Journal of Biological Control, 28(1): 24–30, 2014

Research Article

Characterization of *Lysinibacillus sphaericus* C3-41 strain isolated from northern Karnataka, India that is toxic to mosquito larvae

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ABSTRACT: One of the eco-friendly approaches to control mosquitoes is by the use of *Lysinibacillus sphaericus. L. sphaericus* (previous name *Bacillus sphaericus*) C3-41 (CP000817) was isolated from Rabbanahalli village of Yadgir district, Karnataka, India and was identified by biochemical and molecular means. Biochemical tests established its identity as *L. sphaericus*. It is also clear from the phylogenetic analysis that 16S ribosomal RNA nucleotide sequence of *L. sphaericus* C3-41 (CP000817) showed 99.73 per cent similarity with reference strain. Bioassay of *L. sphaericus* against second instar larvae of *Culex quinquefasciatus* recorded significantly higher mortality of 100 per cent at 0.075 % CSM. When assayed against second instar larvae of *Anopheles subpictus* mortality was 83.33 per cent at 0.075 % CSM. This new strain of *L. sphaericus* isolated from Northern dry zone of Karnataka, where the temperature ranges from 38°C to 46°C, could be a better biological pesticide to tackle the Culex mosquito and prevent diseases transmitted by it, particularly in Northern Karnataka.

KEY WORDS: *Lysinibacillus (Bacillus) sphaericus*, biochemical tests, 16S rRNA gene sequence, *Culex quinquefasciatus*, bioassay

(Article chronicle: Received: 20-11-2013; Revised:28-02-2014; Accepted: 01-03-2014)

INTRODUCTION

Mosquitoes and mosquito borne diseases are major culprits to cause deaths of around one million people every year throughout the world (Rawlings *et al*., 1995). The mosquito borne diseases include the most painful and horrendous tropical diseases like malaria, lymphatic filariasis, dengue, chikungunya, yellow fever and brain fever. India spends around 100 million dollars alone annually to control malaria. Conversely, the diseases continue to burst out sporadically as these disease causing pathogens have developed resistance to medicines and vectors developed resistance to pesticides. There is a need for more effective and eco-friendly control measures such as use of bio-agents like viruses, fungi, bacteria, protozoa, nematodes, invertebrate predators and fishes for the management of *Culex quinquefasciatus*.The discovery of bacteria like *Lysinibacillus sphaericus* and *Bacillus thuringiensis* serovar *israelensis* have opened up the possibility of their use as potential bio-agents in mosquito control programs (Poopathi and Abidha 2010, Poopathi *et al*., 2002, Poopathi and Tyagi, 2002).These bacteria produce proteins, which are highly toxic to larvae of mosquito. In contrast, the genetic diversity and efficiency of toxins of *L. sphaericus* differ from region to region. Hence, there is enormous scope to identify and explore indigenous *L.sphaericus* against local mosquito population.

Development of gene amplification and sequencing of 16S rRNA gene has simplified the identification and detection of specific bacteria (Sharma, 1995) particularly those lacking distinguishable phenotypic characteristics. In the present study, an attempt was made to isolate *L.sphaericus* from soil, confirm its identity by biochemical and molecular assay using 16S rRN Analysis and also test its toxicity against *Culex quinquefasciatus and Anopheles subpictus*.

MATERIALS AND METHODS

Isolation of *Bacillus* sp. from soil

Soil from paddy ecosystem of Yadgir district was collected in sterile plastic bag, air dried, mixed well and 1 gm of soil was taken for isolation of *Bacillus* species. The protocol for isolation was followed as described by Travers *et al.*(1989) and Andrezejczak and Lonc (2008) with some modifications. One gram of air dried soil sample was taken in a conical flask containing Luria broth buffered with sodium acetate (0.25mM). The mixer was shaken

well for 4h at 250 rpm at 30°C. The samples were heated at 80°C for 20 minutes in water bath. The suspension was serially diluted, spread on Luria agar and incubated at 30°C for 48h. Randomly samples of colonies were picked on to M9 medium (6 g Na_2HPO_4-$7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 0.5 g NH_4C , 0.024 g $MgSO_4$, 0.0001 g $CaCl_2$,20g Agar, 1000mL distilled H_2O). The colonies showing the symptoms of irregular edges, dull white and flat colonies were selected and plated onto Luria agar medium. The selected colonies were subjected to Gram's and endospore staining for confirmation. The colonies which showed round spores were identified as *L. sphaericus*. Initial bioassay was conducted for selected isolates against second instar larvae of *C. quinquefasciatus* at 0.075 % crystal spore mixture (CSM) and virulent isolate was chosen for further biochemical and molecular characterization.

