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A report on novel mosquito pathogenic *Bacillus* Spp. isolated from a beach in Goa, India

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Abstract
Nine *Bacilli* were isolated from 3 spatially diverse zones *viz*., tidal, intertidal and seashore at Miramar beach in Goa, India. The isolates were assessed for mosquito larvicidal activity against 3rd instar larvae of *Anopheles stephensi*, *Culex quinquefasciatus*, *Aedes aegypti* and *Aedes albopictus*. Colony and biochemical characteristics of the isolates showed similarity to *Bacillus sphaericus Neide 2362* and *Bacillus thuringiensis* var. *israelensis* H-14 commercial strains. Preliminary toxicity screening indicated that four isolates (B1C, C1A, C2A and C2C) possessed mild to high toxicity (60-100% mortality) and were further assayed to obtain LC_{50} and LC_{90} values following a 24 and 48 hour treatment period. Phylogenetic analysis revealed that these isolates clustered with mild and highly toxic strains of *Bacillus*, *Lysinibacillus* and *Aneurinibacillus* sp. This is the first lead on the presence of mosquito pathogenic bacterial isolates native to Miramar beach in Goa which can be further explored for development of formulations for vector control.

Keywords: Seashore, larvicidal bacteria, high toxicity, formulations

1. Introduction
Mosquitoes transmit a variety of diseases that pose serious public health challenges worldwide [1]. The most important disease vectors belong to the following two subfamilies i.e. Anophelinae (*Anopheles* mosquitoes) which transmit malaria and Culicinae (*Culex* mosquitoes) that transmit filariasis, West Nile virus and Japanese encephalitis and *Aedes* mosquitoes responsible for dengue, chikungunya, yellow fever and Zika transmission. In order to prevent the proliferation of these mosquito borne diseases and to improve the quality of life, vector control is essential.

Goa is a popular domestic and international tourist destination in India. Among the vector borne diseases, Goa has witnessed widespread outbreaks of malaria, dengue, chikungunya and Japanese encephalitis, peaking during the monsoon season, especially along the coast since the mid-1980s [2].

Resurgence of vector borne diseases due to favourable environmental conditions, human lifestyle changes, rapid urbanization, development of resistance in vectors to routinely used public health insecticides, changes in public perception against mosquito nuisance favour adoption of safer and effective methods of vector control [3, 4]. The World Health Organization advocates Integrated Vector Management (IVM), where emphasis is placed on the application of alternative cost effective, environment friendly and sustainable methods of vector control [5]. Among the alternate control tools, biological control agents such as larvivorous fish and several strains of spore forming bacteria (*Bacillus sphaericus* & *Bacillus thuringiensis* var. *israelensis*) have shown effectiveness for vector control in several field evaluations [6]. Besides, *Bacillus subtilis*, was also found to be a promising candidate for mosquito vector control [7]. Currently, these microbial agents are formulated as an aqueous suspension, wettable powder, water dispersible granules, briquettes as well as tablets to suit the application in the various breeding habitats of mosquitoes [8]. However, there is a risk of development of resistance in the vectors to these bio control agents as a result of their long-term use. There are reports of development of resistance by the vectors against *B. sphaericus* and *B. thuringiensis* var. *israelensis* [9], which calls for continued search for new bio control agents of mosquitoes.

Hence in the present study, we explored the coastal region of Goa i.e.

Hence in the present study, we explored the coastal region of Goa i.e. Miramar beach for bio-larvicidal bacteria and it resulted in the isolation of some novel, previously unreported strains. These strains were identified and characterised through morphological, biochemical and molecular methods. Their larvicidal efficacy was assessed against the four mosquito vector species and the results are presented in this communication.

2. Materials and Methods

2.1 Bacterial Isolation and Biochemical characterization

Soil samples were collected from 9 locations at Miramar beach, Goa, India. The sampling location was at 15.4827° N and 73.8074° E. Approximately, ten grams of soil samples were collected from near the sea shore, intertidal and tidal zones aseptically with a spatula and transferred into a sterile falcon tube. The top layer of the soil was collected aseptically and samples were transported to the laboratory for further processing. For isolation of mosquito pathogenic bacteria from the soil samples, three methods were followed. Method 1 involved inoculation of 1 gram of soil sample in 10 ml Nutrient broth (NB) followed by incubation on a shaker at 100 rpm for 48 hours. One ml of the supernatant was then screened for mosquito larvicidal activity. Method 2 consisted of direct inoculation 0.5 grams of soil in 100 ml of Nutrient yeast sporulating (NYSM) medium followed by incubation on a shaker at 100rpm for 48 hours. Method 3 was that proposed by Dhindsa *et al*. in 2002 [10]. Accordingly, 0.5 g of soil sample was added to LB broth (10ml) (buffered with sodium acetate) and incubated on a shaker at 200 rpm for 4 hours at 30˚C. One milliliter of this aliquot was heat shocked at 65˚C for ten minutes in a pre-warmed 5 ml glass tube from which 0.1 ml of sample was removed, added to 1 ml of LB broth and incubated for 24 hours at 30˚C. To the same glass tube, one ml of NYSM (sporulating medium) was added and incubated for two days at 30˚C. Sample (0.1ml) was withdrawn for preliminary toxicity testing against the 3rd instar larvae of the mosquito vector species. The active soil samples were then processed by serial dilution and spread plated followed by picking up the morphologically different colonies. Pure colonies were maintained on Nutrient Agar (NA) plates. Colony, morphological and biochemical characteristics of the isolates were studied as per the tests listed in Bergey's Manual of Systematic Bacteriology [11]. Morphological characteristics observed were colony morphology, Gram staining and endospore staining. Biochemical characteristics tested were nitrate, catalase, indole, oxidase, sugar fermentation, MR-VP, gelatin liquefaction, starch hydrolysis, tyrosine degradation, hydrolysis of casein, hydrolysis of tween, hydrolysis of arginine, Hugh leifsons test and growth conditions. Sensitivity against antibiotics (Kanamycin, Streptomycin and Erythromycin) was also assessed.

