Tropical Biomedicine 32(1): 84–97 (2015)

An investigation on the diversity of mosquitocidal bacteria and its relationship with incidence of vector borne diseases

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Received 13 November 2013; received in revised form 25 March 2014; accepted 6 June 2014

Abstract. Control of mosquitoes is the most important aspect of public health, as mosquitoes transmit many human diseases, including the fatal infection, Japanese encephalitis. This paper addresses the isolation of new mosquitocidal bacteria from soil samples in the Union Territory of Pondicherry, India, where, no clinical cases of vector borne infections have been reported. Bacterial isolates from soil samples were screened for potential mosquitocidal strains and bioassays against mosquito vectors (*Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*) were carried out. Genomic DNA of potential mosquitocidal isolates was amplified and species identification was carried out using BLASTn program (NCBI). Phylogenetic analysis of 16S rRNA sequences of mosquitocidal bacteria revealed seven potential isolates. SDS-PAGE results have shown that there was considerable difference in the protein profiles. Numerical analysis revealed 4 distinct groups at similarity level 25%. The relationship between VBDs and prevalence of soil mosquitocidal bacteria in the study sites has elicited considerable interest in the diversity of mosquitocidal bacteria and their application for mosquito borne diseases control.

INTRODUCTION

Insect vectors are recognized as organisms causing diseases of public health importance in both developing and developed countries. Although mosquito borne diseases *viz*: filariasis, malaria, yellow fever, West Nile, and Japanese encephalitis have declined in many parts of the world, dengue infection continue to be a major public health problem in tropical countries. Chikungunya pandemic that spread to many parts of the world during the last decade affected the quality of life of the infected individuals. In the early days, chemical pesticides were widely used in vector control programme. Since usage of chemical pesticides for long time and development of insecticidal resistance in insect vectors are environmental concern, it is important to search and discover biological agents for control of mosquito vectors. Usage of bacterial agents in mosquito control started from discovery of *Bacillus sphaericus* (*Bs*) 1593 (Singer, 1974) and a highly toxic strain of *Bacillus thuringiensis* serovar *israelensis* (*Bti*) in 1977 (Goldberg, 1977; Mwangangi *et al.*, 2011). These bacterial agents produce sporulation and crystalline-endotoxin, specific to Coleopteran and Dipteran organisms (Ellar *et al.*, 1985; Hofte & Whitley, 1989; Gleave *et al.*, 1992; Poopathi *et al.*, 2002; Rodriguez *et al.*, 2003; Poopathi & Tyagi, 2006; Raghavendra *et al.*, 2011). A large number of strains from these species have been isolated hitherto.

Extensive research on screening of mosquitocidal bacteria resulted in isolation of over 300 *B. sphaericus* strains. Among them, 17 strains are highly toxic to mosquito larvae (de Barjac, 1990). *Bacillus sphaericus* strain 2297 from Sri Lanka (Wickremeshighe, 1980), 1593M from Indonesia (de Barjac &

Sutherland, 1990; Singer, 1990), 2362 from Nigeria (Weiser, 1984), and C3-41 from China (Liu *et al.*, 1989) were studied extensively and are now available commercially as mosquito larvicides.

In addition to existing isolates, only *B. sphaericus*, *B. thuringiensis*, *Brevibacillus laterosporus*, *Streptomyces* and *Clostridium bifermentans* are known to be mosquitocidal (Thiery *et al.*, 1992; Orlova *et al.*, 1998; Federici *et al.*, 2006; Park *et al.*, 2007; Patil *et al.*, 2013; Thenmozhi *et al.*, 2013). The *B. thuringiensis* subsp. *israelensis* is the most potent and effective, and produces 4 major mosquitocidal toxins of Cry4A, Cry4B, Cry11A and Cyt1A in a parasporal body (Federici *et al.*, 2006; Park *et al.*, 2006; Frankenhuyzen, 2009; Singh & Prakash, 2009; Wirth *et al.*, 2011; Poopathi & Archina, 2012). On the other hand, putative toxins from *C. bifermentans* include a doublet of 66-68kDa and two other small proteins of 16 and 18kDa (Nicolas *et al.*, 1993). Larvicidal effect of *B. sphaericus* is mainly due to two kinds of toxins (crystal (Cry) and mosquitocidal (Mtx) toxins), which differ in composition and time of synthesis (Baumann *et al.*, 1991). Among these two toxins, crystal toxin is the main toxic factor in the highly larvicidal strains. The crystal toxin is made up of two polypeptides with molecular weight of about 51 and 42 kDa (Charles *et al.*, 1997; Dias *et al.*, 1999; Poopathi & Abidha, 2009). Different methods have been used for typing bacterial species as follows: Serotyping, analysis of cellular fatty acid content, native-PAGE, and small-subunit ribosomal RNA sequencing and genome analysis (Ash *et al.*, 1991; Berber & Cokmus, 2001).

All these years, field studies were carried out to know the efficacy of these strains against the vectors. As most of the bacteria are from the soil or aquatic environment, it is imperative to address the relationship between their presence in the environment and vector borne diseases. In the present study, an attempt has been made to investigate the diversity of mosquitocidal bacteria isolated from natural soil and its relationship with endemicity of mosquito borne diseases in the study sites (rural villages) around union territory of Pondicherry, India. In addition, it was envisaged to study the phylogenetic relationship of these new isolates. The outcome of the study is likely to expound a different approach on the correlation of mosquitocidal bacteria and vector borne disease control and an insight into evolutionary relationships with known *Bacilli*.

