Article

Evaluation of larvicidal activity of soil microbial isolates (*Bacillus* and *Acinetobactor* Sp.) against *Aedes aegypti* (Diptera: Culicidae) - the vector of Chikungunya and Dengue

D. Radhika, A. Ramathilaga, C. Sathesh Prabu, A.G. Murugesan
Manonmaniam Sundaranar University, Sri Paramakalyani Centre of Excellence in Environmental Sciences, Alwarkurichi-627 412, Tamil Nadu, India
E-mail: agmspkce@rediffmail.com

Received 28 June 2011; *Accepted 3 August 2011*; *Published online 20 November 2011*
IAEES

Abstract
To isolate and identify the natural non- harmful microbial population from the soil sample for the control of many epidemiological disease causing vector of *Aedes aegypti* mosquito larvae via larvicidal activity. Heat treat and non heat treated soil samples were used for the isolation of spore forming and non- spore forming microbial isolates through spread plate technique. Preliminary and quantitative larvicidal activity was performed against the mosquito larvae. Protein analyses of best microbial isolates were carried out by SDS-PAGE technique. For the screening assay ten microbial isolates were used and five were active against chosen mosquito larvae which were identified as *B. megaterium, B. sphaericus, B. cereus, B. subtillis* and *Acinetobactor sp*. under laboratory conditions. The two isolates (*B. megaterium* and *Acinetobactor sp.)* were considered as most toxic strains followed by *B. sphaericus, B. cereus,* and *B. subtillis* with LC_{90} values 4.1 ± 0.39, 2.8 ± 0.17, 3.6 ± 0.37, 2.5 ± 0.71, 3.6 ± 0.71 mg/ml respectively under laboratory conditions and ensuring mortality rate was 97% at 48 hrs exposure tests. This study concludes that non spore formers of common microbial isolates from the natural environment were also able to kill the larvae of *A.aegypti* through their secondary metabolites which are non – toxic to human population.

Keywords *Aedes aegypti*; *Bacillus thuringiensis*; *Bacillus sphaericus*; lethal concentration.

1 Introduction

Mosquitoes are the disease causing vectors within almost all tropical and subtropical countries are responsible for the transmission of pathogens causing some of the most life threatening and debilitating diseases of man, like malaria, yellow fever, dengue fever, chikungunya, filariasis, encephalitis, etc. (Chandra et al., 2008) which has put 55% of the world's population at risk in 124 countries (Beatty et al., 2007). *A. aegypti* is the major vector for chikungunya and dengue (Sourisseau et al., 2007). Till 10 October 2006, 151 districts of eight states/provinces of India have been affected by Chikungunya fever (Pialoux et al., 2007) and also, where there were 140, 000 cases in 2007. There is no specific treatment for these vector borne diseases. The present outbreak, which has seen more than 40 people die in the past two weeks in the south and central district of Kerala. According to sources, more than 100,000 people are down with fever in the south and central districts

of the state and the disease is now spreading to the northern districts as well. According to a study conducted by the Indian Institute of Management in Ahmadabad, *Aedes* mosquito-borne diseases primarily dengue fever and chikungunya costs India alone a hefty US$ 1.3 billion every year, 95% of that due to illness (WHO, 2009).

Around the world, the medical and economic burden caused by vector-borne diseases continues to grow as current control measures fail to cope. There is an urgent need to identify new control strategies that will remain effective, even in the face of growing insecticide and drug resistance (Achs and Malaney, 2002). Vector control strategies include chemical based control measures, non - chemical based control measures and biological control agents (Poopathi and Tyagi, 2006). Repetitive use of man-made insecticides for mosquito control disrupts natural biological control systems and lead to reappearance of mosquito populations. It also resulted in the development of resistance, detrimental effects on non-target organisms and human health problems and subsequently this initiated a search for alternative control measures (Das et al., 2007; Zhang et al., 2011). The use of biological control agents such as predatory fish (Legner, 1995), bacteria (Becker and Ascher, 1998), protozoa (Chapman, 1974), fungus (Murugesan et al., 2009) and nematodes (Kaya and Gaugler, 1993) had shown promising results to control mosquito populations. The development of new strategies, including naturally occurring larvicides to control mosquitoes, is important in order to counter the evolution of resistance in target populations and the possible effects on non target organisms (Cetin and Yanikoglu, 2006). *Bacillus thuringiensis subsp. israelensis (Bti)* and *Bacillus sphaericus (Bsp)* are entomopathogenic bacteria that are produce a parasporal crystal, were toxic to some invertebrates, mostly insects and nematodes (Feitelson et al., 1992). The purpose of the present study is to explore the larvicidal activity of natural microbial isolates of *B. megaterium, B. sphaericus, B. cereus, B. subtillis* and *Acinetobactor* species which are isolated from soil against the life threatened disease causing mosquito vector *A. aegypti.*