2.2 Molecular Characterization

2.2.1 DNA extraction

Pure colonies of the isolates were sub-cultured onto Nutrient Agar plates and following 24hrs incubation the bacterial genomic DNA extraction was carried out by phenol-chloroform method [12].

2.2.2 PCR amplification

The 16S rRNA gene amplification was carried out using the universal primers (S-D-Bact-0011-a-5-17: 5`-GTTTGATCCTGGCTCAG-3`) and (S-*-Univ1392-b-A-15: 5`-ACGGGCGGTGTGTNC-3`) [13]. PCR reaction mixture consisted of 15µl of PCR Master mix 2X concentrated solution (0.05U/µl taq DNA polymerase, reaction buffer, 4mM $MgCl_2$, 4x 1.25 ml nuclease free water), 1 µl of forward primer and 1 µl of reverse primer and 1.5 µl of the extracted bacterial genomic DNA template of each sample was added in sterile PCR tubes and the final volume was made up to 30 µl by using nuclease free water. Without DNA template control was also maintained. The samples were amplified in a thermal cycler (Bioer XP Cycler). The PCR conditions were initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94° C for 1 min, annealing at 55°C and extension at 72°C for 2 min respectively, followed by final extension at 72°C for 5 min. To check for the amplification, 8µl of PCR amplified products were run on a 1% agarose gel. To verify the size of the PCR product, 500bp ladder was loaded along with the sample. The amplified PCR products were purified using Qiagen Q1A quick PCR purification kit (Cat. No. 28104) and sent for sequencing to Eurofins India Genomics Pvt. Ltd., Bangalore. The sequences obtained were edited using the Bio edit sequence alignment editor version 7.0.4.1 software and compared with sequences of other closely related species retrieved from the GenBank database http://www.ncbi.nlm.nih.gov/BLAST and identified based on sequence homology.

2.2.3 Phylogenetic analysis

The phylogenetic analysis and tree construction was carried out using neighbour-joining method. ClustalX version 2.0. was used to generate multiple sequence alignment between closely related species. The tree was obtained with 100 seeds and 1000 bootstraps. The final tree was rooted and drawn using MEGA4 [14].

2.3 Bioassays

2.3.1 Source of Mosquito Larvae

The mosquito colonies of the 4 test vector species (*An. stephensi, Cx quinquefasciatus, Ae. aegypti* and *Ae. albopictus*) were maintained at ambient laboratory conditions i.e. temperature of 27±2 ºC, relative humidity 70± 5% and a 12hrs day and night cycle. A pinch of larval food (Cerelac™ and finely ground fish meal in a 1:1 ratio) was provided to the larvae daily until the pupae stage. From the laboratory bred immature mosquitoes, healthy 3rd instar larvae were used for bioassays to screen the bacterial isolates for larvicidal activity.

2.3.2 Preliminary toxicity screening

Preliminary toxicity screening was carried out in sterile bioassay bowls containing ten laboratory reared larvae of each of the test species in 100 ml of water. 0.1ml of a bacterial culture grown in NYSM broth (sporulating medium) was used for screening for checking larvicidal activity. Uninoculated NYSM broth was used in the control. Mortality was scored after 24 hours and 48 hours of the treatment by counting the number of the dead larvae in the respective bioassay bowls.
The percentage mortality was calculated using the formula:

$$\% \text{ Mortality} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \text{ X } 100$$

If the % mortality was > 5% in the control larvae, the corrected mortalities were determined by Abbot's formula [15].

$$\% \text{ Mortality} = \frac{(\% \text{ mortality in the experiment}) - (\% \text{ mortality in the control})}{100 - (\% \text{ mortality in the control})} \text{ X } 100$$

2.3.3 Purification and storage of the isolates

The isolates that showed potent activity were further maintained by streaking onto Nutrient Agar plates and slants which were kept in the refrigerator at 4 °C. They were purified by sub-culturing followed by Gram staining of individual colonies in order to confirm purity. A pure sample of each isolate was kept safe at -20 °C in 20% (v/v) glycerol.

2.3.4 Preparation of lyophilized powder for bioassay

The bacterial isolates were grown in a 250ml conical flask containing 100ml of NYSM broth at 28°C on a rotary shaker for 72-96 hours [16]. Spore crystals were harvested by centrifugation at 8000rpm for 20 minutes and the pellet was washed twice with sterilized distilled water. The final pellet obtained was lyophilized and stored at 4 ˚C until use.

2.3.5 Main Bioassays

The bioassays of the bacterial isolates were carried out against laboratory reared 3rd instar larvae of *An. stephensi, Cx. quinquefasciatus, Ae. aegypti* and *Ae. albopictus* mosquitoes according to WHO protocol [17]. Stock dilutions in parts per million (ppm) were prepared by dissolving the weighed lyophilized powder in sterile distilled water. A series of doses were prepared based on the preliminary toxicity screening results. Four replicates of 25 3rd instar larvae from each mosquito species were taken in 500ml plastic bowls containing 250ml of distilled water and different doses were administered. Concurrent controls were maintained under similar conditions. The mortality was scored by counting the number of dead larvae after 24 hours and 48 hours of treatment. Efficacy of the isolates in terms of LC_{50} and LC_{90} was calculated by analyzing dose mortality responses of individual strains by Probit analysis using SPSS version 16 software [18].