MATERIALS AND METHODS

Study sites and soil sample collection

An open cross sectional study was carried out in two groups of villages in the Union Territory of Pondicherry (11°59'N and 79°50'E). Group 1 included three villages, Poraiyur, Koodappakkam and Pilliyarkuppam in which there were no reported cases of VBDs and group 2 included another three villages, Suthukeny, Santhaipudukuppam and Lingareddipallayam in which VBDs were reported (Figure 1). A quantum of soil samples (1-2 gms) from 10 different locations from each village were collected in vials containing 30% sterile glycerol. To obtain samples with minimal effect of external factors, samples were collected about 2-3 cm below the surface of habitat. The samples were transported to the laboratory for further analysis.

Bacterial strain isolation

The number of soil samples collected from different study sites was pasteurized at 80ºC for 15 minutes (Poopathi *et al.*, 2014) and after serial dilutions (10^{-6}), 100µl of aliquot was poured into plates of Luria-Bertani agar (peptone, yeast extract, NaCl and Agar 2:1:2:2, pH 7.8). The plates were incubated at 30ºC for 24 hrs and different morphological colonies were selected. They were subcultured and finally maintained as 30% glycerol stock until further use.

Bacterial sample preparation

The bacterial colonies isolated were inoculated into 500 ml conical flask containing 50 ml of LB broth and incubated for 72 hours in orbital shaker. As soon as the cultures were completely sporulated, the

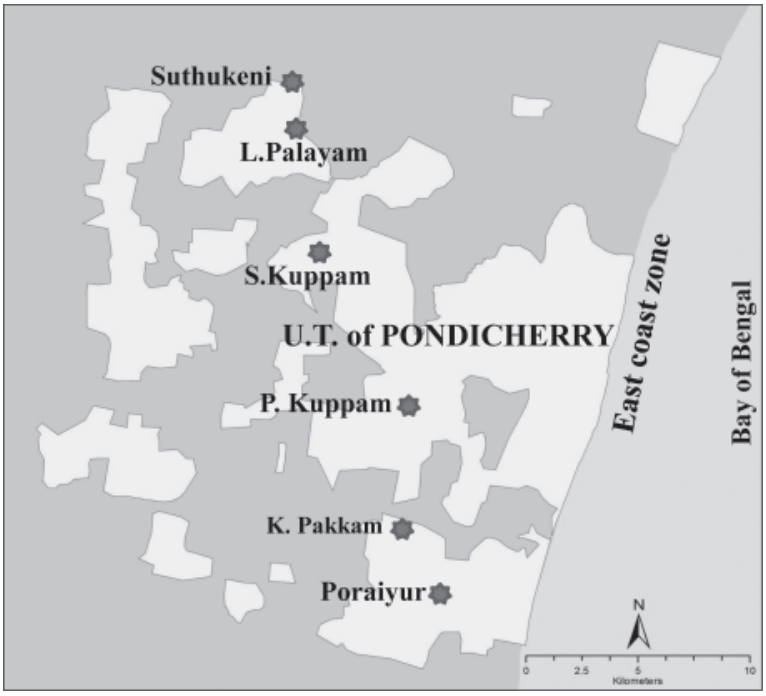

Figure 1. Map showing the study sites at Pondicherry where soil samples were collected for microbial characterization

biomass containing the spore/crystal toxin complex were recovered by centrifugation (10,000g/30 min/4°C) using refrigerated high-speed centrifuge (Kendro, USA) (Poopathi *et al.*, 2014), freeze dried (Edwards Freeze Dryer, E2M5, England) and preserved at -20°C for further analysis.

Bioassays

Culex quinquefasciatus, *Anopheles stephensi* and *Aedes aegypti* mosquito larval species used in the present study were obtained from the mosquito colony maintained in the insectary of the rearing and colonization section (R&C), Vector Control Research Centre (ICMR), Pondicherry, India. The bioassay procedure followed was essentially that recommended by the World Health Organization (WHO, 1985). Bioassays were conducted in 300 ml disposable wax quoted paper cups. A stock solution was prepared from the new isolates (5mg/l0ml water), and serial dilutions were made (0.02 to 2mg/l). Twenty five early 3rd instar larvae from each mosquito species were introduced separately into each of the test concentrations. The bioassays were conducted at room temperature (28°C) and larval mortality was assessed. If the mortality in control larvae was between 5 and 20%, Abbott's formula was used to correct the mortality (Abbott, 1925). The moribund larvae were counted as dead.

Amplification of 16S rRNA gene

Total genomic DNA from isolates showing mosquitocidal activity was extracted using GenElute™ Bacterial Genomic DNA Kit (SIGMA-ALDRICH) and quality/quantity were determined by agarose gel electrophoresis, followed by Ethidium Bromide (EtBr) staining (0.5µg/ml). The genomic DNA was subjected to PCR amplification of the 16S rDNA using forward (8F) and reverse (1942R) primers respectively (5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3'). The polymerase chain reaction was carried out using the following program: 95°C for 5 minutes followed by 35 cycles of 94°C for 1 minute, 50°C for 45 seconds and 72°C for 1 minute, final extension was carried out at 72°C for 5

minutes (Poopathi *et al.*, 2014). The amplified product was confirmed by agarose gel electrophoresis, followed by Ethidium Bromide staining.

