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Current Trends in the Control of Mosquito Vectors by Means of Biological Larvicides

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Abstract

Bacillus sphaericus Neide (B) and *B. thuringiensis* serovar *israelensis (Bti)* de Barjac provides effective alternatives to broad spectrum larvicides in many situations, with little or no environmental impact. Taking into account environmental benefits including safety for humans and other non-target organisms, reduction of pesticide residues in the aquatic environment, increased activity of most other natural enemies and increased biodiversity in aquatic ecosystems, their advantages are numerous. In addition to recombinant bacteria used as larvicides, research is also underway to develop transgenic algae and *cyanobacteria* using larvicidal endotoxins of *Bti* and *Bs*. The advent of recombinant DNA technology is now having an enormous impact on agriculture and medicine, and it is appropriate that the ability to manipulate and recombine genes with this technology be applied to improving larvicides for vector control. These new recombinant bacteria are as potent as many synthetic chemical insecticides, yet are much less prone to resistance, as they typically contain a mixture of endotoxins with different modes of action. The existing recombinants also have what can be considered disadvantageous, in that they do not show significantly improved activity against aedine and anopheline mosquitoes, in comparison to *Bti*. But it may be possible to overcome this limitation using some of the newly discovered mosquitocidal proteins, such as the Mtx proteins and peptides such as the trypsin-modulating oostatic factor, which could be easily engineered for high expression in recombinant bacteria. While other microbial technologies such as recombinant algae and other bacteria are being evaluated, it has yet to be shown that these are as efficacious and environment friendly as *Bti* and *Bs*. By combining the genes from a variety of organisms, it should ultimately be possible to design 'smart' bacteria that will seek out and kill larvae of specific vector mosquitoes. Thus, recombinant bacteria show excellent promise for development and use in operational vector control programs, hopefully within the next few years.

Keywords: *Bacillus sphaericus*; *B. thuringiensis* serovar *israelensis*; Endotoxins; Bio-pesticides; Mosquito control; DNA technology; Resistance; Histopathology; Mode of action; Binding kinetics; Transgenic mosquitoes; Cost-effective technology.

Introduction

Mosquitoes are a source of great nuisance to human beings and pose a threat to public health, as vectors of diseases like malaria, filariasis, dengue, Japanese encephalitis, West Nile fever [1,2]. Annually about 500 million people are estimated to be infected by malaria, a major killer disease, which threatens 2,400 million (about 40%) of the world's population [3,4]. Correspondingly, lymphatic filariasis transmitted by *Wuchereria bancrofti* affects about 100 million people worldwide, and the closely related *Brugia malayi* and *B. timori* affect 15 million people in South East Asia. About 25 million people are infected every year by dengue viruses transmitted by *Aedes* mosquitoes, with about 25,000 deaths. The occurrence of mosquito-borne diseases is increasing due to uncontrolled urbanization, creating mosquitogenic conditions for the vector mosquito populations. Therefore, mosquito control forms an essential component for the control of mosquito borne diseases. Dengue and malaria are effectively managed through a combination of vector control, drugs and management of clinical illness. There are numerous cases of insecticide resistance reported for *Anopheles* species. The emergence of mosquito species resistant to insecticides, widely used in malaria and dengue control, has the potential to impact severely on the control of these disease vectors. A limited number of resistance mechanisms, including modification of the insecticides' target site, or changes in rates of metabolism involving esterases, glutathione S-transferases or monooxygenases, operate in all insects. The potential for resistance to develop in vectors has been apparent since the 1950's, but the scale of the problem has been poorly documented [5,6]. Vector control is recognized as an effective tool for controlling tropical diseases. Several strategies have been adopted to control these dipteran pests, and to reduce vector-borne diseases. Synthetic insecticides have been effectively used during the past several decades for mosquito-control operations. But the chemical approach has several demerits, such as the development of insecticide resistance, environmental pollution, bio-amplification of contamination of food chain, and harmful effects to beneficial insects. Hence, there has been an increased interest in recent years, in the use of biological agents for mosquito control.

Bio-Pesticides as Effective Tools for Mosquito Control

In recent years, it has been witnessed, an increased interest in the usage of bio-pesticides as effective tool for mosquito control. Several bio-control agents were screened for their potency, mammalian safety and environmental impact. Many organisms have been investigated as potential agents for vector mosquito control, including bacteria,

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viruses, fungi, nematodes, protozoa, fish and invertebrate predators. However, most of these agents were shown to be of little operational use, largely because of the difficulty in multiplying them in large quantities. Only a few spore forming bacteria, copepods and fish have reached operational use and are undergoing extensive field trials. The discovery of a bacteria like *Bacillus sphaericus* Neide (*Bs*) and *B. thuringiensis* serovar *israelensis* deBarjac *(Bti)*, which are highly toxic to dipteran larvae have opened up the possibility of its use as potential biolarvicides in mosquito eradication programs, the world over [7-9]. These bacteria have some important advantages over conventional insecticides in mosquito control operations, besides being safe to non-target organisms including human beings. Also, it is innocuous to the environment. Besides these bacteria, several other types of bacteria such as *B.t. jegathesan*. subspecies *p. medellin*, B.t. subspecies *P. malaysiensis*, B.t. subspecies *P. canadensis, asticcacaulis excentricus, Clostridium bifermentans* subspecies *P. malaysia* and *synechococcus*, are being examined as effective biological control agents. The *Bti* has been used operationally for the control of mosquitoes for over two decades, and its formulations are highly effective against *Anopheles*, *Aedes*, and *Culex* mosquitoes [10]. No evidence has been found that *Bs* and *Bti* toxins harm aquatic organisms sharing the breeding sites of these vectors, or have an adverse effect on the environment. Although, *Bti* is effective, specific, bio- degradable and possesses a long shelf life, it does not recycle in the environment at levels high enough to provide significant residual activity. It has a limited time of mode of action, usually 24 to 48 hours and must, therefore, be applied at frequent intervals. Moreover, current spore forming *Bti* formulations sink in water and are consequently less efficient in controlling species of mosquito larvae that feed only near the water surface [11-14]. The rate of killing with spores is slow compared with the chemical insecticides, and the toxins have a narrow mosquito host range than the chemicals. *Bacillus sphaericus*, on the other hand, has been shown to recycle in the field conditions and exert larvicidal activity for a long period. However, the spores of *Bti* have the advantage over *Bs*, that *Bti* has a wider spectrum of activities against *Anopheles*, *Culex* and *Aedes* spp, while *Bs* has its effect mainly on *Culex*, for a lesser extent to *Anopheles*, and it is strongly species specific and act against only a few *Aedes* species. Field resistance has been only reported for *Bs*, which was purified. The 42 kDa protein inclusions were found to be toxic to *Culex* larvae, in contrast to the 51 kDa protein inclusions which were not toxic on their own, but a synergistic effect between these two components was observed [15]. Studies conducted with recombinant bacteria expressing these polypeptides individually, have revealed that Bin A could be toxic at high dosage in the absence of Bin B, but this was not in the case for the Bin B alone. However, presence of both Bin B and Bin A in equimolar amounts showed highest toxicity in larvae, since they seem to act in synergy. In addition to the binary toxin, another mosquitocidal protein with molecular weight of 100 kDa, appears to be synthesized in low-toxicity strains, as well as in some of the highly toxic strains and this polypeptide is expressed during the vegetative phase, and is not homologous with the 51 kDa and 42 kDa proteins [16]. The efficient expression of this 100 kDa mosquitocidal toxin in protease deficient recombinant *Bs* was thoroughly studied, and it was concluded that protease negative *Bs* strains expressing Mtx and other toxins may form the basis of an alternative to the natural highly toxic strains for mosquito control. The location of the binary toxin (btx) and mosquitocidal toxin (mtx) genes in *Bs* strains were determined by hybridization of specific gene probes to chromosomal DNA, in Southern blots. The identification and introduction into *Bs* of the *Bt* subspecies *P. medellin* Cyt1 Abt gene, results in higher susceptibility of which are otherwise resistant mosquito larval populations to *Bs*. Apart from *Bs* and *Bti*, the cloning and expression of other mosquitocidal strains such as *Bt* subspecies *P. medellin*, *Bt* subspecies *P. jegathesan* and *Clostridium bifermentans*, have been reported [17]. The binary toxin of *Bs* strains is generally very toxic to *Anopheles* and *Culex* species, but poorly or non-toxic to most *Aedes* species. However, susceptibility appears to depend on the stability of bacterial strains, appropriate methodology, etc. Since these bacteria are safe for animals, environment and cause no health risk to humans, several formulations in the form of wettable powder (WP), water dispersable concentrate (WDC), emulsifiable concentrate (EC), flowable concentrate (FC), granules (G) and dust (D) have been produced to control many species of mosquitoes. These products have been tested extensively in USA, France, Brazil, Zaire, India and in Bangladesh.

