

GERM WARFARE AGAINST MOSQUITOES. WHAT NOW?

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Abstract Mosquitoes and the diseases they transmit are still major public health concerns in many countries in the tropics and subtropics resulting in significant morbidity and mortality in man and animals. The use of chemical control agents is still the major forerunner in mosquito control strategies. Despite success achieved in chemical control, the use of chemicals has resulted in two major impacts, namely, environmental disruption and development of insecticide resistance in target mosquitoes. This dilemma has prompted the search for alternative control agents/methods. Among these, the use of bacterial control agents is the most effective and proven method that have been successfully replicated in many parts of the world. These bacterial agents, *Bacillus thuringiensis* and *Bacillus sphaericus* have now been used widely in various control programmes. Both bacteria are spore-forming rod-shaped bacilli and are known as crystal-forming bacteria as they produce the mosquito-killing toxins as crystals. *Bacillus thuringiensis* is known to consist of >70 serotypes but the most effective mosquitocidal serotype is H-14 or *Bacillus thuringiensis* var. *israelensis* (*Bti*). Similarly, *Bacillus sphaericus* (*Bs*) has a number of serotypes; the H-5 and H-25 serotypes exhibit the highest mosquitocidal activity. Both bacteria are only larvicidal and kill by the crystal toxin they produce. Essentially, the toxins are activated in the gut of the larvae by the specific proteolytic enzymes and alkaline pH and go on to destroy the gut cells resulting in septicæmia (blood-poisoning). Death occurs within 30 minutes at high dosages. Both agents are highly and specific selective only against larvae of mosquitoes. *Bti* is known to be toxic against all mosquito larvae, the most susceptible are *Aedes aegypti* and *Culex quinquefasciatus*, while *Bs* has a narrower spectrum in that it is most effective against *Culex* larvae. Both agents, however, are not harmful to all other non-target organisms. After >20 years of use, no resistance to *Bti* is reported, though *Culex* resistance to *Bs* is known. Today, these agents are formulated as aqueous suspension, wettable powder, water dispersible granules, tablets etc. to suit the application in the various breeding habitats of mosquitoes. Future research will focus on the development of more effective formulation and method of application, isolation of bacteria that exhibit higher mosquitocidal activity and combined use of bacterial agents and other control agents.

Key Words *Bacillus thuringiensis* H-14, *Bti*, *Bacillus sphaericus*, mosquito control

INTRODUCTION

In many Southeast Asian countries including Malaysia, vector-borne diseases especially malaria, filariasis, dengue fever and dengue haemorrhagic fever as well as Japanese encephalitis continue to plague the local populations both in the rural and urban areas. Sporadic outbreaks of these diseases occur repeatedly almost every year, sometimes reaching epidemic proportion with high mortality. All these important tropical diseases are mosquito-borne.

Globally, malaria risk of varying degrees exists in 100 countries with over 40% of the world's population living in areas with malaria risk. The incidence of malaria in the world remains at 300 – 500 million clinical cases annually, with 1.5 – 2.7 million deaths. The vast majority of malaria deaths occur among young children in tropical Africa. Dengue is prevalent in over 100 countries and threatens the health of more than 2.5 billion people, living in tropical and subtropical regions. Dengue is the cause of an estimated 500,000 hospitalizations each year, with some 24,000 deaths each year. Filariasis, most commonly recognised by the elephantiasis and male genital damage it causes, is endemic in at least 73 countries where 120 million people are infected.

Traditionally, the control of the disease-bearing vectors relies heavily on the extensive and intensive use of chemical insecticides. These chemicals are to certain extent quite successful in curbing the diseases concerned. However, the widespread use of chemicals is highly stressful on the environment accompanied with undesirable side-effects on the complex tropical ecosystem and may affect a wide spectrum of non-target organisms. Occasionally, accidental poisoning of human is also reported. Prolonged use of a particular chemical will also eventually lead to the development of insecticide resistance in the vector population (Lee et al., 1987).

To date, there have been no reported cases of resistance development towards mosquitocidal *B. thuringiensis* in the field. Mosquito resistance to *B. thuringiensis* H-14 in the field has not been reported, although it was possible to select for mosquito resistant to individual protein toxin in the laboratory (Goldman et al., 1986). However, such resistance to the intact parasporal inclusion is low. In view of the potential of mosquito to develop resistance to toxins of known microbial control agents, the need to screen novel agents and toxins is further justified (Kawalek et al., 1995).

