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Bacillus sphaericus in the biological control of mosquito vector complex

Vanlalhruaia[1], N. Senthil Kumar[2] and G. Gurusubramanian[1*]

Department of Zoology[1], Department of Biotechnology[2], Mizoram University, Aizawl 796 004, India

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ABSTRACT

Vector control is primordial and very essential means for controlling transmission of filariasis, malaria, Japanese encephalitis and dengue in human society. Over the last few decades, there is growing realization that alternate methods to synthetic chemical control needs to be studied and perfected. Several control strategies have been adopted to control diseases transmitted by mosquitoes. Mosquito control programs worldwide have been evaluating the feasibility to implement biological control strategies by using *Bacillus sphaericus* (*Bs*). A comprehensive review cum research data is presented here to assess the potentiality of *Bs* in mosquito control operation. The major advantages of *Bs* are reduced application cost, safety to environment, human beings, animals and other non-target organisms. This paper explores the importance of *Bs* bacterial toxin in controlling vector mosquitoes.

Key words: *Bacillus sphaericus*; mosquito; mode of action; toxicity; vector.

INTRODUCTION

Mosquitoes transmit some of the world's worst life threatening and debilitating parasitic and viral diseases including malaria (*Anopheles*), filariasis (*Culex, Mansonia* and some *Anopheles* spp.,), Japanese encephalitis (*Culex tritaeniorhynchus*) and dengue and yellow fever (principally *Aedes aegypti*). In 2008, about 9.57 million people were affected by malaria in India.[1] Similarly, lymphatic filariasis caused by *Wuchereria bancrofti* which affects about 496 million people worldwide and the closely related *Brugia malayi* and *B. timori* affect 12.5 million people in south-east Asia. About 20 million people are infected every year by dengue virus transmitted by *Aedes* mosquitoes with about 24,000 deaths and 294 Japanese encephalitis (JE) cases reported in the year 2008.[2]

In Mizoram, out of 6081-10644 cases of incidence of malaria, *Plasmodium falciparum* was found to be the main causative agent [4189-9421 cases] and deaths [75-120 cases] due to malaria is showing a fluctuating trend in Mizoram during 2006-2010. State Health departments have intensified the efforts to

Corresponding author: G. Gurusubramanian
Phone. +91-0389-2331021/2330754
E-mail: gurus64@yahoo.com

reduce malaria mortality by DDT spray, distributing mosquito nets treated with insecticide, establishing proper effective referral mechanism and treatment facilities for severe cases. Other vector-borne diseases namely filaria, kala-azar, JE, dengue and chikungunya are not endemic in the states.[3,4] However, the incidence of mosquito-borne diseases in the Mizoram region is increasing due to uncontrolled urbanization creating mosquitogenic conditions for the vector populations. Therefore, mosquito control forms an essential component for the management of mosquito-borne diseases. The use of chemical insecticides has been greatly impeded due to development of physiological resistance in the vectors, entrenched with stable malaria, particularly *P. falciparum* with growing drug resistance,[5,6] environmental pollution resulting in bio-amplification of food chain contamination and harmful effects on beneficial non-target animals. Therefore, the need of alternate, more effective and environment-friendly control agents became urgent.

The last decade has evidenced an increased interest in biological control agents. More number of biocontrol agents was screened for their efficacy, mammalian safety and environmental impact. Many organisms have been investigated as potential agents for vector mosquito control, including viruses, fungi, bacteria, protozoans, nematodes, invertebrate predators and fish. 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 *Bacillus sphaericus* Neide (*Bs*) which is highly toxic to dipteran larvae have opened up the possibility of its use as potential biolarvicides in mosquito eradication programs the world over.[7,8] Mosquitocidal bacteria currently represent a tiny fraction of the biopesticide market, which in turn is still only a small fraction of the annual worldwide pesticide market.

This paper focuses on the current approaches in relation to the general features, isolation, characterization, assessment of toxicity, formulation and use of *Bs* to tackle the rising emergence of mosquito vectors.