Biochemical characterization

The most virulent bacterial strain was selected and subjected for biochemical assay and confirmed according to Bergey's Manual of Systematic Bacteriology 1& 2 (Palleroni, 1986; Sneath, 1986). Biochemical tests such as H_2S, urease, citrate, methyl red, indole tests, carbohydrate fermentation (glucose, lactose, mannitol, and sucrose), starch hydrolysis, nitrate reduction, gelatin, casein, catalase test, oxidase test, NaCl at 5,7 and 10% and motility tests were conducted to characterize the selected virulent bacterial strain (Robert *et al*., 2002).

Toxicity of *Lysinibacillus sphaericus* against larvae of *Culex quinquefasciatus*

Mosquito populations for bioassay

The egg raft of *C. quinquefasciatus* was collected from field and released into small plastic containers with water, which was kept in the insect rearing cage, one egg raft per container was maintained. Hatching of the egg was monitored daily and fish feed was administered daily as a food. The second instar larvae were selected for conducting bioassay. Larval population of *Anopheles* sp. was brought from small pond, where suspected *Anopheles* was breeding. The adults of both mosquito species were sent to Dr. A. R. Rajavel, Scientist,Vector Control Research Centre, Puducherry, for identification and confirmed as *C. quinquefasciatus* and *A. subpictus*.

Bacterial suspension preparation and determination of CFU/mL

The bacterial cells were suspended in 25 mL nutrient broth for 96 h in conical flask, from this one mL of culture was taken for serial dilution and CFU/mL was counted. The turbid solution was centrifuged at 10,000 rpm for 10 min at 4°C. The pellet and supernatant were used for conducting bioassay.

Estimation of protein by Lowry's method

Standard method was followed to estimate the protein in the pellet or Crystal Spore Mixture (CSM). The CSM was mixed with 0.1 per cent Triton X, incubated for 2 h, centrifuged at 10,000 rpm for 10 min at 4°C, supernatant was used for estimating protein (Lowry *et al*., 1951).

Koch's postulate

The CSM was used for conducting bioassay. The CSM was suspended in 1 mL of distilled water. Known concentration of CSM was dispensed into 100 mL of distilled water in 150 mL capacity plastic cups. Ten second instar larvae of *C. quinquefasciatus* were released into each container. The containers were wrapped with rubber bands, holes were made on the paper and larval food added. Observations on number of dead larvae was recorded at 24 and 48 h after treatment.

The dead larvae were surface sterilized with 0.1 % sodium hypoclorite by keeping for 1 min, washed three times with distilled water. The dead larvae were dissected with fine sterilized needle and the haemolymph was streaked on to nutrient agar. The culture was compared with parent isolate and its identity was confirmed using 16srRNA analysis and biochemical tests.

Bioassay

The CSM was obtained as previously described and suspended in 1 mL of distilled water, from which different concentrations of CSM were dispensed into the 100 mL of distilled water in 150 mL capacity plastic cups. Ten second instar *C. quinquefasciatus* or *A. subpictus* larvae were released into each container, five replications were maintained for each concentration. The containers were wrapped with tissue paper with rubber band, holes were made on the paper and larval food was added. Observation on the number of dead larvae was recorded at 24 and 48 h after the treatment.

Similarly, the bioassay against pupae of *C. quinquefasciatus* was also conducted using the culture filtrate. In this case, the filtrate after centrifugation was taken for determining the toxicity studies. The supernatant was added to the plastic cups containing distilled water at different concentrations (0.5%, 1.0%, 2.0%, 3.0%, 4.0% and 5.0%). Observations on the number of larvae dead were recorded at 24 and 48 h after treatment.

Identification by 16SrRNA sequence analysis

Genomic DNA of the bacteria was extracted using GeneElute Genomic DNA isolation Kit (Sigma, USA) as

per the manufacturer's instructions and used as template for PCR. A combination of universal primers was chosen to sequence the nearly complete gene (Poopathi *et al*., 2002). Forward primer FDD2[CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG] and reverse primer-RPP2 [RPP2–CCAAGCTTCTAGACGGITACCTTGTTACGACTT] were used to amplify entire 16S rRNA gene sequence (Poopathi *et al*., 2002).