Sequencing of 16s rRNA and bacterial identification

PCR product of 16S rDNA was purified using Qiaquick PCR purification kit (QIAGEN, USA). Sequencing reactions were carried out in both directions using same forward and reverse primers used for amplification of 16S rDNA region with BigDye Version 3.1 kit (Applied Bio-systems) on an ABI-PRISM 3730 DNA Sequencer (Applied Bio-systems). Ambiguous sequences from the base called sequences were corrected with Chromas (Version 2.01) and the sequences were assembled with Bio-Edit (Version 7.0.9.0). The sequence of potential isolate for species identification was made using the BLASTn program (NCBI) and the nucleotide sequence has been submitted to GenBank for reference (Table 1).

SDS-PAGE (Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis)

Electrophoretic separation of protein was carried out using small and larger vertical slab gel unit (GENEI, India) (Laemmli, 1970). The resolving gel consisted of 10% acrylamide (30:0.8 acrylamide: Bis), 0.38M Tris (pH 8.8), 0.1% Sodium dodecyl sulphate (SDS), 0.1% Ammonium per sulphate (APS), and 0.07% N,N,N',N'- tetra ethylene dimethylene diamine (TEMED). The concentrations of the stacking gel was 0.4% acrylamide (30: 0.8, acrylamide: bis), 0.13M Tris (pH 6.8), 0.1% SDS, 0.05% APS and 0.7% TEMED. The running buffer consisted of 0.25M Tris (pH 8.3), 0.19M glycine and 0.4% SDS.

A total of 15 µg protein equivalent samples from potential bacterial isolates were estimated (Bradford, 1976), incubated with an equal volume of sample loading buffer (0.125 M Tris-Buffer (pH 6.8), 4% SDS, 20% glycerol, 10% β – mercaptoethanol), boiled (10 min) and finally loaded on the wells of the gel and subjected to electrophoresis (volt: 200V). The gel was stained in Coomassie Brilliant Blue R-250 (0.13% CBB, 50% methanol and 10% glacial acetic acid) overnight and de-stained (methanol: glacial acetic acid: water, 12:7:81%). The protein bands were visualized, photographed and analyzed in a Gel Doc system (SYNGENE, U.K).

Data analysis

The software package 'ASSAY' was used for dosage mortality regression analysis. Phylogenetic analysis of the 16S rRNA sequences of the selected mosquitocidal bacteria was performed with Mega5 and the evolutionary history was inferred using the UPGMA method (Sneath, 1989). The evolutionary distances in the units of the number of base substitutions per site were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2007) and finally, the evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2007). For protein profile analysis, gels were scanned using gel documentation system (Gel Doc. SYNGENE and analyzed with GeneTools software data analysis). Each band was recognized by its length, width and intensity. Accordingly, relative amount of each band quantity was measured and scored. Each band was scored as present (1) or absent (0), and pair-wise comparisons between isolates were used to calculate the Jaccard's coefficient of genetic similarity matrix. Hierarchical cluster analysis to produce a dendrogram was performed using un-weighted pair-group method with arithmetical (UPGMA).

RESULTS

Identification of mosquitocidal bacteria from natural soils

Screening of bacterial colonies from soil samples collected from all experimental sites from group 1 villages where there were no reported cases of VBDs revealed the occurrence of seven potential bacterial isolates (Table 1). These isolates had been identified as new and their respective 16s rDNA sequences had been submitted to NCBI. The isolates had also been submitted to "Microbial Collection Stock Centre" of

Table 1. Novel mosquitocidal bacteria isolated from study villages where, vector borne disease (VBD) prevalence was not reported by the State Health Authorities, Govt. of Pondicherry

Sl. No	Study villages	Potential isolates	Bacterial strains	NCBI-Accession No
1.	Suthukeny	VCRC-B588	*B.thuringiensis*	JQ289052
		VCRC-B589	*B.thuringiensis*	JQ289051
2.	Santhai Pudukuppam	VCRC-B583	*B.thuringiensis*	JQ289047
		VCRC-B593	*B.thuringiensis*	JQ289046
		VCRC-B594	*B.thuringiensis*	JQ289048
3.	Lingareddipallayam	VCRC-B595	*B.thuringiensis*	JQ289049
		VCRC-B596	*Lysinibacillus sphaericus*	JQ289050

Vector Control Research Centre (VCRC), Pondicherry (India) for coding and institutional reference.

Bioassays (larval toxicity assays) were carried out with lyophilized powders of new isolates (*B. thuringiensis* and *B. sphaericus*) cultured from LB media. Three larval species (*Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti*) reared in the laboratory were used for bioassays. The sub-lethal concentration to obtain 50 and 90 percent mortality of mosquito larvae (LC_{50} and LC_{90}) are shown in Table 2. As shown in the results, the isolates of *B. thuringiensis* (JQ289046 to JQ289048) and *B. sphaericus* (JQ289050) showed toxicity against all three mosquito species, whereas the other strains of *B. thuringiensis (*JQ289049, JQ289052) showed toxicity against only two mosquito species (*Cx. quinquefasciatus* and *An. stephensi*). The strain, *B. thuringiensis* (JQ289051) showed toxicity against only one mosquito species (*Cx. quinquefasciatus*). Among the seven isolates, the most promising isolates were *B. thuringiensis* JQ289048 and JQ289046.