Mechanism of Action by *B. sphaericus* against Mosquito Larvae

Crystal toxins from *Bs* are ingested by mosquito larvae, and after solubilization and proteolytic cleavage, the activated toxin interacts with the midgut epithelium, leading to death of larvae. In mosquito larvae, the sequence of events follow in the manner given below, (i) ingestion of spore/crystal toxin (ii) toxin solubilization in the midgut (iii) activation of the protoxin by protease, into active toxin i.e. 42 and 52 kDa of *Bs* to 39 and 43 kDa proteins (iv) binding of active toxin to specific receptors present in the midgut brush border membrane, and (v) putative internalization of toxin and cell lysis. However, the eventual intracellular action of binary toxin in the cells is not completely clarified, except for a few reports on cytopathological effects caused by the action of the toxin [18-20]. In *C. pipiens* larvae, it was shown that Bin B was mainly responsible for the binding to the receptor, while Bin A had very low affinity for the receptor [21]. Recently, the receptor was identified as a 60 kDa protein, attached to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor Micro sequencing, indicated that this molecule had a string homology with insect maltases, and enzymatic activity suggested that it could be a alpha glucosidase [22]. In the course of sporulation, *Bacillus sphaericus* produces an inclusion body which is toxic to a variety of mosquito larvae. The larvicide of *B. sphaericus* is unique, in that it consists of two proteins of 51 and 42 kDa, both of which are required for toxicity to mosquito larvae. There is a low level of sequence similarity between these two proteins, which differ in their sequences from all the other known insecticidal proteins of *Bacillus thuringiensis*. Within the midgut, the 51and 42 kDa proteins are processed to proteins of 43 and 39 kDa, respectively. The conversion of the 42 kDa protein to a 39 kDa protein, results in a major increase in toxicity; the significance of the processing of the 51 kDa protein is not known. In contrast to the results with mosquito larvae, the 39 kDa protein is alone toxic for mosquito-derived tissue culture grown cells, and this toxicity is not affected by the 51 kDa protein or its derivative, the 43 kDa protein. Comparisons of larvae from species, which differ in their susceptibility to the *B. sphaericus* toxin, indicate that the probable difference resides in the nature of the target sites of the epithelial midgut cells, and not in uptake or processing of the toxin [23].

Direct and Homologous Binding Assays of Bacterial Toxins

It was reported from binding kinetics (direct binding and homologous competition assays) of *Bs* binary toxin to the midgut brush border membrane fractions (BBMFs) of larvae, that the radio-labelled toxin is bound specifically to a single class of receptors. Toxin dissociation was fast and almost complete in BBMF of all species

Generations	Mosquito strains	Intercept	Slope ± SE	LC_{50} (g/litre)	LC_{95} (g/litre)	χ^2 (df)	RR (at LC_{50})[c]	RR (at LC_{95})[c]
F_{49}	**Parents** MS[a] 8.32		1.48 ± 0.18	0.006 (0.007-0.005)	0.075 (0.107-0.052)	6.72(6)		
	GR[b]	5.52	1.31 ± 0.17	0.401 (0.48-0.33)	7.22 (11.12-4.69)	2.27(6)	66.83 (96.0-47.14)	96.30 (213.8-43.8)
	Reciprocal crosses MS x GR	6.35	1.24 ± 0.16	0.082 (0.098-0.07)	1.72 (2.69-1.10)	3.40(6)	13.67 (19.6-10.0)	22.93 (51.73-10.3)
	MS x GR	6.37	1.33 ± 0.17	0.094 (0.11-0.078)	1.59 2.43-1.05)	6.22(6)	15.67 (22.0-11.14)	21.2 (46.73-9.81)
F_{50}	**Parents** MS[a] 8.32	8.20	1.39 ± 0.17	0.005 (0.006-0.004)	0.076 (0.11-0.05)	4.56(6)		
	GR[b]	5.33	1.41 ± 0.22	0.58 (0.81-0.42)	8.64 (19.17-3.89)	13.65(6)	116.0 (202.5-70.0)	113.7 (383.4-35.4)
	Reciprocal crosses MS x GR	6.29	1.27 ± 0.17	0.098 (0.12-0.08)	1.92 (3.00-1.22)	6.06(6)	19.6 (30.0-13.3)	25.26 (60.0-11.1)
	MS x GR	6.38	1.36 ± 0.17	0.097 (0.116-0.08)	1.58 (2.37-1.05)	3.78(6)	19.4 (29.0-13.3)	20.79 (47.4-9.55)
F_{51}	**Parents** MS[a] 8.32	8.34	1.44 ± 0.18	0.0049 (0.006-0.004)	0.067 (0.096-0.047)	3.79(6)		
	GR[b]	5.63	1.32 ± 0.17	0.33 (0.39-0.28)	5.83 (8.82-3.86)	3.90(6)	67.3 (97.5-46.7)	87.0 (187.7-40.2)
	Reciprocal crosses MSxGR	6.31	1.27 ± 0.16	0.094 (0.112-0.078)	1.84 (2.86-1.18)	3.87(6)	19.18 (28.0-13.0)	27.46 (60.85-12.29)
	MSxGR	6.41	1.39 ± 0.17	0.096 (0.114-0.081)	1.48 (2.21-0.98)	7.77(6)	19.59 (28.5-13.5)	22.1 (47.0-10.21)
F_{52}	**Parents** MS[a] 8.32	8.26	1.54 ± 0.18	0.007 (0.0091-0.0066)	0.09 (0.128-0.06)	4.43(6)		
	GR[b]	5.55	1.34 ± 0.17	0.39 (0.46-0.32)	6.50 (9.87-4.28)	3.18(6)	55.7 (69.7-35.2)	72.2 (164.5-33.4)
	Reciprocal crosses MSxGR	6.28	1.28 ± 0.17	0.099 (0.119-0.082)	1.91 (2.99-1.22)	4.26(6)	14.14 (18.0-9.01)	21.2 (49.83-9.53)
	MSxGR	6.36	1.39 ± 0.17	0.105 (0.124-0.088)	1.61 (2.40-1.079)	1.63(6)	15.0 (18.79-9.67)	17.89 (40.0-8.43)

Table 1: Dose/mortality results for the progeny of *Bacillus sphaericus* (*Bs*) susceptible, resistant and reciprocal crosses of *Culex quinquefasciatus* in bioassays with *Bs* toxin.
Strains of *Culex quinquefasciatus* : [a]*Bs* susceptible and [b]*Bs* resistant strains; [c] resistance ratio at LC_{50} and LC_{95} levels calculated by substracting the values from resistant and susceptible strains.

studied. Studies showed that resistance is correlated with a reduction or absence of affinity of the toxin, for the membrane receptor. The resistant strain lost the functional receptor for the *Bs* toxin [24].The resistance is encoded by a recessive gene-linked to the sex locus on chromosome, and it is not associated with any loss of binding affinity between BBMF and *Bs* radiolabelled toxin. Binding affinity of the *Bs* binary toxin to a specific receptor on the midgut brush border membrane, from geographically different mosquito species of *Cx. Quinquefasciatus* (Indian strain) of resistant, susceptible, F1 progeny and back-crosses to susceptible and resistant strains, have been studied recently [7]. Toxicity assays in the larvae of these strains confirmed that the resistance was inherited by partially recessive gene. The similarities in susceptibilities of *Bs* susceptible and the progeny from back-crosses strain with F5 may be expected, which may reflect lack of any susceptibility variations between these two strains, whereas, the susceptibility of F1 offspring was higher than that of susceptible parent but lower than that of resistant parent, indicating that resistance was being controlled by partially recessive gene. SDS-PAGE studies confirmed the presence of a new polypeptide (MW:80 kDa) in *Bs* resistant strains. Nielsen-LeRoux et al. [24] have found that the *Bs* resistance was due to a single recessive gene in mosquitoes.

However, others have reported a partially recessive inheritance of resistance gene to *Bt* Cry IC and phosphine, along with *Bt* toxins in *Spodoptera littoralis, Tribolium castaneum* and *Ostrinia nubilalis* [25, 26]. Results of Poopathi et al. [20] also complied with the above studies (Table 1). Validation tests for four consecutive generations of *Cx. Quinquefasciatus* (F49 to F52), regarding toxicity of *B. sphaericus* against susceptible (MS) and resistant (GR) larvae; their F1 progeny derived from reciprocal parental crosses (MSxGR; MSxGR) also concurred the report of partially recessive inheritance of resistance (Table 2). The LC50 and LC95 in *Bs* susceptible parental strain (MS) was very low, whereas high for *Bs* resistant parental strain (GR). SDS-PAGE profile of the GR strain showed an additional protein band (M.wt, 80 kDa) that possibly linked to resistance development. A similar protein band was also visualized in back-cross offsprings from resistant parents (F3xGR), although lacked in back- cross offsprings developed from susceptible parent (F3xMS). The studies indicated that the levels of resistance were found to be high in *C. quinquefasciatus* larvae, maintained by selection

