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*Full Length Research Paper*

# An evaluation of the larval toxicity, fecundity and mosquito longevity in Coimbatore, India

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**In India, the most important diseases transmitting mosquitoes belong to the genera Anopheles, Culex, Aedes and Mansonia. The indiscriminate use of synthetic insecticides creates multifarious problems like environmental pollution, global warming, insecticide resistance and is hazardous to humans. In the present study, bacterial pesticide, *Bacillus sphaericus* a soil dwelling bacteria, isolated from the sludge samples collected from Bharathiar University, Coimbatore, India cultured with required substrates and used to control the malaria vector, *Anopheles stephensi*. Bioassay was conducted to test the larval toxicity, fecundity and mosquito longevity. $LC_{50}$ and $LC_{90}$ values were calculated for the larval instars of the malaria vector, *A. stephensi*. The fecundity and adult longevity were highly reduced after the treatment with *B. sphaericus*. Field trials were also conducted in different breeding sites of *A. stephensi* with different range of temperature and altitude. Percentage reduction of larval population was maximum (100%) at 96 h of treatment. Toxicity efficacy of *B. sphaericus* was varied according to the temperature and altitude of the study area. The bacterial pesticide *B. sphaericus* proved that it is an effective mosquito control agent and can be used for further integrated pest management programmes.**

**Key words:** *Bacillus sphaericus*, larval toxicity, lethal concentration, *Anopheles stephensi,* field studies.

## INTRODUCTION

Mosquitoes are vectors of several diseases like malaria, filariasis, dengue fever, yellow fever, etc., causing serious health problems to human beings. The present resurgence of these diseases are due to the higher number of breeding places in today's `throw away' society. Further, the indiscriminate use of synthetic insecticides is creating multifarious problems like environmental pollution, insecticide resistance and toxic hazards to humans. Globally, there have been conscientious efforts to overcome these problems and great emphasis has been placed recently on enviro-friendly and economically viable methodologies for pest control. In recent years, microbial pesticide, *Bacillus sphaericus* have received much attention as potent bioactive compounds against various species of mosquitoes (Tom Floore and Robert Ward, 2009; Skovmand et al., 2009).

The known vectors of malaria which are common in India include *Anopheles stephensi*, *An. culicifacies, An. fluviattis, An. minimmus, An. sudanicus and An. philippinensis*. Malaria is caused by the protozoan parasite plasmodium, viz: *P. flaciparam, P. malaiae, P. ovale and P. vivax.* Their primary host and transmission vector are female mosquitoes of the genus Anopheles;

humans act as intermediate hosts. Man develops the disease after 10 to 14 days of being bitten by an infective female Anopheles. Presently, 420 species of Anopheles mosquitoes have been recorded throughout the world out of which 50 are known vector of malaria. In India, 58 species of Anopheles are present among them, 6 are primary vector of malaria. Among the Anopheles species, *Anopheles stephensi* is recognized as a major vector of urban malaria in India (Mittal et al., 2005). *An. stephensi* Liston (Diptera: Culicidae), a major malaria vector, breeds in wells, overhead or ground level water tanks, cisterns, coolers, roof gulters and artificial containers (Herrel et al., 2001).

There were over 1 million cases of malaria in India in 2008, as per the National Vector Borne Disease Control Programme (NVBDCP). Malaria is by far the most important insect transmitted disease remaining a major health problem in many parts of the world and is responsible for high childhood mortality and morbidity in Africa and Asia (Kleinschmidt et al., 2000; Pates and Curtis, 2005). Insect-transmitted diseases remain a major source of illness and death worldwide. Mosquitoes alone transmit diseases to more than 700 million people annually (Taubes, 2000). Although mosquito-borne diseases currently represent a greater health problem in tropical and subtropical climates, no part of the world is immune to this risk (Fradin and Day, 2002). Control of such diseases is becoming increasingly difficult because of increasing resistance of mosquitoes to pesticides (Ranson et al., 2001).