Figure 2 shows PCR products of 1.5kb (1500bp) from all the selected isolates. Further, the isolates were identified to species level with BLASTn analysis. Homology search against non- redundant nucleotide database identified all isolates VCRC-B583, B588, B589, B593, B594 and B595 as *B. thuringiensis* and VCRC-B596 as *Lysinibacillus sphaericus*.

The homology of 16S rDNA gene sequences of new *Bacillus* isolates investigated in the present study were compared with 16S rDNA gene sequences of closely related *Bacillus* strains from Genbank database and a rectangular phylogenetic tree based on topological algorithm was assessed. The result revealed that presence of similar mosquitocidal *B. thuringiensis* strains from all three study sites from group 2 (Figure 3). Analysis of phylogenetic tree with branch length 0.035 revealed three distinct groups. Phylogeny resulted in identification of a distinct isolate of *B. thuringiensis* were observed in the study site, Santhaipudukuppam (*Bt*: JQ289046) with most potential placed as group II in the phylogeny. Bacterial isolate from other study site, Lingareddipalayam (*Bs*: JQ289050) was observed among separate group of genus *Lysinibacillus*, with more identity to *B. sphaericus*. Further observation of the phylogeny revealed *B. thuringiensis* similar to *B. thuringiensis* serovar *israelensis* and other serovars were present naturally in soils of all three villages (JQ289047 to JQ289049 and JQ289051, JQ289052) as group I. The sequence alignment of 16S rDNA gene (JQ289047 to JQ289049 and JQ289051, JQ289052) showed the maximum homology with *B.t.* serovar *asturiensis*, *B.t.* serovar *finitimus*, *B.t.* serovar *sotto*, *B.t.* serovar *kurstaki*, *B.t.* serovar *fukuokaensis* and *B.t.* serovar *israelensis* and *B.t. navarrensis* respectively. Thus, sequencing of 16S rDNA gene helped in rapid identification of *B.t.* at sub-species level. Mosquitocidal *Lysinibacillus sphaericus* isolate from Lingareddipalayam in the phylogeny revealed a distinct group among different *Lysinibacillus* and it was observed to be more similar to *L. sphaericus* 16S rRNA

Table 2. Toxicity of newly isolated potential mosquitocidal bacteria against major mosquito vectors

Bacterial strains	Mosquito species	Intercept	Slope ± SE	LC_{50} (Mg/l) (LCL-UCL)	LC_{90} (Mg/l) (LCL-UCL)	x^2df
B. thuringiensis (JQ289047)	Cq	6.73	0.82 ± 0.07	0.120(0.10-0.14)	0.57(0.45-0.74)	7.24
	An	6.70	0.83 ± 0.07	0.12(00.11-0.14)	0.60(0.48-0.75)	10.07
	Aed	6.58	0.77 ± 0.07	0.128(0.11-0.14)	0.673(0.52-0.85)	6.84
B. thuringiensis (JQ289048)	Cq	7.80	0.81 ± 0.07	0.031(0.02-0.036)	0.15(0.11-0.19)	2.01
	An	7.62	0.81 ± 0.07	0.04(0.03-0.046)	0.19(0.15-0.24)	12.68
	Aed	6.67	0.82 ± 0.07	0.110(0.10-0.13)	0.54(0.43-0.69)	3.30
B. thuringiensis (JQ289046)	Cq	7.53	0.77 ± 0.07	0.037(0.032-0.043)	0.19(0.15-0.25)	3.65
	An	6.83	0.82 ± 0.07	0.108(0.09-0.12)	0.51(0.40-0.64)	3.02
	Aed	6.77	0.82 ± 0.07	0.11(0.10-0.13)	0.54(0.43-0.69)	2.40
B. sphaericus (JQ289050)	Cq	6.62	0.66 ± 0.08	0.085(0.072-0.101)	0.596(0.45-0.78)	7.82
	An	6.57	0.77 ± 0.07	0.13(0.113-0.152)	0.68(0.53-0.87)	5.92
	Aed	6.57	0.80 ± 0.07	0.14(0.122-0.162)	0.68(0.546-0.870)	5.85
B. thuringiensis (JQ289052)	Cq	6.87	0.87 ± 0.069	0.11(0.10-0.13)	0.505(0.40-0.63)	1.46
	An	6.75	0.82 ± 0.07	0.12(0.104-0.139)	0.566(0.44-0.72)	6.92
B. thuringiensis (JQ289049)	Cq	6.79	0.73 ± 0.08	0.08(0.07-0.10)	0.5(0.38-0.65)	4.03
	An	6.51	0.75 ± 0.07	0.134(0.11-0.15)	0.72(0.56-0.93)	6.29
B. thuringiensis (JQ289051)	Cq	6.80	0.77 ± 0.07	0.096(0.082-0.11)	0.50(0.39-0.65)	9.73

