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# Molecular Characterization and Evaluation of Two Potential Mosquitocidal *Lysinibacillus* Strains from Himalayan Valley Kashmir

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**In an exertion to isolate natural non-harmful mosquitocidal bacteria, 158 samples of soil were collected from various habitats of Himalayan valley Kashmir. A total of 450 bacteria were screened for mosquitocidal activities against three epidemiological disease causing vectors: *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi* larvae/pupae. Out of 450 bacteria screened, none had shown pupicidal activity. However, two isolates KS2-15 and KS2-13 exhibited mosquito larvicidal activity against *C. quinquefasciatus* (LC50: 1.36 × $10^3$ spores/mL; 1.41 × $10^3$ spores/mL respectively) and *A. stephensi* (LC50: 2.14 × $10^3$ spores/mL; 2.11 × $10^3$ spores/mL correspondingly). These two isolates were identified, morphologically, biochemically and comparative investigation of 16S rRNA gene sequences, as *Lysinibacillus sphaericus* (previously *Bacillus sphaericus*). Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of spore crystal mixture of each strain revealed two major bands of around 51.4 kDa and 41.9 kDa. PCR of mosquitocidal toxin genes showed the presence of *binA* and *binB* genes in both the strains. Comparative amino acid sequence analysis revealed that the BinA (41.9 kDa) and BinB (51.4kDa) proteins of KS2-13 and *L. sphaericus* 2362 differ by 3 (K89E, E104A, Y176D) and 6 (A69S, K70N, I110T, N248H, H314L and L317F) amino acids respectively. Similarly BinA and BinB proteins of KS2-15 and *L. sphaericus* 2362 strains vary by 1(E104A) and 3 (H109P, N248H and P274S) amino acids respectively. The varied amino acid sequences could be reason for the difference in activity. These two strains can act as good candidates for insecticidal formulation. Moreover, we reported for the first time the isolation of mosquitocidal *Lysinibacillus* strains from Kashmir valley.**

**Keywords:** Bin proteins; *bin* genes; Kashmir valley; *Lysinibacillus*; SDS-PAGE.

Vector-borne diseases cause main public health tribulations and their control is largely accomplished with usage of synthetic insecticides against carrier insects (Baird 2000). Although the application of chemical insecticides proved lucrative to effectively control mosquitoes for many decades, the usage of chemical pesticides in long term will result in malicious effect on human and environment. There are many health problems associated to pesticide usage which vary from abdominal pain, dizziness, headaches, nausea, vomiting, as well as skin and eye problems to cancer and developmental defects as well (Lorenz, 2009). Side effects on environment range from non-target organism killing (harmless insects, birds, amphibians and fishes) to increasing resistance to mosquito (Denholm *et al*. 2002). Effects on non-target organisms and concern about accumulation

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of chemicals in the environment have hastened the requirement to develop substitutes. Control of mosquitoes by means of entomopathogenic bacteria is a potential environmental friendly alternative to chemical insecticides (Park and Federici. 2009). *L. sphaericus* is an aerobic, endospore-forming gram-positive bacterium (Suryadi *et al*. 2016). This bacterium shows high toxicity against mosquito larvae and has been used extensively in some countries as biopesticide (Poopathi and Abidha, 2010). The larvicidal properties of this bacterium are mainly attributed to the presence of Binary proteins (BinA 41.9 kDa and BinB 51.4 kDa) expressed during sporulation stage (Broadwell *et al*. 1990; Berry, 2012). Cry48/ Cry49 toxin expressed during sporulation in some strains play significant in the activity against Culex mosquitoes (Jones *et al*. 2007) and Mtx proteins (100-kDa toxin) formed during vegetative growth (Priest *et al*. 1997; Wirth *et al*. 2014). However Bin toxins are the key factors responsible for larvicidal activity. These Bin toxins after intake by susceptible larvae dissolve in the alkaline midgut and get activated by gut proteases. The 41.9 kDa BinA protein is cleaved to 39 kDa, and 51.4 kDa BinB is changed to 43 kDa (Baumann *et al*. 1991). BinB binds to the specific receptors present on larval midgut brush border membranes (Silva-Filha *et al*. 1999), while activated BinA brings the toxicity by working together with BinB (Oei *et al*. 1992; Lekakarn *et al*. 2015).

