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Laboratory evaluation on the efficacy of *Bacillus thuringiensis* var. *israelensis* Berliner 1915 (Bacillaceae) on the instars of the dengue vector *Aedes aegypti* Linnaeus 1762 (Diptera: Culicidae) and against the adults of *Diplonychus indicus* Venk. & Rao (Hemiptera: Belostomatidae) as non-target organism

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Abstract
Mosquito control agents based on bacterial insecticides are the most widely used microbial pesticides as they are environment friendly alternatives to chemical insecticides and hold tremendous potential with improved efficacy. On the other hand, it is also necessary to preserve the non-target organisms, especially those that predate upon target mosquito larvae. Since, chemical larvicides are largely non-selective owing to their deleterious role on non-target organisms, bacterial insecticides when used do not cause harm to them. Therefore, in the present investigation, the toxicity of *Bacillus thuringiensis* var. *israelensis* was evaluated on all the larval instars of *Aedes aegypti* at concentrations of 0.1, 0.2, 0.3 and 0.4%. The LC_{50} values after 24, 48 and 72 hours against the first, second, third and fourth instars were 0.20, 0.19 and 0.17%; 0.16, 0.15 and 0.15%; 0.19, 0.18 and 0.16%; 0.14, 0.12 and 0.12% respectively. Further, the adults of *Diplonychus indicus* which were tested as the non-target organism was exposed to the same concentrations of bacterial formulation and the results indicated that they did not have toxic effect and that the treated adult bugs were normal with high predatory potential. The present research indicated that integration with appropriate biocides may not interfere with the biocontrol potential of the predator in controlling mosquitoes.

Keywords: *Bacillus thuringiensis* var. *israelensis*, *Aedes aegypti,* larvicidal, *Diplonychus indicus*

Introduction
The most important event in the 20th century in the history of vector control was the discovery of mosquitocidal strains of bacterial insecticides. Mosquito control agents based on bacterial insecticides are the most widely used group of microbial pesticides as they are environment friendly alternatives to chemical insecticides [1]. Considerable research has been conducted on bacterial insecticides over the last decades, and major successes have been obtained. The significant advantages of bacterial insecticides over chemical insecticides have been responsible for their fast introduction into large-scale routine operations for mosquito control [2]. *Bacillus* species are potent pathogens of mosquitoes [3-5] with increased toxicity against its larval stage [6], as they hold tremendous potential with improved efficacy since they produce parasporal crystal proteins (endotoxin) during sporulation which are considered to possess mosquitocidal activity against the larval populations of *Aedes*, *Anopheles* and *Culex* species [7-9]. On the other hand, it is also necessary to preserve the diversity of organisms, especially those competitors or predators upon target mosquito larvae; and bacterial insecticides when used do not cause harm to the non-target organisms. Therefore, the present investigation was carried out to evaluate the toxicity of *Bacillus thuringiensis* var. *israelensis* on the larval instars of the dengue vector, *Aedes aegypti*, and against the adults of *Diplonychus indicus* as the non-target organism.

2. Materials and Methods
2.1. Microbial collection and preparation
Bacillus thuringiensis var. *israelensis* was obtained from Inbiotics laboratory, Nagercoil, Kanyakumari district, Tamil Nadu, India. The required quantity of this microbe was thoroughly mixed with distilled water for bioassay.

2.2. Collection of *Diplonychus indicus*

Diplonychus indicus adults collected from a nearby pond at Nagercoil, Kanyakumari district, Tamil Nadu, India were brought to the laboratory, maintained in a glass aquaria and were fed with mosquito larvae. The eggs were allowed to hatch into the instars and the adults were reared.

2.3. Larvicidal bioassay

Larvicidal bioassay was carried out as per the guidelines of World Health Organization with minor modifications [10]. Eggs strips of F_1 generation of laboratory colonized *Aedes aegypti* mosquitoes obtained from Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, India were allowed to hatch. The study was conducted by introducing 25 first instar larvae into glass beakers (500mL) containing formulations of *Bacillus thuringiensis* var. *israelensis* (0.1, 0.2, 0.3 and 0.4%) each. The same procedure was performed for the second, third and fourth instars. Distilled water as control was maintained separately and run simultaneously. Mortality was observed 24, 48 and 72 hours after treatment. A total of six replicates per trial and a total of three trials for each concentration were carried out. Moribund larvae were scored dead when they showed no signs of movement when probed by a needle at their respiratory siphon.