3. Results

3.1 Isolation and Characterization of potential larvicidal bacterial strains

A total of 9 soil samples from three different zones i.e. tidal (Sample A1, B1, C1), intertidal (Sample A2, B2, C2) and sea shore (Sample A3, B3, C3) were collected and processed further. Among the three methods used for screening the soil samples, the method proposed by Dhindsa *et al.* [10] showed the best results in terms of mosquito pathogenic activity with sample B1 collected from the tidal zone showing 100% mortality against the 3rd instar larvae of all the test vector species. Sample C1 obtained from tidal and C2 from the intertidal zone showed 100% mortality against 3rd instar *Culex quinquefasciatus* larvae. The rest of the soil samples showed mortality of 40% and below. All the samples processed by Method 1 and 2 showed a mortality of 20% and below against the tested vector species (Figure 1, 2, 3).

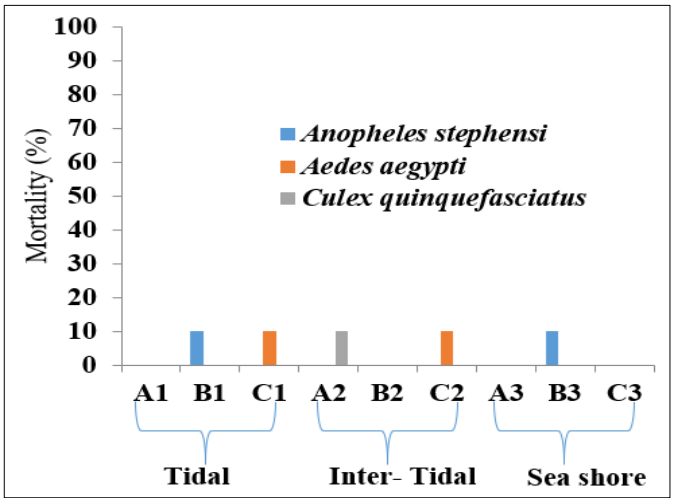

Fig 1: Screening of soil sample by Method 1 using Nutrient broth (NB)

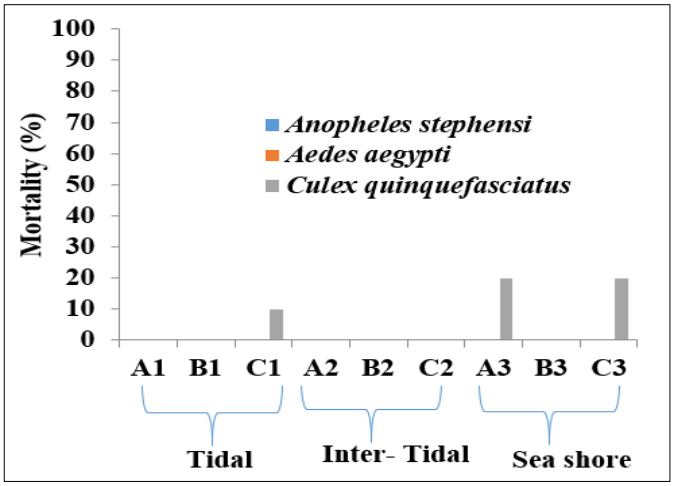

Fig 2: Screening of soil sample by Method 2 using Nutrient Yeast Sporulating medium

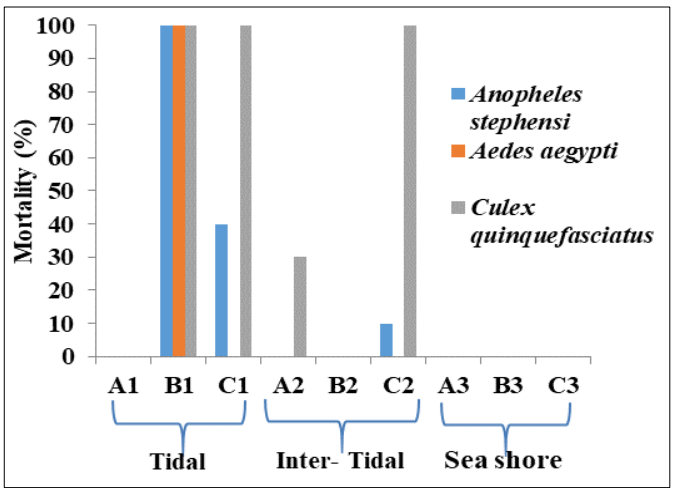

Fig 3: Screening of soil sample by Method 3 using Dhindsa *et al.* 2002 method

The 3 soil samples (B1, C1, C2) which showed significant larvicidal activity were considered to be ideal for isolation of mosquito pathogenic *Bacilli*. From these soil samples nine morphologically distinct bacterial isolates were obtained and coded as B1A, B1B, B1C, C1A, C2A, C2B, C2C, C2D, C2E. Preliminary screening showed that 4 out of the 9 isolates i.e. C1A, C2A, C2C and B1C showed moderate to high toxicity (60-100%) against the 3rd instar larvae of *An. stephensi*, *Cx. quinquefasciatus*, *Ae. aegypti* and *Ae. albopictus*. Subsequently, these isolates were bio assayed at different doses to determine their LC_{50} and LC_{90} values as seen in Table 1.