Cq: *Culex quinquefasciatus*; An: *Anopheles stephensi*; Aed: *Aedes aegypti*; LCL: Lower confidential limit; UCL: Upper confidential limit

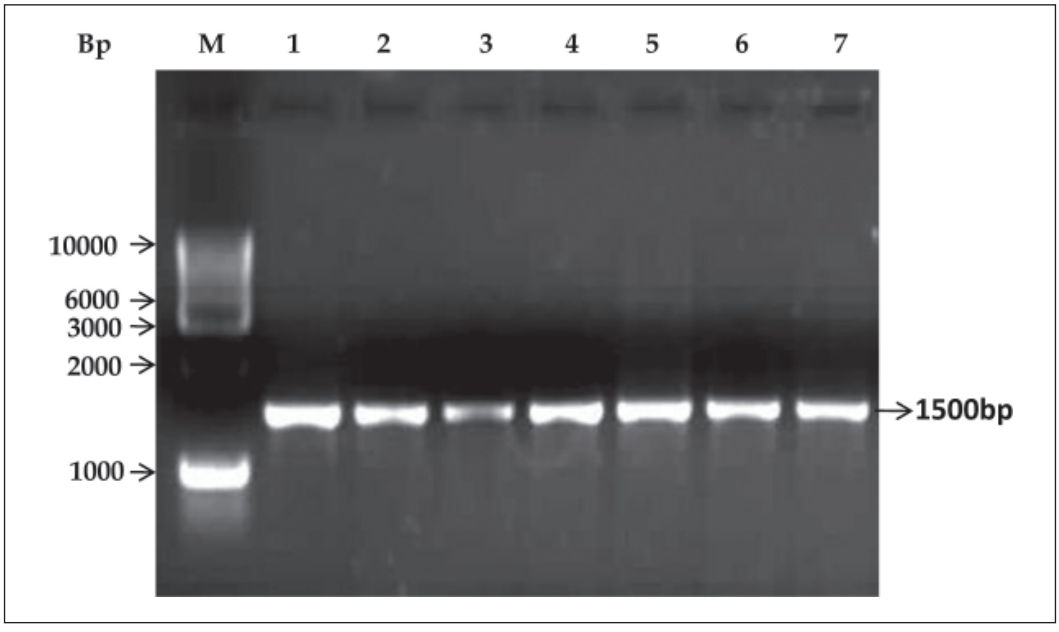

Figure 2. Amplified 16S rRNA genes from selected mosquitocidal bacterial isolate
Lanes: M = Ladder (1kb); 1 to 6 = *Bt*: (JQ289046 to JQ289049, JQ289051, JQ289052), 7 = *Bs*: (JQ289050)

