this survey. Therefore, at least 12 cockroach species can be found in Thailand.

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