Valley Kashmir that is often referred as Terrestrial Paradise on Earth is situated at northern western tip of Himalayan biodiversity hot spot (Mittermeier *et al*., 2005). It is located roughly between 32Ú.152 and 37Ú.052 North latitude and 72Ú.352 to 83Ú.202 East longitude, with complicated geomorphologic characteristics (i.e. snow clad mountains, vast meadows full of flowers, thick forests, small mountains, valley lakes and numerous serpentine rivers). These distinctive features associated with variety of animal forms ranging from higher groups such as vertebrates, including mammals, birds, reptiles, amphibians to lower groups like invertebrates including insects and even unicellular micro-organisms provide the opportunity to isolate novel mosquitocidal bacterial strains. In this study, we reported isolation and characterization of two highly mosquitocidal *B. sphaericus* strains from Himalayan valley Kashmir, active against larvae of *C. quinquefasciatus* and *A. stephensi* epidemiological disease causing vectors.

## MATERIALS AND METHODS

**Reference strain**

*L. sphaericus* 2362 was obtained from *Bacillus* Genetic Stock Center (Columbus, Ohio) which served as positive control.

**Mosquito cultures**

The cultures of *Culex quinquefasciatus, A. stephensi* and *A. aegypti* were procured from the Centre for Research in Medical Entomology (CRME), Madurai, Tamil Nadu and maintained at 28±2 ºC and 75% to 85% relative humidity under a photoperiod of14L:10D in our laboratory. Larvae were reared in chlorine free water and fed with dog yeast and biscuits at 2:3 ratio. For all bioassays, third-instar larvae of the size and same age were used.

**Sample collection**

A total of 158 samples from different divisions (Anantnag, Kulgam, Pulwama, Shopian, Budgam, Srinagar, Ganderbal, Bandipora, Baramulla, Kupwara) of the Himalayan valley Kashmir, India, were used for isolation of mosquitocidal bacteria. To our best knowledge, microbial insecticides had not been formerly applied in the sampled areas. All the soil samples (each ~ 5 g) were collected from 2 to 4 cm below the surface after scrapping off the surface material with a sterile spatula.

**Isolation of Bacteria**

Isolation of bacterial strains from the soil samples was performed according to the method described by Geetha *et al* (2007) with slight modification. In the laboratory, One gram of soil sample was suspended in 10 mL of sterile distilled water ($10^{-1}$) in a boiling tube. One ml from this suspension was added to nine ml of water which gives $10^{-2}$ dilution. Similarly, dilutions were made up to $10^{-5}$ and 0.1 ml from each dilution was spread on nutrient yeast salt mineral agar (NYSM) comprising of 5 g peptone, 5 g yeast extract, 3 g beef extract, 5 g NaCl, 5 g glucose, 103 mg $CaCl_2$, 10 mg $MnCl_2$ and 203 mg $MgCl_2$, (Hi-Media, India) per liter of distilled water. The bacterial suspensions were subjected to pasteurization before plating, expecting only gram-positive bacteria. The bacterial colonies which appeared on NYSM plates

after incubation at 30 ºC for 48 h and were further purified using NYSM agar. Purified colonies were then sub-cultured on NYSM agar slants and stored at 4 ºC. These bacterial isolates were tested for mosquito larvicidal activity.

**Determination of larvicidal activity**

A loopful of bacterial culture from each NYSM medium slant was inoculated to 3 ml of NYSM broth. This was incubated in a shaking incubator (Hasthas Scientific Instruments, India) maintained at 30 °C and 200 rpm till e"90% sporulation ( 4 72 h). After incubation, 1 ml (about $10^9$ spores) from the whole culture was utilized to screen for mosquito larvicidal activity. Bioassays with bacterial suspension (1 mL) were carried out in wax coated paper cups each containing 25 third instar larvae and 25 pupae of *C. quinquefasciatus/ A. stephensi/ A. aegypti* in 125 ml chlorine-free tap water at a temperature of 28±2 ºC and 75% to 85% relative humidity under a photoperiod of14L:10D. In control cups 1 ml of un-inoculated NYSM broth only was used and in another control 1ml of *L. sphaericus* 2362 was added. Mortality in the individual cups was noted down by counting the number of live larvae or pupae present subsequent to 24 h of culture introduction. Based on the mosquitocidal activity two strains were selected, code named as KS2-13 and KS2-15 and further characterized. Further bioassays with larvicidal active isolates (KS2-13 and KS2-15) and *L. sphaericus* 2362 were performed using seven different concentrations ($10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$ and $10^8$) of bacterial spore crystal suspension obtained by serial dilution. All the bioassays were performed in ten replicates in wax coated paper cups each containing 25 third instar larvae of *C. quinquefasciatus/ A. stephensi* in 125 ml chlorine-free tap water at 28±2 ºC and 75% to 85% relative humidity under a photoperiod of 14L:10D. A control group, tested on NYSM broth without bacterial suspension, was included in each experiment. No source of food was added during bioassays. The number of dead larvae in each cup was counted after 24 h of culture introduction. Probit regression analysis was performed with SPSS 20.0 for windows software and LC50 and LC90 as well as their 95% fiducial limits were determined (Geetha *et al*. 2007; Tranchida *et al*. 2011).