DISCOVERY OF *BACILLUS SPHAERICUS* (*BS*)

The discovery of *Bs* which is highly toxic to dipteran larvae has opened up the possibility of its use as potential biolarvicide in mosquito eradication programs the world over.[7] *Bs* was discovered to have larvicidal activity against mosquito species[9] and around 300 mosquitocidal strains have been described.[10] Strain 2362, isolated from *Simulium* in Nigeria,[11] is not toxic to black flies, but it is regarded as the most promising isolate for field use against mosquitoes.[12] Abbott Laboratories has recently formulated a commercial product (Vectolex) of *Bs* 2362 and has some advantages that its toxicity is not loss even in polluted water.[13] *Bs* has longer duration of efficacy due to persistence (present in the environment with its spore/crystal complex containing larval toxin) and recycling (replication and sporulation of this bacterium in mosquito cavaders) or their aqueous environment with subsequent larvicidal activity in the same habitat.[12]

TAXONOMY OF *BS*

The name was coined by Neide in 1904. *Bs* is an aerobic, rod-shaped, endospore forming Gram positive soil bacterium, producing terminal spherical spores[14] belonging to the fam-

Kingdom	:	Bacteria
Phylum	:	Firmicutes
Class	:	Bacilli
Order	:	Bacillales
Family	:	Bacillaceae
Genus	:	*Bacillus*
Species	:	*sphaericus*

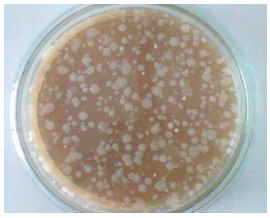

Figure 1. *B. sphaericus* isolate from Mizoram.

ily Bacilliaceae, commonly isolated from the soil[15] also found in water and other substrates in nature[13].

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *BS*

Many strains of *Bs* grow with acetate as the only major source of carbon which is available in soil and decaying plant material. Most of these strains also require biotin or thiamine, or both, for growth, and some are stimulated additionally by glutamate. *Bs* was found to grow poorly on glucose when provided as sole carbon source, which is a confirmatory biochemical test to identify the species (Table 1).[14] This bacterium was found to be unable to transport glucose or sucrose into the cell and it lacked glucokinase and hexokinase activities, phosphoglucoisomerase, phosphofructokinase and glucose-6-phosphate dehydrogenase. They are unable to ferment glucose, denitrify, or reduce nitrate to nitrite. Extracellular enzymes such as amylase, gelatinase, chitinase, and lecithinase are lacking.[15] It was found that *Bs* was able to grow on citrate and 5% NaCl, the cultured colony turned from red to purple which indicates oxidase activity, and the presence of bubbles in the colony indicates catalase activity. Moreover, all the other biochemical tests were found negative (Table 1). *Bs* can be identified by performing different biochemical tests (Table 1) and formation of terminal spherical spores, long rod, motile white/creamy mucoid colony (Fig. 1) and gram positive.[16]

ISOLATION OF *BS* FROM SOIL SAMPLES

Soil samples were mixed in NaCl (0.85%) solution and submitted to thermal shock (80° C, 12 min; ice, 5 min). Aliquots of the solution were placed on plates in a nutrient agar medium (meat extract 3 g/l, peptone 5 g/l, and agar 15 g/l) and incubated at 30°C for 48 h. Colonies were identified by morphology of spores and by observation on a phase contrast light microscope.[17] Medium A3 (*Bs* specific media) was used for isolation; it contained 5 g of sodium acetate trihydrate per liter (37 mM acetate) unless stated otherwise. Supplements (when added) are (milligrams per liter): L-glutamate, 1,000; thiamine, 10; biotin, 0.001 (Fig. 1).[18]

Growth/culture media

Bs strains can be cultured in MBS[19] and NYSM media,[20] their composition (Table 2). Culture strains reached stationary growth phase at 12-14 hr, and completed sporulation at 24 hr (more than 10^9 cells per ml), with many of the sporangia lysed, liberating free spores with attached parasporal bodies. At 15°C, 20°C and 30°C, a sporulation yield of >95% was achieved. However, at 40°C *Bs* grew only vegetatively.