Each reaction mixture contained 10ng of DNA, 2.5 mM $MgC1_2$, 1x PCR buffer, 200µm each dCTP, dGTP, dATPand dTTP, 2picmol of each forward and reverse primer and 1 unit of Taq DNA polymerase (Merck Biosciences) in a final volume of 20µl. FDD2 and RPP2 primers were used to amplify almost entire 16S rRNA gene, as described previously (Poopathi*et al*., 2002). The PCR was performed using the Eppendorf Gradient Master cycler system with a cycle of 94 for 5min; 30 cycles of 94°C, 60°C, and 72°C for 1 min each; and final extension at 72°C for 10min, and the mixture was held at 4°C. The PCR Product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed thrice using 70% ethanol and dissolved in Tris–HCL (10mM, pH 8.0).

The ABI prism Big Dye Terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.) was used for sequencing the PCR product. The sequencing reaction template preparation was performed and purified in accordance with the directions of the manufacture (Applied Biosystems). Samples were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

The 16s rDNA sequence of the test strain was aligned manually against corresponding sequences of representative *Pseudonocardiaceae* family strains obtained from the Ribosomal Database Project release 4 with the AL16S program. Pairwise similarity values were calculated and converted to distances with the algorithm of Jukes and Cantor in the DNADIST program (in PHYLIP, version 3.5). A phylogenetic tree was generated by the neighbor- joining method. In order to determine the stability of the resultant phylogenetic tree, the sequence data were resampled 1,000 times for bootstrap analysis using the SEQBOOT program (PHYLIP, version 3.3). The sequencing output was analyzed using the accompanying DNA sequence analyzer computer software (Applied Biosystems). The sequence was compared with National Center for Biotechnology information GenBank entries by using the BLAST algorithm.

RESULTS AND DISCUSSION

Isolation and screening of *L. sphaericus* for new and highly potent strains has become inevitable as one of the strategies for mosquito resistance management. Many reports on the frequent occurrence of *L. sphaericus* isolates in the natural environment showed the high possibility of isolating a novel strain.Therefore, an attempt was made to isolate a virulent strain of *L. sphaericus* in Yagdir district of Karnataka, India.

Collection and Isolation

Total numbers of soil samples collected were 15, total numbers of bacilli like colonies isolated were 102 and total number of bacilli like colonies identified were 4 (Table.1). Average Bs index was 0.24. The bacilli colonies showing irregular edges, flat and dull white characters were chosen, pure cultured, subjected to Gram's and endospore staining. Initial bioassay against second in star larvae of *C. quinquefasciatus* showed that the *L. sphaericus* collected from Rabbanahalli caused maximum mortality of 100.00 per cent at 0.075 per cent CSM. The remaining isolates showed the mortalities ranging from 35.00 to 50.00 per cent. The isolate collected from Rabbanahalli was selected for the further study.

Biochemical characterization

The isolate was found positive for catalase, oxidase, urease and motility tests. The isolate showed negative reaction to H_2S, citrate, methyl red, indole, carbohydrate fermentation (glucose, lactose, mannitol, and sucrose), starch hydrolysis, nitrate reduction, gelatin and casein tests. Tolerance studies to growth in NaCl at 5, 7 and 10 % indicated that the bacterium could tolerate 5 % NaCl. Based on the cultural characteristics and biochemical tests, the bacteria was identified as *L. sphaericus* (Table 2). The study was inline with reports of Rhadika *et al*. (2011) and Olga *et al*.