Table 1: LC_{50} and LC_{90} values in ppm of the most active bacterial isolates following 24 hours and 48 hours of treatment against the 3rd instar larvae of test vector species

Isolate No.	Test Vector species	LC_{50} 24 hours	48 hours	LC_{90} 24 hours	48 hours
Isolate C1A	*Anopheles stephensi*	39.42 (17.38-89.36)	30.68 (12.36-80.88)	303.70 (133.96-688.49)	261.28 (184.36-461.46)
	Culex quinquefasciatus	0.08 (0.038-0.169)	0.032 (0.012-0.078)	0.87 (0.423-1.854)	0.45 (0.174-1.183)
	Aedes aegypti	13.18 (7.81-22.26)	3.65 (2.01-6.63)	61.15 (36.21-103.24)	23.22 (12.78-42.17)
	Aedes albopictus	23.58 (12.54-50.08)	20.593 (9.57-44.273)	636.723 (151.24-1156.54)	190.259 (88.497-409.034)
Isolate C2A	*Anopheles stephensi*	95.74 (39.43-232.42)	38.28 (9.85-148.68)	408.76 (280.28-820.07)	299.17 (149.44-445.80)
	Culex quinquefasciatus	0.30 (0.174-0.502)	0.16 (0.102-0.300)	1.32 (0.77-2.24)	0.62 (0.34-1.00)
	Aedes aegypti	50.98 (24.82-104.68)	14.21 (7.73-26.10)	399.95 (194.7-821.27)	96.31 (52.42-176.93)
	Aedes albopictus	16.500 (9.56-28.46)	12.729 (7.68-21.07)	89.769 (52.03-154.88)	60.629 (36.62-100.37)
Isolate C2C	*Anopheles stephensi*	54.22 (17.39-89.37)	29.35 (12.36-80.89)	323.78 (133.96-688.49)	144.33 (54.81-358.68)
	Culex quinquefasciatus	0.25 (0.13-0.45)	0.15 (0.08-0.28)	1.37 (0.75-2.51)	0.70 (0.37-1.33)
	Aedes aegypti	42.30 (27.89-64.29)	17.17 (11.21-26.32)	145.74 (95.88-221.53)	60.52 (39.49-92.73)
	Aedes albopictus	41.848 (28.61-61.20)	24.907 (16.40-37.82)	124.802 (85.334-182.523)	92.703 (61.044-140.780)
Isolate B1C	*Anopheles stephensi*	2.011 (0.83-4.85)	0.37 (0.18-0.75)	50.2 (20.79-121.17)	3.99 (1.96-8.11)
	Culex quinquefasciatus	0.162 (0.11-0.23)	0.13 (0.091-0.197)	0.528 (0.36-0.76)	0.461 (0.31-0.68)
	Aedes aegypti	0.14 (0.10-0.19)	0.13 (0.09-0.17)	0.384 (0.28-0.52)	0.315 (0.23-0.43)
	Aedes albopictus	0.189 (0.131-0.272)	0.161 (0.112-0.233)	0.660 (0.458 - 0.951)	0.530 (0.368-0.763)
**Bti* commercial strain	*Anopheles stephensi*	0.34 (0.19-0.61)	0.21 (0.09-0.46)	1.83 (1.02-3.25)	0.63 (0.38- 1.02)
	Culex quinquefasciatus	0.0002 (0.0001-0.0021)	0.00009 (0.00002-0.00031)	0.02 (0.002-0.31)	0.003 (0.001-0.012)
	Aedes aegypti	0.00104 (0.00021-0.00508)	0.00252 (0.00057-0.01121)	0.09 (0.02-0.48)	0.020 (0.0042-0.101)
	Aedes albopictus	0.00252 (0.00057-0.01121)	0.00015 (0.0002-0.00129)	0.271 (0.06-1.20)	0.048 (0.005-0.421)
**Bs* commercial strain	*Anopheles stephensi*	1.86 (0.72-4.82)	0.88 (0.31-2.45)	20.23 (7.83-52.26)	18.25 (7.45-44.68)
	Culex quinquefasciatus	0.00006 (0.00001-0.00029)	0.00002 (0.00001-0.0001)	0.007 (0.001-0.034)	0.0049 (0.00074-0.03267)
	Aedes aegypti	0.12 (0.04- 0.35)	0.05 (0.01- 0.16)	2.411 (0.872- 6.663)	0.995 (0.319- 3.100)
	Aedes albopictus	0.23 (0.09- 0.59)	0.02 (0.008- 0.091)	3.594 (1.439- 8.977)	0.380 (0.110-1.313)

*All values are in parts per million (pp)

*Bti- *Bacillus thuringiensis israelensis* strain *H-14** Bs- *Bacillus sphaericus Neide 2362*

Microscopic examination following Gram staining and endospore staining showed all isolates to be gram positive, rod shaped and endospore forming. The scanning electron microscopy (SEM) images also revealed similar morphological features i.e. rod shaped and endospore formers as depicted in (Figure 4a, 4b, 4c and 4d). Biochemical tests were performed along with commercial strains of *B. thuringiensis* H-14 and *B. sphaericus Neide* 2362. The morphological and biochemical characteristics of these isolates are presented in Table 2. Based on Gordon (1973) and Berkeley (1984) simplified key for *Bacillus* species and Bergeys Manual of Systemic Bacteriology Vol 4, Bacteria:

Firmicutes, these isolates were found to be belonging to *Bacillus* sp. [11, 19, 20].