Mosquito strains	Intercept	Σλοπε ± ΣE	LC_{50} (g/litre)	LC_{95} (g/litre)	χ^2 (df)	RR (at LC_{50})[c]	RR (at LC_{95})[c]
Inbreeding (F_2)[a] F_1xF_1	6.30	1.15 ± 0.15	0.075 (0.091-0.06)	2.015 (3.29-1.23)	5.73(6)	15.0 (22.75-10.0)	26.5 (65.8-11.18)
Reciprocal and back-crosses F_1xGR	5.77	1.49 ± 0.18	0.305 (0.36-0.26)	3.88 (5.56-2.70)	7.71(6)	61.0 (90.0-43.3)	51.1 (111.2-24.55)
F_1xGR	5.77	1.59 ± 0.19	0.33 (0.38-0.28)	3.55 (4.93-2.56)	1.84(6)	66.0 (95.0-43.3)	46.7 (98.6-23.27
F_1xMS	6.83	1.34 ± 0.17	0.04 (0.05-0.036)	0.73 (1.09-0.48)	5.70(6)	8.0 (12.5-6.0)	9.6 (21.8-4.36)
F_1xMS	6.85	1.34 ± 0.17	0.042 (0.05-0.035)	0.71 (1.06-0.47)	4.88(6)	8.4 (12.25-5.83)	9.34 (21.2-4.27)
Inbreeding (F_3) F_2xF_2	6.59	1.05 ± 0.15	0.029 (0.037-0.024)	1.12 (1.94-0.65)	12.32(6)	5.92 (9.25-4.0)	16.7 (41.28-6.77)
Reciprocal and back-crosses F_2xGR	5.56	1.42 ± 0.18	0.401 (0.47-0.34)	5.81 (8.58-3.93)	2.58(6)	81.8 (117.5-56.7)	86.72 (182.55-40.94)
F_2xGR	5.54	1.43 ± 0.18	0.42 (0.49-0.36)	5.90 (8.67-4.02)	3.54(6)	85.7 (122.5-60.0)	88.1 (184.5-41.9)
F_2xMS	6.76	0.96 ± 0.16	0.015 (0.025-0.009)	0.77 (2.64-0.22)	24.52(7)	3.06 (6.25-1.50)	11.49 (56.17-2.29)
F_2xMS	6.63	0.95 ± 0.15	0.019 (0.028-0.013)	1.04 (2.70-0.39)	14.14(7)	3.88 (7.0-2.17)	15.5 (57.4-4.06)
Inbreeding (F_4) F_3xF_3	6.62	1.08 ± 0.15	0.03 (0.04-0.026)	1.06 (1.80-0.63)	11.31(6)	3.90 (5.91-2.86)	11.78 (30.0-4.92)
Reciprocal and back-crosses F_2xGR	5.62	1.52 ± 0.19	0.39 (0.46-0.33)	4.69 (6.73-3.28)	5.05(6)	5.06 (69.7-36.3)	52.11 (112.2-25.6)
F_2xGR	5.57	1.60 ± 0.19	0.44 (0.51-0.38)	4.67 (6.56-3.32)	3.65(6)	5.71 (77.3-41.76)	51.9 (109.3-25.9)
F_2xMS	6.76	1.00 ± 0.16	0.018 (0.027-0.011)	0.77 (2.24-0.27)	19.66(7)	0.23 (4.09-1.21)	8.56 (37.3-2.11) 2.29)
F_2xMS	6.69	1.01 ± 0.13	0.021 (0.026-0.017)	0.89 (1.50-0.53)	10.22(7)	0.27 (3.94-1.87)	9.89 (25.0-4.14)

Table 2: Analysis of the *Bacillus sphaericus* resistance of various offsprings (inbreeds, reciprocals and back-crosses) from *Culex quinquefasciatus* larvae.
A Progeny of three successive inbreeds, reciprocals and back-crosses developed from *Bacillus sphaericus* susceptible and resistant mosquito strains.

pressure with *Bs* toxin. Table 3 presents *in-vitro* binding competition experiments by using 125_I labeled *Bs* binary toxin with brush border membrane fractions (BBMFs) from *C. quinquefasciatus* larval midgut. In *Bs* susceptible (MS) strain, clear specific binding of radiolabeled toxin of *Bs* to receptors of BBMF was found. The binding capacity was 1.74 p mole/mg BBMF protein at 150nM concentration level, whereas in *Bs* resistant strain, it was significantly low due to limited specific binding of radiolabelled toxin to receptors.

Histopathological Effects by Bacterial Toxins

Transmission electron microscopic (TEM) studies showed that the midgut epithelial cells of *Bs* susceptible and resistant strains of *C. quinquefasciatus*, had well defined microvilli in a parallel line on the outer boundary. Each microvillus contained a microfibrillar core, and it extended below the plasma membrane to form a terminal web. It has been reported that *Bs* and *Bti* treatments bring about some changes in the midgut structure of the mosquitoes [27-29]. Before *Bs* treatment, the nuclei of midgut epithelial cells were packed with nucleolar granules, inside the nucleoplasm. The nucleolemma was well defined on the outer boundary. The mitochondria, rough endoplasmic reticulum, lysosome and golgi body were also visible in the cytoplasm. The binary toxin from *Bs* and the multiple toxins from *Bti* after being a *Bs* orbed into the gut, cells exert their effects on the midgut epithelium by causing disruption, separation and ploughing of columnar epithelial cells into the gut lumen. It has been argued that disruption and swelling of the midgut causes the death of the insect, following *Bs* or *Bti* poisoning. *Bacillus sphaericus* toxin is a slow acting larvicide that does not paralyze mosquito larvae, until 24 to 48 hours treatment. However pathological lesions in the midgut of toxin treated larvae are also observed, as early as 7 to 10 hours after treatment. This causes a delayed paralysis, and death of *Bs* exposed larvae was a certainty [29]. *Bacillus thuringiensis* subsp. *israelensis* toxin destroys the structure of the cells in the midgut epithelium, whereas *Bs* toxin does not and takes a longer time to disintegrate [18,19]. The difference in the toxin effect is probably due to variation in the size of active toxins from the two bacteria. Ultrastructural variations were also found to be similar in both *Bs* resistant and susceptible larval strains [20].

Mechanism of Resistance to Bacterial Toxins

The entomopathogenic bacteria *Bacillus thuringiensis* subspecies *israelensis* and *Bacillus sphaericus* have gained importance due to the rising trend in the development of resistance of mosquitoes to chemical pesticides, as well as due to their deleterious effects to man and the environment worldwide. *B. sphaericus* is advantageous to *B. thuringiensis* subspecies *israelensis* due to the increased duration of larvicidal activity against certain mosquito species, especially in organically enriched larval habitats. There is also evidence of spore recycling in dead mosquito larvae, in certain environments. *Bacillus*

Mosquito strains	Specific binding (pmole toxin/mg BBMF protein)		
	8 nM	50 nM	150 nM
Madurai (MS)	1.14 (1.19–0.022)[a]	1.48 (1.58–1.39)[a]	1.74 (1.84–1.65)[a]
Gandhinagar (GR)	0.065 (0.13–0.004)	0.48 (0.65–0.31)	0.67 (1.10–0.24)
MSxGR	0.06 (0.08–0.038)	0.39 (0.58–0.21)	0.37 (0.62–0.11)
F_3xGR	0.116 (0.13–0.096)	0.25 (0.27–0.24)	0.097 (0.21–0.02)
F_3xMS	0.95 (1.20–0.82)	1.18 (1.33–0.91)	1.44 (1.63–1.32)

Table 3: Direct-binding assay of ^{125}I labeled *Bacillus sphaericus* (*Bs*) binary toxin to *Culex quinquefasciatus* larval BBMF from *Bs*-susceptible, resistant and their back-crosses. A 95% fiducial limits of upper and lower at different concentrations.

sphaericus (Bs) has been recognized as an effective mosquito larvicide, since its discovery 20 years ago. Various strains of this agent such as 2362, 2297, 1593 and C3-41 have been developed, formulated, and field-evaluated against mosquito larvae in different countries. Their high efficacy in controlling mosquitoes breeding in various habitats, especially those in polluted water has been documented. *B. sphaericus*, therefore, has been considered a promising agent for mosquito control, especially for *Culex* spp. in urban environments. However, recent reports have shown that microbial larvicides based on *B. sphaericus*, leads to resistance in mosquitoes in some areas of the world. This is mainly because under continuous selection pressure, mosquito populations develop resistance to *B. sphaericus* binary toxin (Bin), both in the laboratory and in the field. It has been demonstrated that *Culex quinquefasciatus* can develop from 35-150,000 and from 10-10,000-fold resistance to *B. sphaericus* in the laboratory and in the field, respectively [30]. Laboratory studies have shown that the resistance developed to certain strains of *B. sphaericus*, confers more or less cross-resistance to other strains of the same species of toxin-producing organisms. Therefore, the resistance of mosquito populations to *B. sphaericus* Bin toxin would seriously threaten the sustainability of current mosquito control programs, using this microbial insecticide. Selection of resistance in two distinct *Culex quinquefasciatus* populations to commercial *B. sphaericus* strains, 2362 and C3-41 is possible under laboratory conditions. However, *B. sphaericus* strain IAB59 appeared to induce a different evolution of resistance, causing much more slow evolving and lower resistance, in both the field-collected susceptible colony and the low-level-resistant colony, after approximately the same number of generations were subjected to selection [31]. A laboratory investigation was undertaken, to study the cyclic usage of field recommended doses of *Bacillus thuringiensis israelensis (Bti)*, *Bacillus sphaericus* (*Bs*) and combination of *Bti* and *Bs* (half the recommended dose of each) with deltamethrin to attain better control of mosquito larvae. The results revealed that *Bti* excels *Bs*, as it recorded 54% mortality only on 17th day after application. The other salient finding of this study is LC50 of deltamethrin is sufficient to follow the biopesticides application, for an effective control of *Culex* larvae [32].Though, *B. sphaericus* spore/crystal toxins are powerful tools to control mosquito vectors, and the recent development of resistance in *Culex* species has impeded progress in mosquito control operations. The magnitude of *Bs* cross-resistance to different strains of *Bs* and *Bti* in filarial vector of *Culex quinquefasciatus*, has been reported [27,28,33-35]. The resistance ratio recorded between *Bs* resistant and susceptible larvae were several thousand folds at the LC_{50} and LC_{95} levels. These results indicated a need for judicious use of appropriate strains of *Bs* and *Bti*, in the event of biopesticide resistance for mosquito control.

