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Biolarvicides in vector control : challenges and prospects

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Biolarvicides, based on mosquitocidal toxins of certain strains of *Bacillus sphaericus* and *Bacillus thuringiensis* var *israelensis* H-14 (*Bti*) are highly effective against mosquito larvae at very low doses and safe to other non-target organisms. During past two decades various biolarvicide formulations produced in India and abroad have been tested at Malaria Research Centre and some formulations have undergone large-scale operational trials. Biolarvicide formulations of *B. sphaericus* are useful in the control of *Culex* and certain *Anopheles* spp, such as *An. stephensi* and *An. subpictus,* but not much effective against *An. culicifacies* and almost ineffective against *Aedes aegypti*. Repeated application of *B. sphaericus* in the same habitat, however, results in the development of resistance in larvae of target mosquitoes. In view of its low specificity for *An. culicifacies* and the potential for resistance in *An. stephensi, B. sphaericus* has limited prospects for control of malaria vectors. However, with some resistance management, *B. sphaericus* can still be used against *Culex* mosquitoes. On the other hand *Bti* formulations, which have broader spectrum of activity against *Aedes, Culex* and *Anopheles* spp, have not shown significant development of resistance in mosquitoes but their activity in field, particularly against surface feeding anopheline larvae is affected by various bioenvironmental factors, thus requiring weekly application in most habitats. To overcome this problem development of slow release formulations and genetically engineered biolarvicides by transplanting mosquitocidal toxin genes of *Bti* and *B. sphaericus* in some other environmentally compatible organisms have been investigated by different scientists.

Key words Biolarvicides – *Bacillus thuringiensis israelensis* – *B. sphaericus* – limitations & prospects – mosquito larvae – vector control

Extensive use of chemical insecticides against vector mosquitoes, for the control of malaria and other mosquito borne diseases, for about four decades, have caused development of resistance in vector mosquitoes to these insecticides and hazards to the environment. In spite of the sustained and prolonged use of chemical insecticides, these diseases are not only still prevalent but also outbreaks into epidemics. Therefore, to minimise the dependency on chemical insecticides, efforts have been made for the search and development of alternative methods for the control of vector mosquitoes. In this respect various biological control agents have been thoroughly investigated with the support of United Nations Development Programme/World Health Organization Special Programme for Research and Training in Tropical Diseases (WHO/TDR). Certain strains of bacteria, especially *Bacillus thuringiensis* var *israelensis* (*Bti*) and *B. sphaericus* have been found to be highly effective for the control of larvae of mosquitoes and some other dipterans. These bacterial agents have been developed as larvicides, which are commonly known as biocides or biolarvicides. These biolarvicides are highly effective against mosquito larvae at very low doses and completely safe to other non-target organisms, environment, man and wild life, and are suitable for

community use. During past two decades various biolarvicide formulations produced in India and abroad have been tested at Malaria Research Centre (MRC), and some of these formulations have undergone large-scale operational trials. Though some of these biolarvicides have been found highly effective against target mosquito vectors and can be used as eco-friendly alternatives to synthetic chemical insecticides, there are many limitations in their usage. Based on the experience of trials carried out at MRC, challenges and prospects for the use of biolarvicides in vector control has been discussed in this paper.

Characteristics of biolarvicides

Bacillus thuringiensis var *israelensis* (*Bti*): *Bti*, an aerobic spore forming, entomopathogenic bacterium specific to dipterans (particularly against Culicidae and Simulidae) was isolated for the first time in 1976 in Israel[1]. At present, it is regarded as the most promising microbial control agent against mosquitoes and black flies, which can be used alone, or as a component in integrated vector control programme. It is a gram-positive bacterium, which grows in culture in chains of 3–3.5 μm long cells. During spore formation a protein inclusion is formed in the cell as a crystal (para sporal body), which is composed of several distinct proteins ranging in size from 27 to 138 kDa and are commonly called as delta-endotoxins[2]. Though all of these purified proteins are mosquitocidal, they alone are not as toxic as the intact spore-crystal. The high toxicity of the whole spore crystal complex is due to a synergistic interaction between the 25 kDa protein (proteolytic product of the 27 kDa protein) and one or more other proteins[3]. When the spore-crystal of *Bti* containing toxic proteins (protoxins) is ingested by larvae of a susceptible species, the pro-toxins are solublised in alkaline pH of the larval gut and get activated in the form of toxins. The primary target of these toxins is the plasma membrane of the midgut epithelium. The interaction of *Bti* toxin with specific receptors in plasma membrane, cause a detergent-like rearrangement of the lipids, leading to disruption of membrane integrity and cytolysis.