In view of some of the serious side-effects of chemical agents used in vector control, interest in microbial control agents especially those effective against mosquito larvae has been revived since the discovery of highly mosquitocidal *Bacillus sphaericus* (Singer, 1974) and *B. thuringiensis* serotype H-14 (Goldberg and Margalit, 1977). Several advantages i.e., high larvicidal activity; non-toxic to non-target organisms; cost-effectiveness and long shelf life of these agents have enabled their widespread use especially *B. thuringiensis* H-14. In this region, the use of microbial agents for vector control is a relatively recent development, although such use in many other countries is commonplace. However, this particular important field is now the focus of study of several workers.

MICROBIAL CONTROL

Bacillus thuringiensis var. *israelensis*

The potential of *B. thuringiensis* as a microbial control agent was recognised since its discovery in 1902 by the Japanese scientist Ishiwata and later by Berliner in 1911 in Thuringia, Germany. The first isolate was designated *B. thuringiensis* var. *thuringiensis* and this particular variety was also the first insect microbial pathogen commercialised for the control of agricultural, forest and store product lepidopterous pests since the early 1950's. Since then, many other varieties of *B. thuringiensis* were isolated and produced. However, most of these isolates are highly active against several agricultural pests but only moderately active against mosquito larvae. In 1977, Goldberg and Margalit reported for the first time the isolation of a strain of *B. thuringiensis* from dead *Culex pipens* demonstrating exceptional high larvicidal activity against mosquitoes. This new serotype was subsequently designated as *B. thuringiensis* var *israelensis* (serotype H-14) or Bti. Since then, this new serotype is being studied intensively and commercialised.

Bacillus thuringiensis H-14 is an aerobic, gram positive, endospore- and crystal-forming bacterium in the family Bacillaceae. The toxic moiety of this serotype is the thermo-labile endotoxin (delta-endotoxin) resided in the parasporal inclusion or the so-called crystals which may be produced in different shapes and sizes during sporulation. Each bacterium may contain 1-3 of these proteinaceous crystals. The actual detail mode of killing action of the endotoxin on mosquito larva is far from clear, but the sequence of toxin activation is well-studied (WHO, 1979). When bacteria are ingested by mosquito larvae, the crystals are activated in the naturally alkaline environment of the larval midgut. The larval proteolytic enzymes then break down the endotoxin resulting in the release of a polypeptide fraction with killing activity. This fraction acts rapidly on the midgut cells causing them to swell, lyse and slough into the lumen of the gut; causing death to affected larvae. Death usually occurs rapidly within 6-30 minutes at high dosages and about 24 hours at lower bacterial concentrations. *B. thuringiensis* H-14 is known to be larvicidal to all species of mosquitoes, blackflies and chironomid larvae (WHO, 1979).

Recently more information on the mode of action of *B. thuringiensis* toxin is available. *B. thuringiensis* H-14 parasporal body consists of at least 4 major proteins of 27 kDa, 65 kDa and a doublet of 130 kDa, all of which are mosquitocidal (Visser et al., 1986; Ibarra and Federici, 1986; Ward et al., 1986; Ward and Ellar, 1988). Mode-of-action studies have been confined to the 25 kDa protein (derived from proteolysis of the 27 kDa protein). The reasons that this protein was initially described as the principal toxin and because of its wide range of target cells in vitro. The first step in toxicity consists of binding of the toxin to specific receptors on the cell surface, but the nature of these binding sites is still uncertain. Ellar et al. (1985) showed that the 25 kDa protein was the only parasporal body protein that was inserted into lipid bilayers. Thomas and Ellar (1983) reported that lipids extracted from *Ae. albopictus* cells inactivated the alkali-soluble parasporal body protein, while Gill et al. (1987) confirmed a specific interaction between the 24/25 kDa toxins and certain unsaturated phospholipids. Despite this evidence, the possibility still exists that in cells sensitive to the toxin and additional receptor may be present. A glycoprotein receptor is proposed to be involved (Chilcott et al., 1990).

Several theories were postulated to explain the subsequent action of the toxin after binding to receptors. Recently, Chilcott et al. (1990) proposed that after binding to receptors in the plasma membrane, the toxin generate small pores in the membrane. These pores will disrupt the permeability barrier of the plasma membrane, leading to equilibration of ions across the membrane, disturbing the colloid-equilibrium and resulting in a net

influx of water into the cell. The consequent cell swelling will cause a further disruption of membrane integrity, eventual lysis and the subsequent death of the larvae.

Bacillus sphaericus

This bacterium was first isolated from mosquito larvae in 1973 (Singer, 1973). Early studies of the larvicidal potential of this agent were mainly conducted on strain SSII-I (strain 1321) isolated from India. However, this strain was highly unstable and continued sub-culturings were required to maintain its activity. More recent work has concentrated on strain 1593, 2297 and 2362 isolated from Indonesia, Nigeria and Sri Lanka respectively.