Management of this disease vector using synthetic chemicals has failed because of insecticide resistance, vector resurgence and environmental pollution. An alternative approach for mosquito control is the use of natural products such as plant and microorganisms. The microbial pesticides have undergone extensive testing prior to registration. They are essentially nontoxic to humans, so there are no concerns for human health effects with *Bacillus thuringiensis* or *B. sphaericus*, when they are used according to label directions. Extensive testing shows that microbial larvicides do not pose risk to wildlife, non-target species or the environment (EPA, 2000).

*B. sphaericus* is an aerobic, mesophilic, spore-forming bacterium with terminal swollen sporangia and spherical spores. Closely related species are *Bacillus pasteurii, B. fusiformis, B. loehnisii and B. rotans*; the last three species are now usually regarded as variants of *B. sphaericus* (Gordonr et al., 1973). These five species are among the most numerous representatives of the genus Bacillus found in soils (Buchanan and Gibbons, 1974). As a consequence of the specific toxicity to mosquito larvae of binary toxin (Bin) and mosquitocidal toxins (Mtxs) produced during the sporulation and vegetative stages, respectively, some toxic strains have been widely used for many years as biopesticides in the field in mosquito control programs (Bei et al. 2007). In this present study we report the larvicidal, fecundity and longevity efficacy of *Bacillus sphaericus* on the malarial vector *Anopheles stephensi*. This study also reports the field efficacy of *B. sphaericus* in *A. stephensi* breeding sites with varied temperatures and altitude.

## MATERIALS AND METHODS

### Collection of eggs of mosquitoes

The eggs of *Anopheles stephensi*, were collected from local (in and around Coimbatore districts) drinking water bodies and water stored container with the help of 'O' type brush, for the laboratory bioassay. These eggs were brought to the laboratory and were transferred to size enamel trays (18 × 13 × 4 cm) containing 500 ml of water and kept for larval hatching.

### Maintenance of larvae

The mosquito (*Anopheles stephensi*) larval cultures were maintained in our laboratory at (27 ± 2°C, 75 to 85% RH, under 14L: 10D) photoperiod cycles. The mosquito larvae were fed *ad labitum* on (0.5 g) powdered liver and glucose at a ratio of (3:2) wt:wt as enhanced method of Roberts (1998). The feeding was continued till the larvae were transformed into the pupa stage.

### Maintenance of pupae and adult

The pupae were collected from the culture trays and were transferred to plastic containers (12 ×12 cm) containing 500 ml of water with the help of a dipper. The plastic jars were kept in (90 × 90 × 90 cm) size mosquito cage for adult emergence. The adults were fed with 10% sugar solution for a period of three days before they were provided an animal for blood feeding.

### Blood feeding of adult *Anopheles stephensi*

The adult female mosquitoes were allowed to feed on the blood of a rabbit (exposed on the dorsal side) for two days. After blood feeding, enamel trays with water from the culture trays were placed in the cage for the adults to lay eggs. Both females and males were provided with 10%glucose solution on cotton wicks. The cotton was always kept moist with the solution and changed every day.

### Isolation and Identification of *Bacillus sphaericus*

Extracts were prepared by mixing 1 g sludge sample collected from in and around Bharathiar University in sterilized distilled water (10 ml) and filtered. The filtrate was diluted and appropriate dilutions were heated at (70°C) for 15 min before inoculating a liquid medium. The flask was incubated on a rotary shaker at (30 ±1°C). Spores formed after 30 h of incubation were diluted and suitable dilutions plated on nutrient agar in Petri plates after heat treatment at (70°C). The colonies developed in the Petri plates after 30 h of incubation were grown individually in liquid medium for 24 h followed by centrifugation at 10,000 rpm for 10 min and the pellet was viewed under microscope for identification and cultured for bioassay studies. Different concentrations (0.001, 0.01, 0.1, 1.0 and 10 ppm) of *B. sphaericus* were used for toxicity bioassay against different larval instars *of A. stephensi mosquitoes.*

### Cultural conditions

The organism was grown in a liquid medium containing (g/litre of distilled water): - $FeSO_4.7H_2O$, 0.01; $MnSO_4$, 0.1; $MgSO_4.7H_2O$,