Bacillus sphaericus (B. sp.): Certain strains of *B. sphaericus*, another spore forming aerobic bacterium, are also highly insecticidal against mosquito larvae[4]. The first insecticidal strain of *B. sphaericus* was isolated in 1965 from USA[5]. The various strains of *B. sphaericus* are divided into different serotypes based on H-antigen. The most insecticidal strain belongs to serotype H5a & 5b (strain 1593, 2362). *B. sphaericus* grows in culture as rods of 2–3 μm length which form sphaerical spores during sporulation at the end of the rod. Insecticidal protein is located in the spore wall and also in a granule, analogous to the crystal inclusion of *Bti*. The strains, which are less insecticidal, lack this crystal. The major components of the crystal are two proteins—51 and 42 kDa which act as binary toxins as both the proteins are jointly required for toxicity [6]. Solublisation of the crystal with alkali reduces its toxicity. Like *Bti*, the mode of action of *B. sphaericus* is through larval gut. After the crystal-spore cell is ingested by susceptible mosquito larvae, the inclusions are rapidly solubilised in the larval mid gut by alkaline pH. The 51 and 42 kDa proteins which act as protoxins are processed (activated) to 43 and 39 kDa proteins respectively. These protein toxins bind to the cells of the gastric caecum and posterior mid-gut. Symptoms of intoxication start appearing with in 30–60 min by some unknown mechanism. In case of *B. sphaericus,* in contrast to *Bti*, there does not appear to be a general dissolution of mid-gut cells. The specificity of the *B. sphaericus* toxin is in part due to differences in the number of binding of target sites[6]. The binding of the protein toxin to the gastric caecum and posterior mid-gut has been observed in *Culex pipiens* (a susceptible species) but not in the resistant *Aedes aegypti*.

Efficacy of biolarvicides: The efficacy of *B. sphaericus* and *Bti* preparation against mosquito larvae depends on the formulation suited to the biology and habitat of the target mosquito species. Various formulations of *Bti* H-14 and *B. sphaericus* have been tested for their efficacy against different vector mosquitoes at Malaria Research Centre (Table 1).

Table 1. List of some biolarvicide formulations tested at MRC in field conditions

Bacillus thuringiensis var *israelensis* (*Bti*) H-14	*Bacillus sphaericus*	
Products/Formulations	Products/Formulations	Strains
BMP-144-2X AS	Biocid-S HIL-8 WP	1593M
Moskiture WP	Biocid-S HIL-9 WP	1593M
Deltafix G	Biocid-S HIL-10 Dust	1593M
Teknar HPD (liquid conc.)	CDRI WP	1593
VectoBac 12 AS	Solvay AS	2362
VectoBac G	Vectolex AS	2362
VectoBac Tablets	Vectolex G	2297
Wockhardt WP	Spherimos AS	2362
Bacticide/Bactoculicide WP	Spherix WP	B-101

AS—Aqueous suspension; WP—Wettable powder; G—Granules.

B. thuringiensis israelensis (Bti): In general, *Bti* formulations were found more effective against larvae of *Aedes* and *Culex* species than *Anopheles* spp and among the two anopheline species tested in the laboratory, *An. stephensi* was more susceptible than *An. culicifacies* to different *Bti* formulations (Fig. 1). The efficacy of different *Bti* formulations in field conditions, lasted for 2–7 days against *An. culicifacies* in fresh water pools, 2–14 days against *An. stephensi* in tanks, 2–7 days against *Cx. quinquefasciatus* in polluted pools and drains and 7–28 days against *Ae. aegypti* in desert coolers and industrial scrapes (Fig. 2).

Among different formulations tested in field conditions at MRC, bactoculicide, a powder formulation of *Bti* (strain 164) imported from Russia, was evaluated in