Bacillus sphaericus is a complex of aerobic spore-forming bacterial strains with cosmopolitan distribution in soil and aquatic environment. Both the insecticidal as well as inactive strains are known to exist. Some of the insecticidal strains produce parasporal bodies (inclusions). There are at present over 30 known insecticidal strains originating from more than 11 countries. Based on the use of flagellar antigenic characteristics, these strains are classified into 4 serotypes i.e. H1, H2, H5 and H25.

The sequence of toxin activation is thought to be similar to that of *B. thuringiensis* endotoxin, although much remains to be studied. When a susceptible mosquito larva ingests *B. sphaericus* spores, symptoms of intoxication appear within 30-60 minutes. The midgut epithelium swells and distends which later is destroyed, resulting in larval death after 4 hours at high dosages or after 48 h at lower dosages (Davidson, 1984). Unlike *B. thuringiensis*, the spores of *B. sphaericus* germinate in the midgut of dead larva and multiply vegetatively to produce more spores. This important aspect of recycling is vital as the activity of the bacteria is extended in field applications, resulting in longer residual effects.

Much information on the toxins of *B. sphaericus* and their activation have been obtained recently. There is now ample evidence that the mosquitocidal activity of *B. sphaericus* is due to the presence of binary toxins of 51.4- and 41.9-kDa protein toxins, produced during sporulation, both of which are required for toxicity (Payne and Davidson, 1984; Baumann et al., 1985; Charles et al., 1988; Broadwell et al., 1990; Baumann et al., 1991). Recent studies indicated that the action of the crystal toxins on *Cx. quinquefasciatus* larvae involves the following steps: 1) ingestion of toxin by the larva; 2) solubilisation of crystal in midgut by the alkaline pH; 3) proteolytic processing of the 51- and 42-kDa protoxins to 43- and 39-kDa proteins respectively; 4) binding of processed proteins to epithelial cells of the gastric caecum and posterior midgut; and 5) incorporation of both toxins. Toxicity is exerted by an unknown mechanism involving the appearance of areas of low electron density, vacuolation and mitochondrial swelling and (vi) lysis of cells (Baumann et al., 1991; Oei et al., 1992).

STUDIES ON MICROBIAL CONTROL AGENTS

In the past decade, Malaysian researchers had shown great interests in the study of microbial control agents of mosquitoes and their potential in vector control. Many of these studies focused on two highly mosquitocidal bacteria, viz. *B. thuringiensis* H-14 and *B. sphaericus* H-5a5b and H-25.

Against malaria Vectors

In Malaysia, both *Bacillus thuringiensis* H-14 and *B. sphaericus* were evaluated against *Anopheles* larvae under laboratory conditions. Foo and Yap (1982) reported that *Anopheles balabacensis*, a major malaria vector in northern Peninsular Malaysia and Sabah was highly susceptible to a standard preparation of *B. thuringiensis* H-14 (IPS-78 standard from Pasteur Institute, France) lyophilised powder and to 2 other commercial preparations. Lee and Cheong (1985) showed that this mosquito species was also highly susceptible to IPS-78 preparation, with LC₅₀ and LC₉₀ values of 0.79 and 2.09 mg/L respectively. Tests were also conducted on the toxicity of *B. sphaericus* on *Anopheles* larvae. In a later study, Cheong and Yap (1985) indicated that larvae of *An. balabacensis* were moderately susceptible to a laboratory culture of *B. sphaericus* strain 1593, with LC₅₀ of 120 x 10⁴ spores/mL and LC₉₀ of 700 x 10⁴ spores/mL in the first 24 hours. Lee et al. (1986a) reported bioassay results from the testing of 3 strains of *B. sphaericus* (1593, 2297 and 2362) against *An. maculatus*, a principal malaria vector in Peninsular Malaysia. All 3 strains of *B. sphaericus* were found to moderately toxic to *An. maculatus*.