2 Materials and Methods

2.1 Mosquito culture

The *A. aegypti* eggs were collected from the ICMR- Vector control Laboratory, Madurai. Then the strain has been maintained in our laboratory for further studies of biological control of mosquito larvae through naturally isolated bacteria.

For culturing the mosquito strains, egg pad was placed in an enamel plastic tray (50×25×7cm) containing 2L of tap water and a pinch of larval fed on dried yeast or mouse diet, depending on the growth stages (Saitoh et al., 1998). These were reared under Photoperiod of 14:10 (Light- dark) at 27±2ºC. Larvae were reared to the 4th instars and allowed to pupae. Then pupae were transferred into beaker with tap water and placed in screen cage for adult maintenance. Adults were provided with 10% sucrose solution (Suenaga et al., 1987) and on day emergence, females were allowed to feed on restrained 5 month old white mouse (Saitoh et al., 1998). Early third instar larvae were selected for the experiments.

2.2 Soil sample collection

Different soil samples were collected from different regions in and around Alwarkurichi area. Each 1g of the sample was suspended in 9ml of sterile distilled water and shaken vigorously for about 2min. The samples were heated at 80°C for 30 min in a water bath to destroy all vegetative microbial cells for the isolation of *Bacillus* spp.

2.3 Identification of Bacillus spp.

Isolated strains were identified based on their morphological and biochemical characteristics, and confirmed according to Bergey's Manual of systematic Bacteriology 1& 2 (Palleroni, 1986; Sneath, 1986).

2.4 Preparation of bacterial inoculum

A single-dose test was performed, where the main aim was to identify the microbial isolates showing at least 50% toxicity against *A. aegypti* mosquito (Saitoh et al., 1996). All the microbial isolates were grown in nutrient agar medium and tested against third- instar larvae of *A.aegypti*. The cultures were grown for 48 h at 28ºC. During larval testing each culture were grown in LB (Louria Berthani) broth and incubated for two days and centrifuged at 5000g for 10 minutes. Each bacterial cell pellets were collected and washed two times with sterile distilled water and resuspended in sterile distilled water. The bacterial concentration was determined by dry cell weight (Baumann et al., 1991).

2.5 Bioassay

1ml of each bacterial concentration was added in to the 200ml container in triplicates with 100ml of distilled water and 25 larvae of tested mosquito strains. One container without bacterial suspension was used as control. After 24 and 48 hrs numbers of dead larvae were evaluated. The strains that killed more than 50% of the larvae were considered as pathogenic (Monnerat et al., 2001). Five microbial isolates were examined quantitatively for larvicidal activity against *A. aegypti*, using various concentrations (1ml, 2ml and 3ml) of microbial suspensions.

Mortality was scored after larval exposure for 24 and 48 hrs and the LC_{50}, LC_{90} values were determined by profit analysis (Finney, 1971). Data from mortality were expressed as the mean of three replications and transformed percentages were subjected to analysis of variance (ANOVA). Differences between the three treatments were determined by Tukey-Kramer HSD test ($P<0.05$) by using Minitab®15 software package.

2.6 Analysis of protein profile

Microbial isolate was cultured in the nutrient broth and incubate at 28°C for 4 days. 10ml of these cultured broths were centrifuged at 10,000g for 20 min at 4°C and the pellets of each isolates were washed three times with distilled water. Then add 2ml of EBC buffer [50 mM Tris pH 8.0,120 mM NaCl, 0.5% NP-40) + 5 mM DTT (Dithiothrietol) + protease inhibitors (2 µg/ml aprotinin, 1 µg/ml leupeptin, 2.5 mM PMSF) + 2 mg/ml lysozyme] into the pellet and heated up to 10 minutes in water bath and the proteins are harvested by centrifugation at 10,000g for 20min at 4°C. Then add 50µl of 1X sample buffer into the centrifuged pellets. Protein concentrations were measured by the method of (Lowry et al., 1951) with Bovine serum albumin used as the standard.