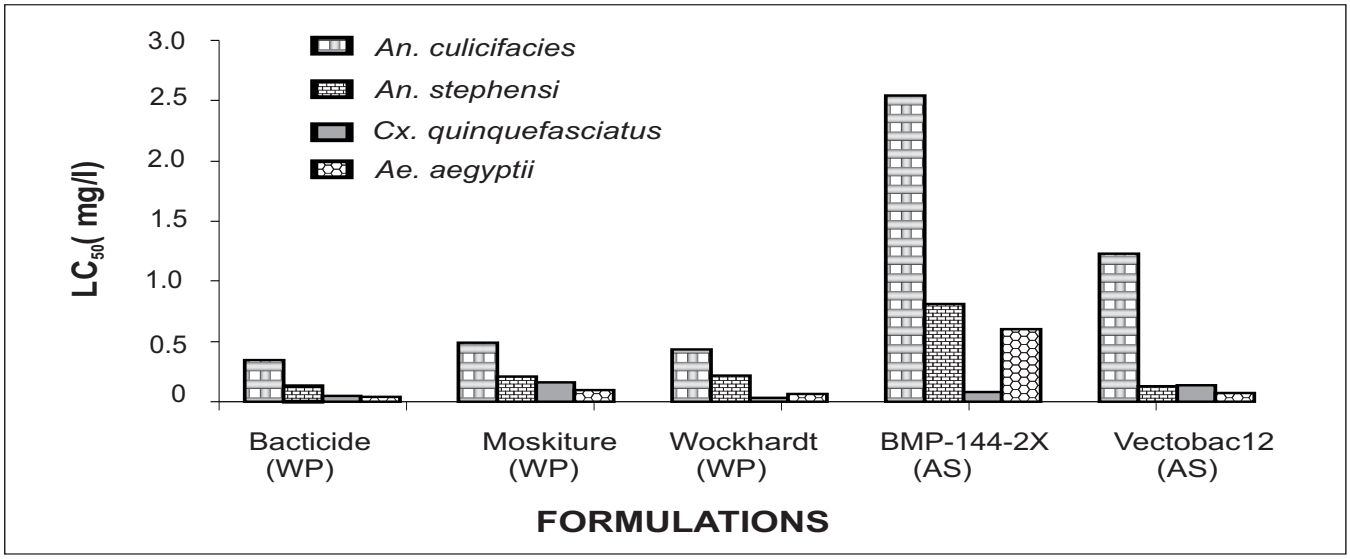

Fig. 1: Laboratory efficacy of *B. thuringiensis* H-14 (*Bti*) formulations against mosquito larvae

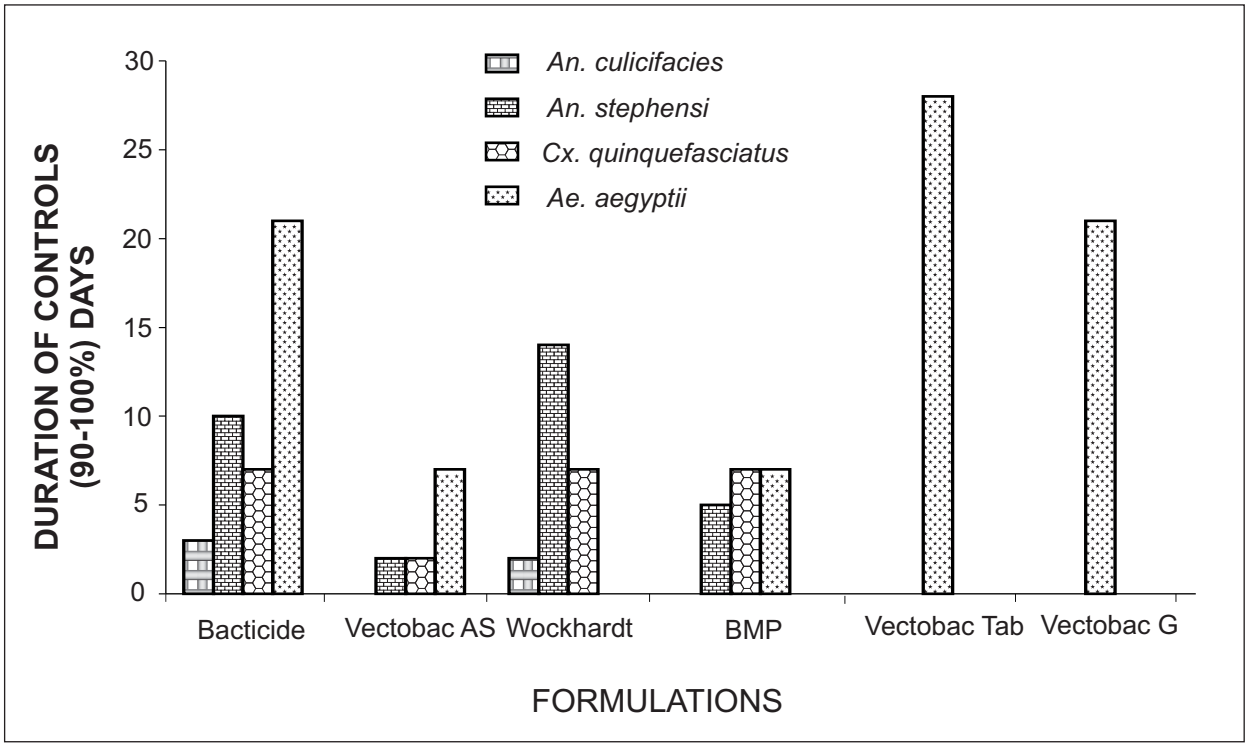

Fig. 2: Efficacy of some *Bti* formulations against mosquito larvae in field conditions