These 2 microbial agents were evaluated in field trials for malaria vector control since laboratory results were promising. Lee et al. (1990) reported that field evaluations of the efficacy of indigenous isolate IMR-BT-8 (*B. thuringiensis* H-14) and IMR-BS-4 (*B. sphaericus* H5a5b) were conducted against *Anopheles karwari* near the foothill of an abandoned quarry in Ipoh in the state of Perak. Although this mosquito is not a known vector of malaria in Malaysia, its larval habitats and biology closely resembles *An. maculatus*, the principal malaria vector in Peninsular Malaysia. Data from such trial can therefore be used as a model for testing purposes. These results indicated that in plots sprayed with a commercial preparation of *B. sphaericus* strain 2362

or a mixture of IMR-BT-8 and IMR-BS-4, the initial larval population was totally eliminated 24 h post-treatment and remained almost mosquito-free throughout the trial period of 16 days. In plots sprayed with BACTIMOS (commercial *B. thuringiensis* H-14) or IMR-BT-8 only, a control period of 5 days was achieved; while IMR-BS-4 and *B. sphaericus* strain 2362 was effective for 16 days. Bacterial counts of samples from plots sprayed with these agents confirmed the presence of *B. thuringiensis* H-14 spores. Unfortunately, owing to the clumping of the colonies, spore counts of *B. sphaericus* could not be quantified and hence the possible recycling mechanism of this agent could not be studied. Subsequently, a large scale trial was conducted in Grik, Perak for the control of malaria. A commercial formulation of *B. thuringiensis* H-14 was applied at 2g/minute into the streams where the vector (*An. maculatus*) bred. Six months after the treatment, the malaria slide positivity rate was reduced from 5% to less than 1% and the vector population was greatly reduced (Lee et al., 1994).

Against dengue vectors

Both *Aedes aegypti* and *Ae. albopictus* were found to be highly sensitive to *B. thuringiensis* H-14 in the laboratory at very low dosages (Foo and Yap, 1982; Lee and Cheong, 1985) indicating that *B. thuringiensis* H-14 is a highly promising microbial control agent since it is also very safe. In actual facts, *Aedes aegypti* and *Ae. albopictus* were most susceptible to *B. thuringiensis* H-14 in comparison to all the vector mosquito tested so far in Malaysia. A small-scale study conducted in the laboratory under simulated field conditions using a wettable powder of *B. thuringiensis* H-14 for the control of *Ae. aegypti* showed that at a target dosage of 50 mg/L it was possible to maintain the persistency of *B. thuringiensis* H-14 for up to approximately 2 months even though the water in the containers were removed and replenished daily by half or one-third (Lee et al., 1986). This experiment also showed that persistency of *B. thuringiensis* H-14 was not affected by the amount of water removed or by the presence of chlorine in the tap water. The extremely high target dosage could be reduced if appropriate formulation was used. Subsequently, Lee and Cheong (1987) conducted a small-scale field evaluation of the efficacy of *B. thuringiensis* H-14 against *Ae. albopictus*. In this trial, 2 laboratory-prepared formulations of sand granules applied at target dosages of 1 and 2 mg/L were able to prevent mosquito breeding for 5 and 6 weeks respectively, while at a dosage of 5 mg/L no breeding was observed throughout the trial period. In automobile tyres, a dosage of 1 mg/L sand granule was able to prevent *Ae. albopictus* larvae from breeding for 5 weeks, on doubling this dosage an 8-week control was achieved. Generally, the pellets showed extremely poor results. Although *B. thuringiensis* H-14 did not show a long residual effects compared to temephos, it could be used alternately with chemical larvicides to delay the development of resistance in the mosquito population. All the 3 strains of *B. sphaericus* (1593, 2297 and 2362) were also evaluated against *Ae. aegypti* in the laboratory. It was shown that *Ae. aegypti* larvae were highly tolerant to *B. sphaericus* (Cheong and Yap, 1985; Lee et al., 1986a). In another study, a Malaysian isolate IMR-BT-20 (*B. thuringiensis* H-14) was evaluated simultaneously with other isolates against container-breeding *Ae. albopictus* (Lee and Seleena, 1992). In 2.5 L earthen pots and at a dosage of 1.0 mg/L, the *Aedes* population was almost eliminated 24 hours post-treatment and remained suppressed for about 3 weeks (Fig 24). When the dosage was increased to 2.0 mg/L, the infested containers remained mosquito-free for more than 3 weeks. A commercial *B. thuringiensis* H-14 preparation (VECTOBAC) showed similar trend. However, in automobile tyres, the effectiveness of both isolates was much reduced.

Against filariasis vectors

Only *Mansonia uniformis* were tested against both *B. thuringiensis* H-14 and *B. sphaericus*. Preliminary results showed that the *Mansonia uniformis* larvae were highly susceptible to both agents. However, other important *Mansonia* species were not bioassayed. Subsequently, Foo and Yap (1983) carried out field trials using a suspension concentrate of *B. thuringiensis* H-14 against laboratory-reared *Ma. uniformis* and naturally occurring *Mansonia* larvae on small plots in swampy ditches on Penang Island. Six dosages ranging from 1.1 to 11.40 kg/hectare were used in 2 experiments. In general, higher dosages of the *B. thuringiensis* H-14 formulation were needed to achieve control of the *Mansonia* larvae when compared with other vector mosquitoes. Yap (1985a) reported that 2 formulations of *B. sphaericus* strain 2362 applied at 1 and 11 kg/ha induced 81%-94% reduction of *Mansonia* larval population. The residual effects ranged from 3 to 14 days.