BIN AND MTX TOXINS

The insecticidal activity of *Bs* is due to a binary toxin protein crystal (Btx/Bin) and mosquitocidal toxin (Mtx). Btx is absent during exponential-growth phase and forms during stage III of sporulation and is located next to spore within exosporium,[14] and Mtx is synthesized during exponential-phase growth and is proteolytically degraded as the cells enter the stationary phase. Many high-toxicity strains synthesize both Mtx and Btx toxin, while others synthesize only the Btx toxin. Low-toxicity strains synthesize only Mtx or neither toxin.[21] The crystal toxin is made up of two polypeptides with molecular weights of about 51 kDa (Bin B) and 42 kDa (Bin A). The different Mtx toxins have molecular masses of protein 100 kDa (Mtx1) and 32 and 36 kDa (Mtx2 and Mtx3) are expressed during the vegetative growth phase. Unlike the Bin toxin, Mtx do not form crystals and, therefore, are degraded quickly upon synthesis during the vegetative stage but are not as toxic as the Bin toxin.[22] The distribution of toxic gene in some strains of *Bs* is shown in

Table 1. Colony morphological, physiological and biochemical characters of *Bacillus sphaericus*[a,b].

Character or test	*Bacillus sphaericus*
Shape (Gram staining)	Rods with terminal sphaerica spores
Spore (Spore staining)	Positive (Oval)
Crystal staining	Positive (Oval)
Sporangium	swollen
Form	Circular
Colour	White
Colony Elevation	Flat
Colony Margin	Entire
Gram stain	Positive
Methyl Red	Negative
Growth on glucose	Negative
Growth on mannitol	Negative
Growth on citrate	Positive
Vogues Proskauer	Negative
Esculin,	Negative
Tryptophan	Negative
Indole	Negative
anaerobic growth	Negative
Arginine dihydrolase	Negative
Starch hydrolysis	Negative
Growth with 7% Nacl	Negative
Casein	Positive
Urease	Positive
Oxidase	Positive
Catalase	Positive
Nitrate reduction	Negative
Mean population in Mizoram soil (CFU/0.5 gm/ml $\times 10^2$)	18.6 ± 2.14-36.2 ± 3.54
Vegetative cells (length - µm)	4.35 ± 1.99-6.52 ± 2.98
Vegetative cells (Breadth µm)	2.44 ± 1.12-2.99 ± 1.35
Spores (length - µm)	0.86 ± 0.05-2.78 ± 0.19
Spores (Breadth - µm)	0.19 ± 0.01-1.56 ± 0.46
Crystals (length - µm)	0.85 ± 1.02-3.15 ± 0.74
Crystals (Breadth - µm)	0.26 ± 0.51-3.48 ± 0.05
Larvicidal toxicity	
Culex quinquefasciatus	85-98%
Anopheles stephensi	75-80%

[a]Soils samples (25 nos.) were collected from Tanhril, Lengpui, Chawnpui, Ramrikawn, Kanan, Vaivakawn, Dawrpui, Zonuam, Luangmual, Chawlhhmun, Zotlang, Tuivamit, Chanmari West.
[b]Standard strain Bsp – 1593 was used as a reference.

Table 2. Composition of MBS and NYSM media.