**Table 1.** Larval and pupal toxicity of *Bacillus sphericus* on Malaria vector, *Anopheles stephensi*.

| Larval and pupal stages | % of larval and pupal mortality (Mean ± SD) Concentration (ppm) | | | | | Standard error | $LC_{50}$ ($LC_{90}$) (ppm) | 95% Confidence limit | | $X^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.001 | 0.01 | 0.1 | 1 | 10 | | | $LC_{50}$ LCL-UCL (ppm) | $LC_{90}$ LCL-UCL (ppm) | |
| I | $36 \pm 1.2^{cd}$ | $41 \pm 2.2^{c}$ | $56 \pm 2.3^{b}$ | $94 \pm 1.5^{ab}$ | $100^{a}$ | 0.223 | 0.119(0.824) | 0.041 - 0.194 | 0.677 - 1.054 | 3.538 |
| II | $32 \pm 1.6^{de}$ | $38 \pm 1.1^{d}$ | $51 \pm 1.2^{c}$ | $88 \pm 1.2^{b}$ | $100^{a}$ | 0.186 | 0.203(1.050) | 0.114 - 0.293 | 0.868 - 1.338 | 3.675 |
| III | $24 \pm 0.7^{de}$ | $29 \pm 1.2^{d}$ | $44 \pm 0.9^{c}$ | $77 \pm 1.2^{b}$ | $100^{a}$ | 0.164 | 0.402(1.401) | 0.170 - 0.769 | 0.950 - 2.909 | 5.719 |
| IV | $21 \pm 1.2^{de}$ | $26 \pm 0.7^{d}$ | $40 \pm 1.6^{c}$ | $73 \pm 1.1^{b}$ | $100^{a}$ | 0.161 | 0.489(1.516) | 0.258 - 0.910 | 1.305 - 3.103 | 5.506 |
| Pupae | $8 \pm 0.7^{cd}$ | $9 \pm 0.7^{cd}$ | $13 \pm 0.7^{c}$ | $22 \pm 2.1^{b}$ | $39 \pm 0.5^{a}$ | 0.015 | 12.819(26.937) | 7.434 - 78.946 | 15.480 - 187.012 | 7.761 |

Means (± Standard deviation) followed by same letter within rows indicate no significant difference in Duncan's multiple range test (P<0.05 value).

0.2; $CaCl_2$, 0.08; $K_2HPO_4$, 0.025; yeast extract, 2; peptone, 4; D-glucose, 1 and casein, 5. Solutions of yeast extract, peptone casein, D-glucose, $K_2HPO_4$ and $CaCl_2$ were separately prepared, sterilized and added before inoculation. The pH of the medium was adjusted to 7.1 before sterilization.

### Larval toxicity test

Laboratory colonies of mosquito larvae/pupae were used for larvicidal/pupicidal activity. The *B. sphaericus* was evaluated at 0.001, 0.01, 0.1, 1.0 and 10 ppm, (v/v) ml concentrations. Untreated distilled water treatment served as control. Each treatment was replicated five times. Twenty actively swimming *A. stephensi* larvae were sieved out from different rearing trays to maintain uniformity of batches of larvae and exposed to 100 m leach concentration of *B. sphaericus* and untreated control held in separate (250 ml) capacity plastic containers (WHO, 1996). The larvae were fed *ad labitum* on 0.5 g powdered liver and glucose at a ratio of 3:2 (wt: wt) as enhanced method of Roberts (1998) larval mortality was assessed after 24 h of exposure by probing the larvae with needle and moribund larvae were counted as dead (Azmi et al., 1998).

The control mortalities were corrected by using Abbott's formula (1925):

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment - Observed mortality in control}}{\text{100 - Control mortality}} \times 100$$

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae pupae}}{\text{Number of larvae pupae introduced}} \times 100$$

$LC_{50}$ and $LC_{90}$ values were calculated from toxicity data by using SPSS software package (version 17).

### Fecundity studies

The fecundity experiments were conducted by taking an equal number of male and female mosquito larvae which had emerged from the control and treated sets. They were settled in the cages of 30 × 30 cm dimension individually of each concentration (0.001, 0.01, 0.1, 1.0 and 10 ppm). Three days after the blood meal, eggs were collected daily from the small plastic bowls containing water kept in ovitrap in the cages. The fecundity was calculated by the number of eggs laid in ovitrap divided by number of females let to mate (20 nos.) (The death of adults in the experiments was also considered).