large-scale multicentric trials against *Anopheles, Culex* and *Aedes* spp, the vectors of malaria, filariasis, Japanese encephalitis (JE) and dengue respectively, in different types of breeding habitats in different areas of India[7–12]. Application of bactoculicide @ 0.5 g/m^2 (5 kg/ha) in industrial scrapes produced 100% reduction of III and IV instar larvae of *Ae. aegypti* and *Ae. albopictus* mosquitoes for 4–5 weeks[7], while against *Cx. quinquefasciatus*, 90–100% reduction was observed for 3–14 days in drains[9,11,12] . However, in fresh water pools against *An. culicifacies,* bactoculicide produced 90–100% reduction in III and IV instar larvae for 2–7 days only[10,12]. Kumar *et al*[8] showed the control of *An. stephensi* breeding in construction sites, abandoned tanks and overhead tanks by spraying bactoculicide (@ 5 kg/ha) and found that pupal production was completely checked for 3,18 and 21 days respectively in those habitats (Table 2).

Bacillus sphaericus: Though various formulations of *B. sphaericus*, both indigenous as well as imported, have been evaluated against vector mosquitoes in different habitats at MRC during past two decades[9,10,13–20], one of these formulations—Spherix (*B. sphaericus,* serotype H5a & 5b, strain B101) imported from Russia has been evaluated in large-scale multicentric trials in different parts of India[12,14,18–20]. Laboratory studies with different strains and formulations of *B. sphaericus* revealed that *B. sphaericus* preparations are more effective against larvae of *Culex* sp than *Anopheles* sp (Fig. 3). Among the two anopheline species, *B. sphaericus* formulations produced better effect against *An. stephensi* than *An. culicifacies*. However, *B. sphaericus* was not effective against *Ae. aegypti.* Similar results have been reported by others[21,22]. The lack of effectiveness of *B. sphaericus* toxins against *Aedes* species particularly *Ae. aegypti* has been reported to be due to the absence of functional receptors in this species [6,22] .

Small-scale field trials with different formulations of *B. sphaericus* against larvae of *Culex* and *Anopheles* mosquitoes carried out in different habitats, revealed

Table 2. Summarised results of field trial with Bacticide/Bactoculicide, a *B. thuringiensis* H-14 formulation applied @ 0.5 g/m^2 against larvae of different mosquito species

Mosquito spp	Habitat	Duration of impact (90–100% reduction in larval density)	Reference (No.)
Aedes aegypti *Ae. albopictus*	Industrial scrap	5 weeks	Dua *et al*[7]
Anopheles culicifacies	Fresh water, Pools	3 days	Anon[12]
An. culicifacies	Seepage irrigation channels	2–7 days	Anon[12]
An. culicifacies	River bed pools, Quarry pits	3–7 days	Anon[12]
An. fluviatilis	Tanks, Ponds	3–7 days	Shukla *et al*[10]
An. stephensi	Burrow pits, Cement drains	2–3 weeks, 1 week	Anon[12]
An. stephensi	Cement tanks, Overhead tanks	2–3 weeks	Kumar *et al*[8]
An. stephensi	Masonry tanks	7 days	Biswas *et al*[11]
An. sundaicus	Clear water pits	3 days	Anon[12]
Cx. quinquefasciatus	Drains	1–2 weeks	Kar *et al*[9]
Cx. quinquefasciatus	Pools	7 days	Anon[12]
Cx. quinquefasciatus	Blocked cement drains	3–7 days	Anon[12] Biswas *et al*[11]