Dynamics of Resistance

Resistance to *Bs* has been reported in *Culex* pipiens complex, in both laboratory colonies and natural populations. During field trials on *Bs* water-dispersible granules (WDG) against natural populations of *Culex quinquefasciatus* in a low-income community, Thailand, control failure occurred within 4 months after 5 treatments, using VectoLex WDG at the dosages of 50-200 mg/m. The resistance ratios (RR) at LC_{50}, depending on reference colonies were 21,100-28,100-fold, against *Bs* WDG against *Bs* technical-grade material. These *Bs* -resistant mosquitoes however, were completely susceptible to *Bacillus thuringiensis* serovar *israelensis*, *(Bti)* preparations, LC_{50} ranging from 0.017 ppm for technical material with 7,000 ITU/mg to 0.052 ppm for water-dispersible granules with 3,000 ITU/mg. But, addition of *Bti* to *Bs* subspecies substantially enhanced the mosquitocidal activity (synergism) against these highly Bs-resistant *Culex quinquefasciatus* [36].

For B, the Bin toxin has to be considered as a one site-acting molecule, because of the single receptor interaction with Bin B component (at least in *C. pipiens*). Resistance to *B. sphaericus* has been reported in *B. sphaericus*-treated field populations of the *C. pipiens* complex in Brazil and India, and *C. pipiens* pipiens in France and China. *Bs* resistance has been recorded during the last four years in Brazil (10 fold resistance [37], in India (150 fold) [38] and in France on *C. pipiens* (10,000 fold) [24]. Reports from China (25,000) and Tunisia (2,000 fold) confirmed that resistance to *Bs* may develop in the field, when this bacteria is used intensively. Before, records of field resistance to *Bs* active laboratory selections for resistance had been done in two different laboratories in California (>100,000 fold) [39,40]. Studies were done to investigate the evolution of resistance to *B. sphaericus* strains C3-41, 2362, and IAB59, in field-collected populations of *C. quinquefasciatus* from China and Brazil, under laboratory conditions. Particular attention was paid to strain IAB59 for its toxicity against *B. sphaericus*-resistant mosquito larvae, with the aim of investigating whether this strain could be an alternative to the already commercialized *B. sphaericus* strains. The stability of resistance in the selected mosquito colonies and their cross-resistance to *B. sphaericus* strains C3-41, 2362, and IAB59 and *B. thuringiensis* subspecies *P. israelensis*, were also investigated. Two independent laboratory selections with California mosquitoes (*C. pipiens*, *C. quinquefasciatus*) have also led to resistance. Levels of stable laboratory-selected resistance of between 35-fold and more than 100,000-fold have been reported, suggesting that there may be different resistance mechanisms. Investigations of the mechanisms and genetics of resistance to *B. sphaericus*, have been carried out for some of the resistant populations. All of the *B. sphaericus*-resistant *C. pipiens* populations were selected on strain 2362, 1593, or C3-41; all of these strains belong to the same serotype and have identical genes encoding the binary toxin. However, there are small differences in the amino acid sequences of the B.*sphaericus* Bin toxins, which may be important in the structure and function of the toxin-receptor complex, and therefore for larvicidal activity [15]. All these studies would help to understand the inheritance of resistance and to develop approaches for resistance detection and monitoring, as well as for management strategies for resistant mosquito colonies [31].

Mode of Action of Bacterial Toxins

Binding studies (in vitro) between the toxin and midgut BBMF (brush border membrane fractions) from three resistant *Culex* populations, gave some knowledge about the mechanisms of resistance. For the high level resistant population from France and the low-level resistant population from Brazil (both field-selected), no changes were found in binding kinetics, meaning that the receptor was not functional [24]. Further, the gut proteases from this colony were able to proteolyse the protoxins to the activated forms. Then, if the *Bs* crystal toxin has selected highly resistant individuals possessing a mutation

influencing the initial toxin-binding in one case, in the other case the same toxin selected highly resistant individuals expressing their resistance at another level of the intoxication process. However, the receptor molecule could also be involved in the resistance from France, but at another site than the binding site. This indicates that different genes can be involved in the resistance to *Bs*, depending on various factors like the origin of *Culex* populations, the frequency of the resistance genes and the conditions of selection [41]. The use of *Bacillus sphaericus* (Bs) as a potential biolarvicide in India is limited, due to development of resistance by the target mosquito species. Observations on the biological processes of development and resistance in the *Bs* susceptible population of *Culex quinquefasciatus*, have provided good insight towards developing a better control strategy for vector mosquitoes. In a laboratory evaluation, *C. quinquefasciatus* susceptible to *Bs* attained a high resistance level (70 and 90.5 fold) at LC_{50} and LC_{95} levels, with several important underlying factors involving binding of *Bs* toxic molecules to the receptor proteins, at the site of action. The resistant larvae showed insignificant variation from susceptible larvae in biological features, especially pre-oviposition period, number of egg rafts laid, incubation period, hatching percentage, stadial period, adult longevity and mortality rate. However, *in vitro* binding assays showed a significant reduction in the affinity of *Bs* toxin, for the membrane receptors in the resistant strain compared to the susceptible strain [8].

Resistance Heritage

The molecular basis of *Bs* resistance have been investigated on the two high-level resistant populations, from France and from California, by crossing homozygous resistant colonies with susceptible homozygous and back-cross experiments between F1 and the resistant colonies. This indicated that resistance was due to one major gene, sex linked for the colony from France but autosomal for the colony from California, by crossing homozygous resistant colonies with susceptible homozygous, and backcross experiments between F1 and the resistant colonies [24,42-44]. In other populations such as the low-level Brazilian one, resistance is also supposed to be recessive, because of the fast decline in resistance when *Bs* treatments were interrupted. Although, resistance is recessive in all studied cases, high-level resistance may constitute a major threat to the future use of *Bs* toxins for mosquito control. However, it seems that in some areas, even with intensively field applications (e.g. in Cameroon, Tanzania, Brazil and India), decrease in susceptibility has not occurred. In southern France, *Bs* had been used for eight years from March to October with 1-2 treatments per month. Resistance occurred faster in closed breeding sites. This was also the case in Tunis, meaning that in such breeding sites, only low migration of susceptible *Culex* individuals from non-treated areas could occur. In Recife (Brazil), the 10 fold resistant populations was found in open drains and covered cesspits in a small area, where all breeding sites were treated during a two year period with a total of 37 treatments [43]. In Cochin (India), resistance occurred in different kinds of open breeding sites after about two years (35 treatments), and in Doungguan (China) after eight years, with about 36 treatments per year [37]. This shows that the key elements for appearance of resistance are the selection pressure in time, and in dose and the genetic background of the populations.

Key Elements for Cross-Resistance

In the above mentioned treated areas, only three different *Bs* strains were used 1593, 2362 and C3-41, all belonging to serotype H5a5b, which express the same crystal toxin (identical amino acid compositions). These strains are used in most commercial *Bs* formulations. Investigations on the level of cross-resistance among natural *Bs* strains have been done by testing the toxicity of several highly active *Bs* strains on some of the above mentioned *Bs* resistant *Culex* colonies. For the laboratory selected low-level resistant colony from California, cross-resistance was found to strain 2297 [40]. This was also the case for the field-selected population from India [45]. There is no cross-resistance to *Bti* within the populations resistant to Bs, and there is even evidence for an increased susceptibility to *Bti* [37,46]. This is in agreement with the finding that the crystal toxin from *Bs* and the crystal toxins from *Bti*, do not compete for the same binding sites. In all cases of binding site modification, resistance seems to be inherited as a single recessive or partially recessive major gene, and the resistance levels are high. In these cases, cross-resistance seems to be very limited and extends only to ICPs binding to the same binding site. In contrast, in those cases where resistance is due to another as yet unknown modification, inheritance was found to follow an additive pattern; levels of resistance were moderate and at least in one case, a more general cross-resistance was observed [47]. *Bacillus sphaericus* IAB 872 has high toxicity against susceptible *Culex* spp. and medium larvicidal activity against binary toxin-resistant *Culex* spp. Sequence analysis revealed that the sequence of the binary toxin gene from IAB872 was totally identical to that of the reference strain 2362. Mosquito larvicides based on the bacteria *Bacillus thuringiensis* subspecies *P. israelensis (Bti)* or *B. sphaericus* (Bs) are effective in many habitats, but use is limited by their high cost. Moreover, mosquito resistance evolves rapidly to Bs, where it is used intensively [48,49]. *Bacillus sphaericus* 1593M resistant larvae of *Culex quinquefasciatus* were reared in the laboratory, since 1995. Resistance in the larvae was monitored by subjecting selection pressure, using *B.sphaericus* 1593 M at every generation. Bioassays were conducted with different strains of *B.sphaericus* (Bs 2297, *Bs* 2362 and *Bs* IAB 59), and confirmed cross-resistance in the present study. The level ranged between 27.3 to 18.2-fold, in comparison with susceptible larvae.