Component	Concentration (g/l)
MBS Medium	
Tryptone	10.0
Yeast extract	2.0
$MgSO_4$	0.3
$CaCl_2$	0.2
$Fe_2(SO_4)_3$	0.02
$MnSO_4$	0.02
$ZnSO_4$	0.02
NYSM Medium	
Nutrient broth	8.0
Yeast extract	0.5
$MgCl_2.6H_2O$	0.2
$MnCl_2.4H_2O$	10.0 mg
$CaCl_2.24H_2O$	0.1

Table 3. Highly toxic strains contain both Mtx and Btx gene while less toxic strains lack both of them.[21]

MODE OF ACTION

When the crystal is ingested by mosquito larvae, the protein crystal matrix (parasporal matrix) is dissolved in the anterior stomach, midgut proteinases and alkaline pH (pH 9-10) slowly convert protoxin 42 to a 39 kDa active form, and rapidly cleave protoxin 51 to a 43 kDa active form. Both proteins are needed for larval toxicity. The 51 kDa acts as a binding protein, enabling the entry of the 42 kDa protein into the midgut cells of the larval gut. It is modified by the larval gut proteases (consisting of chymotrypsin like and trypsin like enzymes, which remove six additional amino acids from the N terminus and approximately 20 amino acids from the C termi-

Table 3. Origin, serotype and distribution of Btx and Mtx genes of *Bs* strains.

Strain	Origin	Serotype	Btx gene	Mtx gene	Strain	Origin	Serotype	Btx gene	Mtx gene
K	US	1a	-	+	SSII-1	India	2a2b	-	+
Q	US	1a	-	+	1889	Israel	2a2b	-	+
9002	Indonesia	1a	+	+	1883	Israel	2a2b	-	+
9201	Indonesia	1a	+	+	4b 1	Nicaragua	2a2b	-	-
9301	Indonesia	1a	+	+	LP24-4	Singapore	2a2b	-	-
BS 197	Indonesia	1a	+	+	LP35-6	Singapore	2a2b	-	-
BDG2	France	3	-	-	17N	Caledonia	2a2b	-	ND[b]
SL 42	US	3	-	-	COK 1	US	2a2b	-	-
IAB 881	Ghana	3	+	-	K 8908	Indonesia	2a2b	-	-
LP1-G	Singapore	3	+	-	1593	India	5a5b	+	+
LP7-A	Singapore	3	+	-	1691	ElSalvador	5a5b	+	+
LP12-AS	Singapore	3	+	-	2017.3	Romania	5a5b	+	+
LP14-8	Singapore	3	+	-	2362	Nigeria	5a5b	+	+
LP20-e	Singapore	3	+	-	2317.3	Thailand	5a5b	+	+
IAB 59	Ghana	6	+	+	2500	Thailand	5a5b	+	+
BM1	US	6	+	+	BSE 18	Scotland	5a5b	+	+
S06 015	Iraq	6	-	-	COK 31	Turkey	9a9c	-	+
IAB 481	Ghana	6	+	+	COK 34	Turkey	9a9c	-	+
IAB620.1	Ghana	6	+	+	2173	India	26a62b	-	-
IAB 460	Ghana	6	+	+	2315	Thailand	26a62b	-	-
B55	Indonesia	6	-	-	2377	Indonesia	26a62b	-	-
2279	Sri Lanka	25	-	-	LB 29	CZ	26a62b	-	-
2627	Israel	25	-	-	BM2	US	26a62b	-	-
IMR 6	Malaysia	25	-	-	S26 009	US	26a62b	-	-
1602	Canada	25	-	-	18W1.2	Iraq	26a62b	-	-
IMR 66.1	Malaysia	48	-	-	IAB 872	Ghana	48	+	+
Pr-1	Scotland	48[c]	+	+					

ND[b]-Not done, 48[c]-Allocation based on pulsed-field gel electrophoresis of *sma*-I digested chromosomal DNA.

nus), resulting in a 54-fold increase in the toxicity of the protein. The mode of action of the binary toxin in the sequence of events is given as:

(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 (Fig. 2). [23]

Bs exerts its toxic effect in the midguts of mosquito larvae. Midgut damage starts as soon as 15 minutes after ingestion of the spore