The vector of urban filariasis (*Wuchereria bancrofti*) *Cx. quinquefasciatus* was also found to be highly susceptible to both *B. thuringiensis* H-14 and all 3 strains of *B. sphaericus* (Foo and Yap, 1982; Lee and Cheong, 1985; Cheong and Yap, 1985; Lee et al., 1986a). This species, however, was more susceptible to *B. sphaericus* which was more effective in the habitats of *Cx. quinquefasciatus* consisting mainly of polluted water with high organic contents. *B. sphaericus* is known to be able to recycle in polluted water and its persistency is much longer than *B. thuringiensis* H-14. In container-breeding *Culex* mosquitoes, *B. sphaericus* can also be applied

together with the predatory larvae of *Toxorhynchites* to provide more efficient control since tests in Malaysia show that larvae of the indigenous *Toxorhynchites splendens* are not affected by *B. sphaericus* at the target dosages (Lee et al., 1986a). As yet, no field trials were conducted to investigate the possibility of using *B. sphaericus* against *Cx. quinquefasciatus*.

Against Japanese encephalitis vector

Culex pseudovishnui, a potential vector of Japanese encephalitis was found breeding in pools of mud water near a residential area adjacent to a rubber estate during a larval survey. A limited field testing of IMR-BT-8 was conducted in a selected plot of the breeding site (Lee and Seleena, 1990). A dosage equivalent to 1.5 Kg/ha of a primary powder was applied after the initial visual counts of the larvae. The results showed that IMR-BT-8 was highly effective in suppressing the larval population of *Cx. pseudovishnui*. Over 80% reduction of the population was achieved 24 and 48 hours after spraying. The presence of IMR-BT-8 and the indigenous *B. thuringiensis* occurring naturally were confirmed. After 48 hours, the spore count of IMR-BT-8 was much reduced. This was probably due to the rapid settling of the spores as well as ingestion of the spores by the mosquito larvae and other organisms such as tadpoles, water beetles etc. It was noted that IMR-BT-8 has no observable effects on these non-target organisms found naturally with the larvae.

SCREENING INDIGENOUS MICROBIAL CONTROL AGENTS

Very little work has been conducted in this area, and there are few publications on the isolation and screening of local strains of bacteria and other microorganisms for possible use as microbial insecticide. The World Health Organisation has recommended that indigenous strains of these agents are more desirable from the viewpoint of ecological considerations and the feasibility of production in developing countries (WHO, 1982). It was considered by WHO that the isolation and use of indigenous microbial control agents in a specific ecotype may exert minimal impact on the environment (WHO, 1982). However, at present, there are no field data to support this assumption, although Lee and Seleena (unpublished data) had observed that the use of Malaysian isolates of *B. thuringiensis* in mosquito control did not affect indigenous non-target organisms such as tadpoles, water beetles and fish found concurrently in mosquito breeding sites.

Collection of samples for isolation

Since the terrestrial and aquatic environment are favourable habitats of bacteria and fungi, samples of soil, water and debris were collected from all possible mosquito breeding habitats in Malaysia which included forest, beaches, stream, pool etc. As the use of mosquitocidal microbial agents in Malaysia is experimental only and limited to known areas, all these sites of collection are considered free from introduced microbial agents. At each spot, about 10 g of the soil and debris were collected using sterile plastic spoons into screw-capped sterile plastic containers; while 10 ml of water was collected with sterile disposable Pasteur pipettes into similar containers. Both soil and water samples were collected about 2-3 cm below the surface where the effect of ultra-violet light was minimal. Samples were collected from all states in Peninsular Malaysia and Sabah. The samples were transported back to the laboratory as soon as possible and stored at 4°C until analysed. All the samples were analysed to obtain the mosquitocidal strains of bacteria.