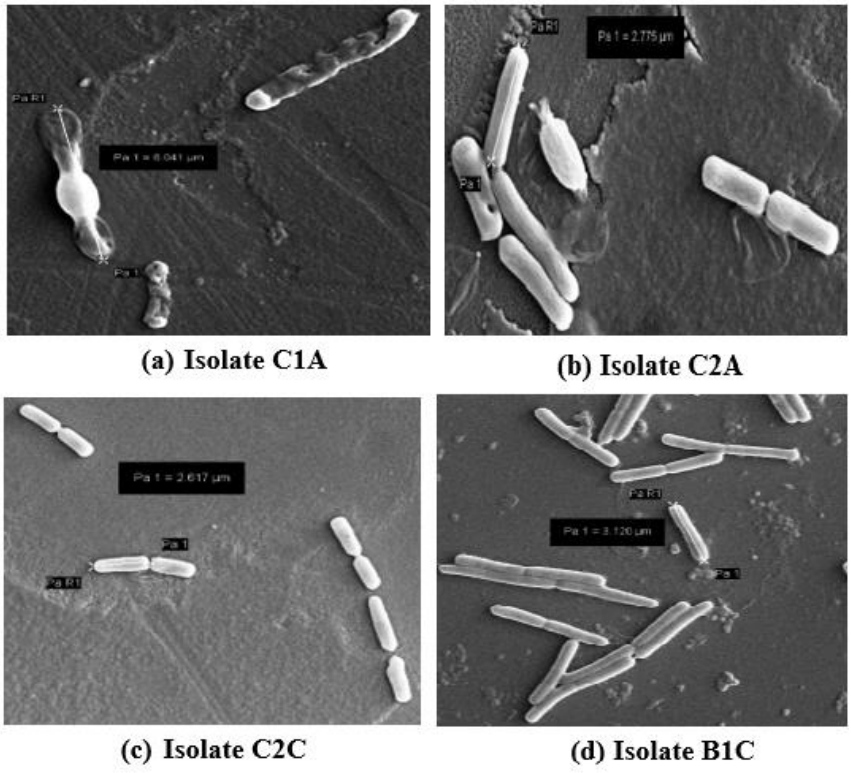

Fig 4 (a, b, c, d): Scanning Electron micrographs of mosquito-pathogenic bacilli isolated from a coastal region sampling site showing long rods and endospores (a) Isolate C1A (b) Isolate C2A (c) Isolate C2C (d) Isolate B1C

Table 2: Biochemical characteristics of Isolates

TEST	Isolate					
	C1A	C2A	C2C	B1C	*Bti* H-14	*Bs* 2362
Gram staining	+	+	+	+	+	+
Endospore	+	+	+	+	+	+
Indole	-	-	-	-	-	-
Hugh Leifson	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Motility	+	+	+	+	+	+
Growth at 65°	-	-	-	-	-	-
MR	-	-	-	+	+	-
VP	-	-	-	+	+	-
Nitrate	+	-	-	+	+	-
Sugar (Glucose) fermentation	-	-	-	+	+	-
Arginine hydrolysis	-	-	-	+	+	-
Tyrosine	+	-	-	-	-	-
Casein Hydrolysis	-	+	+	-	+	+
Growth in 2% NaCl	-	+	+	+	+	+
5% NaCl	-	+	+	+	+	+
7% NaCl	-	-	-	-	-	-
Growth at 50°C	-	-	-	+	+	+
Sensitivity to Streptomycin(10mcg)	+	-	-	+	+	+
Sensitivity to Kanamycin (30mcg)	-	+	+	+	+	+
Sensitivity to Erythromycin (15mcg)	+	+	+	+	+	+
Tentative identification	*Bacillus sp.*	*Bacillus sp.*	*Bacillus sp.*	*Bacillus sp.*	*Bacillus thuringiensis*	*Bacillus sphaericus*

*Bti- *Bacillus thuringiensis israelensis* strain *H-14* Bs- *Bacillus sphaericus Neide 2362* +: Positive-: Negative

Further molecular characterization of these isolates by PCR amplification of the 16S rRNA gene yielded amplicons of the size 1500 bps. The PCR amplicons were custom sequenced and the nucleotide sequences obtained were blasted against the NCBI database using BLASTN. Isolate C1A showed 99.93% sequence similarity with *Aneurinibacillus* sp., while Isolate C2A and C2C showed high similarity (99.2% and 100% respectively) with *Lysinibacillus* sp. Isolate B1C showed 100% sequence similarity with *B. thuringiensis* strain. All sequences have been submitted to the NCBI GenBank

database with accession id's MN595035, MN606113, MN606137 and MN606138 respectively. The phylogenetic tree was constructed based on the comparison of the 16S rRNA sequences generated in this study with sequences of species belonging to *Bacillus* genus, and other closely related organisms. The isolate C1A formed a coherent branch with *Aneurinibacillus* group. While the isolates C2A and C2C formed a branch with *Lysinibacillus* group. Isolate B1C aligned with *B. thuringiensis* group (Figure 5).