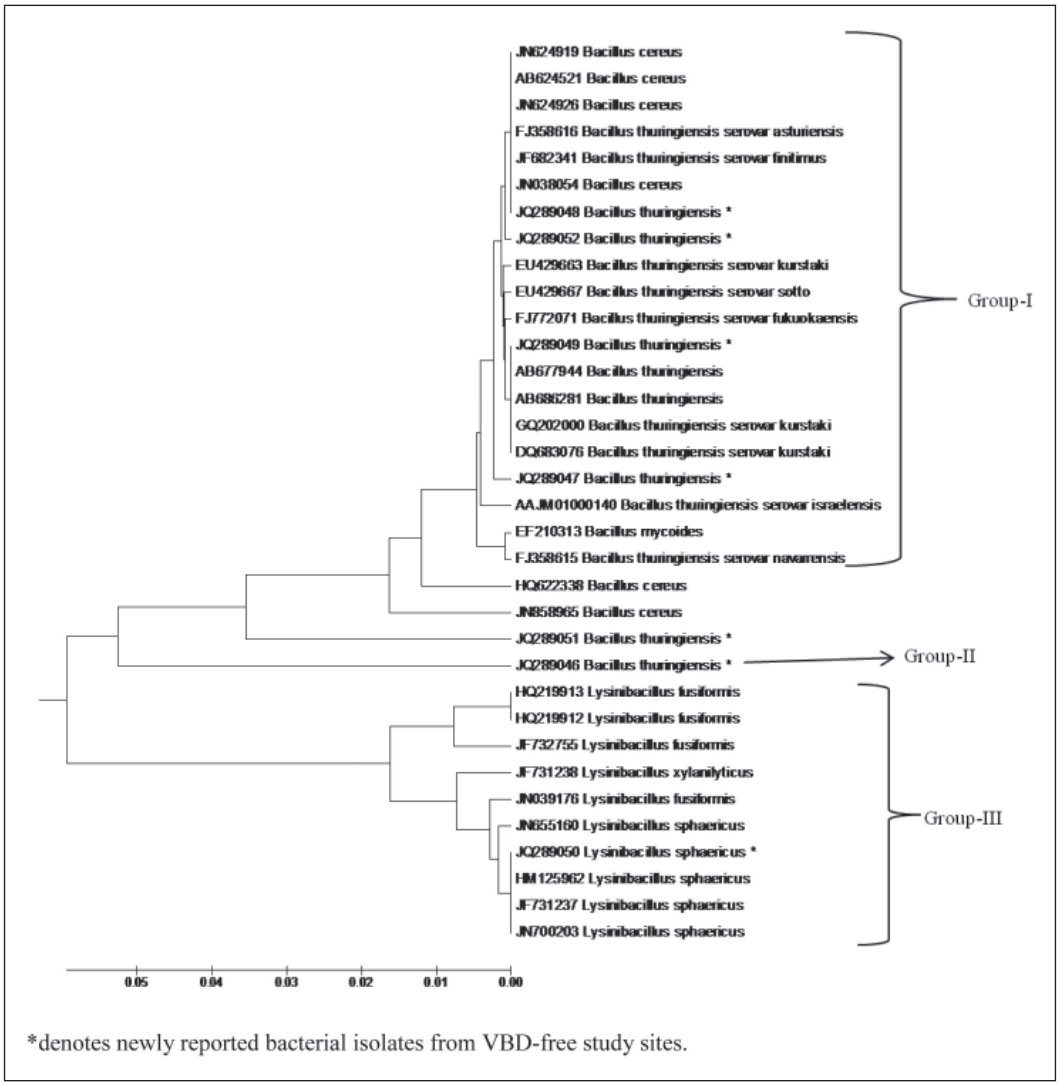

Figure 3. Dendrogram showing the relatedness of 16S rDNA between novel mosquitocidal bacterial isolates (NCBI-Acc. No: JQ289046 - JQ289052) and selected reference isolates derived from Genbank database

sequences (HM125962, JF31237 and JN700203).

In order to describe the relationship between the newly isolated mosquitocidal strains and the known ones, protein profiling of the sporulated cultures were performed with SDS-PAGE analysis. Figure 4 shows the whole-cell protein profiles of mosquitocidal bacterial strains. The results depicted that there were considerable differences among the protein profile pattern at the regions of 20-200 kDa. The above protein profile was analyzed and a dendrogram produced after numerical analysis of the whole-cell protein profiles using the Pearson product-moment correlation coefficient and un-weighted pair group method with arithmetic averages algorithm (UPGMA) as shown in Figure 5. Numerical analysis revealed clearly four distinct groups at a similarity level of 25% in the dendrogram. *Bacillus thuringiensis* isolates from Lingareddipalayam (JQ289049) and Sandhaipudukuppam (JQ289048) in Group I was similar to *B. thuringiensis* serovar *israelensis*. *Bacillus thruinginensis* from Suthukennai (JQ289051, JQ289052) and

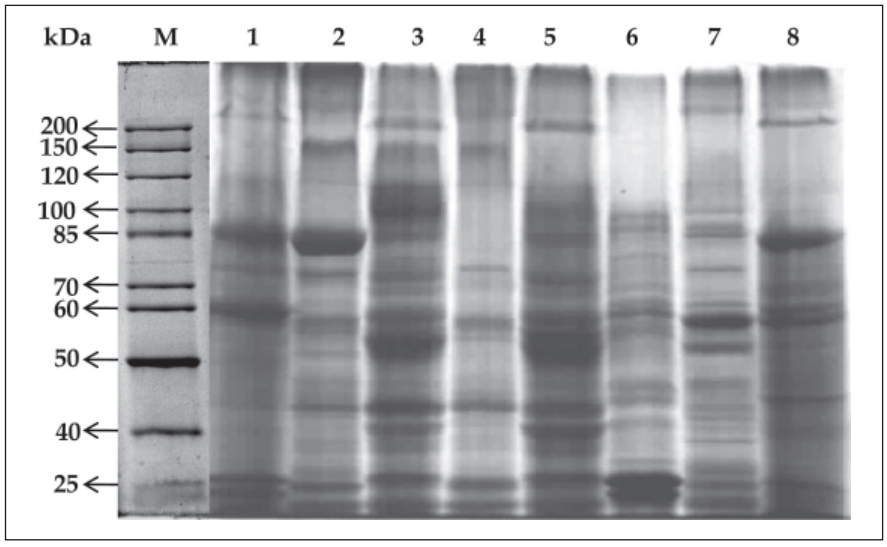

Figure 4. Protein profiles of Potential mosquitocidal strains
M–Protein marker; 1–*Bs* (JQ289050); 2 to 7–*Bt* (JQ289046-JQ289049, JQ289051, JQ289052