Microbial Isolates

A total of 876 samples comprising 562 soil, 309 water samples and 5 dead insects were collected from 35 types of habitats. These samples were mostly collected from fresh-water swamp (147), forest (134) and stream (128). All collected samples were transported back to the laboratory for analysis. At the end of the programme, samples were obtained nationwide from all states in Peninsular Malaysia as well as Sabah and Sarawak. All samples collected were analysed and a total of 3,922 bacterial and 486 fungal colonies were isolated and available for screening against *Aedes aegypti* and *Culex quinquefasciatus* in the laboratory. A total of 29 *Bacillus thuringiensis* isolates were obtained which included 25 isolates of serotype H-14, 1 isolate each of serotype H-7, H-8a8b, and an isolate related to H-28. In addition, an untypable isolate tentatively named as subspecies *Bacillus malaysianensis* or IMR-BT-20a was also isolated. This isolate was not reactive with H-1 to H-24 antiserum at the time of serotyping. The other serotype identified as H-28 is a new serotype and designated as *B. thuringiensis jegathesan*. Of all the H-14 isolates, only IMR-BT-16 was isolated from mangrove swamp.

Totally, 8 larvicidal isolates of *B. sphaericus* comprising 4 each of serotype H-5a5b and H-25 were obtained. Of special interest is the discovery that IMR-BS-7 also exhibited significant anti-microbial activities against colonies of pathogenic *Salmonella paratyphi* A and *Acinetobacter* species in addition to its mosquitocidal characteristics (Seleena and Lee, 1993). Studies should be conducted to identify the antimicrobial-like product(s)

and the effects on mosquito larvae.

Other microbial control agents serotyped included a new anaerobe of *Clostridium bif fermentans* which was individualised as serovariety *C. malaysia*. This new agent produces in addition to spores, proteinic parasporal inclusion bodies containing similar amino acid contents as *B. thuringiensis* H-14 and *B. sphaericus* crystals (de Barjac et al., 1990). The absence of toxicity of the sporulated cells to mice was confirmed by different routes of administration.

RECENT DEVELOPMENTS

Simultaneous Adulticiding and Larviciding

Presently, the interruption of dengue transmission is dependent on the use of malathion, an adulticide which is highly effective when delivered by thermal or ULV fogging; while larviciding is effected through the use of temephos in containers. Traditionally, adulticiding is conducted by the health authorities and the application of larvicide is left to the community. However, this compartmentisation of adult and larval control is unfortunately ineffective. Lee (1991) found in a nation-wide survey that only about 30% of the households has ever used temephos at least once. Most households did not apply the larvicide regularly which was often under- or over-dosed. An ideal and effective dengue vector control operation should therefore incorporate both activity in a single operation. This concept of simultaneous adulticiding and larviciding is not new and has been known and used for many years in the control of other vectors such as malaria. Seleena et al., (1996) found that ULV fogging of *B. thuringiensis* H-14 was highly effective in *Aedes* larval control and when used together with malathion was able to induce also complete adult mortality. However, the discharge dosage used was 1.6 L/min of *B. thuringiensis* H-14 and this was considered excessively high. In another trial, *B. thuringiensis* H-14 was mixed with malathion (9V:1V) and discharged at 250 mL/min in a residential area in Pandamaran, Klang, Malaysia. It was found that complete mortality of caged *Ae. aegypti* and *Ae. albopictus* adults left indoor and outdoor was achieved. Similarly, high larval mortality of both species was obtained (Lee et al., 1997). Trials conducted in construction sites using a mixture of malathion-Bt induced complete adult and larval mortality in *Ae. aegypti* (Lee and Seleena, unpub data). A field trial in Johor, Malaysia using malathion-Bt mixture at a ratio of 1:1 and 3:7 indicated that the 3:7 ratio was more effective in inducing adult and larval mortality (VBDCP, unpub data). From these trials, it is obvious that the original ULV machines are able to disperse the bacteria, residual larvicidal activity was seen, and the cost-effectiveness of this method need to be assessed.