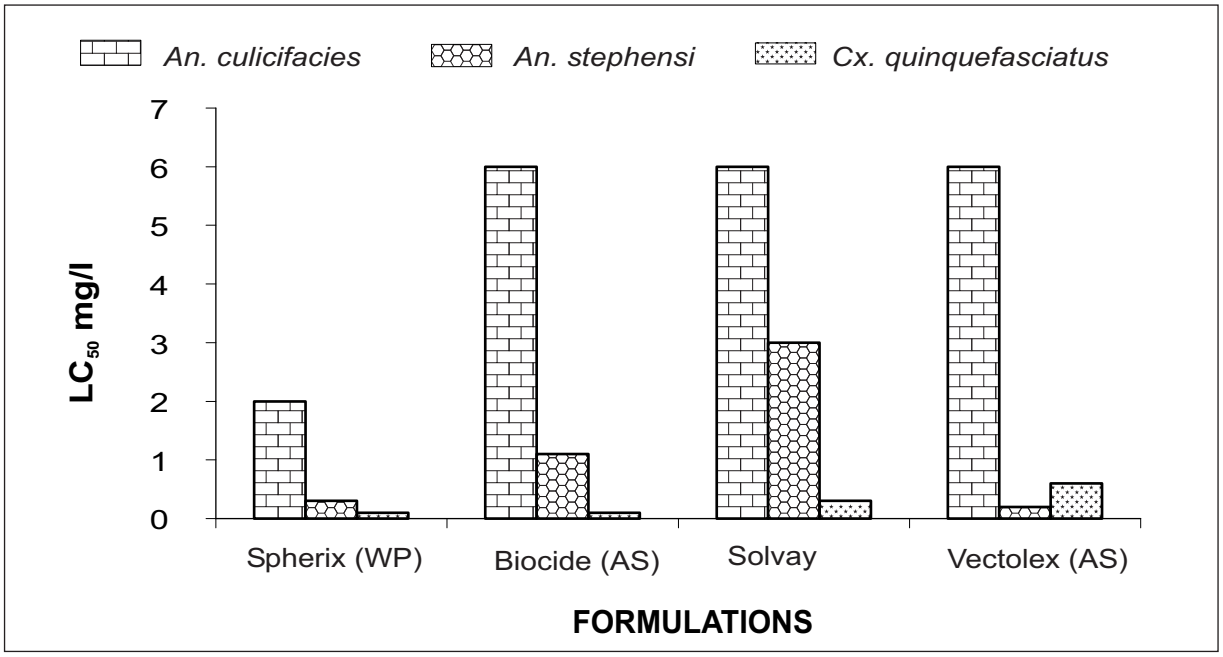

Fig. 3: Efficacy of *B. sphaericus* formulations against different mosquito larvae

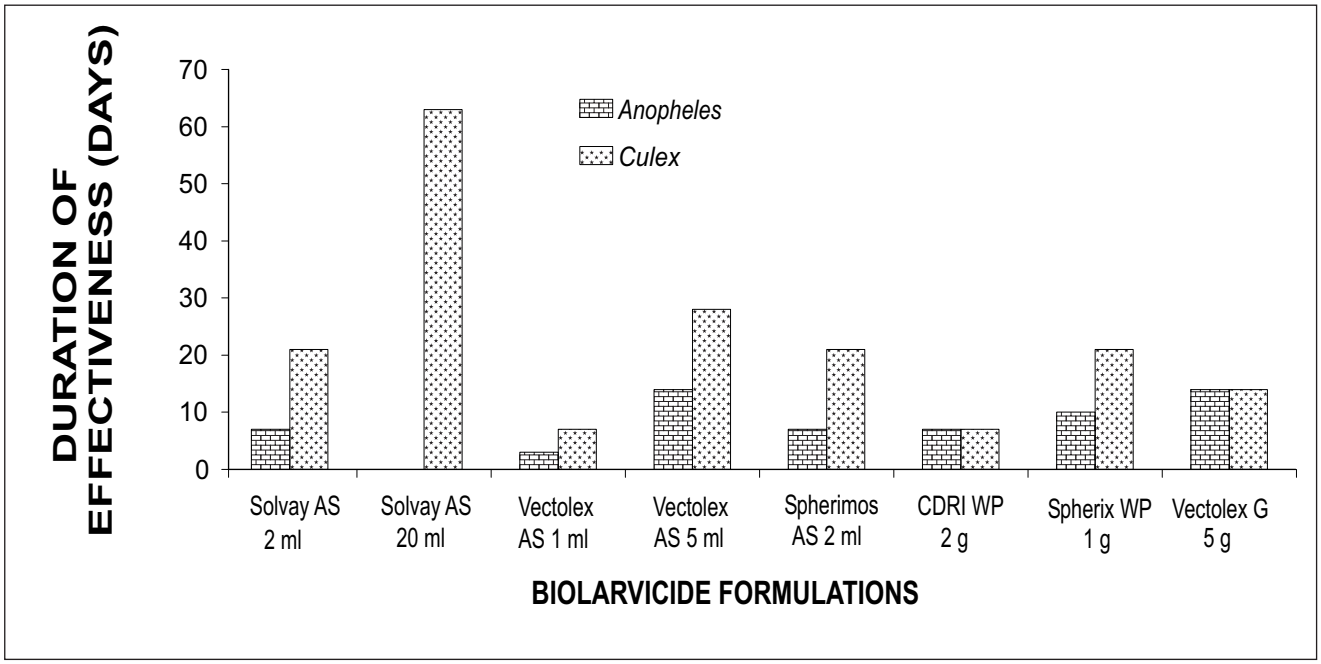

Fig. 4: Efficacy of *B. sphaericus* formulations against larvae of *Anopheles* and *Culex* species in the field trials