**Characterization of the potential bacterial strains**

Two potential strains were studied for morphological, biochemical and physiological characteristics according to Bergey's Manual of Systematic Bacteriology (Sneath, 1986). Biochemical tests namely, fermentation of arabinose, glucose, mannitol, xylose and malonate, hydrolysis of starch, decarboxylation of lysine, ornithine, arginine dihydrolase, utilization of citrate, degradation of tyrosine, deamination of phenylalanine, nitrate reduction, decomposition of urea, indole and acetyl methyl carbinol production were performed. The capability to grow on different concentrations (2%, 5%, 7% and 10%) of NaCl was also studied.

**Molecular characterization of the mosquitocidal bacterial strains**

Genomic DNA was extracted from KS2-13 and KS2-15 using HiPurA™ Bacterial and Yeast Genomic DNA Purification Spin Kit (Hi-Media, India). To determine the sequence of the 16S rRNA gene, a DNA fragment of ~ 1.5-kb was amplified by PCR from the genomic DNA of the samples using universal eubacteria-specific primers: 27F (5Œ§-AGAGTTTGATCMTGGCTCAG-3Œ§) and 1492R (5Œ§-GGYTACCTTGTTACGACTT-3Œ§), synthesized at Xcelris Labs Ltd, Gujarat, India. For polymerase chain reactions (PCR), 0.1 µg of total DNA from each isolate was mixed with 10µl of 2X PCR Master Mix (GeNei™ Bengaluru, India) consisting of dNTPs, Taq polymerase and PCR buffer. Forward and reverse primers were used at a concentration of 1µM. The final volume was made upto 20µl with sterile double distilled water. PCR amplification was performed in a thermal cycler (cyber cycler-P series PCR peltier model p96+ USA) using the program: a 5 min denaturation step at 94 ºC, 30 amplification cycles of 1 min at 94 ºC, 1 min at 56 ºC, and 1 min at 72 ºC, with a final extension step of 10 min at 72 ºC. The amplified PCR products were purified using GeneJET™ PCR purification kit (Fermentas life science Mumbai India) and sequenced by automated sequencer (ABI 3730xl Genetic) at Xcelris Labs Ltd, Gujarat, India. The forward and reverse sequences were edited using Bioedit program (Hall, 1999). The sequences obtained from strains KS2-13 and KS2-15 were compared to 16S ribosomal DNA (rDNA) gene

sequences available in the databases of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) by BLASTN homology search, as described by Altschul *et al*. (1997). Phylogenetic analysis included the 16S rDNA gene sequences of the local isolates KS2-13 and KS2-15 and the reference strains of *L. sphaericus*, and *L. fusiformis* obtained from GenBank. *Alycyclobacillus cycloheptanicus* was used as an outgroup (Nakamura. 2000). Neighbor-Joining method (Saitou and Nei. 1987) was used to infer the evolutionary history. Cluster support was assessed through 1,000 bootstrap replicates (Felsenstein. 1985). The branch lengths of the drawn tree were in the similar units as those of the evolutionary distances used to deduce the phylogenetic tree. A Kimura 2-parameter method was used to compute the evolutionary distances and is in the units of the number of base substitutions per site (Kimura. 1980). The analysis entailed 17 nucleotide sequences. All positions having missing data and gaps were removed. There were a total of 1366 nucleotides positions in the final dataset. Phylogenetic and Evolutionary analyses were conducted in MEGA5 (Tamura *et al*. 2011). Sequences were submitted in GenBank and accession numbers were obtained.