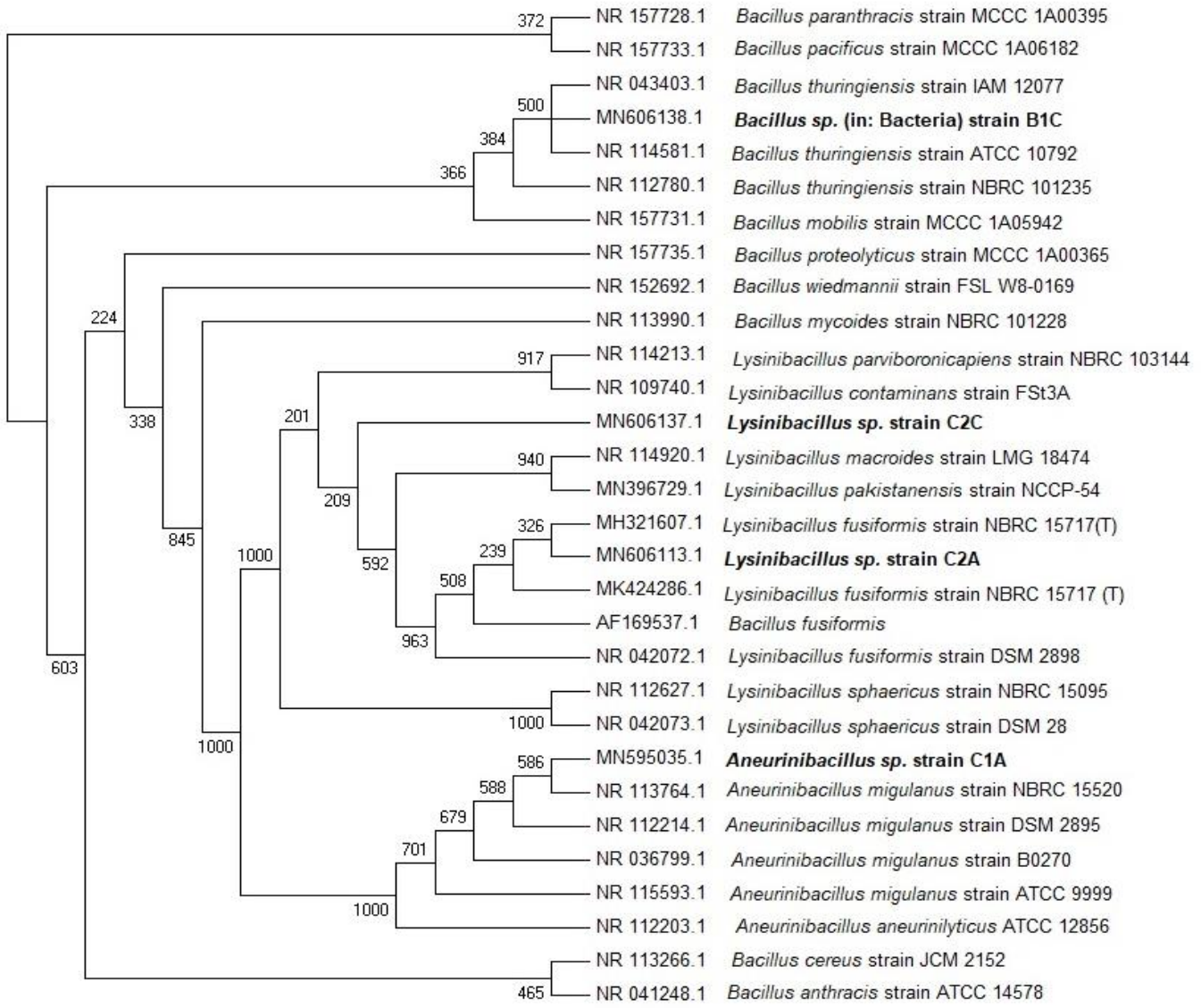

Fig 5: Phylogenetic tree showing the phylogenetic relationships among isolate *Anuerinibacillus* sp strain C1A., *Lysinibacillus* sp. strain C2A, *Lysinibacillus* sp. strain C2C, *Bacillus thuringiensis* strain B1C and their close relatives inferred from 16S rRNA gene sequences from NCBI GenBank.

3.2 Larvicidal activity of the bacterial isolates

All the toxic isolates showed a similar pattern with the mortality increasing with an increase in the dose (ppm). *Aneurinibacillus* sp., showed the highest toxicity against the 3rd instar larvae of the test vector species with *Cx. quinquefasciatus* being most susceptible (LC_{50} = 0.08ppm) followed by *Ae. aegypti* (LC_{50} = 13.12ppm), *An. stephensi* (LC_{50} = 39.42ppm) and *Ae. albopictus* (LC_{50} = 23.58ppm). When treated with *Lysinibacillus* sp., strain C2A, *Culex* species showed the highest mortality (LC_{50} = 0.30ppm) followed by *Aedes* species (LC_{50} = 16.50ppm and 50.98ppm against *Ae. albopictus* and *Ae. aegypti*, respectively). *An. stephensi* showed least susceptibility (LC_{50} = 95.74ppm). A similar pattern of susceptibility was observed upon treatment with *Lysinibacillus* sp., strain C2C. *Culex* species showed highest mortality (LC_{50} = 0.25ppm) followed by *Aedes* species (LC_{50} = 41.84ppm and 42.30ppm against *Ae. albopictus* and *Ae. aegypti* respectively. *An. stephensi* showed the least susceptibility (LC_{50} = 54.22ppm) on 24 hours of treatment. Lastly, *Bacillus thuringiensis* sp., strain B1C showed highest toxicity against *Ae. aegypti* larvae (LC_{50} = 0.14ppm) followed by *Cx. quinquefasciatus* and *Ae. albopictus* larvae with LC_{50} = 0.162ppm and 0.189ppm respectively and LC_{50} of 2.011ppm against *An. stephensi* larvae. The mode of action of the bacterial isolates was observed to be due to lysis of the midgut of the larvae which is well known, resulting in death (Figure 6, 7, 8, 9).