2.7 Gel electrophoresis

SDS-PAGE (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis) of the inclusion proteins was performed as described by Laemmli (1970) using 10% separating and 4% stacking gels. After electrophoresis the gels were stained with 0.1% (w/v) Coomassie blue. The molecular masses of proteins were determined by using protein standards (Helini biomolecules).

3 Results and Discussion

Natural soil sample is an excellent residence for plentiful microbes have ability to produce secondary metabolites applied in industrial production processes and biocontrol activities. Basis of this, natural as well as harmless microbial isolates screened and isolate from the soil sample for larvae control programm under laboratory condition. In screening assay ten bacterial cultures were used for larvicidal activity of *A. aegypti* mosquito among which the five of the isolates have been effective. The effective isolates were identified as based on their biochemical and morphological structures were found as *B. megaterium*, *B. sphaericus, B.*

cereus, B. subtillis and Acinetobactor spp. respectively. The biochemical characteristics of screened microbial isolates were shown in Table 1.

Table 1 Biochemical characterizations of microbial isolates.

S.No	Identification tests	*B. megaterium*	*B. sphaericus*	*B. cereus*	*B. subtilis*	*Acinetobacter sp.*
Preliminary tests						
1	**Grams staining**	Positive	Positive	Positive	Positive	Negative
2	**Spore staining**	Positive	Positive	Positive	Positive	Negative
3	**Shape**	Rod	Rod	Rod	Rod	Coccobacilli
4	**Motility**	Positive	Positive	Positive	Positive	Negative
Biochemical tests						
1	**Indole**	Negative	Negative	Negative	Negative	Negative
2	**Methyl red**	Negative	Negative	Positive	Negative	Negative
3	**Voges Proskauer**	Negative	Negative	Negative	Positive	Negative
4	**Citrate**	Positive	Negative	Positive	Positive	Positive
5	**Urease**	Negative	Positive	Positive	Negative	Negative
6	**H_2S**	Negative	Negative	Positive	Positive	Negative
Carbohydrate fermentation tests						
1	**Glucose**	A^-G^+	A^-G^-	A^+G^-	A^+G^-	A^-G^-
2	**Mannitol**	A^-G^+	A^-G^-	A^-G^-	A^+G^-	A^-G^-
3	**Lactose**	A^-G^+	A^-G^-	A^-G^-	A^-G^-	A^-G^-
4	**Sucrose**	A^+G^-	A^-G^-	A^+G^-	A^+G^-	A^-G^-
A^+G^- - Acid positive, Gas negative A^-G^- - Acid Negative, Gas negative **Other tests**						
1	**Starch agar test**	Positive	Negative	Positive	Positive	Negative
2	**Nitrate broth reaction**	Positive	Negative	Positive	Positive	Negative
3	**Gelatin**	Positive	Negative	Positive	Positive	Negative
4	**Caesin**	Positive	Negative	Positive	Positive	Negative
5	**Catalase**	Positive	Positive	Positive	Positive	positive
6	**Oxidase**	Negative	Positive	Negative	Positive	Negative

The percentage mortality of third instar larvae of *A. aegypti* mosquito by *B. megaterium, B. sphaericus, B. cereus, B. subtillis and Acinetobactor* spp. were represented in the Fig. 1. Although the isolated microbes were effective in control of *A. aegypti* the *B. megaterium* was found to highly effective which exhibit 87 ± 4% mortality. The percentage mortality was varied with concentration of isolated microbial suspension and the incubation time.

The initial cell concentration (1ml) of five microbial isolates shows the mortality about 50%, further the mortality rate was found to be higher with the increased concentration were significant to each other mortality range was about 50-75% within 24 hrs. The mortality rate was gradually increased with the incubation time range between 70-90% in 48 hrs which is represented in the Fig.1. Previous studies proved that *B. thuringiensis* subsp. *israelensis* (*Bti*) and *B. sphaericus* (*Bsp*) were entomopathogenic bacteria that have ability to control the larvae of *A. aegypti* mosquitoes (Das and Amalraj, 1997). Our study reports that the isolated *B. megaterium, B. sphaericus, B. cereus, B. subtillis* also have the ability to control *A. aegypti* larvae effectively in addition to their *B. thuringiensis* subsp. *israelensis* (*Bti*) and *B. sphaericus* (*Bsp*), not only the *Bacillus* spp but also the *Acinetobactor spp* control the *A. aegypti* larvae effectively.