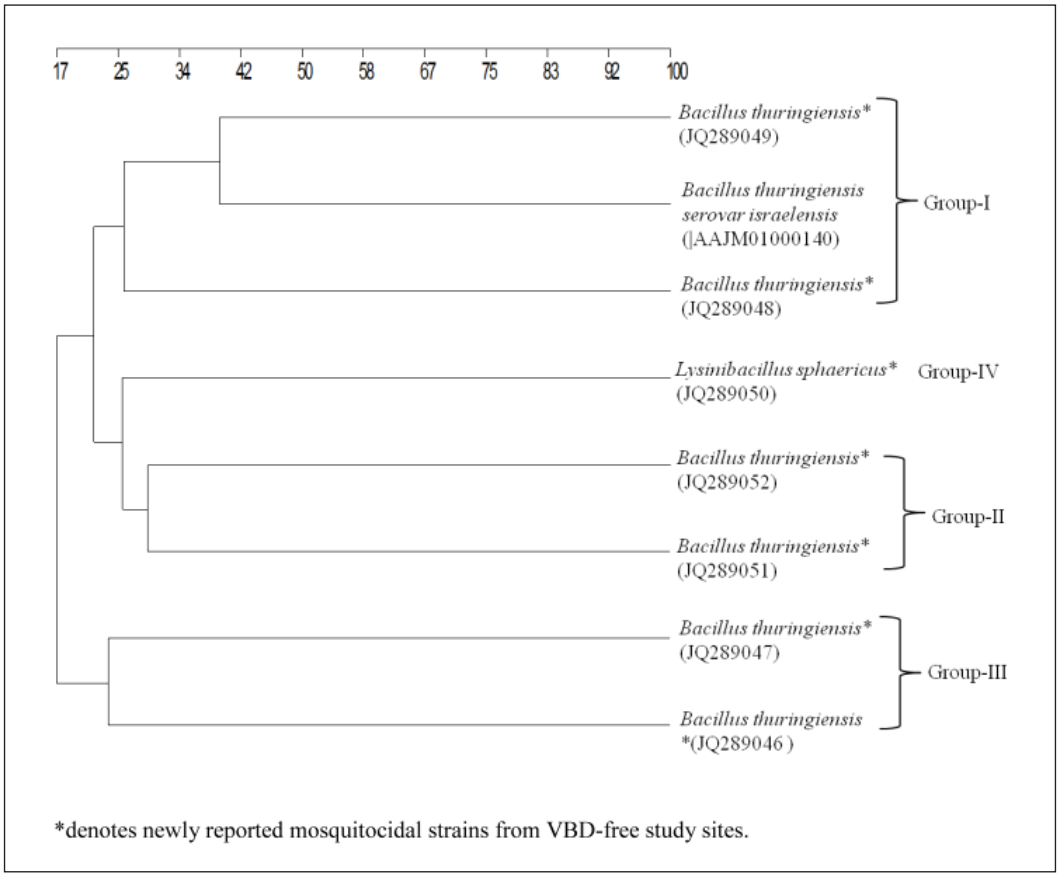

Figure 5. Dendrogram showing relatedness of protein profile of seven potential mosquitocidal isolates (NCBI Acc No: JQ289046-JQ289052) with reference strain of *Bacillus thuringiensis* serovar *israelensis* H14

Sandthaipudukuppam (JQ289046, JQ289047) form a distinct group II and IV respectively, which were distinct from the standard *Bti* stain; whereas, the identified *L. sphaericus* from the soil of Lingareddipalayam (JQ289050) was noticed as a separate group III.

DISCUSSION

Mosquito species *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* are the major vectors for the spread of the most common VBDs *viz.* filariasis, malaria, dengue affecting the communities of lower socio-economic groups in tropical countries. Since, the true burden of these are not known, it poses a major public health challenge in disease endemic countries. Biological control techniques aimed at suppressing mosquito populations or reducing their capacity to transmit disease may be a useful addition to traditional vector control strategies, especially if resistance to chemical insecticides in mosquito populations continues to rise (Ranson *et al.*, 2009). *Bacillus sphaericus* (*Bs*) and *B. thuringiensis* serovar *israelensis* (*Bti*) are the aerobic, mesophilic, spore-forming and gram-positive bacteria, commonly isolated from soil samples. Most of the strains are pathogenic to mosquito larvae and have been widely used as bio-control agents for disease transmitting mosquitoes (Foschino *et al.*, 2004). The larvicidal activity of *Bs* and *Bti* mainly originates from the crystal toxins (*Bs*: 42, 51 kDa; *Bti*: 27, 67, 127, 135 kDa), which are produced during sporulation (Wassim *et al.*, 2011). In the application of the above mentioned *Bacillus* species for insect biological control, only limited efforts have been directed towards identifying genetically diverse strains that have novel toxic activities towards insect pests (Raffel *et al.*, 1996). Since there is always a demand for selecting the most promising and most potential bacterial strains for producing biological insecticides, genetically diverse collections of strains from different environmental sources are advocated. In addition to that, the formulation of these organisms might have potential medicinal and agricultural applications. In the present study, the genotypic analysis of *Bacillus* species (*B. cereus*, *B. thuringiensis*, *B. thuringiensis kurstaki*, *B. thuringiensis israelensis*, and *B. sphaericus*) was carried out to identify similarities and differences that exist between the newly isolated and previously reported strains. Further, on the relationship between the existence of mosquitocidal bacteria and the prevalence of vector borne diseases (VBD) in the rural villages around Pondicherry (India) was also studied. The result depicted that similar mosquitocidal *B. thuringiensis* (*Bt*) strains from all three sites (Group 2) and phylogenetic analysis revealed the distinct groups. The sequence alignment of 16S rDNA gene from *Bt* showed the maximum homology with other *Bt* strains (*B.t. asturiensis*, *B.t. finitimus*, *B.t. sotto*, *B.t. kurstaki*, *B.t. fukuokaensis* and *B.t. israelensis* and *B.t. navarrensis*). The isolate of *L. sphaericus* from Lingareddipalayam also revealed a distinct group and showed homology with strains of *Lysinibacillus*.