Resistance Management Tactics

Resistance is believed to be a complex, genetic, evolutionary and ecological phenomenon. Resistance management tactics are most likely to succeed, if they are directed at reducing the single-factored selection pressure that occurs with conventional biocide or chemical control. During a pesticide change, two factors are pivotal for the dynamics of the resistance genes [50]. The effectiveness of resistance management is central for maintaining adequate pest control. Critical evolutionary factors determine the dynamics of pesticide resistance in the field. One of the factors is the fitness cost required to induce a rapid reversal in the frequency of resistance genes, when the selecting pesticide is withdrawn from pest-control programs. For species like insects, adaptation results from an alteration in ICP binding, henceforth, resistance management strategies should consider combinations (either simultaneously or in rotation) of ICPs with different binding site specificity. Obvious counter measures include: (i) rotation or alternation of *Bs* or *Bt* toxins with other toxins, insecticides or cultural or biological control strategies (ii) reducing the frequency of biocide treatments, (iii) avoiding insecticides with prolonged environmental persistence and slow-release formulations, (iv) avoiding treatments that apply selection pressure, and (v) incorporating source reduction method. The combination of these principles is essentially a blue print for integrated pest management (IPM), which will successfully delay or prevent the development of resistance in vector population. Theoretically, integrated pest management (IPM) helps delay resistance

by providing multiple sources of pest mortality. Combined application of neem based biopesticides with microbial agents, also revealed that the neem biopesticide showed synergistic interaction with the *Bs* toxin against resistant larvae *C. quinquefasciatus* [51]. There is the evidence for development of resistance to any bacterial toxin, as soon as its mode of action implies only one toxin, or toxins with identical mode of action (binding on the same receptor); *Bs* belongs to this category. This microbial insecticide has therefore, to be used in a reasonable way in integrated control program. Monitoring of the susceptibility of the treated mosquito populations before and during treatments is necessary. Other measures to be taken are to multiply the control methods and/or insecticides. *Bti* could be used as an alternative in certain conditions and formulations. In addition, other *Bs* strains or recombinant *Bs* expressing additional toxins from other mosquitocidal bacteria have to be considered. Nevertheless, there is a risk in introducing the *Bs* crystal toxin genes alone into natural mosquito larval food (e.g. *Cyanobacteria*), because this would expose the larvae to a continuous selection pressure. Besides this, further understanding on the mode of action on the receptor identification for other mosquito species, and on the putative intracellular activity of the *Bs* crystal toxin, may give good tools to identify other mechanisms of resistance, in order to predict and reduce resistance [38]. Genetic analysis revealed that *B. sphaericus* resistance was inherited as a recessive trait and controlled by a single major locus. *B. sphaericus*-resistant mosquito colonies remained highly susceptible to *B. thuringiensis israelensis*, suggesting that *Bti* would be of value in the management of *B. sphaericus*-resistant *Culex quinquefasciatus* colonies [52]. The 2362 strain of *Bacillus sphaericus*, which produces a binary toxin highly active against *Culex* mosquitoes, has been developed recently as a commercial larvicide.

It is being used currently in operational mosquito control programs in several countries, including Brazil, France, India, and the United States. Laboratory studies have shown that mosquitoes can develop resistance to *B. sphaericus*, and low levels of resistance have already been reported in field populations in Brazil, France, and India. To develop tools for resistance management, the Cyt1A protein of *Bacillus thuringiensis* subspecies *P. israelensis* de Barjac was evaluated for its ability to suppress resistance to *B. sphaericus*, in a highly resistant population of *Culex quinquefasciatus*. Synergism was observed between the Cyt1A toxin and *B. sphaericus* against the resistant mosquito population, and accounted for the marked reduction in resistance. However, no synergism was observed between the toxins against a nonresistant mosquito population. These results indicate that Cyt1A could be useful for managing resistance to *B. sphaericus* 2362 in *Culex* populations, and also provide additional evidence that Cyt1A may synergize toxicity by enhancing the binding to and insertion of toxins into the mosquito microvillar membrane [43,44]. The 2362 strain of *Bacillus sphaericus* (Bs) Neide is a highly mosquitocidal bacterium used in commercial bacterial larvicides, primarily to control mosquitoes of the genus *Culex*. Unfortunately, *Bs* is at high risk for selecting resistance in mosquito populations, because its binary toxin apparently only binds to a single receptor type on midgut microvilli. A potential key strategy for delaying resistance to insecticidal proteins is to use mixtures of toxins that act at different targets within the insect, especially mixtures that interact synergistically. This hypothesis was tested for delaying the phenotypic expression of resistance, by exposing *Culex quinquefasciatus* say larvae to *Bs* alone or in combination with Cyt1A, from *Bacillus thuringiensis* subspecies P. *israelensis*. Two laboratory lines of *Culex quinquefasciatus*, one sensitive to *Bs* and the other containing *Bs* resistance alleles, were subjected to intensive selection pressure for 20 generations, with either *Bs* 2362 or a 3:1 mixture of *Bs* 2362+Cyt1A. At the end of the study, the sensitive line had evolved >1000-fold resistance when selected with *Bs* alone, whereas the parallel line selected with *Bs* +Cyt1A exhibited only low resistance toward this mixture (RR95, 1.4). Similar results were observed in the lines containing *Bs* resistance alleles. Both lines selected with *Bs* +Cyt1A, exhibited substantial resistance to *Bs* in the absence of Cyt1A. Although, selection with *Bs*+Cyt1A did not prevent the underlying evolution of resistance to Bs, these results suggest that a mixture of *Bs* with other endotoxins, particularly one like *Bs*+Cyt1A in which the components interact synergistically, would provide longer lasting and more effective mosquito control than *Bs* alone [53].

Bacillus thuringiensis Serovar *Israelensis (Bti)*

A bacterial mosquito pathogen was isolated for a first time [54] and was designated as *Bacillus thuringiensis* serovar *israelensis (Bti)* [55]. Laboratory bioassays and field applications of this entomopathogen have shown biological control of several mosquito species and black flies [56-58]. There are 34 recognized subspecies of *B. thuringiensis* – some of the most commonly used include subspecies kurstaki (against Lepidoptera), subspecies *israelensis* (against Diptera, primarily mosquitoes and blackflies), and subspecies tenebrionis (against Leptinotarsa decemlineata, the Colorado potato beetle) [59]. Two general groups of insecticidal crystal proteins made by this wide variety of subspecies have been identified; Cyt (cytolysins) and Cry (crystal delta-endotoxins. Hofte and Whiteley [58] define four classes of Cry genes and two classes of Cyt genes. Cry I and Cry II toxins are active against lepidopterans, CryII and Cry IV against dipterans, and Cry III against coleopterans [60]. While Cry III toxins are produced by subspecies tenebrionis and tolworthi and Cry IV by *israelensis*, generally very little correlation between certain toxins and certain subspecies exists. *Bti* crystals are composed of four major polypeptides with molecular weights of 125, 135, 68 and 28 kDa, now referred to as Cry IVA, Cry IVB, Cry IVD, and Cyt A, respectively.

Like *B.sphaericus*, *B.thuringiensis* serovar *israelensis (Bti)* is also a spore forming Gram-positive soil bacterium, since its discovery two decades ago [54], more than 50,000 isolates have been screened and tested in insect control. This bacterium synthesizes proteins during sporulation that assemble into crystals, which are toxic to mosquitoes. Crystal development during sporulation of *Bt* strains has been studied extensively. The crystals are composed of four polypeptides (M.wt. 125, 135.68 and 28 kDa proteins) referred to as Cry IVA, Cry IVB, Cry IVD and CytA. These genes encoding these Cry toxins are located on a 72 kDa resident plasmid, and they have been cloned and expressed in various hosts. Chromosomal Cry genes have also been reported in some *Bt* strains, and the role, structure and molecular organization of genes coding for the parasporal delta endotoxin of Bt. A review of the biochemical mechanisms of resistance of insects to *Bt* indicates that altered proteolytic processing of *Bt* crystal proteins may be involved in one case of resistance in mosquitoes. The presence of IS240 elements responsible for mosquitocidal action was investigated in sixty nine *Bt* strains. A PCR-based approach for detection of Cry genes in Bt, has been reported. Since the toxins of this bacterium are highly potent for mosquito control, evaluation of the activity of *Bt* preparations is currently carried out by bioassay with a target insect, and compared to a defined standard.

Binding kinetics of *Bti*

Genes encoding these polypeptides are located on a 72 MDa resident plasmid, and have all been cloned and expressed in various hosts. Expression of *Bti* genes, either individually or in combination in crystal-negative *Bt* strains, as well as disruption of genes by *in-vivo* recombination from toxic strains, have led to the conclusion that 1) Cry IV A, Cry IVB, and Cry IVD are to various extents, involved in the toxicity towards mosquitoes, although displaying different specificities, depending on the mosquito species tested. Cyt A is not a key factor for toxicity but can potentiate the activity of the toxins, and synergistic interactions seem to account for the high toxicity of the wildstrain [61]. Cry toxins bind to specific receptors on cells in the insect midgut. Cyt genes are active against dipteran and coleopteran pests, and additionally have shown action against hemipterans (true bugs) and dictyopterans (roaches and termites) [62,63]. Cyt toxins unlike Cry toxins do not recognize specific binding sites. *Bt* directly causes mortality in insects, and isolates of the toxin from different strains follow similar modes of action. After the delta-endotoxin crystals are ingested, they are dissolved in the insect midgut liberating the protoxins of which they are made. These are proteolytically processed into fragments, one of which binds to cells of the midgut epithelium. The activated protein disrupts the osmotic balance of these cells, by forming pores in the cell membrane causing the cells to lyse [64]. The gut becomes paralyzed and the insect stops feeding; most insects will die within a few hours of ingestion [65]. The binding affinity of these toxin fragments is often directly related to the toxicity, though binding does not assure toxicity [59].