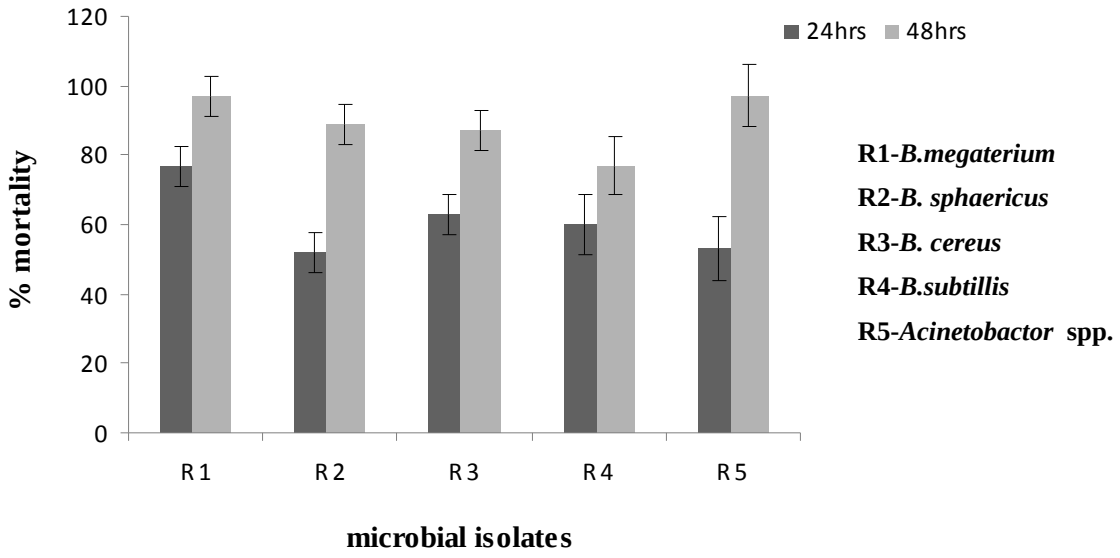

Fig. 1 Percent mortality of third instar larvae of *A. aegypti* treated with the bacterial isolates

The two microbial isolates (*B. megaterium* and *Acınetobactor sp.*) were effective which cause 97% larval mortality at 48 hr incubation in the bacterial concentrations of 4.1 ± 0.39 and 3.6 ± 0.71 mg/l. our results were correlated with the previous studies revealed that *B. cereus* and *B. megaterium* were ineffective against the third instar larvae of *A. aegypti*. The mortality rate was not observed even after 48 hrs of larval treatment (Corey et al., 1986). Dacre and colleagues (1997) studied the mutant type of *B. megaterium* strains produce toxic protein that was very effective against mosquito species than the wild type strains.

B. sphaericus is a spore forming aerobic bacterium, several strains of which are pathogenic for mosquito larvae. In the present study found that *B. sphaericus* exhibit 87 ± 6% mortality rate compared to the other investigations reduced level lethality occurred when treated cell concentration of 5 ml/l. *B. sphaericus* was used to control *Culex pipiens* and *C. quinquefasciatus* mosquito larvae and in some areas it is also used to

control *Anopheles* spp. (Surendran et al., 2011). The local mosquito species *C. thalassius, C. nebulosus, C. perfuscus, C. pocilipes* showed high susceptibility to a *B. sphaericus* formulation (VectoLex(R) WDG) in a standardized field test. A dosage of 1 g/m^2 was effective to achieve 100 percent mortality rate for *C. quinquefasciatus* late instar larvae in a sewage habitat, with a residual effect of up to 7 days (Lingenfelser et al., 2010). *B. sphaericus* against the first to fourth instar larvae and pupae had the following LC_{50} values: I instar was 0.051%, II instar was 0.057%, III instar was 0.062%, IV instar was 0.066%, and for the pupae was 0.073% (Kovendan et al., 2011). A flowable concentrate of *B. sphaericus* (Neide) strain 2362 was applied against *Anopheles gambiae* Giles *s.l.* mosquito larvae in small plot field trials in Bobo-Dioulasso area, Burkina-Faso. Third and fourth instar larvae were controlled for 10–15 days with a dosage of 10 g/m^2, 3–10 days with 1 or 0.1 mg/m^2, and 2 days with 0.01 g/m^2 (Nicolas et al., 1987).