2.4. Effect of *Bacillus thuringiensis israelensis* on non-target organism

Diplonychus indicus adults were selected as non-target organism and were acclimatized to the laboratory condition, where they were kept in an environment similar to their natural habitat. Five adults were exposed to the same concentrations of the bacterial formulation kept in a glass trough (1L). Distilled water without the bacterial formulation served as control was maintained separately and run parallel. Numbers of dead adults were recorded after 24, 48 and 72 hours of exposures and percentage mortality was calculated. A total of six replicates per trial and a total of three trials for each concentration were carried out.

2.5. Statistical analysis

The per cent larval mortality was calculated and corrections for control mortality (5-20%) if necessary was done using Abbott's formula [11]. Statistical analysis of all mortality data were subjected to probit analysis. Chi-square and regression analysis tests were used to determine if the mortality in treated bioassays significantly differed from that of the controls and at which doses in particular and the differences were considered as significant at P=0.05 level. All statistics were conducted in IBM SPSS Statistics v22 with significance set at 95% confidence [12].

3. Results

No larval mortality was observed in control. The mean and percentage mortality of *Aedes aegypti* larval instars treated with the *Bacillus thuringiensis* var. *israelensis* after 24, 48 and 72 hours are presented in Figure 1 and Table 1. One hundred per cent larval mortality was achieved at the highest concentration at 72 hours against the first, second and third instars and at 24 hours against the fourth instar. The LC_{50} values after 24, 48 and 72 hours against the first, second, third and fourth instars were 0.20, 0.19 and 0.17%; 0.16, 0.15 and 0.15%; 0.19, 0.18 and 0.16%; 0.14, 0.12 and 0.12% respectively. Chi-square analysis revealed the data to be significant at P=0.05 level. Regression analysis revealed that mortality rates were positively correlated with the periods of exposure and R^2 values neared the value of 1. The same concentrations of *Bacillus thuringiensis* var. *israelensis* formulation when tested against the non-target organism did not have toxic effect even after 72 hours. The treated adults of *Diplonychus indicus* were normal and the predatory potential was high.