Problem of resistance to *Bti*

While *Bt* is very unlike other insecticides in its origin, mode of action and use, it still shares some of the problems of any insecticide. One major problem with insect control via insecticides is the evolution in insects of resistance to those insecticides. The first reported cases of insecticide resistance to early synthetic insecticides occurred over 50 years ago. About thirty years later, in 1979, the United Nations Environmental Programme declared pesticide resistance, one of the world's most serious environmental problems. Its seriousness to the environment stems from problems of human nutrition due to crop loss, spread of disease by resistant insects, in addition to the environment of new and potentially dangerous insecticides after resistance has developed, and application of greater and greater amounts of chemicals to which pests have already gained resistance [66]. Insecticide resistance is a major problem – not only in agriculture, but also in health and economics. The development of resistance to *Bacillus thuringiensis* toxins is however, particularly unfortunate. *Bt* toxins are more pest-specific and environmentally safe than conventional pesticides, yet as effective against problem insects [67].

In 1985, the first evidence of resistance developing in the field against *Bt* delta-endotoxins was published. Low levels of resistance were found in *Plodia interpunctella*, the Indian meal moth, in storage bins of *Bt*-treated grain [67]. Recognition of the potential of the *Bt* resistance problem became greater, when the first reports of high resistance to *Bt* toxins in the field came in 1990 from Hawaii, Florida, and New York in the United States – thirty years after its commercial debut here. The species found to be losing susceptibility to *Bt* toxin was *Plutella xylostella*, the diamondback moth, treated with spray formulations of the toxins. At about that same time, resistance was detected in *P. xylostella* after intensive use in several other countries, including Japan, China, the Philippines and Thailand [68]. Malaysia also reported *Bt* resistance in the diamondback moth in 1990; interviews with local farmers confirmed their personal experiences with this unfortunate situation [69]. Thus, far *P. xylostella* is still the only insect species in which considerable resistance has been found to develop outside of the laboratory. In the fifteen years since *Bt* resistance was discovered in *P. interpunctella*, *Bt* resistance has been selected in laboratory populations of a total of thirteen insect species. Eleven of these species have developed resistance to various strains of *Bt* toxin in the laboratory, but not in the field: *Ostrinia nubilalis* (the European corn borer), *Heliothis virescens* (the tobacco budworm), *Pectinophora gossypiella* (the pink bollworm moth), *Culex quinquefasciatus* (the mosquito), *Caudra cautella* (the almond moth), *Chrysomela scripta* (the cottonwood leaf beetle), *Spodoptera exigua* (the beet armyworm), *Spodoptera littoralis* (the Egyptian cotton leafworm), *Trichoplusiani* (the tiger moth), *L. decemlineata* (the Colorado potato beetle) and *Aedes aegypti* (the yellow fever mosquito) [26,62,69-72]. Many other species have been tested in the lab, but retained susceptibility to *Bt* [59]. While none of the species listed here has yet developed resistance in the field, these laboratory studies show that the potential to develop resistance is real. No records of field resistance have been found to *Bti* because of the presence of the four different toxins with putative different modes of action. But, *Bacillus thuringiensis* serovar *israelensis* strains (*Bti* PG14 and *Bti* 426) did not show any cross-resistance in the larvae, and it emphasized a need to study the mode of action of *B. sphaericus* toxin that induced cross-resistance in the larval strain [73]. Wei et al. [72] have studied the toxicity and delayed effects of a mosquitocidal toxin (Mtx1) and a binary toxin (Bin) produced in *Escherchia coli* E-TH21 and *Bacillus thuringiensis* B-CW1, respectively on *Culex quinquefasciatus (Diptera: Culicidae)*. Bioassay results showed that both E-TH21 powder and B-CW1 sporulated culture were highly toxic against susceptible *Culex quinquefasciatus*, with LC_{50} values of 0.65 and 1.70 mg/liter against third and fourth instars at 48 h, respectively [74]. After initial 48-h exposure of larvae to different concentrations of Mtx1 and Bin, significant continued mortality could be observed in larval, pupal, and emergence stages of *Culex quinquefasciatus*. Importantly, the Mtx1 could induce higher cumulative larval and pre-adult mortalities than Bin toxin on the target mosquito. This finding is important in understanding the mode of action of Mtx1 and Bin toxins, and for developing a new bioassay procedure for evaluation of toxicity of *Bacillus sphaericus* Neide; some strains of which produce Mtx1 and Bin, in the laboratory and field.

Resistance heritage: how it happened

Insects can and have developed resistance to nearly every type of insecticide. Resistance to other insecticides is in fact, one of the many reasons. *Bacillus thuringiensis* has come into common use today. Insecticide resistance develops due to genetic variation in large insect populations. A few individuals in the original insect population are unaffected by a given insecticide. Generally, unaffected (resistant) individuals differ from affected (susceptible) individuals, either in the nature of the insecticide's target molecules in the insect, or in the method the insect uses to break down toxin molecules [75]. When the insecticide is applied, individuals who are unaffected by it are those who survive to pass their genes on to following generations. Over time, a greater and greater proportion of the insect population is unaffected by the insecticide [76]. Insecticides based on *Bacillus thuringiensis* subspecies *israelensis* have been used for mosquito and black fly control for more than 20 years, yet no resistance to this bacterium has been reported. Moreover, in contrast to *B. thuringiensis* subspecies *israelensis*, toxic to coleopteran or lepidopteran larvae, only low levels of resistance to *B.thuringiensis* subspecies *P. israelensis* have been

obtained in laboratory experiments, where mosquito larvae were placed under heavy selection pressure for more than 30 generations. Selection of *Culex quinquefasciatus* with mutants of *B. thuringiensis* subspecies *P. israelensis* that contained different combinations of its Cry proteins and Cyt1Aa, suggested that the latter protein delayed resistance. These results indicated that Cyt1Aa was the principal factor responsible for delaying the evolution and expression of resistance to mosquitocidal Cry proteins [53].

Reason for resistance

There are several factors that increase the rate at which insecticide resistance is generally developed. Some factors are related to the insect population itself: species with higher reproductive rates, shorter generation times, greater numbers of progeny, and larger, more genetically varied local populations develop a large resistance population more quickly [66]. Whether the genetic basis of insect resistance is dominant or recessive is also of importance [77]. Other factors are dependent upon the insecticide. Resistance develops more rapidly to more persistent insecticides; their staying power in the environment increases the chance that susceptible individuals are exposed to the toxin and die, thus not passing on their insecticide-susceptible traits to the next generation. This selects more strongly on resistant insects because only the resistant insects thrive. By similar logic, frequent application of non-persistent insecticides has the same effect [78]. Insect populations with little immigration into the gene pool of new, non-exposed susceptible individuals, also develop resistance more readily [79]. Populations that have in the past been exposed to an insecticide with a mode of action similar to that of a new insecticide are quick to develop resistance to the new toxin. This phenomenon is known as cross-resistance.

Underlying mechanism on resistance

Central to learning to curb resistance to *Bti* understands the mechanism by which an insect resists the toxins.

Mechanisms by which insects resist the lethal effects of *B. thuringiensis* toxins are naturally, closely related to the mode of action of *Bt*. As stated earlier, *Bti* protoxins are activated by proteases in the insect midgut; after activation, they bind to receptors on the epithelium. Thereafter, a number of steps lead to the death of the insect. The specifics of the mode of action are complex and varied among insect and *Bt* strains, so complex in fact that prior to 1985, it was thought that the complexity itself would prevent the evolution of resistance [59]. Mechanisms of resistance are equally complex. Because so many steps are involved in the full process of *Bti*'s mode of action, many ways of stopping the process and resisting the toxin, are possible. Thus, far studies have most commonly shown the resistance mechanism to involve a change in the membrane receptors, to which activated *Bti* toxins bind [71].

Objective to overcome problem of resistance

It will be necessary to counter resistance, in order to preserve the efficacy of Bt. There are three goals of resistance management: avoiding resistance where and if possible, delaying resistance as long as possible, and making resistant populations revert to susceptibility [80]. Several possible resistance programs have been conceived in the past 25 years, most of which could potentially be used in conserving susceptibility to Bt. The transgenic plant forms of *Bti*, the use of which is on the rise, are especially prone to resistance development. Transgenic plants expose insects to toxins continually, even at times when they are not causing economic damage [81].

Resistance management programs generally use one of just three basic approaches to delay resistance. One approach seeks to minimize exposure to toxins, and/or allow for mating between resistant insects and a large population of susceptible insects, to keep susceptible traits continuing in the gene pool. These strategies include tissue-specific and time-specific expression of toxins, mixtures, mosaics, rotations, refuges, and occasional release of susceptible males into the field. Another approach focuses on combining pest-control techniques, and is based on the assumption that an insect is more likely to develop resistance to just one type of control than more than one type of control, simultaneously. Strategies in this category include gene stacking, high doses, combinations of toxins with completely different modes of action, and combinations of low toxin dose and natural enemies.

Can The Method of Release of Susceptible Insect Population Solve The Problem?