During sporulation *B. sphaericus* able to produce two crystal proteins 51.4 and 41.9KDa which were encoded by highly conserved chromosomal genes. Nevertheless during vegetative growth some strains produce mosquito larvicidal proteins of 100 and 30.8KDa (Mixed toxins) the mode of action of which is still unknown (Charles et al., 1996). In the present study, two proteins were obtained (Fig. 2, Lane 3) which are might be related to the toxicity against mosquito larvae. Monnerat et al. (2004) found that the effective strains of *B. sphaericus* were isolated from the soil sample against *A. aegypti*. Baumann et al. (1987) had found that cell suspension containing 174 ng (dry weight) of the more toxic recombinant *B. sphaericus* 2362 strain per ml killed 50% of the larvae during sporulation, produce a parasporal crystalline protein which is toxic for the larvae of a number of mosquito species, but the present study explore the mortality rate of *B. sphaericus* 87 ± 6% at 48 hr incubation period at dry weight (0.011 ± 0.001g/l) cell suspension. LC_{50} and LC_{90} values, using a find whole culture against the target mosquito species, were subsequently determined for the five isolates that presented in (Fig. 3) and explore various activities.

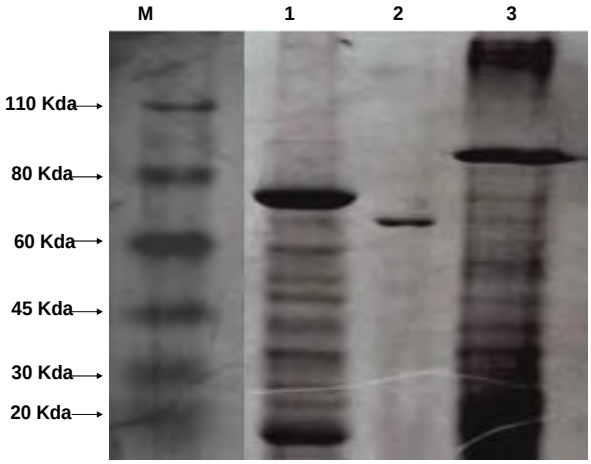

Fig. 2 Two proteins. Plate-1 SDS – PAGE protein profile for microbial isolates: M- Marker; Lane 1-*B. megaterium;* Lane 2-*B. subtillis*; Lane 3- *B.sphaericus.*

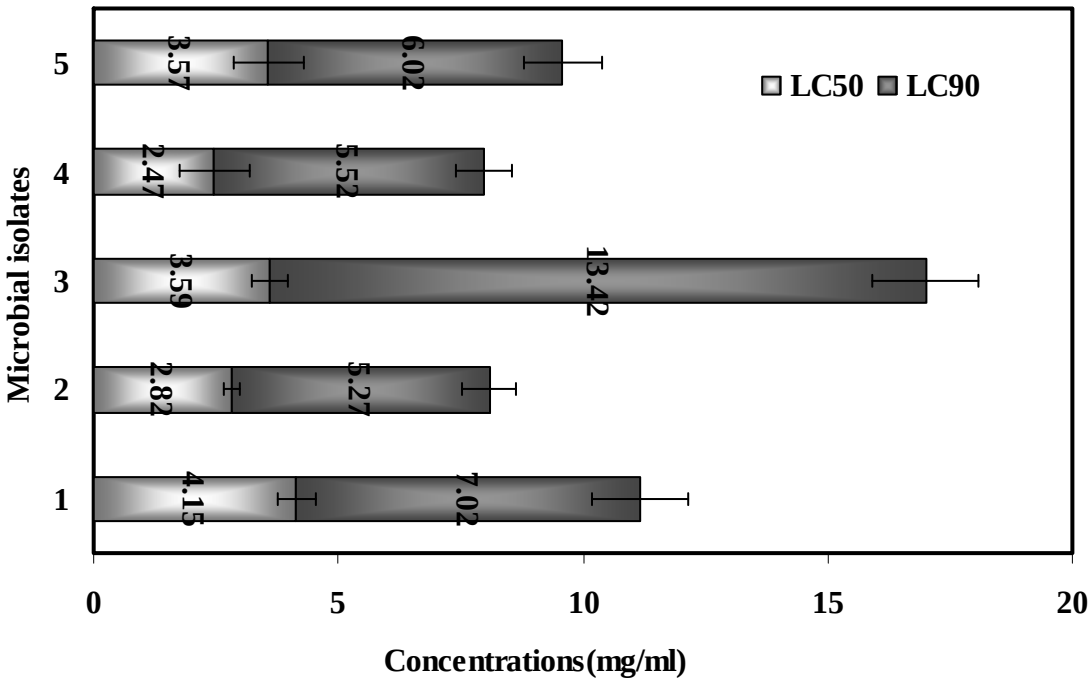

Fig. 3 LC_{50} & LC_{90} values of third instar larvae of *A. aegypti* treated with the bacterial isolates