that the efficacy of *B. sphaericus* formulations lasted for 1–4 weeks against *Cx. quinquefasciatus* at 1–2 g/m^2 in polluted water habitats, and three days to two weeks against *Anopheles* spp in fresh water habitats[15] (Fig. 4). Ansari *et al*[16] reported 60 to 93% control of *Culex* spp larvae for three weeks in pools with a single application of solvay liquid formulation of *B. sphaericus* 2362 at a dose 2.5 ml/m^2, while the same formulation at a higher dosage (10 ml/m^2) produced 99–100% control of *Culex* spp for three weeks in pools and 84–100% control for nine weeks in unused wells. Another formulation—spherimos @ 2 ml/m^2, showed over 99% reduction in *Culex* larvae for one week in pools and for three weeks in the wells[16]. The impact of vectolex, another *B. sphaericus* formulation on *Culex* sp in the field, lasted for 2–4 weeks in the pools[16] @ 2–5 ml/m^2 and for six weeks in unused wells @ 5–10 ml/m^2. A water dispersible powder formulation of *B. sphaericus* 1593 developed by CDRI, showed 85–94% reduction[17] of *Culex* spp for 7–21 days in pits and pools at a dose of 2 g/m^2 .

Multicentric field trials with spherix carried out at different field stations of MRC (Table 3), revealed that the application of spherix at a dosage rate of 1 g/m^2 against larvae of *Anopheles* and *Culex* spp in pools, pits and drains, produced 90–100% for a period of 1–4 weeks. Large-scale trials with repeated application of spherix at an interval of 1–2 weeks produced effective control of *Culex* spp initially for few months but the impact particularly against *Cx. quinquefasciatus* started declining thereafter[9,19]. Kumar *et al*[18] demonstrated the control of *An. stephensi* and malaria in construction sites in Panaji, Goa by spraying spherix @ 1 g/m^2 (10 kg/ha) at fortnight intervals.

Potential for the development of resistance to biolarvicides

B. sphaericus: Since these bacterial agents are natural products and produce biologically degradable toxic proteins, it was initially thought that resistance to bacterial agents will not develop very fast in mosquitoes. However within a year, application of spherix (*B. sphaericus)* against *Cx. quinquefasciatus* resulted in the development of 10–155 fold tolerance in this species from different areas (Fig. 5). Further selection of these field collected strains of *Cx. quinquefasciatus*

Table 3. Summarised results of field trials with Spherix *(B. sphaericus)* formulation applied @ 1 g/m^2 against larvae of different mosquito species

Mosquito spp	Habitat	Duration of impact (90–100% reduction in larval density)	Reference (No.)
Anopheles culicifacies	Ponds	3 days	Shukla *et al*[10]
An. culicifacies	River bed pools	2 weeks	Anon[12]
An. fluviatilis	Ponds	3 days	Shukla *et al*[10]
An. stephensi	Burrow pits, Cement drains	1–4 weeks	Mittal *et al*[14]
	Cement tanks, Curing water tanks	2–3 weeks	Kumar *et al*[18]
An. sundaicus	Marshy areas	2–3 weeks	Anon[12]
Culex quinquefasciatus	Ponds, Pools, Drains,	1–2 weeks	Anon[12]
	Polluted drains, Cesspits	1–3 weeks	Mittal *et al*[14]
	Drains	2 weeks	Kar *et al*[9]
	Crowding pits, Septic tanks, Rice fields	1–2 weeks	Yadav *et al*[20]

in the laboratory with spherix at LC_{90} concentration resulted in very high degree of resistance (>100,000 fold) within 5–6 generations[23]. Inheritance studies on the nature on resistance to *B. sphaericus* in *Cx. quinquefasciatus* showed that resistance to *B. sphaericus* is genetically inherited, autosomal and recessive in nature[23]. Since then various reports of resistance to different strains and formulations of *B. sphaericus* in *Cx. quinquefasciatus* from different countries have been published [24–28], which showed that continuous exposure to *B. sphaericus* would result in the development of moderate to high level of resistance in *Cx. quin-*