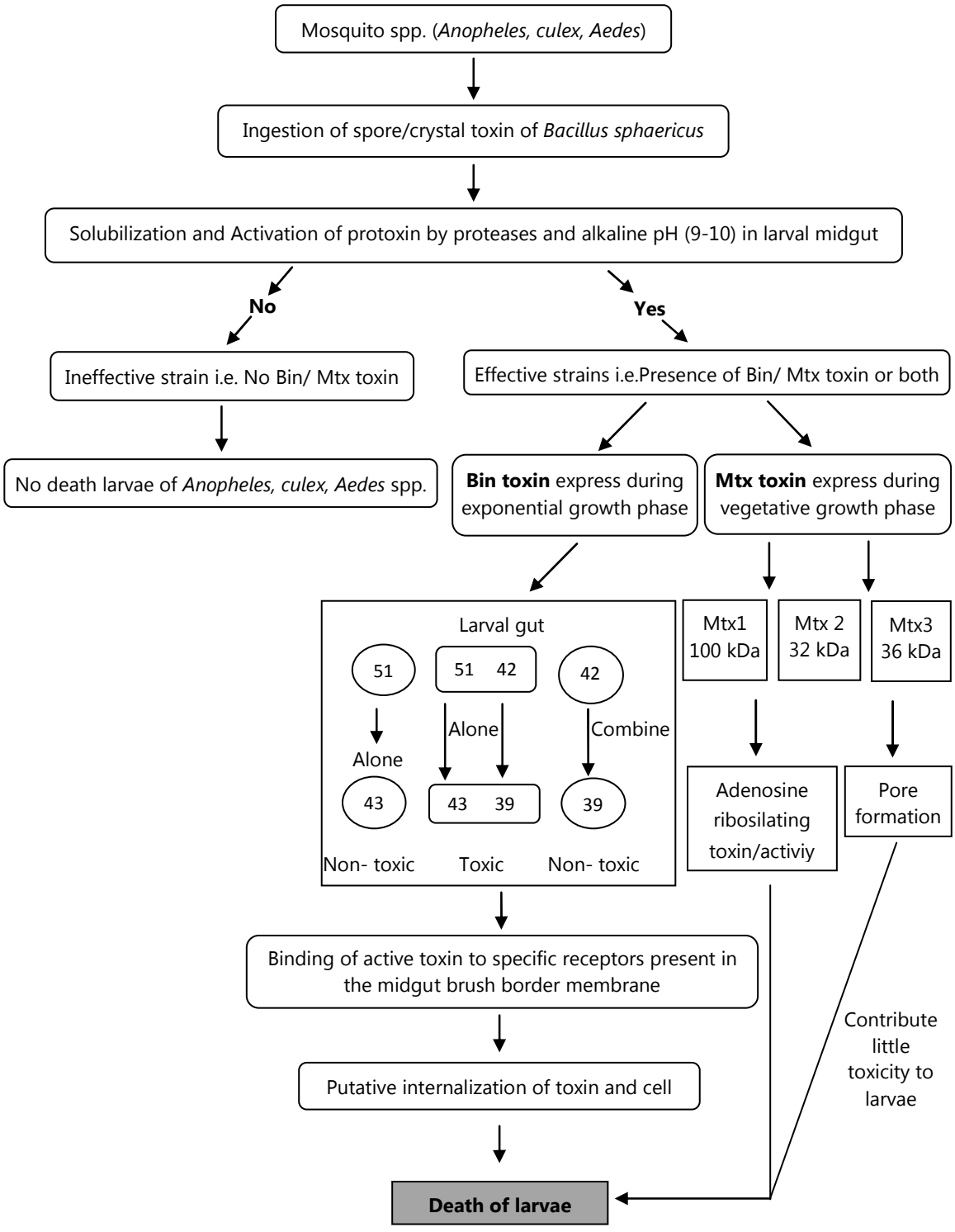

Figure 2. Mode of action of *B. sphaericus* protoxin.

Table 4. Mosquitocide proteins from *B. sphaericus*.

Mol mass (kDa)	No. of amino acids	Mol mass of protoxin (kDa)	Mol mass of gut processed or activated toxin (kDa)	Reference
51.4	448	51	43	14
41.9	370	42	39	39
100.6	870	100	?	40

Table 5. Different serotypes of *B. sphaericus*.