Insecticide Mixtures

Trials were also conducted using pyrethroid and other insecticides mixed with Bti. Field trials were conducted in three unoccupied single story houses to study the compatibility of dispersing *Bacillus thuringiensis* serovar *israelensis* (*Bti*) simultaneously with chemical insecticides for the control of *Aedes aegypti* adult and larvae mosquitoes. The trials were conducted using the following insecticides : commercial aqueous *Bti* formulation, Vectobac 12AS[®] (Abbott Laboratories) containing 1200 ITU/mg against *Ae. aegypti* ; Actellic 50EC[®] (a.i. pirimiphos - methyl 48.1 % w/w); Aqua Resigen (a.i. s-bioallethrin 0.14 % w/v, permethrin 10.26 % w/v and piperonyl butoxide 9.79 % w/v); Resigen EC (a.i. s-bioallethrin 0.8 % w/w, permethrin 18.7 % w/w and piperonyl butoxide 63.7 % w/w); and Fendona SC[®] (a.i. alphacypermethrin 1.47 % w/w). The dosage of the insecticides used in the trials were based on the manufacturer recommendations for an ULV indoor application. A portable mist -blower, Mist Blower MD 300, Maruyama MFG. CO., INC.TM with ULV attachments was used to disperse the insecticides. The discharge rate of the insecticides was maintained at 60 ml per min. The fogging trials were conducted on 9 different days, dispersing 9 different formulations. The effectiveness of each trial was evaluated by measuring 3 different parameters: larval mortality, adult mortality and ULV droplet analysis at varying distances from the fogging machine, i.e. 3 m, 6 m and 9 m from the fogging machine. The significant difference of the larval mortality among trials was analysed using the Student t-test computer software program. The droplet size for each trial was in the range of 50.0 - 60.0 μ m. Commercial aqueous *Bti* achieved 95.0 - 100 % mortality for *Ae. aegypti* larvae in all the 0 h and 7 d post fog test samples. The chemical insecticides also exhibited larvicidal activity. However, complete larval mortality was only achieved in 0 h post fog test samples placed 3 m from the fogging machine. The larvicidal activity was hardly exhibited in the 7 d post fog test samples. All 4 chemical insecticides mixed well with Vectobac 12AS and the mixtures flowed through the nozzle of the fogging machine smoothly. The larvicidal activity of the mixtures was not significantly different from fogging with the chemical insecticides only for the 0 h post fog test samples ($p > 0.05$) but it was significantly different in the 7 d post fog test samples except for the mixture of Actellic 50EC and Vectobac 12AS[®] ($p < 0.05$). This indicated that the chemical insecticides by themselves do provide larvicidal activity but the activity is not stable, it degrades with time. But the activity, of Vectobac 12AS is stable even

after 7 d post fogging. Thus, insecticide mixtures with Vectobac 12AS did exhibit larvicidal activity in the 7 d post fog test samples. Aqua Resigen, Resigen EC and Fendona SC achieved 100 % adult mortality in all the cages placed within 9 m from the fogging except for Actellic 50EC. Complete adult mortality was also achieved in all the mixtures except for the mixture of Actellic 50EC and Vectobac 12AS. Thus, indicating that Vectobac 12AS does not have any adverse effects on the adulticidal activity of the chemical insecticides. Vectobac 12AS is only known to provide larvicidal activity and the activity is found to be stable for 7 d post fogging. On the other hand the chemical insecticides provide both larvicidal and adulticidal activity, but the larvicidal activity degrades with time. So for an effective and efficient *Aedes* mosquito control will be when Vectobac 12AS is dispersed simultaneously with the chemical insecticides. The recommended insecticides will be Aqua Resigen, Resigen EC and Fendona SC and not Actellic 50EC as Actellic 50EC was found to reduce the larvicidal activity of Vectobac 12AS. Moreover, Actellic 50EC did not exhibit complete adulticidal activity as Aqua Resigen, Resigen EC and Fendona SC (Seleena et al., 1999). Trials were also conducted using a local *Bti* product (MOSBAC®) mixed with Aqua Resigen and dispersed by ULV. The results were promising.

Compatibility of *Bacillus thuringiensis* H-14 and chemical insecticides

If *Bti* is to be mixed with chemical insecticides and used in dengue vector control, the compatibility of the bacteria with the chemicals need to be ascertained. Seleena and Lee (1999) recently reported laboratory studies on the compatibility of *Bti* with malathion 96% TG; primiphos-methyl 50EC and Aqua-Resigen (S-bioallethrin+permethrin+piperonyl butoxide). Malathion was found to be the most compatible insecticide with *Bti*. There was no loss in the larvicidal activity of *Bti* on mixing with primiphos-methyl. However, after 7 days the *Bti* toxicity was reduced by 3 folds. Larvicidal activity of *Bti* in Aqua-Resigen was not observed at all, probably due to the rapid knock-down of the larvae by the pyrethroids. These limited tests indicated that certain insecticides can be mixed with *Bti* and used for dengue vector control.

ULV Application

Microbial agents, especially *B. thuringiensis*, are highly effective against larvae of *Aedes*. However, the use of *Bti* in dengue vector control is not widespread due to difficulty in applying the bacteria effectiveness. In this respect, ultra-low-volume cold fogging is ideal for the mass scale dispersal of the bacteria for larviciding purposes. Lee et al., (1996) conducted a series of ULV-*Bti* tests to attempt to determine the optimal effective dosage. They found that a flow-rate of 0.3 L/min was not effective, but at 0.5 L/min, very high larval mortality was achieved with long residual activity of the bacteria in test cups and tyres. Subsequently, a real-life trial using an aqueous solution of *B. thuringiensis* H-14 (Vectobac AS) was conducted in a residential area in Selangor, Malaysia. The field dosage used was 0.5 L/min. At this dosage, complete larval mortality was achieved in outdoor containers and those containers placed about 5 ft. indoor (Lee et al., 1996). Obviously, to achieve more significant results, a higher flow-rate of probably > 0.5 to 1 L/min of *Bti* should be used only for larviciding. A local isolate of *Bt* H-14 dispersed by a portable mist-blower at a discharge rate of 50 mL/min was highly effective in *Aedes* control and the residual activity of the bacteria last for about 1 month (Lee et al., 1977).