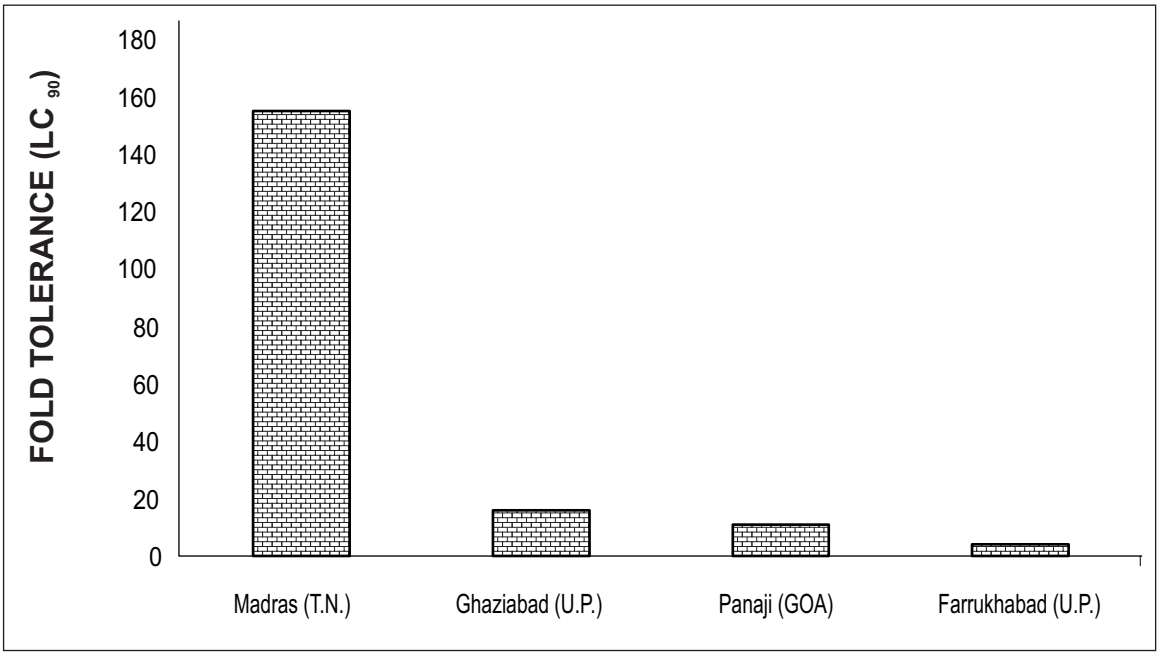

Fig. 5: Development of resistance to *B. sphaericus* in field populations of *Cx. quinquefasciatus*

quefasciatus. These studies indicate that resistance to *B. sphaericus* in *Cx. quinquefasciatus* will precipitate very fast if constant selection pressure is applied. Development of resistance to *B. sphaericus* has also been demonstrated in *An. stephensi* under laboratory selection[29].

Bti: Though there are various reports on the development of resistance to *B. sphaericus* there is hardly any report on development of resistance to *Bti* in any of the mosquito species in field conditions. Laboratory selection studies in *Cx. quinquefasciatus* showed only three fold increase in the tolerance to *Bti* after 20 generations (MRC unpublished data). Other reports have also shown similar results in *Ae. aegypti* and *Cx. quinquefasciatus*[30,31]. Gill *et al*[32], however, reported high level of resistance in *Cx. quinquefasciatus* after selection with a purified Cry IV D toxin of *Bti*, but only a slight increase in tolerance to whole complex of *Bti* toxins was observed. The complex mode of action of *Bti* may partly explain the relative absence of resistance. The lethal changes in the mid-gut cells are induced only by the synergistic effects of the different protein toxins present in the parasporal body of *Bti*. This combination reduces the likelihood of resistance.

Factors influencing efficacy of biolarvicide toxins: The efficacy of bacterial preparations against target mosquitoes is influenced by various physico-chemical and biotic factors such as temperature, water pH, sunlight, sedimentation rate of spores, organic pollution, larval stage, density, etc[21,33–36].

Temperature: Temperature is an important factor, which influences the toxicity of these bacterial preparations. The efficacy of spherix (*B. sphaericus)* especially against anopheline larvae was greatly reduced in laboratory bioassays at 21ºC as compared to 31ºC (Fig. 6), which indicated that biolarvicide will not be effective in colder months. Similar results were obtained with bactoculicide (*Bti*) formulation but with a lower degree of difference.

Water pH: In addition to temperature, pH of the water has also been found to influence the activity of bacterial preparations. Water pH higher than 10 greatly reduced the activity of spherix (*B. sphaericus*) and bactoculicide (*Bti*) against larvae of *An. stephensi* in laboratory bioassays (Fig. 7).

Exposure to sunlight: Protein toxins of *Bti* and *B. sphaericus* and highly sensitive to UV radiations (sunlight) which reduces the activity of biolarvicides. In laboratory bioassays, activity of spherix and bactoculicide against larvae of *An. stephensi,* was reduced in experimental bowls kept under direct sunlight for six hours as compared to those kept inside the room in dark conditions for the same duration (Fig. 8).

Various other factors such as presence of organic particulate matter, stage and number of larvae and the type of biolarvicide formulation (with respect to surface or bottom feeding behaviour of larvae) also influences the activity of bacterial preparations in laboratory bioassays (unpublished data).