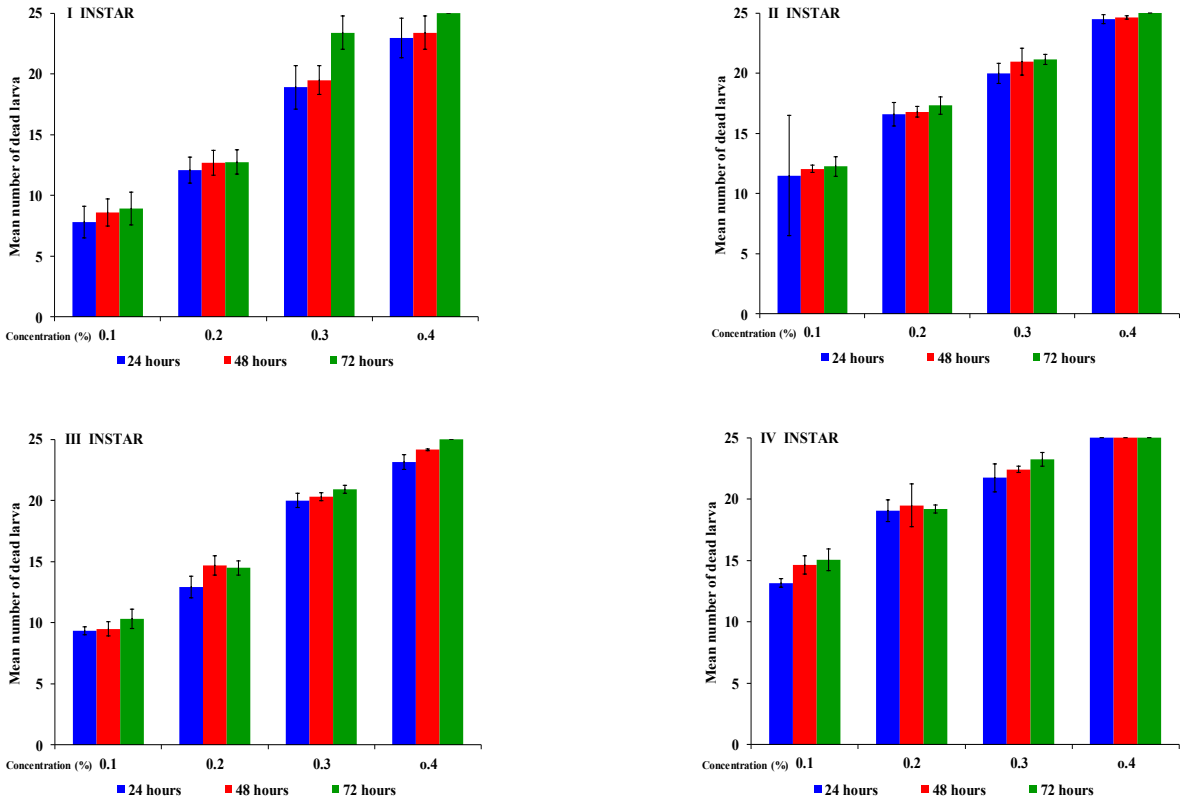

Fig 1: Mean mortality of *Aedes aegypti* larval instars on exposure to *Bacillus thuringiensis israelensis*

Table 1. Statistical inferences on the mortality of *Aedes aegypti* larval instars on exposure to *Bacillus thuringiensis israelensis*

Hours	Concentration (%)				LC_{50} (%)	95% Confidence Limit		LC_{90} (%)	95% Confidence Limit		Slope ±SE	Intercept ±SE	χ^2
	0.1	0.2	0.3	0.4		LL	UL		LL	UL			
I instar													
24	31.22 ±1.32	48.33 ±1.08	75.65 ±1.78	91.92 ±1.64	0.20	0.16	0.24	0.37	0.32	0.45	7.63 ±1.15	-1.55 ±0.27	3.34*
48	34.32 ±1.12	50.80 ±1.02	78.00 ±1.18	93.64 ±1.38	0.19	0.15	0.22	0.36	0.31	0.43	7.62 ±1.14	-1.46 ±0.26	4.10*
72	35.66 ±1.33	51.00 ±1.00	93.64 ±1.38	100.00 ±0.00	0.17	0.14	0.19	0.29	0.25	0.35	10.52 ±1.59	-1.78 ±0.31	4.66*
II instar													
24	46.00 ±5.00	66.32 ±0.98	80.00 ±0.82	98.00 ±0.38	0.16	0.03	0.25	0.32	0.23	0.66	7.95 ±1.20	-1.30 ±0.25	6.08*
48	48.23 ±0.32	67.21 ±0.45	83.83 ±1.12	98.64 ±1.56	0.15	0.02	0.25	0.31	0.22	0.67	8.09 ±1.22	-1.25 ±0.25	6.38*
72	49.00 ±0.83	69.32 ±0.72	84.62 ±0.40	100.00 ±0.00	0.15	0.01	0.25	0.29	0.20	0.69	9.06 ±1.37	-1.37 ±0.27	7.11*
III instar													
24	37.33 ±0.33	51.66 ±0.88	80.00 ±0.58	92.66 ±0.60	0.19	0.15	0.22	0.36	0.31	0.43	7.62 ±1.14	-1.46 ±0.26	4.10*
48	38.00 ±0.57	58.72 ±0.80	81.30 ±0.32	96.64 ±0.88	0.18	0.14	0.21	0.33	0.29	0.40	8.29 ±1.23	-1.49 ±0.27	3.74*
72	41.33 ±0.80	58.00 ±0.58	83.64 ±0.32	100.00 ±0.00	0.16	0.13	0.19	0.30	0.26	0.36	9.33 ±1.39	-1.55 ±0.28	5.04*
IV instar													
24	52.66 ±0.36	76.33 ±0.88	87.00 ±1.15	100.00 ±0.00	0.14	-0.08	0.26	0.28	0.18	0.99	9.10 ±1.39	-1.27 ±0.26	9.06*
48	58.60 ±0.76	78.00 ±1.76	89.66 ±0.26	100.00 ±0.00	0.12	-0.20	0.27	0.26	0.17	1.36	9.36 ±1.46	-1.20 ±0.26	9.90*
72	60.32 ±0.88	76.82 ±0.32	93.00 ±0.56	100.00 ±0.00	0.12	-0.07	0.23	0.25	0.16	0.83	10.15 ±1.59	-1.26 ±0.27	8.33*