Thermal Application

It was long thought that because of the heat-labile *Bti* larvicidal toxins, *Bti* and other microbial agents could not be applied thermally. However, Seleena et al., (2001) reported the successful application of an aqueous suspension of *Bti* with and without pyrethroids using a thermal fogger without loss of any larvicidal activity. This was made possible by diluting the *Bti* suspension with water. It was found that contact with the heat was generally very brief and *Bti*, being protected by the water, did not loss the activity. The effectiveness of this technique was confirmed by Chung et al., (2001) and Yap et al., (2002). Thus *Bti* can now be applied by both cold and thermal fogging generators.

Tablet Application

Water stored for household use permits the breeding of dengue vectors, *Aedes aegypti* and *Ae. albopictus*. VectoBac DT®, a tablet formulation of *Bacillus thuringiensis israelensis* (*Bti*) was evaluated for the control of dengue vectors in varied types of potable containers (Seleena et al., 2004). The tablet on introduction into the container sinks to the bottom and the *Bti* toxins are concentrated at the sides and the base of the container; and the treated water column is devoid of *Bti* toxins within 24 h. In a study where the containers were kept covered and laboratory-bred larvae were used to determine the efficacy of the tablet, it was found that the efficacy and the persistency of the tablet was significantly longer in earthen containers in comparison to HDPE (in full) and plastic containers. Effective efficacy and persistency was observed in earthen containers for a

minimum of 5.5 months without water replenishments; for a minimum period of 5.5 months with weekly 50 % water in volume replenishments; and for a maximum period of 2.2 months with daily 50 % water in volume replenishments. In plastic and HDPE containers, the tablet had a persistency of 2 months without water replenishments and of 1.8 months with weekly 50 % water in volume replenishments. The efficacy and persistency of the VectoBac DT was significantly longer in earthen containers, with or without regular treated-water exchange, due to *Bti* toxins being embedded into the porous earthen container surfaces which protects them from rapid degradation and also lesser toxin amounts are removed from the water column during water exchange. The efficacy of the tablet was also evaluated for the control of natural infestation of *Aedes* larvae which were resistant to temephos at the WHO diagnostic dosage of 0.012 mg/L. The tablet significantly reduced the pupal density by 8 folds in earthen containers for 67 days and 5 folds in HDPE containers for 55 days in comparison to untreated containers ($p < 0.05$). In the plastic containers the tablet was effective for shorter period of time of 25 days post tablet introduction due to fungal infestation in the treated plastic containers. VectoBac DT is an easy to use *Bti* formulation in potable containers, and has proven to be effective in reducing *Aedes* larvae in varied container types. Nevertheless, there is a need to determine the efficacy of VectoBac DT to reduce the dengue vector population to a threshold that will prevent dengue outbreak in a dengue endemic site.

CONCLUSION

Although many studies on microbial control agents have been conducted in the past decade in Malaysia, there is still a lack of field data. For the practical use of these agents, field trial data are indispensable for actual planning of mosquito control strategy. The availability of some field data in this study may be used as baseline information for future large-scale trials. Although a standard field trial protocol for field assessment of microbial control agents has been proposed by WHO (1981), it is difficult to standardise trial procedures due to differences in vector biology, ecotypes, formulations etc.

Though advances thus far achieved in the research of microbial control agents in Malaysia have been impressive in the past several years, there are still gaps remain in present day knowledge. The fate of these agents after application need to be ascertained since such information is invaluable in terms of residual activity. Some studies were conducted towards this end (Lee and Seleena, 1993). Moreover, the cost-effectiveness of using these agents needs to be looked into. Above all, large-scale field trials involving both entomological and epidemiological studies should be conducted. There is also a pressing need to mass-produce these agents using locally available wastes through biofermentation. To ensure continuous supply at affordable cost, scale-up production as well as improved down-stream processing of fermented products and quality control of these end products is also urgently required. In spite of these yet-to-be solved problems, it is reasonable to believe that these indigenous agents will eventually be integrated into the present vector control programme in the not-too-far future and hopefully such use may effect a more favourable outcome in the control of these dreadful human diseases in the tropics.

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