B. subtilis is a ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. The bacterium produces an endospore that allows it to endure extreme conditions of heat and desiccation in the environment. The culture supernatant of a strain of *B. subtilis* subsp. *subtilis* isolated from mangrove forests was found to kill larval and pupal stages of mosquitoes through their secondary metabolite surfactin (Geetha et al., 2010; Geetha and Manonmani, 2008). *B. subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. Kishore and Ashis (2006) concluded that *B. subtilis* secret cyclic lipopeptides (CLPs) that have the mosquito larvicidal potency. LC_{50} values of the crude CLPs secreted by *B. subtilis* DM-03 and DM-04 strains against third instar larvae of *C. quinquefasciatus* was recorded as 120.0 ± 5.0 and 300.0 ± 8.0 mg/l, respectively for 24 h treatment. Our study found that *B. subtillis* isolated from the soil shows percentage mortality about 77% at 48hrs treatment and the LC_{90} value was 0.03 ± 0.003 mg/l.

B. cereus also a gram positive, spore forming rod shaped bacteria used for biological control agent widely available in soil environment. *B. cereus* is a natural facultative mosquito pathogen (Krattiger, 1997; Cooping and Menn, 2001; Wirth et al., 2004; Teng et al., 2005, Chatterjee et al., 2008). *B. cereus* strains are able to colonize in the guts of the mosquito larvae (Plearnpis et al., 2001). Insecticidal activity of spores of *B. cereus* against *A. aegypti* has been determined (Tyrell et al., 1981). Significant larval reduction was observed using *B. cereus* as a facultative pathogen for *A. subpictus* Grassi larvae in the natural environment (Chatterjee et al., 2010). The control of *A. aegypti* was found to be significant at the cell concentration of 0.010 ± 0.001g/l and cause 77 ± 6% mortality rates.

Acinetobactor sp. is common soil borne coccoid bacteria that also give 97 ± 5% mortality rates. Sezen, and Demyrbag (2007) found that *Acinetobactor sp*. active against summer cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae) is one of the pests. Furthermore, *Acinetobactor spp.* can be potent for the control of mosquito larvae. The results from this study show that the microbial isolates of those *Bacillus sp.* and *Acinetobactor sp*. act as effective control agents against the chikungunya and dengue vector of *A. aegypti* mosquito.

The SDS-PAGE analyses of crude extra cellular proteins from the three selected microbial isolates of *B. megaterium*, *B. sphaericus*, and *B. subtillis* were shown in the plate 1. Chemical pesticides are very active against mosquito larvae but at the same time they are highly toxic to non targeted organisms. Control (without microbial suspensions) showed no larval mortality after 48hr treatments. Triplicate values were used for all the statistical analysis.

4 Conclusion

Mosquito borne diseases are extensively spreads in the world population and it influence the global economy also. Consequently it should be eradicated from the world through the usage of the non-polluted mosquitocidal agents like microbial metabolites. For the basis of these concerns we isolate five microbial species from the soil have ability to kill *A. aegypti* mosquito larvae. This study just reflects the larvicidal potential of the some natural microbial isolates and their preliminary protein analysis, which now being further investigated at the molecular level.

References

Achs J, Malaney P. 2002. The economic and social burden of malaria. Nature, 15: 680- 685

Baumann MA, Clark L, Baumann P. 1991. *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxins. Microbiology Reviews, 55(3): 425-436

Baumann P, Baumann L, Bowditch RD, et al. 1987. Cloning of the gene for the larvicidal toxin of *Bacillus sphaericus* 2362: Evidence for a family of related sequences. Journal of Bacteriology, 169(9): 4061–4067

Beatty ME, Letson W, Edgil DM, et al. 2007. Estimating the total world population at risk for locally acquired dengue infection. Proceedings of 56th Annual Meeting of American Society of Tropical Medicine and Hygiene, Philadelphia, Pennsylvania, USA, 4-8