**SDS PAGE analysis of spore crystal mixture**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of spore crystal mixture was carried out by the method of Laemmli (1970) using 10% running and 4% stacking gels. The gels were stained with 0.4% Coomassie blue R250. The molecular mass of proteins was determined by using higher range protein molecular weight marker (myosin rabbit muscle 205kDa, phosphorylase b 97.4kDa, bovine serum albumin 66kDa, ovalbumin 43kDa and carbonic anhydrase 29kDa) obtained from GeNei™ Bangalore, India.

**Screening for the presence of *L. sphaericus* toxin genes**

Total cellular DNA isolated from indigenous *L. sphaericus* strain (KS2-13 and KS2-15) was used for identification of the dipterans-specific binary genes. Primers (Table1) designed by Hire *et al*. (2010) for the detection of binary toxins was used. The PCR amplification was performed in a thermal cycler (cyber cycler-P series PCR peltier model p96+ USA) in a 20µL reaction volume containing 100 ng DNA, 0.5 mM of primers, 2X PCR Master Mix consisting of dNTPs, Taq polymerase and PCR buffer (GeNei™ Bangalore, India). The amplified PCR products were purified using GeneJET™ PCR purification kit (Fermentas life science, Mumbai India).

**Cloning and sequencing of binary genes**

InsTAclone™ PCR Cloning Kit (Fermentas Life Science, Mumbai India) was used for cloning of purified PCR products. The PCR amplified *binA* and *binB* sequences were ligated in pTZ57R/T vector as per instructions in user manual. The recombinant vectors pTZ57R/T-binA and pTZ57R/T-*binB* were transformed into *Escherichia coli* DH5α. Positive clones were identified by blue white screening. The nucleotide sequences of two positive clones each from the pTZ57R/T-*binA* and pTZ57R/T-*binB* constructs were confirmed by complete sequencing of *binA* and *binB* using an automated DNA sequencer (ABI 3730xl Genetic) at Xcelris Labs Ltd, Gujarat, India. The forward and reverse sequences were edited using Bioedit program (Hall. 1999) and blast performed using BLASTN. Sequences were submitted in GenBank and accession numbers were obtained.

**Comparative analysis of deduced amino acid**

The amino acid sequences were deduced from the complete DNA nucleotide coding sequences of binary genes (KS2-13 and KS2-15) using online ExPASy translation tool. These were compared with the sequences of BinA and BinB proteins of reference strain *L. sphaericus* 2362 by Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) an online multiple sequence alignment program for amino acid variations.

## RESULT

A total of 450 bacteria from 158 samples were selected randomly and screened for mosquitocidal activity. Preliminary screening with 1 ml of culture demonstrated that two bacterial strains isolated from soil samples have mosquito larvicidal properties with 100% mortality towards *C. quinquefasciatus* and *A. stephensi* after 24 hours of exposure. However, none of the isolate showed any activity against pupae of mosquito cultures. Subsequent bioassays of these two strains against larvae of *C. quinquefasciatus* and *A. stephensi* with different culture concentrations obtained by serial

dilutions showed promising activities at very low concentrations (Table 2) which were comparable to control *L. sphaericus* 2362 strain. These strains, code named as KS2-13 and KS2-15 were selected and studied further.

**Characterisation of the potential mosquitocidal bacterial strains**

Both the mosquitocidal bacterial isolates were found to be gram-positive, aerobic, motile, rod-shaped bacteria with terminal spherical sporangium. Table 3 summarizes results of the morphological and biochemical tests performed. On the basis of morphological and physiological characters, strains KS2-13 and KS2-15 were identified as *L. sphaericus*. PCR amplification of the 16 S rRNA genes of KS2-13 and KS2-15 yielded amplicon of ~1.5 kb size. BLASTN (NCBI) analyses of KS2-13 sequence indicated a 99% similarity with other *L. sphaericus* strains (e.g., strain C5, accession no. KF523303.1; strain Ot4b.39, accession no. JQ744626.1 and strain VCRC B543, accession no. JN377786.1). Similarly, BLASTN result of KS2-15 also showed a 99% similarity with other *L. sphaericus* strains (e.g. strain Y73, accession no. JX067902.1; strain C5, accession no. KF523303.1 strain Ot4b.39, accession no. JQ744626.1). The 16S rRNA genes of KS2-13 and KS2-15 were submitted in GenBank under accession numbers KJ183073.1 and KJ183074.1 respectively. Next, we determined the degree of relatedness of our isolates to different *L. sphaericus* and *L. fusiformis* species through a phylogenetic analysis. The NJ tree shows a close relationship between the strains isolated in the present study and Group I of Nakamura (strains B-23269 and B-23287) (Fig. 1).