4. Discussion

Bacillus thuringiensis var. *israelensis* are ideal biological control agents against mosquitoes and blackflies due to their efficacy and relative specificity [13, 14]. The efficacy of *Bacillus thuringiensis* var. *israelensis* formulations has been demonstrated in a variety of habitats against a multitude of mosquito species. Since the discovery of *Bacillus thuringiensis* serovariety *israelensis* [15, 16], formulations of these bacteria have become the predominant non-chemical means employed for control of mosquito larvae. The toxins of *Bacillus thuringiensis* var. *israelensis* and their mode of action, efficacy and factors that affect larvicidal activity, development of resistance, safety, and their roles in integrated mosquito control has opened a new way in mosquito control [17-20]. In the present study as the concentration of *Bacillus thuringiensis* var. *israelensis* increased the mortality rate of *Aedes aegypti* also increased. *Bacillus thuringiensis* var. *israelensis* is toxic to mosquitoes and their toxicity is commonly imputed to the parasporal endotoxins which are produced during sporulation time. These endotoxins are assimilated by the larvae to accomplish toxicity. *Bacillus thuringiensis* var. *israelensis* also produce different insecticidal crystal proteins (endotoxins), and their toxicity has been determined [21, 22]. These toxins, when ingested by the larvae, damage the gut tissues, leading to gut paralysis, thereafter the infected larvae stop feeding and finally die due to the combined effects of starvation and impairment of midgut epithelium [23, 24]. Nevertheless, biotic and abiotic factors influence the larvicidal activity of *Bacillus thuringiensis* var. *israelensis* based on mosquito species, their respective feeding strategies, rate of ingestion, age and density of larvae, habitat factors (temperature, depth of water, turbidity), formulation factors (type of formulation, toxin content, how effectively the material reaches the target, and settling rate), storage conditions, means of application and frequency of treatments [25]. Amalraj *et al*. [26] reported that when it was applied for operational field applications the dipteran species were affected, but had no effect on Cladocerans. In the present study too, they caused toxicity to *Aedes aegypti* larvae and were not toxic to the instars and adults of *Diplonychus indicus*. Russel *et al*. [27] reported that water dispensable *Bacillus thuringiensis* var. *israelensis* formulation were toxic to the third instars of *Aedes aegypti*, *Culex sittens, Culex annulristris* and *Culex quinquefasciatus* but had no effect on the adults of *Diplonychus indicus* and the same trend was observed in the present study also. Nonetheless, Boisvert and Boisvert [28] reviewed the effect of *Bacillus thuringiensis israelensis* on target and non-target organisms and stated that, general predictions about the effect of *Bacillus thuringiensis israelensis* on non-target organisms may be difficult to make due to differences in (i) species tested, (ii) laboratory conditions and field methodology, and (iii) different formulations used.

5. Conclusion

The present research indicated that *Diplonychus indicus*, a good biocontrol agent especially on mosquito larva is not affected by biocides like *Bacillus thuringiensis* var. *israelensis*. This large safety margin of this bacterial insecticide for non-target organisms indicates their suitability for mosquito control programmes in areas where protection of the natural ecosystem is important. Therefore, its integration with appropriate biocides may not interfere with the biocontrol potential of the predator in controlling mosquitoes.

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