Becker N, Ascher KRS. 1998. The use of *Bacillus thuringiensis subsp.israelensis (Bti)* against mosquitoes, with special emphasis on the ecological impact. Israel Journal of Entomology, 32: 63-69

Cetin H, Yanikoglu A. 2006. A study of the larvicidal activity of *Origanum* (Labiatae) species from southwest Turkey. Journal of Vector Ecology, 31 (1): 118-122

Chandra G, Bhattacharjee I, Chatterjee SN, et al. 2008. Mosquito control by larvivorous fish. Indian Journal of Medical Research, 127: 13-27

Chapman HC, 1974. Biological control of mosquito larvae. Annual Review of Entomology, 19: 33-59

Charles JF, Nielson-LeRoux C, Delecluse A. 1996. *Bacillus Sphaericus* Toxins: Molecular Biology and Mode of Action. Annual Review of Entomology, 41: 451-472

Chatterjee SN, Das D, Dangar TK. 2008. Efficacy of *Bacillus sphaericus* against *Aedes* (*Stegomyia*) *aegypti*, *Mansonia Indiana* (Edward) and *Culex vishnui* under laboratory and field conditions. Entomon-Trivandrum, 33(4): 181-188

Chatterjee S, Subhra Ghosh T, Das S. 2010. Virulence of *Bacillus cereus* as natural facultative pathogen of *Anopheles subpictus* Grassi (Diptera: Culicidae) larvae in submerged rice-fields and shallow ponds. African Journal of Biotechnology, 9(41): 6983-6987

Cooping LG, Menn JJ. 2001. Biopesticides: a review of their action, applications and efficacy. Pest Management Science. 56: 651-676

Corey JW, Graham AC, Mary AP, et al. 1986. Analysis of mosquito larvicidal potential exhibited by vegetative cells of *Bacillus thuringiensis subsp. Israelensis.* Applied and Environmental Microbiology, 52: 650-653

Dacre FE, Robert JP, Stephen FD, et al. 1997. Isolation of *Bacillus megaterium* mutants that produce high levels of heterologous protein, and their use to construct a highly mosquitocidal strain. Journal of Current Microbiology, 35(2): 71-76

Das NG, Goswami D, Rabha B. 2007. Preliminary evaluation of mosquito larvicidal efficacy of plant extracts. Journal of Vector Borne Diseases, 44: 145–148

Das PK Amalraj DD. 1997. Biological control of malarial vectors. Indian Journal of Medicine, 106: 174-197

Feitelson JS, Payne J, Kim L 1992. *Bacillus thuringiensis*; Insects and beyond. Biotechnology, 10: 271-275

Finney DJ. 1971. Probit Analysis (3rd edition). Cambridge University Press. UK

Geetha I, Manonmani AM. 2008. Mosquito pupicidal toxin production by *Bacillus subtilis* subsp. *Subtilis.* Biological Control, 44(2): 242-247

Geetha I, Manonmani AM, Paily KP. 2010. Identification and characterization of a mosquito pupicidal metabolite of a *Bacillus subtilis* subsp. *subtilis* strain. Applied Microbiology and Biotechnology, 86(6): 1737-1744

Kaya HK, Gaugler R, 1993. Entomopathogenic nematodes. Annual Review of Entomology 38: 181-206

Kishore D, Ashis KM, 2006. Assessment of mosquito larvicidal potency of cyclic lipopeptides produced by *Bacillus subtilis* strains. Acta Tropica, 97(2): 168-173

Kovendan K, Murugan K, Vincent S, et al. 2011. Studies on larvicidal and pupicidal activity of *Leucas aspera* Willd. (Lamiaceae) and bacterial insecticide, *Bacillus sphaericus*, against malarial vector, *Anopheles stephensi* Liston. (Diptera: Culicidae). DOI: 10.1007/s00436-011-2469-2

Krattiger F. 1997. Insect resistant crops: A case study of *Bacillus thuringiensis* (BT) and its transfer to developing countries. ISAAA Briefs, 2: 42

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature, 227: 680-685

Legner EF. 1995. Biological control of Diptera of medical and veterinary importance. Journal of Vector Ecology, 20: 59- 120

Lingenfelser A, Rydzanicz K, Kaiser A, et al. 2010. Mosquito fauna and perspectives for integrated control of urban vector-mosquito populations in Southern Benin (West Africa). Annals of Agricultural and Environmental Medicine, 17(1): 49-57