Type	Serotype	Toxicity
1	1a	Less
	3	High
	6	High
	48	High
2	5a5b	High
3	25	High
4	3	High
-	26a62b,9a9c	Less

Table 6. LC_{50} of some of the highly toxic strains of *B. sphaericus* against 3rd instar larvae of *Anopheles* and *Culex* spp.

Strain	LC50 (ppm)	
	Anopheles	***Culex***
IB15	0.040	0.025
S116	0.048	0.016
IB19	0.048	0.018
IB16	0.052	0.014
S265	0.057	0.017
2362	0.057	0.065

-crystal complex. Binding of the binary toxin to midgut epithelium causes lipid membrane pores causing cellular osmotic disruption, swelling of mitochondrial and endoplasmic reticula and enlargement of vacuoles, followed by lysis of epithelial cells, midgut perforation, and the death of larvae.[24] Late damage to neural tissue and skeletal muscle are also reported. Mtx is synthesized during exponential-phase growth and has low toxicity.[10] Mtx1, Mtx2, and Mtx3 genes which share sequence homology with the family of bacterial adenosyl-ribosylating toxins. Mtx1protein (100 kDa) has ADP-ribosylating activity and is responsible for several morphological changes and mortality especially in *Culex* spp. while Mtx-2 is responsible for pore formation in larval midgut. Group II was further subdivided into groups IIA and IIB. Some strains of Group IIA can produce insecticidal protein, which are active against mosquito larvae. Mosquitocidal *Bs* strains are all found within DNA subgroup IIA and in association with nine serotypes (H1, H2, H3, H5, H6, H9, H25, H26 and H48). *Bs* is highly toxic to *Culex* and *Anopheles* spp., but less toxic to *Aedes* spp. Some strains show high toxicity to *Culex* and *Anopheles* spp. while others strains show high toxicity to *Aedes* spp. (Table 5).

Larvicidal activity is not present in all strains and those which are effective against larvae can be sub-divided according to their degree of toxicity. All highly toxic strains contain a parasporal crystalline inclusion composed of a protein which is solubilized under alkaline conditions,[14] whereas strains with low toxicity lack a crystal. *Bs* strains are classified into 4 types depending on toxicity (presence or absence of Btx gene and Mtx gene). Analysis of DNA homology between strains indicated five major groups (I to V), each probably corresponding to a separate species (Table 5). Lethal concentration (LC_{50} ppm) of some of the highly toxic strains of Bs against third instar larvae of *Anopheles* and *Culex* spp.[10] are given in Table 6. The LC_{50} ranged between 0.040 and 0.057 ppm for *Anopheles* sp. and 0.014 and 0.065 ppm for *Culex* sp.

Table 7. A review of field tests of *B. sphaericus* (Strain 2362) against mosquito vectors.

Mosquito species	Habitat (country)	Product used	Effective dose	Duration of control	Reference
An. gambiae and *C. quinquefasciatus*	Irrigation ponds *(Anopheles),* sewage ponds, gutters *(Culex)*	Vectolex-G (ABG-6185) granule	10–30 kg/ha	5–7 days	33
	Swamps and rice fields in Suburban village (Kinshasa, Zaire)	Same as above	10 kg/ha	7 days	26
	Ponds (village, Senegal)	Spherimos FC and locally produced granular form compared in both studies	30 L/ha for FC, 30 kg/ha for granules	15 days (granules), 5 days (FC) for Senegal study	35
	Rain puddles *(Anopheles),* cesspits *(Culex)* Ouagadougou, Burkina Faso)	ABG 6185 granule		10 days for both forms for Burkina Faso study	25
	Ditches, puddles and naturally flooded areas in periurban Maroua, Cameroon	Suspension	10 kg/ha	Not measured (6 months)	27
An. arabiensis	Natural pools, rice fields, man-made ditches-highlands, Madagascar	ABG 6185 granule	2.5–18 kg/ha	Less than 5 days	36
An. albimanus, *C. quinquefasciatus* and *Ae. taeniorhynchus*	Ponds, dams, river, and water pits – Santa Cruz del Norte, Cuba	Liquid formulation	100 L/ha (using backpack sprayer/plane)	Up to 5 months in water without current	37
An. albimanus and others	Rural Peru and Ecuador	Vectobac TP	1 kg/ha	7–10 days	38
		Bactimos WP	2 kg/ha		