### Mosquito longevity test

The adult longevity of male and female mosquitoes was also recorded. This was calculated by the number of days lived by the adult. The emergence day and mortal days of the adults were recorded and the means were calculated to give the mean longevity in days.

### Field trial on breeding sites of *A. stephensi*

Field applications of *B. sphaericus* were done uniformly with the help of a knapsack sprayer on the surface of the water in each habitat. Sampling of larvae was undertaken before treatment and 24, 48, 72 and 96 h after by dipper sampling and counting. A separate sample was taken to determine the species composition of each larval habitat. Six trials were conducted for each area with similar temperature and altitude. The required quantity of *B. sphaericus* was determined by calculating the total surface area and the required concentration was prepared by multiplying ten times the observed laboratory $LC_{50}$ values. Laboratory $LC_{50}$ values of third instar larvae (Murugan et al., 2003).

## RESULTS

Bacteria were recognized as *B. sphaericus* by their rodlike shape (about 1 µm), lack of cytoplasmic inclusions and aerobic formation of a spherical spore with obvious terminal swelling of the sporangium.

The percentage larval and pupal mortality values of malaria vector, *A. stephensi* after the treatment of *B. sphaericus* at different concentrations (0.001, 0.01, 0.1, 1.0 and 10 ppm) are shown in Table 1. The mortality values ranged between 21 to 100% for the larvae stages and were reduced for the pupal stage. At the higher concentration (10 ppm), no larvae were found alive and 100% mortality was recorded. The $LC_{50}$ values ranged from 0.119 to 0.489 for the larval stages and were increased to 12.819 ppm for the pupae.

**Table 2.** Fecundity and Longevity of *Anopheles stephensi* after the treatment of *Bacillus sphaericus*

| Treatment | Adult longevity(In days) | | Fecundity |
|---|---|---|---|
| | Male | Female | |
| Control | 20 | 35 | 140 |
| 0.001 | 16 | 31 | 118 |
| 0.01 | 13 | 27 | 99 |
| 0.1 | 10 | 16 | 68 |
| 1.0 | 5 | 9 | 33 |
| 10 | 1 | 2 | 14 |

**Table 3.** Effect of *Bacillus sphaericus* against larval density of mosquito vectors at the breeding sites (Drinking water) of *Anopheles stephensi*.

| S. No | Larval density (%) | | | | |
|---|---|---|---|---|---|
| | Before treatment | After treatment | | | |
| | | 24 h | 48 h | 72 h | 96 h |
| 1 | 45 | 20 | 12 | 4 | 0 |
| 2 | 50 | 25 | 16 | 6 | 0 |
| 3 | 30 | 13 | 6 | 1 | 0 |
| 4 | 36 | 17 | 7 | 1 | 0 |
| 5 | 44 | 20 | 10 | 4 | 0 |
| 6 | 33 | 15 | 7 | 2 | 0 |
| Total | 238 | 110 | 58 | 18 | 0 |
| Average | 39.66 | 18.33 | 9.66 | 3 | 0 |
| % Reduction | | 53.78% | 75.63% | 92.43% | 100% |

Place : Coimbatore, Tamil Nadu, India. Habitat: Over head tanks (Drinking water bodies), Species: Anopheles stephensi, Stage: Larvae, Size: 1.6 x 1.6 m, Depth: 1.5 m, Required quantity: 1.6 x 1.6 x 1.5 = 2.56 x 1.5 = 3.84 L, Required Concentration: 0.402 x 10 = 4.02 ppm, Temperature: 32.2°C, Altitude: 409 m.

Table 2 shows the effect of *B. sphaericus* on the adult longevity and fecundity of *An. stephensi*. The adult longevity of both male and female was considerably reduced by the treatment of *B. sphaericus*. The longevity was reduced to 16 days in male and 31 days in female at 0.001 ppm concentration, 13 days in male and 27 days in female at 0.01 ppm concentration, 10 days in male and 16 days in female at 0.1 ppm concentration, 5 days in male and 9 days in female at 1.0 ppm concentration and 1 days in male and 2 days in female at 10.0 ppm concentration. The fecundity was also highly reduced after the treatment of *B. sphaericus*. The number of eggs laid was inversely proportional to the concentration in treatment. The number of eggs was reduced from 118 to 14 as the concentration was increased.