It is known that prokaryotic microorganisms are widespread in all environments, establishing diverse interactions with many eukaryotic taxa, including insects. These associations may be symbiotic, pathogenic and vectoring (Sanchez-Chontreras & Vlisidou, 2008). Majority of the isolates with mosquitocidal property reported in the present study were identified as *B. thuringiensis*, a ubiquitous soil micro-organism, but it can also be found in other environmental niches, including phylloplane and the insect host intestinal system, rarely causing natural epizootic episodes (Munk *et al.*, 1998; Jensen *et al.*, 2003). Occurrence of mosquitocidal strains of *B.t.* from different continents (except, the America and Australia) from sources such as soils/sediments, plants (rhizoplane of aquatic plants, phylloplanes, etc.), insects (mosquito larvae, stem borer, etc.), animal feces (wild mammals, zoo-animals and deer) and water were identified (Bukhari & Shakoori, 2010). These strains were

identified as toxic to mosquito larvae after selective bioassays and they are advantageous over chemical compounds with similar properties (Lambert & Peferoen, 1992; Monnerat *et al.*, 2005). Strains of *B. thuringiensis* were also isolated from the phylloplane of deciduous and conifer trees using shaken-flask, leaf-lift and leaf-scrub techniques (Smith & Couch, 1991). Thus, the occurrence of mosquitocidal bacteria has diversified habitats other than soils.

Bacterial diversity of larvae and adult midgut microflora were identified from 16S rRNA gene library (Bhatnagar & Bhatnagar, 2005) and with similar approach, using 16S rRNA sequences of the selected mosquitocidal bacterial agents, wide *Bt* serotypes were identified. Recent study on six different serovars of *B. thuringiensis* with mosquitocidal property was identified as serotypes *B.t. tolworthi*, *B.t. alhakam*, *B.t. thompsoni*, *B.t. konkukian*, and *B.t. fukuokaensis* (Bukhari & Shakoori, 2010). Electrophoretic separation of protein can be efficiently used to confer the genomic inter-relationship from 16S rRNA sequence of bacteria. The protein profiles of both whole-cell and extra-cellular proteins help to distinguish most of bacterial genera at species level (Elliott & Facklam, 1993; Sacilik *et al.*, 1998; Berber *et al.*, 2003). Species of *Proteus*, *B. sphaericus* and *Streptomyces* were differentiated at sub-species level with analysis of whole cell proteins using SDS-PAGE (Cokmus & Yousten, 1987; Attalan *et al.*, 2000). The electrophoretic profiles of the strains *B. thuringiensis* showed bands of 130 kDa similar to those found in strains pathogenic against lepidopteran species (Dias *et al.*, 1999). Combination of SDS-PAGE and computerized analysis of protein profiles is an effective approach to investigate the taxonomic relationships among many bacterial species (Kersters, 1985; Coatas, 1992). In the present study, in addition to phylogenetic relationship with 16S rRNA sequence, we have confirmed the variations among the protein profiling at the regions of 20-200 kDa. Thus, the newly isolated mosquitocidal bacteria from soil samples of negatively reported VBD sites have unique contribution in understanding the diversity of mosquitocidal bacteria with disease incidence. Similar comparative analysis on microbial communities associated with mosquito habitats and community organization was reported recently (Ponnusamy *et al.*, 2008).

The results from this study also imply that occurrence of multiple mosquitocidal bacteria, such as, *B. thuringiensis L. sphaericus* from soils of human dwellings play a considerable role on the prevalence of vector borne diseases. It is postulated from the isolation of many species of soil bacterium with mosquitocidal property from villages of non occurrence of vector-borne diseases. This was confirmed with other group of study sites where high incidence of diseases with no report of mosquitocidal bacterial agent The micro-environment of the soil is a dynamic process and it depends on the agriculture practices and the chemical pollution from the industries. The ecological survey on the distribution pattern of soil bacterium in the natural habitat was recently studied which suggested that the modernization of agricultural practices is one of the factors for the significant variations in their mosquitocidal properties(Surendran & Vennison, 2011). Hence, this study is vital to comprehend the feature of surveillance of vectors of mosquitoes in the disease prone areas as well as microbial isolation on the study sites.

In conclusion, the results revealed the interrelationship among the occurrence of mosquitocidal microflora with vector borne disease incidence. 16S rRNA sequencing and phylogenetic analysis of newly isolated bacteria shows their relationship with other bacterial agents. Single dimensional SDS-PAGE for numerical analysis of protein patterns of identified bacterial spores provides useful information towards clarifying relationship within identified mosquitocidal bacterial agents. We conclude that 16S rRNA sequence and numerical analysis of SDS-PAGE of spore proteins are exceptionally useful in taxonomic assessment in studying *Bacillus* species.

Further experiments on the vector biology, surveillance and vector competence are essential features which provide additional data to understand the interrelationship between mosquitocidal bacteria and vector borne diseases.

Acknowledgements. To Dr. P. Jambulingam, The Director, VCRC, Pondicherry-605006, India for permission, Department of Biotechnology (DBT), New Delhi (Project ID: BT/PR13776/PID/06/527/2010), for funding, and to Smt. R. Sundarammal, Sr. Library Information Officer, VCRC, Pondicherry for updating scientific information.

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