Table 1. Isolation of *Bacillus* spp. from soils of Yadgir districts

Sl No	Place of sample collection	No. of soil samples	No. of Bacilli observed	Mosquitocidal isolates	Bs Index	% mortality against *Culex quinquefasciatus*
1	Gundahalli	2	20	1	0.05	35.00
2	Kanapur	3	12	1	0-83	40.00
3	Gogi	3	18	0	0.00	----
4	B, Gudi	1	16	1	0.06	50.00
5	Naikal	4	28	0	0.00	----
6	Rabbnahalli	2	8	1	0.13	100.00
7	Total	15	102	4	0.24	

Table 2. Biochemical characterization of Rabanahalli isolate (C3-41)

Sl.No	Identification tests	Remarks
Preliminary tests		
	Grams staining	+
	Spore staining	+
	Shape	Rod
	Motility	+
Biochemical tests		
	Indole	-
	Methyl red	-
	Voges Proskauer	-
	Citrate	-
	Urease	+
	H2S	-
Carbohydrate fermentation tests		
	Glucose	A-G-
	Mannitol	A-G-
	Lactose	A-G-
	Sucrose	A-G-
Other tests		
	Starch agar test	-
	Nitrate broth reaction	-
	Gelatin	-
	Caesin	-
	Catalase	+
	Oxidase	+
	Nacl 5%	+
	Nacl 7%	-
	Nacl 10%	-

+ : Positive, - : Negative

(2006).The population of the bacterium in the culture used for obtaining CSM had $1.08x10^8$ CFU/mL. The protein concentration of pellet as estimated by Lowry's method was found to be 36.50 mg/mL.

Bioassay

The selected *L. sphaericus* isolate was evaluated at different concentrations of CSM against second in star larvae of *C. quinquefasciatus* and *A.subpictus*. At 24 h after treatment, mortality of larvae of *C. quinquefasciatus* increased with increasing concentration of CMS (0.025 to 0.1 per cent). At 24 h after treatment, significantly highest mortality of 76.66 per cent was recorded at 0.1 per cent concentration of CSM. However, 48 h after treatment, significantly highest mortality of 100.00 per cent was recorded at 0.075 and 0.1 per cent concentration of CSM (Table 3).

Mortality of larvae of *A. subpictus* ranged from 10.00 to 30.00 per cent at 24 h after treatment. Maximum mortality of 83.33 per cent was recorded at 0.10 per cent concentration of CSM at 48 h after treatment (Table 3).

Table 4. Toxicity of culture filtrate against second instar larvae of *Culex quinquefasciatus* mosquito

Culture Filtrate (%)	% Mortality of Larvae	
	24 Hrs	48 Hrs
0.25 %	0.00 (0.50)f	3.33 (10.34)h
0.50%	0.00 (0.50)f	6.66 (14.93)g
1.00%	10.00 (18.42)e	36.66 (37.26)e
2.00%	50.00 (44.99)d	70.00(56.80)d
3.00%	53.33 (47.49)c	80.00 (63.47)c
4.00%	70.00(56.79)b	86.66(68.66)b
5.00%	75.00 (60.03)a	100.00 (89.50)a
D water	10 (18.42)e	12.33 (20.44)f
CD P = 0.01	2.43	3.54

Figures in parenthesis are angular transformed values

Table 3. Toxicity of crystal- spore mixture (CSM) obtained from C3-41 isolate against second instar larvae of *Culex quinquefasciatus* and *Anopheles subpictus* (36.50 mg/mL toxin protein and $1.08x10^8$ CFU/mL).

CSM (%)	% Mortality of Larvae			
	Culexquinquefasciatus		*Anopheles subpictus*	
	24 Hrs	48 Hrs	24 Hrs	48 Hrs
0.025%	6.66 (14.95)e	48.88 (43.86)c	10.00 (18.36)d	19.00 (25.82)d
0.050 %	24.44 (29.62)c	73.33 (58.92)b	18.00 (25.08)c	35.00 (36.23)c
0.075 %	57.70 (49.44)b	100.00 (89.35)a	22.00 (27.96)b	53.00 (46.73)b
0.10 %	76.66 (61.12)a	100.00 (89.35)a	30.00 (33.19)a	83.33 (65.93)a
Distilled water	10 (18.38)d	12.33 (20.53)d	8.88 (17.30)d	10.50 (18.40)e
CD P = 0.01	2.56	1.65	3.69	3.59

Figures in parenthesis are angular transformed values

Since the culture filtrate also contains other toxins, it was evaluated against second instar larvae of *C. quinquefasciatus*. Significantly highest mortality was recorded at 5.00 % culture filtrate with 75.00 per cent at 24 h after treatment. However 48 h after treatment, maximum mortality of 100.00 per cent was observed with 5.0 % filtrate (Table 4).