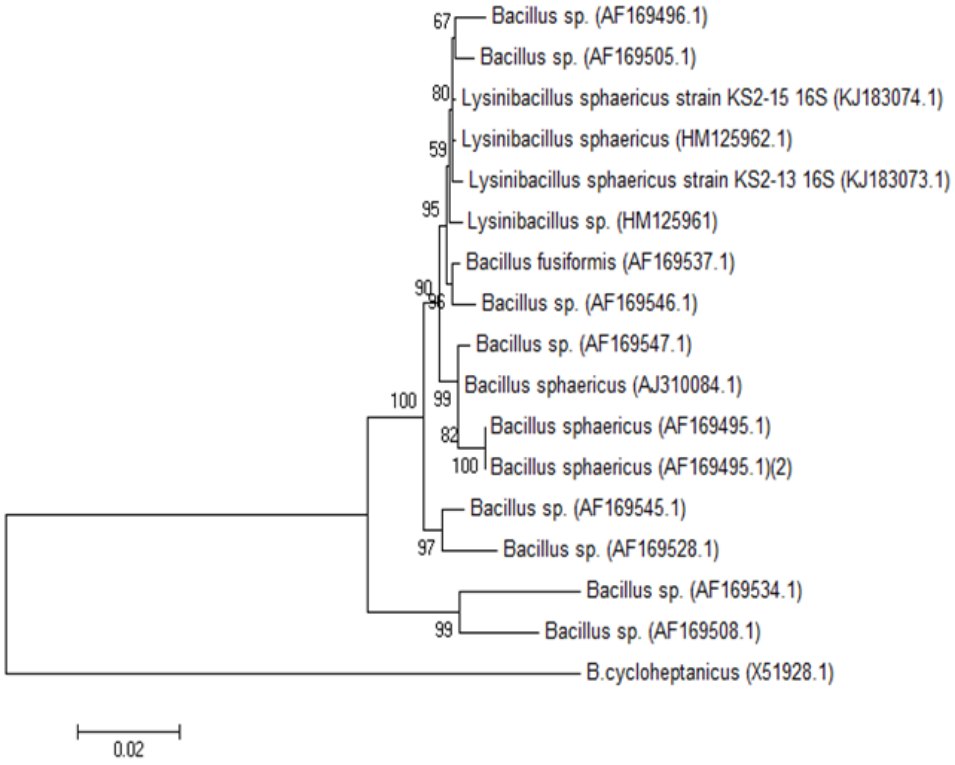


**Fig. 1.** Phylogenetic relationships are based on 16S rRNA gene sequence analysis of members of local isolates (KS2-13 and KS2-15) and the *Lysinibacillus* species. Evolutionary distances were calculated using the Kimura two parameters method, and the topology was inferred using the neighbor joining (NJ) method. Numbers above branches represent percentage bootstrap values based on 1,000 replicates. The 16S rRNA gene sequences of *Bacillus cycloheptanicus* was arbitrarily chosen as the out group. Accession numbers are between parentheses

**SDS -PAGE analysis**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of spore mixture revealed two major polypeptide chains of approximately 51.4 kDa and 41.9 kDa which correspond to known consistent sizes of BinA and BinB proteins (Fig. 2).

**Screening for the presence of *L. sphaericus* toxin genes**

Primers for *binA* and *binB* gave rise to fragments of 1.1 kb and 1.3 kb amplicons, corresponding to the *binA* toxin (41.9 kDa) gene and *binB* toxin (51.4 kDa) gene (Fig. 3) respectively. The sequences of *binA* and *binB* genes have showed 99% and 100% identity with the same genes found in *L. sphaericus*. The sequences were submitted to the GenBank under accession no. KJ547612, KJ547613, KJ547614 and KJ547615.