Fig.	Mosquito species	Untreated	Treated with novel isolate *Aneurinibacillus* sp. strain C1A

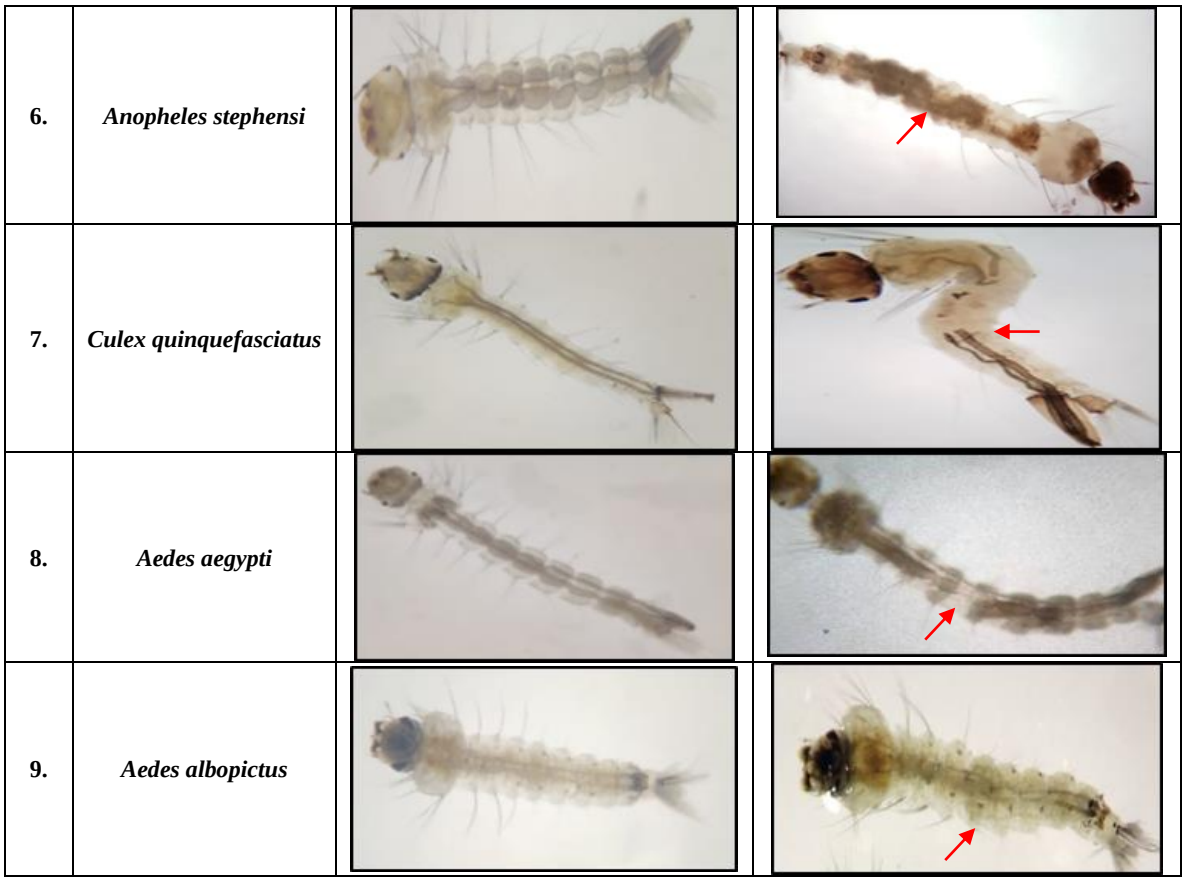

Fig 6-9: Indicating midgut lysis of the 3rd instar larvae following treatment with *Aneurinibacillus* sp.

4. Discussion

Vector mosquitoes pose a significant threat to human health as they have the ability to efficiently transmit disease causing pathogens causing a variety of diseases which afflict millions of people worldwide. They are responsible for infecting over 700 million people every year in more than 80 countries and approximately 20% of the world's population is at risk of acquiring infections of a vector borne-disease [21]. Malaria is the largest single component of disease burden, epidemic form of malaria, in particular, remains a major public health concern in the low income tropical countries [22]. In the past few years, resistance to insecticides and chemical agents has been increasing rapidly, hence there is a persistent demand for development of new insecticidal agents from natural environments.

In this regard, soils have proven to be an excellent source of microbes which can be explored for their bio control potential [23]. *Bacillus* sp., *Streptomyces* sp., *Penicillium* sp., and *Trichoderma* sp. are being developed as effective microbial control agents which can act as successful alternatives to chemical compounds [24].

In a recent study by Suryadi *et al*. [25] four toxic isolates of *B. sphaericus* were isolated from the beach area of Lombok Island, Indonesia. They showed mild toxicity against larvae of three mosquito species such as *Cx. quinquefasciatus*, *An. aconitus* and *Ae. aegypti* with *Culex sp.* being the most susceptible to all the isolates. In another study, Poopathi *et al*. reported the isolation of *Bacillus cereus* strain VCRC-B520, a novel mosquitocidal bacterium from marine soil collected from coastal areas at Pondicherry in Eastern India. The LC_{50} and LC_{90} values for *Cx. quinquefasciatus* were 0.30 and 2.21 mg/L, respectively [26]. Similarly, in this study soil samples from a coastal region of Goa (i.e. Miramar beach) yielded valuable mosquito pathogenic bacilli isolates.

Nabar *et al*. [27] had earlier reported that the extreme natural environments have been consistently generating microbial species which contribute to the control of diseases and their transmission. It has been previously reported by Manonmani *et al*. that *Bacillus* spores are known to settle rapidly in watery areas [28]. In the present study, that of the 3 different sampling zones, mosquito pathogenic isolates were obtained only from tidal and intertidal zones. However, no mosquito pathogenic bacteria could be detected in the soil sample collected from the sea-shore zone as pointed by Manonmani *et al*. [29].