The results are in conformity with reports of Gupta *et al*. (1991) who isolated an indigenous strain of *L. sphaericus* H5a (9001) from diseased larvae of *Culex* sp. and was found to be promising against the fourth instar larvae of *Anopheles culicifacies, A. stephensi, A. subpictus, Aedes aegypti* and *C. quinquefasciatus*. *L. sphaericus* is highly stable and virulent through 25 successive transfers and thus can be effectively used as a biocontrol agent against immature stages of mosquitoes. Surendran and Vennison (2011) observed that, *L. sphaericus* isolates showed a significant level of variation in their larvicidal activity against larvae of *C. quinquefasciatus*.Yadav *et al*. (2010) evaluated *L. sphaericus* and Spherix against *C. quinquefasciatus* and recorded 90% larval mortality at 0.5 and 0.6 ppm.

The LC_{50} and LC_{95} values of *L. sphaericus* against *A. stephensi* at 24 as well as 48 h post-treatment were rather high, exceeding 1 ppm at the LC_{95} level. However, *C. quinquefasciatus* larvae were highly susceptible to *L. sphaericus with* LC_{50} and LC_{95} values of 0.043 and 0.12 ppm, respectively at 24 h, and at 48 h posttreatment, 0.008 ppm (LC_{50}) and 0.11 ppm (LC_{95}) was reported by Jahan and Hussain (2011).

Identification using16S rRNA sequence analysis

From the 16S rRNA analysis, a PCR product of about 1527 nucleotide bases for the isolated bacteria (Rabbanahalli) was found. Figure 1 presents a phylogenetic tree constructed based on comparison of the 16SrRNA sequences generated from the isolated bacterial strain (Rabbanahalli-III) and other bacteria like *Lysinibacillus* spp., *Caryophanon* spp, *Bacillus* spp. and *Viridibacillus* species obtained from GenBank. The isolated bacteria (Rabbanahalli) was clustered with *L. sphaericus* C3-41 (CP000817) in the last group with 99.73 per cent similarity. Hence it is clear from the phylogenetic tree that 16s rRNA sequence of *L. sphaericus* C3-41 (CP000817) showed 99.73 per cent similarity with new strain (Table 5). It is well documented that *L. sphaericus* has mosquitocidal properties.

Our study are in accordance with reports of Nakamura (2000) who grouped *L. sphaericus* strains based on phenotypic analysis of 16S rRNA and concluded that 16s rRNA typing analysis was carried out for identification of highly toxic mosquito larvicidal isolate of *L. sphaericus.* The results are also in agreement with the observations of Xu and Cote (2003) who showed that *Bacillus* species were separated to seven groups (I, II, III, IV, V, VI and X), but *Bacillus circulans* remained ungrouped.