**Comparison of deduced amino acid sequences**

The deduced amino acid sequences of *binA* and *binB* genes of KS2-13 and KS2-15 strains when compared with deduced amino acid sequences of *binA* and *binB* genes of *L. sphaericus* 2362 revealed that the BinA (41.9 kDa) and BinB (51.4kDa) proteins of KS2-13 and *L. sphaericus* 2362 differ by 3 (K89E, E104A, Y176D) and 6 (A69S, K70N, I110T, N248H, H314L and L317F) amino acids respectively. Similarly BinA and BinB proteins of KS2-15 and *L. sphaericus* 2362 strains vary by 1 (E104A) and 3 (H109P, N248H and P274S) amino acids respectively. The varied amino acid sequences may be the reason for difference in activity.

## DISCUSSION

In the course of diverse microbial pesticides, *Bacillus thuringiensis* and *L. sphaericus* are the most widely used alternative control agents for mosquitoes (Geetha *et al*. 2007; Hayes *et al*. 2011; Prabhu *et al*. 2013). *B. sphaericus* strains have been isolated worldwide from diverse habitats, including the soil (Radhika *et al* 2011; Suryadi *et al*. 2016), aquatic habitats (Foda *et al* 2013), excreta of arid birds (Poopathi *et al* 2014) and dead larvae (Tranchida *et al*. 2011). In this study, for the first time, we have reported the isolation and characterization of the mosquitocidal strains from soil samples of Kashmir valley that is situated at northern western tip of Himalayan biodiversity hotspot (Mittermeier *et al*. 2005). A total of 450 bacterial isolates from 158 samples were screened for mosquitocidal activity. However, only two isolates code named as KS2-13 and KS2-15, being toxic were further studied. Level of

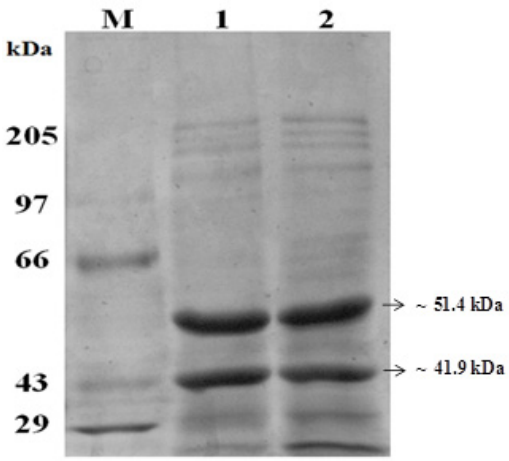


**Fig. 2.** SDS-PAGE analysis of spore crystal mixture of *L. sphaericus* strains: Lane M: High molecular weight marker; Lane 1-2: *L. sphaericus* strains, KS2-13 and KS2-15 respectively

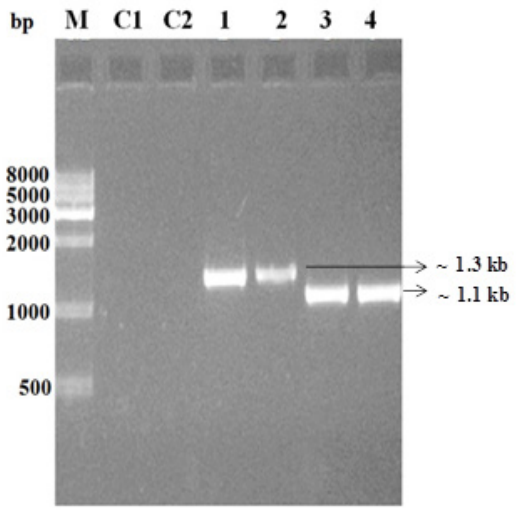


**Fig. 3.** PCR amplification of *binA* and *binB* genes from *L. sphaericus* strains KS2-13 and KS2-15. Lane M, molecular weight marker; Lane C1 and C2: negative controls; Lane 1-2: *binA* genes (KS2-13 and KS2-15 strain); and Lane 3-4, *binB* genes (KS2-13 and KS2-15 strain)