It is noteworthy that the protocol devised by Dhindsa *et al*. [10] for the screening of soil samples for the presence of mosquito pathogenic bacilli prior proved successful in the present study. This method involves the use of LB broth buffered with Sodium acetate and a heat shock step at 65°C. Using this technique for screening, in the present study, three out of nine soil samples were found to contain microbial isolates pathogenic to *An. stephensi*, *Cx. quinquefasciatus*, *Ae. aegypti* and *Ae. albopictus* larvae. Further their identity was confirmed at molecular level following standard method [30].

However, in the recent years, one major challenge for achieving successful mosquito control is the overcoming of insecticide resistance to the commonly used microbial insecticides such as *B. thuringiensis israelensis* and *B. sphaericus* which is a serious threat to their success as bio control agents. Available evidence indicates that *Bti* has a lower risk for resistance development in the target vector species due to the complex makeup of its parasporal crystal, which contains Cyt1A, Cry4A, Cry4B, and Cry11A toxic proteins. Disrupting the toxin complex enables resistance to

evolve, especially in the absence of the cytolytic toxin, Cyt1A. *B. sphaericus* (Bs), on the other hand shows a higher risk for resistance development due to its single-site of action and therefore, operational failures have been reported from several locations worldwide [31].

In the present study, the isolate C1A was identified as *Aneurinibacillus* sp. having highest activity against *Culex quinquefasciatus* 3rd instar larvae (LC_{50} = 0.08ppm) among the isolates tested. This is the first report of *Aneurinibacillus* sp. having mosquito pathogenic activity that could be developed as a novel mosquito pathogenic bacterium with high toxicity to *Cx. quinquefasciatus* larvae. It may be mentioned that Shida *et al.* proposed the genus *Aneurinibacillus* as a novel genus arising from the reclassification of the *Bacillus aneurinilyticus* and the related species in the genus *Bacillus* [32]. Alenezi *et al.* [33] reported that the soil-borne gram-positive bacteria *Aneurinibacillus migulanus* strain *Nagano* shows considerable potential as a bio control agent against plant diseases. However, no prior reports exist of its mosquito larvicidal activity.

Earlier, Mohanty *et al.* had reported isolation of different strains of *Lysinibacillus* (formerly named as *B. sphaericus*) from Goa and grouped them based on their similarity [13]. In the present study two isolates, C2A and C2C were identified as *Lysinibacillus* sp. Though the *Lysinibacillus* genus has been well explored, yet its species have not been commercially formulated as microbial bio control agents and hence gain importance in that context. Following their field testing, a suitable formulation using these *Lysinibacillus* sp., could be developed for the control of *Cx. quinquefasciatus* as these isolates showed high toxicity (LC_{50} = 0.30ppm and LC_{50} = 0.25ppm respectively).

The isolate B1C identified as a strain of *B. thuringiensis* showed a broad spectrum of activity against the 3rd instar larvae of the 4 test vector species *An. stephensi, Cx. quinquefasciatus, Ae. aegypti* and *Ae. albopictus*. It has been reported by Ammouneh *et al.* [34] that screening the environment for new and highly potent strains of *B. thuringiensis* has become inevitable as one of the strategies for insect resistance management. In addition, many reports on the frequent occurrence of *B. thuringiensis* isolates in the natural environment indicate high possibility of isolating novel strains.

The mode of action of these larvicidal strains when studied during the present investigation revealed that the larval mortality occurred because of lysis of the midgut due to the production of insecticidal toxins. This mode of action is well known and has been previously reported by Bauer *et al.* who described the midgut as the primary site of δ-endotoxin activity in *B. thuringiensis israelensis* [35]. Similarly, Baumann *et al.* [36] described the association and binding of the activated BinA and BinB toxin of *B. sphaericus* to the receptor, which is a α-glucosidase on the midgut microvilli, resulting in the lysis of midgut cells after internalization.

Until recently, chemical larvicides were the main components of mosquito immature control strategy in most parts of the world and when compared with biolarvicides, the primary considerations are efficacy and cost. There are reports of extensive isolation of the *Bacillus* genus but these focused mainly on the control of agricultural pests and not so much for mosquito control [37]. Nevertheless, in the last few decades several formulations of biolarvicides for vector control have become commercially available. The major advantage in the use of these organisms is their safety to non-target organisms including humans [38]. However, their continued utilization in vector control programs including integrated vector management, would depend upon better screening methods, isolation of highly virulent indigenous strains showing broad spectrum of activity against mosquitoes, more effective formulations and mass production at an affordable cost [9]. This study for the first time reports the mosquitocidal activity of *Aneurinibacillus* sp. in the world and this bacterial agent can add to the existing armament of bacilli based larvicides.

5. Conclusion

Although a variety of mosquito pathogenic bacteria have been isolated from various geographic regions of the world, there is a pressing need to explore and deploy indigenous strains in vector control programs due to the restrictions imposed on the use of imported strains, the prohibitive cost of formulations and resistance development in target mosquito species. As no mosquito larvicidal strains of *Aneurinibacillus sp.* have yet been described, it is important to add this microbial organism to the list of new agents for bio control of mosquito vectors. Besides, the results from this study suggest that a search for new entomopathogenic bacteria should continue and intensify. Further work is needed in order to study the mode of action and nature of the toxin of the new bacterial isolates as well as validation of the results obtained in the laboratory by small scale field trials followed by phase II and Phase III field evaluation of the formulation based on the promising *Aneurinibacillus* sp. and *Lysinibacillus* sp. isolates discovered in this study for their possible deployment in the public health setting.

6. References

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