Among the oldest strategies are those involving the mating of resistant with susceptible insects. The simplest of these ideas is the periodic release of susceptible males, raised in the lab or collected elsewhere, into a local Bt-treated population. This would theoretically make it possible to keep the frequency of resistance in a population, below a predefined level [82]. This method is best used on populations of insects such as mosquitoes, in which insecticides generally target females [78].

However, *Bt* is not a gender-specific pesticide and there is a risk that many of the susceptible males released would die in the *Bt* field, before mating. Additionally, the feasibility of rearing and transporting large colonies is very questionable.

Synergistic interactions among the multiple endotoxins of *Bacillus thuringiensis* subspecies *P. israelensis* de Barjac, play an important role in its high toxicity to mosquito larvae, and the absence of insecticide resistance in populations treated with this bacterium. A lack of toxin complexity and synergism are the apparent causes of resistance to *Bacillus sphaericus* Neide, in particular *Culex* field populations. The proposed strategies for improving bacterial larvicides are by combining *B. sphaericus* with *Bt* subspecies *P. israelensis*, or by engineering recombinant bacteria that express endotoxins from both strains. These combinations increase both endotoxin complexity and synergistic interactions, and thereby enhance activity and help avoid insecticide resistance [83].

Transgenic Mosquitoes

Genetic engineering techniques have been used to significantly improve mosquito larvicides, based on the bacteria *Bacillus thuringiensis* (Bt), subspecies *P. israelensis (Bti)* and *Bacillus sphaericus* (Bs). By cloning the genes encoding various endotoxins from *Bt* and *Bs* species and engineering these for high levels of synthesis, we have been able to generate recombinant bacterial strains based on *Bti* that are more than 10 times as effective as the conventional strains of *Bti* or Bs, that serve as the active ingredients of commercial bacterial larvicides, currently used for mosquito control. The best of these recombinants contain all major *Bti* endotoxins, specifically, Cry4A, Cry4B, Cry11A, and Cyt1A plus the binary (Bin) endotoxin of Bs, the principal mosquitocidal protein responsible for the activity of this species. The presence of Cyt1A in these recombinants, which synergizes Cry toxicity and delays resistance to these proteins and *Bs* Bin, should enable long term use of

these recombinants, with little if any development of resistance [84]. Recently, however, recombinant DNA techniques have been used to improve bacterial insecticide efficacy by markedly increasing the synthesis of mosquitocidal proteins, and by enabling new endotoxin combinations from different bacteria to be produced within single strains. These new strains combine mosquitocidal Cry and Cyt proteins of *Bacillus thuringiensis* with the binary toxin of *Bacillus sphaericus*, improving efficacy against *Culex* species by 10-fold, and greatly reducing the potential for resistance through the presence of Cyt1A. For example, the recombinant *Bti* species produce Cyt1A, Cry proteins and the *Bs* Bin toxin, each type with a different mode of action. Significantly, Cyt1A adds the important trait of making it difficult for the mosquitoes to develop resistance to these strains, something not achieved with chemical insecticides. Moreover, although intensive use of *B. sphaericus* against *Culex* populations in the field can result in high levels of resistance, most of this can be suppressed by combining this bacterial species with Cyt1A; the latter enables the binary toxin of this species to enter midgut epithelial cells via the microvillar membrane, in the absence of a midgut receptor. The availability of these novel strains and newly discovered mosquitocidal proteins, such as the Mtx toxins of *B. sphaericus*, offers the potential for constructing a range of recombinant bacterial insecticides, for more effective control of the mosquito vectors [84]. Similar to Cyt toxins from *Bti*, Mtx toxins (produced during vegetative growth) can increase the toxicity of other mosquitocidal proteins, and may be useful for both increasing the activity of commercial bacterial larvicides and managing potential resistance to these substances among mosquito populations [85].Thus, there were two obvious strategies for making improved recombinant mosquitocidal bacteria: (1) introduce *Bti* or related mosquitocidal endotoxin genes into the best *Bs* strains and (2) introduce *Bs* toxin genes into *Bti*. Both of these approaches have been to construct a variety of *Bt* and *Bs* recombinants that produce different combinations of *Bt* and *Bs* proteins. Integrative plasmids have been constructed by researches in genetic engineering to enable integration of foreign DNA into the chromosome of *Bacillus sphaericus* 2297, by in vivo recombination. This strategy was applicable with antibiotic resistance selection. Hybridization experiments evidenced two copies of the operon encoding the binary toxin from *B. sphaericus* in the recipient strain. Synthesis of the Cry11A toxin conferred toxicity to the recombinant strains against *Aedes aegypti* larvae, for which the parental strain was not toxic. Interestingly, the level of larvicidal activity of strain 2297 against *Anopheles stephensi* was as high as that of *B. thuringiensis* subspecies P. *israelensis*, and suggested synergy between the *B. thuringiensis* and *B. sphaericus* toxins. The toxicities of parental and recombinant *B. sphaericus* strains against *Culex quinquefasciatus* were similar, but the recombinant strains killed the larvae more rapidly. The production of the Cry11A toxin in *B. sphaericus* also partially restored toxicity for *C. quinquefasciatus* larvae from a population resistant to *B. sphaericus* 1593. In vivo recombination, therefore, appears to be a promising approach to the creation of new *B. sphaericus* strains for vector control [86]. The results suggested that the Cry27A protein is responsible for the *Anopheles*-preferential toxicity of the *B. thuringiensis* serovar high strain [87]. These inclusions exhibited no larvicidal activities against three mosquito species: *Aedes* aegypti, *Anopheles stephensi* and *Culex pipiens molestus*. Likewise, the inclusions contained no cytocidal activity against HeLa cells [88]. A novel mosquitocidal bacterium, *Bacillus thuringiensis* subspecies *P. jegathesan* and one of its toxins, Cry11B in a recombinant *B. thuringiensis* strain were evaluated for cross-resistance with strains of the mosquito *Culex quinquefasciatus*, that are resistant to single and multiple toxins of *Bacillus thuringiensis* subspecies P. *israelensis*. The high levels of activity of *B. thuringiensis* subspecies *P. jegathesan* and *B. thuringiensis* subspecies P. *israelensis*, both of which contain a complex mixture of Cry and Cyt proteins, against Cry4 and Cry11-resistant mosquitoes, suggested that novel bacterial strains with multiple Cry and Cyt proteins may be useful in managing resistance to bacterial insecticides in mosquito populations [89]. The cross-resistance spectra of the mosquitoes were similar to the profiles for recombinant *B. thuringiensis* strains expressing *B. thuringiensis* toxin genes, but with varied toxicity levels. These results indicated that *B. thuringiensis* sp. *israelensis* genes expressed in a heterologous host such as *E. coli* can be effective against susceptible and *B. thuringiensis*-resistant larvae and suppress resistance [85]. The LC50 values were 2.5 and 4.8 mg/ml respectively, against 3-4 instar susceptible and resistant larvae for the final sporulated cultures of recombinants B-pMT9 (Mtx1), and little toxicity was detected for B-pMT4 (Mtx1) [90].

Previous work showed that the resistance to *B. sphaericus* in a *Culex quinquefasciatus* colony is associated with the absence of the approximately 60 kDa binary toxin receptor, in larvae midgut microvilli. Here, the gene encoding the *C. quinquefasciatus* toxin receptor, Cqm1, was cloned and sequenced from a susceptible colony. The deduced amino-acid sequence confirmed its identity as an alpha-glucosidase, and analysis of the corresponding gene sequence from resistant larvae implicated a 19-nucleotide deletion, as the basis for resistance [91]. The toxicities of Mtx1 toxin against dipteran and lepidopteran species showed that Mtx1 has little or no toxicity to the tested lepidopteran species, but has moderate-level toxicity to *Aedes albopictus* Skuse (Diptera: Culicidae) and high-level toxicity to both susceptible and binary toxin-resistant *Culex quinquefasciatus* Say (Diptera: Culicidae). This indicated that Mtx1 has a different mode of action from the binary toxin, and that it could be an alternative toxin to delay or overcome resistance development to binary toxin in *C. quinquefasciatus* [74]. Cry toxins from *Bacillus thuringiensis* (Bt) are used for insect control. Their primary action was

to lyse midgut epithelial cells. In the case of mosquitocidal *Bt* strains, two different toxins participate, Cry and Cyt. These toxins have a synergistic effect and Cyt1Aa overcomes Cry toxin-resistance. Recent findings on the identification of Cry receptors in mosquitoes and the mechanism of synergism summarizes that Cyt1Aa synergizes or suppresses resistance to Cry toxins, by functioning as a Cry membrane-bound receptor. The results obtained in toxicological tests showed significant differences in the larval sensitivities of the four populations, for both insecticides. These differences appeared to be related to the activity of the three main families of detoxifying enzymes: Cytochrome P450 monooxygenases, glutathione-S-transferases (GSTs), and esterases. All three enzyme families were significantly over expressed in the less susceptible larval population, and after multiple regressions, it was found that GSTs and esterases were the most explicative variables of the larval sensitivity. Considering these results and the chemical history of the sites, in terms of insecticide treatments, the

hypothesis of cross-effects of insecticides leading to resistance acquisition to *Bti* in field organisms, emerges. The mechanism of resistance to the binary toxin in a natural population of the West Nile virus vector, *Culex* pipiens, showed that the insertion of a transposable element-like DNA into the coding sequence of the midgut toxin receptor, induced a new mRNA splicing event, unmasking cryptic donor and acceptor sites located in the host gene. The creation of the new intron causes the expression of an altered membrane protein, which is incapable of interacting with the toxin, thus providing the host mosquito with an advantageous phenotype. As a large portion of