All field trials listed achieved 90-100% larval mortality within the first 48 hours after treatment.

A selection of recent field evaluations of *Bs* is summarized in Table 7 and includes several African studies. Skovmand and Sanogo[25] tested *Bs* granules against *A. gambiae* in rain-water puddles in urban and periurban Ouaga-dougou, Burkina Faso, and found that al-though the granules were effective in larger water bodies, the transient nature of the pud-dles, particularly during the rainy season, thwarted this effort. The *Bs* granules were found to remain active as long as 15 days in larger ponds outside a village in Senegal. In a peri-urban village near Kinshasa, Zaire, Karch *et al.*[26] found that biweekly application of *Bs* granules to rice fields and swamps caused a 13.6% decrease in the average *A. gambiae* bites to humans. Although this reduc-tion was too low to consider the *Bs* a success-ful control by itself, it suggests that *Bs* may be useful in some integrated control programs. In urban and periurban Maroua, Cameroon, Barbazan *et al.*[27] found that a large-scale *Bs* spray program targeting *C. quinquefasciatus* delayed the onset of the seasonal malaria

transmission period by two months.

The laboratory and field efficacy of *Bs* against *A. stephensi, A. culicifacies,* and other anophelines as well as *C. quinquefasciatus* has been extensively tested in India.[28] *Bs* formulations were found to be effective against *A. stephensi* and persisted two to four weeks under field conditions.[29] A large-scale trial of weekly applications of *Bs* in Panaji City achieved significant reductions in both *A. stephensi* density and malaria incidence.[30] A comparison study of the control of *A. culicifacies* and *A. fluviatilis* in man-made water containers in India found that *Bs* was superior to *Bti* in cement tanks (*Bs* activity lasted up to six weeks), but *Bti* was more persistent (one week) in ponds.[31]

Formulations of *Bs* are manufactured in the United States, Canada, Russia, India and Cuba (and possibly other countries) and are commercially available. In addition to liquid and water-soluble powder formulations that are similar to many chemical insecticides, *Bs* products available or under development include slow-release granules and briquettes. In India, Balaraman and Hoti[32] found that local production cost of *Bs* in briquette formulation was US $13.34 per batch (enough to treat 0.2 ha).

CONCLUSION

Nature represents a formidable pool of bioactive compounds and is a strategic source for new and successful pesticidal products. It may be concluded that vector control operations for the prevention and control of vector borne -diseases must be carried out at a cost not exceeding what the communities concerned can afford to allocate for such a purpose. Further inputs for developing microbial control agents should therefore be diverted to look for new agents, which have not been encountered so far to improve *Bs* through bioengineering and rDNA techniques on a priority basis. The immediate challenges are (i) to obtain/develop highly toxic strains so as to reduce the bulk of the product and the manufacturing cost, (ii) to develop a stable formulation capable of releasing the toxin in the larval feeding zone for prolonged periods which would obviate the high cost involved in frequent applications and also increase the operational efficiency and (iii) to engineer the toxin coding genes of *Bs* in alternative prokaryotic and/or eukaryotic microorganisms which can proliferate well in aquatic habitats and be readily available in the larval feeding zone. Finally, before declaring an agent as efficient, its activity should be thoroughly evaluated in proper field tests and not in simulated field tests or field conditions. Vector control operations must also be based on the ecological and population dynamics characteristics of vectors concerned. Studies should be pursued towards developing effective and environment friendly "green-technologies."

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