Field trial was conducted at regions with different range of altitude and temperatures namely; Coimbatore (32.2°C and 409 m above sea level), Madurai (35°C and 100.58 m above sea level) and Chennai (38°C and 8 m above sea level) (Tables 3, 4 and 5). The percentage of larval reduction was noted at 24, 48, 72 and 96 h after the treatment of *B. sphaericus*. The percentage of larval Reduction at 24 h of treatment was 53.78% in Coimbatore, 38% in Madurai and 30% in Chennai.

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

Where, C – is the total number of mosquitoes in control.
T – is the total number of mosquitoes in treatment.

## DISCUSSION

The mosquitoes such as Culex, Anopheles, and Aedes are responsible for the transmission of many infectious disease agents. Pathogens transmitted by mosquitoes include West Nile virus, Saint Louis encephalitis virus, Eastern equine encephalomyelitis virus, Everglades virus, Highlands J virus, La Crosse Encephalitis virus in the United States; Dengue fever, Yellow fever, Ilheus virus, and Malaria in the American tropics; Rift Valley fever, Wuchereria bancrofti, Japanese Encephalitis, Dengue fever, Yellow fever, Chikungunya and Malaria in Africa and Asia; and Murray Valley encephalitis in Australia. Mosquito control manages the population of mosquitoes to reduce their damage to human health and economies. Mosquito control is a vital public-health practice throughout the world and especially in the tropics because

**Table 4.** Effect of *Bacillus sphaericus* against larval density of mosquito vectors at the breeding sites (Drinking water) of *Anopheles stephensi*.

| S. No | Larval density (%) | | | | |
|---|---|---|---|---|---|
| | Before treatment | After treatment | | | |
| | | 24 h | 48 h | 72 h | 96 h |
| 1 | 56 | 33 | 25 | 11 | 0 |
| 2 | 61 | 44 | 31 | 18 | 0 |
| 3 | 42 | 25 | 15 | 6 | 0 |
| 4 | 49 | 30 | 18 | 8 | 0 |
| 5 | 53 | 34 | 26 | 13 | 0 |
| 6 | 39 | 20 | 12 | 3 | 0 |
| Total | 300 | 186 | 127 | 59 | 0 |
| Average | 50 | 31 | 21.17 | 9.83 | 0 |
| % Reduction | | 38% | 57.67% | 80.33% | 100% |

Place: Madurai, Tamil Nadu, India. Habitat: Over head tanks (Drinking water bodies), Species: Anopheles stephensi, Stage: Larvae, Size: 1.5 x 1.0 m, Depth : 1.5 m, Required quantity: 1.5x1.0 x 1.5 = 1.5 x 1.5 = 2.25 L, Required Concentration: 0.402 x 10 = 4.02 ppm, Temperature: 35°C,Altitude: 100.58 m.

**Table 5.** Effect of *Bacillus sphaericus* against larval density of mosquito vectors at the breeding sites (Drinking water) of *Anopheles stephensi*.

| S. No | Larval density (%) | | | | |
|---|---|---|---|---|---|
| | Before treatment | After treatment | | | |
| | | 24 h | 48 h | 72 h | 96 h |
| 1 | 59 | 39 | 24 | 11 | 2 |
| 2 | 46 | 31 | 28 | 14 | 2 |
| 3 | 57 | 38 | 22 | 9 | 0 |
| 4 | 68 | 51 | 34 | 19 | 6 |
| 5 | 39 | 27 | 13 | 5 | 0 |
| 6 | 61 | 45 | 29 | 16 | 5 |
| Total | 330 | 231 | 150 | 74 | 15 |
| Average | 55 | 38.5 | 25 | 12.3 | 2.5 |
| % Reduction | | 30 % | 54.54% | 77.58% | 95.45% |

Place: Chennai, Tamil Nadu, India, Habitat: Over head tanks (Drinking water bodies), Species: Anopheles stephensi, Stage: Larvae, Size: 1.5 x 1.2 m, Depth: 1 m, Required quantity: 1.5 x 1.2 x 1.0 = 1.8 x 1 = 1.8 L, Required Concentration : 0.402 x 10 = 4.02 ppm, Temperature: 38$^{o}$C, Altitude: 8 m.

mosquitoes spread many diseases. A program on biological control of mosquitoes, virulence prospecting and evaluation of new isolates around the world is one of the most important steps taken to determine their effect on target populations, and thereby selecting the most promising ones for producing biological insecticides.