insect genome is composed of transposable elements or transposable elements-related sequences, this new mechanism may be of general importance to appreciate their significance as potent agents for insect resistance, as the microbial insecticides [92]. These results indicate that *B. thuringiensis* subsp. *israelensis* genes expressed in a heterologous host, such as *E. coli* can be effective against susceptible and *B. thuringiensis*-resistant larvae and suppress resistance [85]. Mixtures of *B. sphaericus* with either cytolytic toxin were synergistic and *B. sphaericus* resistance in *C. quinquefasciatus* was suppressed from >17,000 to 2-fold, with a 3:1 mixture of *B. sphaericus* and Cyt1Ab. This trait may prove useful for combating insecticide resistance and for improving the activity of microbial insecticides [93]. Synergistic interactions among the multiple endotoxins of *Bacillus thuringiensis* subspecies P. *israelensis* de Barjac, play an important role in its high toxicity to mosquito larvae and the absence of apparent causes of resistance to *Bacillus sphaericus* Neide, in particular *Culex* field populations. To identify endotoxin combinations of the two Bacillus species that might improve insecticidal activity and manage mosquito resistance to *B. sphaericus*, the toxins were tested alone and in combination. Most combinations of *B. sphaericus* and B.t. subspecies *israelensis* toxins were synergistic and enhanced toxicity relative to *B. sphaericus*, particularly against *Culex quinquefasciatus* Say larvae resistant to *B. sphaericus* and *Aedes aegypti* (L.), a species poorly susceptible to *B. sphaericus*. Toxicity also improved against susceptible *Culex quinquefasciatus*. For example, when the CytlAa toxin from B.t. subspecies *israelensis* was added to Bin and Cry toxins, or when native B.t. subspecies P. *israelensis* was combined with *B. sphaericus*, synergism values as high as 883-fold were observed, and combinations were 4-59,000-fold more active than *B. sphaericus*. These data and previous studies using cytolytic toxins validate proposed strategies for improving bacterial larvicides, by combining *B. sphaericus* with B.t. subspecies *israelensis*, or by engineering recombinant bacteria that express endotoxins from both strains. These combinations increase both endotoxin complexity and synergistic interactions, and thereby enhance activity and help avoid insecticide resistance [83]. The 2362 strain of *Bacillus sphaericus*, which produces a binary toxin highly active against *Culex* mosquitoes, has been developed recently as a commercial larvicide. It is being used currently in operational mosquito control programs in several countries including Brazil, France, India and the United States. Laboratory studies have shown that mosquitoes can develop resistance to *B. sphaericus*, and low levels of resistance have already been reported in field populations in Brazil, France, and India. To develop tools for resistance management, the Cyt1A protein of *Bacillus thuringiensis* subspecies P. *israelensis* de Barjac was evaluated for its ability to suppress resistance to *B. sphaericus* in a highly resistant population of *Culex quinquefasciatus* Say. A combination of *B. sphaericus* 2362 in a 10:1 ratio, with a strain of *B. thuringiensis* subspecies P. *israelensis* that only produces Cyt1A reduced resistance by >30,000-fold. Resistance was suppressed completely when *B. sphaericus* was combined with purified Cyt1A crystals, in a 10:1 ratio. Synergism was observed between the Cyt1A toxin and *B. sphaericus* against the resistant mosquito population, and accounted for the marked reduction in resistance. However, no synergism was observed between the toxins against a nonresistant mosquito population. These results indicate that Cyt1A could be useful for managing resistance to *B. sphaericus* 2362 in *Culex* populations, and also provide additional evidence that Cyt1A may synergize toxicity, by enhancing the binding to and insertion of toxins into the mosquito microvillar membrane [43]. Expression of a chitinase gene, chiAC from *Bacillus thuringiensis* in *B. sphaericus* 2297 using the binary toxin promoter, yielded a recombinant strain that was 4,297-fold more toxic than strain 2297 against resistant *Culex quinquefasciatus*. These results show that this chitinase can synergize the toxicity of the binary toxin against mosquitoes and thus, may be useful in managing mosquito resistance to *B. sphaericus* [94]. In the laboratory, three microbial mosquito larvicidal products consisting of *Bacillus thuringiensis* subsp. *israelensis* de Barjac *(Bti)*, *Bacillus sphaericus* (Neide) (Bs) (strain 2362), and the University of California Riverside (UCR) recombinant (producing toxins of both *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* were bioassayed against larvae of *Culex* quinequefasciatus Say (susceptible and resistant to *Bs* 2362), and *Aedes aegypti* (L.). *Bti* proved highly effective against *Culex quinquefasciatus* susceptible and resistant strains. *Bti* was also highly active against *Aedes aegypti* with LC50 and LC90 values of 0.014 and 0.055 ppm, respectively. The UCR recombinant was equally active against both *Bs* susceptible and resistant strains of *Culex quinquefasciatus*. *Bti* and the UCR recombinant essentially showed similar activity against *Bs* -susceptible and resistant strains. *Bs* was highly active against susceptible strain of *Culex quinquefasciatus*, and exhibited little toxicity against *Aedes aegypti* larvae, with no toxicity to *Bs* resistance. In the field, the experimental corn grit formulations of *Bti*, *Bs* and UCR recombinants VBC 60023 in simulated field (microcosms) against *Bs* -susceptible *Culex* mosquitoes were studied. *Bti* and low-concentrate UCR recombinant showed similar initial activity, as well as persistence. Both materials provided high-to-moderate level of control for 2-7 d post treatment, at low treatment rates.

Cost-Effective Technology for Producing Mosquitocidal Bacteria

Each day, vast quantities of bio-organic wastes are discharged from factories, fisheries, poultries and food processing industries globally. Within the food processing sectors, various liquids, sludge and solid biological and organic wastes require remediation and alternative disposal methods are increasingly being investigated [95]. Degrading or handling these wastes as unused disposals without acquiring additional benefits, has led to an idea to develop a suitable technology to utilize bio-organic wastes, by means of simple fermentation technology in mosquito vector control programs [96]. Lately, organic waste treatment system has been developed for utilizing waste materials from food processing industries [97]. These recycling strategies enable the production of biologically active substances, such as soil improvement agents and compost [98]. It would be useful to manufacture and market a biomass fermentation and treatment method, capable of re-utilizing wastes produced by food bio-processing industries into biologically active substances, animal feed, fertilizers and fermentation agents. Such methods would include a system for treating wastes and a new fermentation agent that would be useful in waste decomposition. Use of such methods would reduce waste treatment costs, prevent environmental pollution, improve the soil condition and yield biologically active re-useable substances. Considering the above applications, there has been an identification recently of several bio-organic waste materials from industries, for the production of biopesticides [96,99-103]. All the raw material are rich in nutrient sources (carbohydrate and proteins) and lead to production of bacterial biopesticides (*Bacillus sphaericus*, *Bs* and *B. thuringiensis* subspecies *P. israelensis*, *Bti*), which are well known biocontrol agents used in mosquito vector control programs [17,51,104]. Besides, the fermentation process utilizing agro-industrial residues is nevertheless, an easy to follow, inexpensive technique, efficient enough to produce effective bacterial toxins lethal to disease transmitting mosquito vectors (*Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes* aegypti). Ultimately, this new approach would help to manage solid waste from the environment, in a judicious manner.

Conclusion

The mosquitocidal bacteria such as *Bacillus sphaericus* and *B. thuringiensis* subspecies P. *israelensis*, provide effective alternatives to broad spectrum larvicides in many situations, with little or no environmental impact. Taking into account environmental benefits including safety for humans and other non-target organisms, reduction of pesticide residues in the aquatic environment, increased activity of most other natural enemies and increased biodiversity in aquatic ecosystems, their advantages are numerous. In addition to recombinant bacteria used as larvicides, research is also underway to develop transgenic algae and *cyanobacteria* using larvicidal endotoxins of *Bti* and *Bs*. The advent of recombinant DNA technology is now having an enormous impact on agriculture and medicine, and it is appropriate that the ability to manipulate and recombine genes with this technology be applied to improving larvicides for vector control. These new recombinant bacteria are as potent as many synthetic chemical insecticides yet are much less prone to resistance, as they typically contain a mixture of endotoxins with different modes of action. The existing recombinants also have what can be considered disadvantageous in that, they do not show significantly improved activity against aedine and anopheline mosquitoes in comparison to *Bti*. But, it may be possible to overcome this limitation using some of the newly discovered mosquitocidal proteins such as the Mtx proteins (Delécluse et al. [59]), and peptides such as the trypsin-modulating oostatic factor, which could be easily engineered for high expression in recombinant bacteria. While other microbial technologies such as recombinant algae and other bacteria are being evaluated, it has yet to be shown that these are as efficacious and environmentally friendly as *Bti* and *Bs*. By combining the genes from a variety of organisms, it should ultimately be possible to design 'smart' bacteria that will seek out and kill larvae of specific vector mosquitoes. Thus, recombinant bacteria show excellent promise for development and use in operational vector control programs, hopefully within the next few years. The bio-organic wastes discharged from factories, fisheries, poultries and food processing industries are the good candidate for the production of bio-pesticides in recent years globally.

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