toxicity was higher in case of *C. quinquefasciatus* than *Anopheles stephensi*. The isolate KS2-13 and KS2-15 did not produce mortality in *A. aegypti* larvae. Earlier it had been reported that fluorescent labeled *L. sphaericus* toxin binds proficiently with cultured *C. quinquefasciatus* cells and larval midgut cells in contrast to *A. aegypti* cultured cells and larval midgut cells where it binds slightly or is undetectable (Davidson *et al*. 1987; Davidson 1988). However, Tranchida *et al*. (2011) have reported isolation of *Lysinibacillus* strains from *Culex pipiens* larvae active against larvae of Culex, Aedes, Culex, Ochlerotatus, and Anopheles species. Identification of *B. sphaericus* like organisms is hard and arduous as they cannot be differentiated from each other by conventional phenotypic tests. The sequence of 16S rRNA genes has been widely used to identify an unknown bacterium to the genus or species level (Geetha *et al*. 2008). In present study, two potential mosquito larvicidal toxic strains were identified as *B. sphaericus* by their morphological, biochemical features and 16S rRNA gene sequence. Blast analysis of 16S rRNA gene sequences of KS2-13 and KS2-15 showed 99% identity with other already reported *B. sphaericus*. A typical feature used in the depiction of the *L. sphaericus* species is the lack of ability to utilize pentoses and hexoses as the solitary carbon source. This is due to the absence of the pgi genes encoding enzymes for breaking and transporting these sugars (Hu *et al*. 2008). Our two isolates KS2-15 and KS2-13 exhibited promising mosquito larvicidal activity against *C. quinquefasciatus* (LC50: $1.36 \times 10^3$ spores/mL; $1.41 \times 10^3$ spores/mL respectively) and *A. stephensi* (LC50: $2.14 \times 10^3$ spores/mL; $2.11 \times 10^3$ spores/mL correspondingly) which was comparable to control *L. sphaericus* 2362 (*C. quinquefasciatus* LC50: $1.34 \times 10^3$ spores/mL; *A. stephensi* $2.10 \times 10^3$ spores/mL) under our laboratory conditions. Tranchida *et al*. (2011) reported two *B. sphaericus* strains C107 and C207 that had showed highest mosquitocidal activity against *Culex pipiens* and *Ochlerotatus albifasciatus* with $LC_{50}$: $4 \times 10^4$ spores/mL and $LC_{50}$ of $3.4 \times 10^6$ spores/mL respectively. Recently, Suryadi *et al*. (2016) reported four mosquitocidal *L. sphaericus* strains MNT, SKT, TJL2, and SLG. *L. sphaericus* strain MNT showed $LC_{50}$ of $3.70 \times 10^5$ cell/mL, $1.76 \times 10^7$ cell/mL and $4.45 \times 10^7$ cell/mL against Culex, Anopheles and Aedes respectively. Similarly strains SKT, TJL2 and SLG showed $LC_{50}$ of $1.13 \times 10^5$ cell/mL, $2.85 \times 10^5$ cell/mL, $1.78 \times 10^7$ cell/mL; $1.03 \times 10^5$ cell/mL, $8.94 \times 10^4$ cell/mL, $1.72 \times 10^7$ cell/mL and $9.41 \times 10^5$ cell/mL, 2.39

**Table 1.** Primers used in this study

| Gene | Primer sequence ( 5' to 3') | Size | Reference |
|---|---|---|---|
| *binA* gene | F= AGC TAA AAC ATATGA GAA ATT TGG ATT TTA TTG<br>R= TTG TGG ATCCTTAGT TTT GAT CAT CTG TAATAATC | 1.1 kb | Hire *et al*., 2010 |
| *binB* gene | F= GAT GAA GAA CATATG TGC GAT TCA AAA GAC<br>R= AGT TGG ATC CTT ACT GGT TAA TTT TAG GTA TTA A | 1.3 kb | |

**Table 2.** Larvicidal activity of *Lysinibacillus sphaericus* strain KS2-13 and KS2-15 against third instar larvae of different mosquito species

| Strain | Mosquito species | LC50 (95% CL) | LC90 (95% CL) | $X^2$ |
|---|---|---|---|---|
| 2362 | *Culex quinquefasciatus* | 1.34 (1.09-1.56) | 2.48 (2.15- 3.08) | 0.343 |
| KS2-13 | *Culex quinquefasciatus* | 1.41 (1.14-1.65) | 2.67 (2.29- 3.41) | 0.190 |
| KS2-15 | *Culex quinquefasciatus* | 1.36(1.13-1.58) | 2.47 (2.15- 3.05) | 0.313 |
| 2362 | *Anopheles stephensi* | 2.10 (1.78-2.69) | 3.77 (3.04-5.70) | 0.543 |
| KS2-13 | *Anopheles stephensi* | 2.11 (1.74-2.91) | 4.11 (3.18-7.06) | 0.649 |
| KS2-15 | *Anopheles stephensi* | 2.14 (1.81-2.78) | 3.86 (3.09-5.94) | 0.729 |