In the present study, *B. sphaericus* the bacterial pesticide was isolated from the soil samples in Bharathiar University, Coimbatore, India and used to control the Malaria vector *Anopheles stephensi*. *Bacillus sphaericus* showed a good control over growth and survivability of *A. stephensi* which may be due to the presence of binary toxin (Bin) and mosquitocidal toxins (Mtxs). *B. sphaericus*, a spore-forming, entamopathogenic bacterium, has been shown to possess potent larvicidal activity against several species of mosquito larvae (Yousten et al., 1982; Davidson, 1983). As a consequence of the specific toxicity to mosquito larvae towards binary toxin (Bin) and mosquitocidal toxins (Mtxs) produced during the sporulation and vegetative stages, respectively, some toxic strains have been widely used for many years as biopesticides in the field in mosquito control programs (Bei et al., 2007). In the present study, the soil bacterium, showed varied mortality rates related to the larval stages and concentrations. The younger larval stages were more susceptible than the later ones. Active strains of *B. sphaericus* are known to produce considerable quantities of at least two sets of proteinaceous mosquito larvicidal factors (MLF) at the onset of stationary phase ( Souza et al., 1988; Baumann

et al., 1991; Porter et al., 1993). Larvicidal activity was observed in all strains of *B. sphaericus* from Amazonia in differentiated toxicity levels (Eleilza et al., 2008).

In the present study, *B. sphaericus* treatment reduced the larval and pupal duration and introverted the adult emergence. Those treated larvae escaped from mortality showed reduced longevity. The adult which emerged from affected (abnormal) pupae were morphologically normal but showed a great reduction in fecundity. Some of the adults emerged also showed defective flight movement. Murugan et al. (2002) reported changes in fecundity after treatment with *B. thuringiensis*. Combined treatment of *Bacillus thuringiensis* with neem and pongamia showed a 76% adult mortality and reduction in fecundity (Poopathi and Tyagi, 2002). The reduction in adult longevity (17 males and 27 females) in *Culex quinquefasciatus* after the treatment with *B. sphaericus* (GR strain) (Makowski, 1993).

*B. sphaericus* also proved a withstanding toxicity effect on the field level. An inclusive larval reduction was shown after 96 h and no larvae were found for another 72 h. The study also deals with the efficacy of *B. sphaericus* at different agro-climatic regions. The field toxicity effect of *B. sphaericus* was tested against the mosquito larvae at regions with varied altitudes and temperatures. The result showed that the bioefficacy of bacterial toxicity was reduced as the temperature was increased. The larval reduction rate was very low in region with lower altitude and greater temperature (Chennai – 38°C and 8 m). This may be due to the susceptibility of bacterial toxin to high temperature. However, efficacy varied from 30 to 100% depending on environmental conditions. Field trials indicated that up to 100% of round-leaved mallow plants can be controlled with BioMal regardless of temperature and weed growth (Makowski, 1993; Mortensen, 1988). Dew periods of greater than 20 h and temperatures between 10 and 25°C result in the greatest efficacy Makowski (1993).

Vector borne disease such as malaria, still cause thousands of death per year. Malaria is by for the most important insect transmitted disease. For the control of malaria, first we have to control the malarial vector. Using synthetic chemical has field because of insecticide resistance, vector resurgence and environmental pollution. In addition, these synthetic chemicals y are toxic and have some side effects on environment and public health. *B. sphaericus* is an obligate aerobe bacterium and has good potential to be used as a biopesticide and is eco-friendly.

## Conclusion

From the present study we conclude that *B. sphaericus* proved good larvicidal agent against *An. stephensi* immature in laboratory and in their breeding sites. It also reduced the larval durations and egg productions.

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