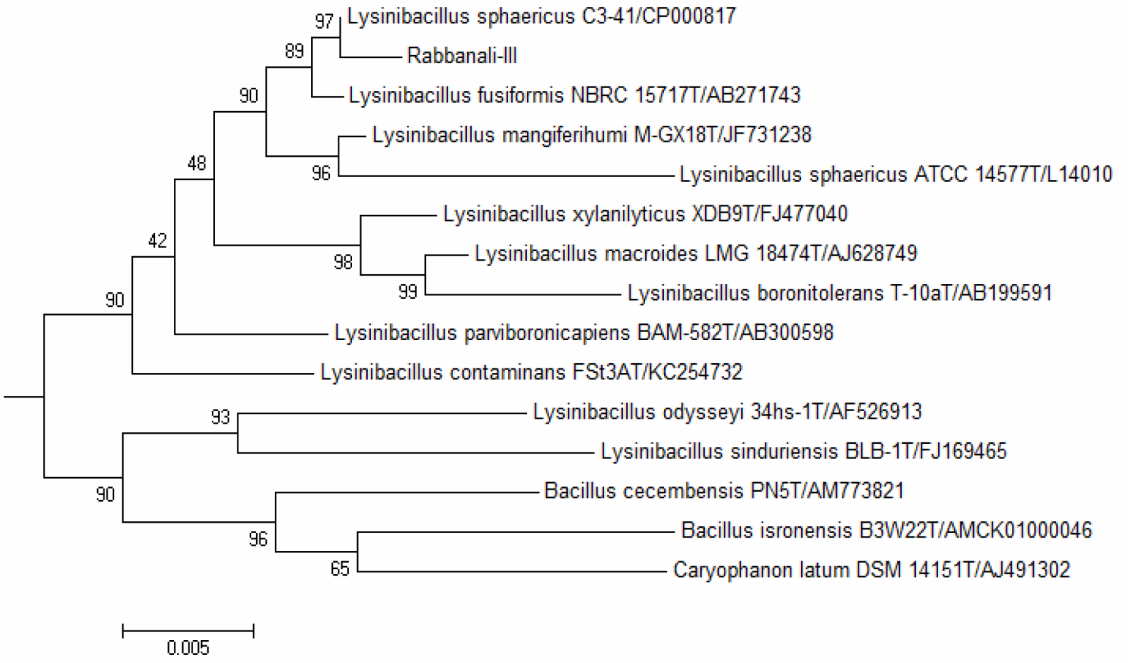

Fig. 1. Phylogenetic tree based on 16S rRNA gene nucleotide sequence comparison (1532nt) indicating the position of bacterial strain (Rabbanahalli) within representatives of the *Bacillus sphaericus*-like group. Numbers at nodes are bootstrap values based on 1000resamplings, 0.002 nucleotide changes per nucleotide position. It correlates higher similarity with the partial sequence of 16S rRNA of the other *Lysinnibacillus sphaericus* C3-41 (CP000817)

Table 5. Closest phylogenetic affiliation

Strain Designation	Closest phylogenetic affiliation	Max Identity
Rabbanahalli-III	*Lysinibacillus sphaericus* C3-41 16S ribosomal RNA gene sequence (CP000817)	99.73%

Gene sequence of 16SrRNA of *Lysinibacillus sphaericus* (>Rabbanali-III)

>Rabbanali-III

CGACAGAGTTTGATCGTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGAGAAGGA
GCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTACCTTATAGTTTGGGATAACTCCGG
GAAACCGGGGCTAATACCGAATAATCTNTTTCACCTCATGGTGAAATATTGAAAGACGGTTTCGGCTGTCGCTATAG
GATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGG
TGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCG
AAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGATTTCGGTTCGTAAAACTCTGTTGTAAGGGAAGAACAAGT
ACAGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGC
CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGATAGTGGAATTCCAAGGGTAG
CGGTGAAAATGCGTAGAGANTTGGAGGAACCCCCAGTGGCGAAGGGGNCTATCTGGTCTGTAACTGACACTGAGGCG
CGAAAGCGTGGGGAGCAAAATCAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAANNTTAGGGG
GTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTT
GACATCCCGTTGACCACTGTAGAGATATGGTTTCCCCTTTCGGGGGCAACGGTGACAGGTGGTGCATGGTTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGG
CACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGG
GCTACACACGTGCTACAATGGACGATACAAACGGTTGCCAACTCGCGAGAGGGAGCTAATCCGATAAAGTCGTTCTC
AGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCAGCCGCGGGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTT
GGAGCCAGCCGCCGAAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAAGGTACCCGTCTAA

L. sphaericus has several advantages, including low environmental toxicity due to specific action of *L. sphaericus* toxins, high levels of efficacy, environmental persistence and the ability to overcome resistance development. Only a few of the highly larvicidal *L. sphaericus* strains are sold commercially; strain 2362 (for example, VectoLex and Spherimos) is sold in United States and Europe, strain 1593 (for example, Biocide-S) is sold in India and strain C3-41 is sold in the People's Republic of China (Polleroni *et al*., 1986). Similarly Hu *et al*., (2008) reported that *L. sphaericus* C3-41, a highly active strain isolated from a mosquito breeding site in China in 1987, showed toxicity against *Culex* sp., *Anopheles* sp., and *Aedes* sp. and had significantly higher activity against *Culex* sp. than the commercialized *L. sphaericus* strain 2362 (Wang *et al*., 2003). It has been developed as a commercial larvicide (JianBao) and successfully used for the control of mosquito larvae for more than 10 years in China.

However, the fact of development of new and more efficient bacterial strains suitable for application in different environmental conditions cannot be neglected. It is well understood that the bacteria are environmentally sensitive and can perform better in its native environment. Hence, the new strain of *L. sphaericus* isolated from Northern dry zone of Karnataka, where the temperature ranges from 35 to 45°C could be better biological weapon in compassionate with nature.

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