Lethal concentrations (expressed in $10^3$ spores $mL^{-1}$) for 50% ($LC_{50}$) or 95% ($LC_{95}$) of larvae treated after 24 h

$\times 10^5$ cell/mL, 2.08 $\times 10^7$ cell/mL against Culex, Anopheles and Aedes respectively. The toxicity of *L. sphaericus* to mosquitos larvae mainly results from binary toxins (41.9 kDa and 51.4 kDa) encoded by the *binA* and *binB* genes expressed during sporulation stage (Broadwell *et al*. 1990; Tangsongcharoen *et al*. 2015). Another toxin called Mtx (100 kDa,) is present in *L. sphaericus* strains of both low and high toxicity. According to Thanabalu *et al* (1991), the existence of the *mtx* genes does not per se confer toxicity to this bacterial strain against mosquito larvae. Therefore, the low toxicity in some strains could result from either low expression or short lived stability of the binary toxins during sporulation. Recently Prabhu *et al* (2013) reported the molecular characterization of forty two *L. sphaericus* strains isolated from Tamil Nadu, India. Their results revealed genetic heterogeneity between both toxic and non-toxic isolates and pointed out there is a good correlation between the existence of toxin genes and toxicity of the strains. In current study SDS-PAGE analysis of spore crystal revealed two major protein bands of 41.9 kDa and 51.4 kDa in size which correspond to the BinA and BinB proteins. The PCR for *binA* and *binB* genes and their sequence analysis confirmed the presence of two *bin* genes in each strain. Deduced amino acid sequences of *binA* and

**Table 3.** Morphological and biochemical characteristics of mosquitocidal bacterial strains

| Property | KS2-13 | KS2-15 |
|---|---|---|
| Colony morphology | Cream colored, moist irregular | Cream colored , irregular |
| Spores | Cylindrical | cylindrical |
| Utilization of | | |
| Glucose | - | - |
| Starch | - | - |
| Lactose | - | - |
| arabinose | - | - |
| Xylose | - | - |
| Mannital | - | - |
| 2% NaCl | + | + |
| 4% NaCl | + | + |
| 5% NaCl | W | W |
| 7% NaCl | - | - |
| Erythromycin 1b | + | + |
| Erythromycin 2b | - | - |
| Tetracycline 2b | + | + |
| Tetracycline 5b | - | - |
| Chloramphenicol 8b | + | + |
| Citrate | + | + |
| Phenyl alanine | + | + |
| Arginine | - | - |
| Lysine | - | - |
| Ornithine | - | - |
| Tyrosine | - | - |
| $H_2S$ production | - | - |
| Decomposition of urea | W | W |
| Catalase | + | + |
| Oxidase | + | + |
| Indole | - | - |
| Methyl red | - | - |
| Voges-Proskauer | + | + |
| Identified as | *Lysininbacillus sphaericus* | *Lysinibacillus sphaericus* |

+ indicates positive; - indicates negative and W indicates weak

*binB* genes from KS2-13 and KS2-15 strains when compared with amino acid sequences of *binA* and *binB* genes from *L. sphaericus* 2362 revealed that the BinA and BinB proteins of KS2-13 and *L. sphaericus* 2362 are different by 3 (K89E, E104A, Y176D) and 6 (A69S, K70N, I110T, N248H, H314L and L317F) amino acids respectively. Similarly BinA and BinB proteins of KS2-15 and *L. sphaericus* 2362 strains contrast by 1 (E104A) and 3 (H109P, N248H and P274S) amino acids respectively. The varied amino acid sequences may be reason for the difference in activity.

The high larval toxicity existing in some *L. sphaericus* strains, such as 2362 (Weiser. 1984) and IAB59 (de Barjac *et al*. 1988), has resulted into their commercial use as biopesticides against populations of mosquitos. Recent reports of resistance development devoted the attention of researchers worldwide towards the discovery of new isolates from natural resources as an alternative to the existing biopesticides globally. As variability was found between the new isolated *L. sphaericus* strains as well as with *L. sphaericus* strains 2362, we concluded that the strains (KS2-13 and KS2-15) isolated and identified in this study embody good candidates for exploiting in the control of mosquito within the situation to which both the host and the parasite are evenly well adapted.

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