Lowry OH, Rosebrough NJ, Farr AL, et al. 1951. Protein measurement with the Folin phenol reagent. Journal of Biolgical Chemistry, 193: 265-275

Monnerat RG, Silva SF, Silva WJO. 2001. Catalogue of the germplasm bank of bacteria entomopathogenic *Bacillus spp*. Brasilia. Embraca Genetic Resources and Biotechnology, Document 60: 65

Monnerat R, Silva SF, Dias DS, et al. 2004. Screening of Brazilian *Bacillus sphaericus* strains for high toxicity against *Culex quinquefasciatus* and *Aedes aegypti*. Journal of Applied Entomology, 128: 469-473

Murugesan AG, Sathesh Prabu C, Selvakumar C. 2009. Biolarvicidal activity of extracellular metabolites of the keratinophilic fungus *trichophyton mentagrophytes* against larvae of *Aedes aegypti* – a major vector for chikungunya and dengue. Folia Microbiologica, 54 (3): 213–216

Nicolas L, Darriet F, Hougard JM. 1987. Efficacy of *Bacillus sphaericus* 2362 against larvae of *Anopheles gambiae* under laboratory and field conditions in West Africa. Medical and Veterinary Entomology, 1(2): 157–162

Palleroni NJ. 1986. In: Bergey's Manual of Systematic Bacteriology (Vol. 1) (Krieg NR, Holt JG, eds). Williams and Wilkins, Baltimore, USA

Pialoux G, Gauzere B. Jaureguiberry S, et al. 2007. Chikungunya An epidemic arbovirus. Lancet Infectious Diseases, 7: 319–327

Plearnpis L, Haruyuki A, Sakol P, et al. 2001. Isolation of bacterial strains colonizable in mosquito larval guts as novel host cells for mosquito control. Journal of Bioscience and Bioengineering, 92(4): 342-345

Poopathi S, Tyagi BK. 2006. The challenge of mosquito control strategies; from primordial to molecular approaches. Biotechnology and Molecular Biology Reviews, 1 (2): 51-65

Saitoh H, Higuchi K, Mizuki E, et al. 1996. Larvicidal activity of *Bacillus thuringiensis* natural isolates, indigenous to Japon, against two nemotoceron insect pests occuring in urban sewage environments. Microbiological Research, 151: 263-271

Saitoh H, Higuchi K, Mizuki E, et al. 1998. Larvicidal activity of Japanese *Bacillus thuringiensis* against the *Anophelous stephensi.* Medical and Veterinary Entomology, 12: 98-102

Sezen K, Demyrbag Z. 2007. The Investigation of Biological Control Agent of Summer Cockchafer (*Melolontha melolontha*, (Coleoptera: Scarabaeidae). Ekoloji, 63: 34-40

Sneath AP. 1986. In: Bergey's Manual of Systematic Bacteriology (Vol. 2) (Sneath AP, Mair NS, Sharpe MS, Holt JG, eds). Williams and Wilkins, Baltimore, USA

Sourisseau M, Schilte C, Casartelli N, et al. 2007. Characterization of reemerging chikungunya virus. PloS Pathogens, 3:89

Suenaga O, Kurukawa K, Wada Y. 1987. A cube sugar technique for leboratory rearing of adult mosquitoes. Tropical medicine, 29: 47-54

Surendran A, John Vennison S, 2011. Occurrence and Distribution of Mosquitocidal *Bacillus sphaericus* in Soil. Academic Journal of Entomology, 4 (1): 17-22

Teng HJ, Lu LC, Wu YL, et al. 2005. Evaluation of various control agents against mosquito larvae in rice paddies in Taiwan, Journal of Vector Ecology, 30: 126-132

Tyrell DJ, Lee A, Bulla JR, et al. 1981. Characterization of spore coat proteins of *Bacillus thuringiensis* and *Bacillus cereus.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 70(3): 535-539

Wirth MC, Delecluse A, Walton WE. 2004. Laboratory selection for resistance to *Bacillus thuringiensis* subsp. *jegathesan* or a component toxin, Cry 11B, in *Culex quinquefasciatus* (Diptera:Culicidae). Journal of Medical Entomology, 41(3): 435-441

World Health Organization, 2009. Mosquito Wars. 87: 167-168

Zhang WJ, Jiang FB, Ou JF. 2011. Global pesticide consumption and pollution: with China as a focus. Proceedings of the International Academy of Ecology and Environmental Sciences, 1(2): 125-144