The residual efficacy of biolarvicides, in field conditions is also influenced by the type of formulation, vegetation and organic pollution which influences the sedimentation settling rate of spore toxin of biolarvicide. Aqueous suspension or flowable liquid formulation generally produced better results against column feeding *Culex* mosquitoes, while dust formulations or surface spreading formulations were more effective against surface feeding *Anopheles* species and granular and tablet formulations were more effective against *Ae. aegypti.*

Conclusions and future prospects of biolarvicides in vector control

Biolarvicides based on mosquitocidal toxins of *B. sphaericus* and *B. thuringiensis* H-14 have great potential in controlling the breeding of mosquito vectors of various diseases in an integrated vector control

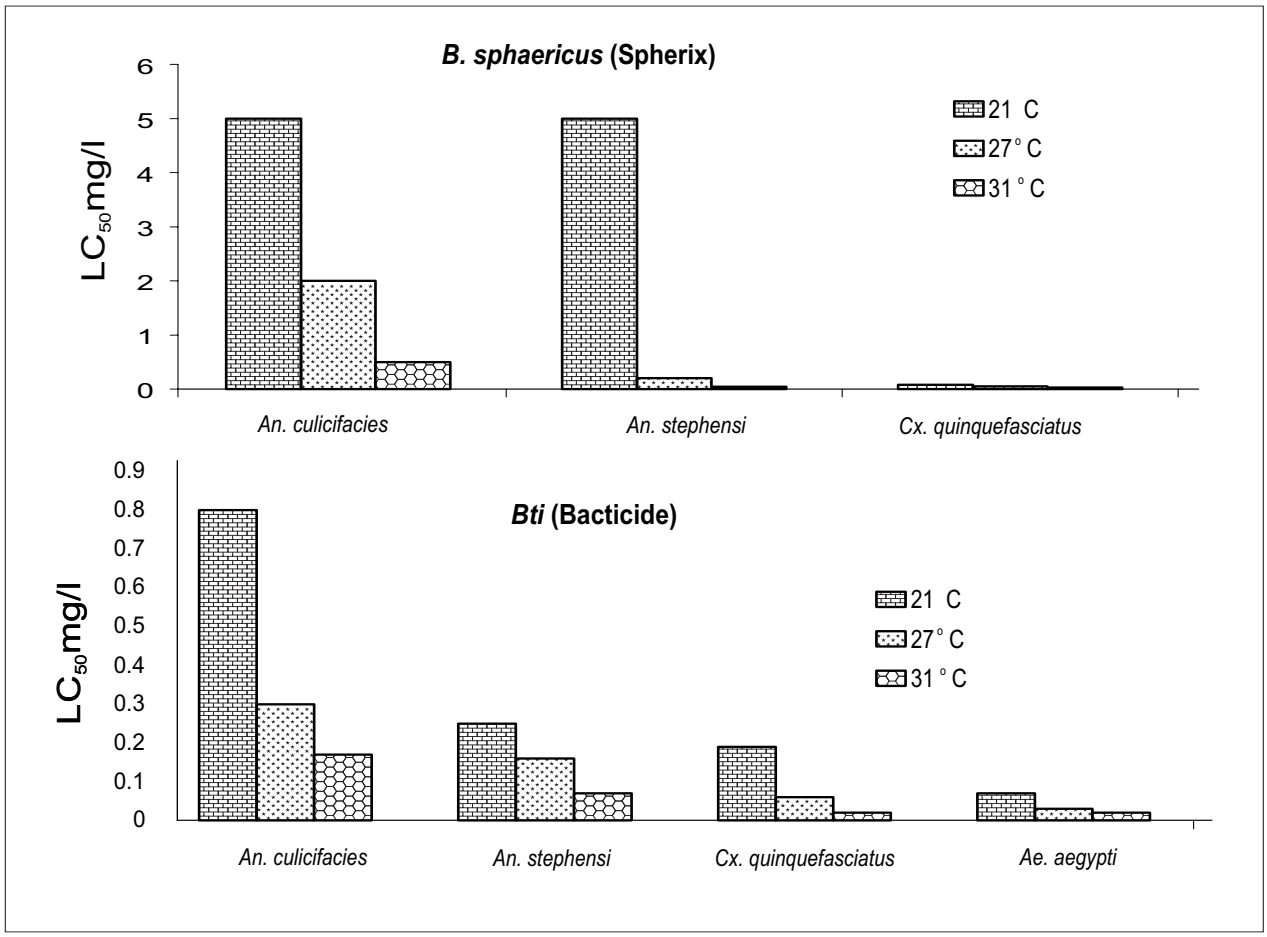

Fig. 6: Effect of temperature on activity of biolarvicides

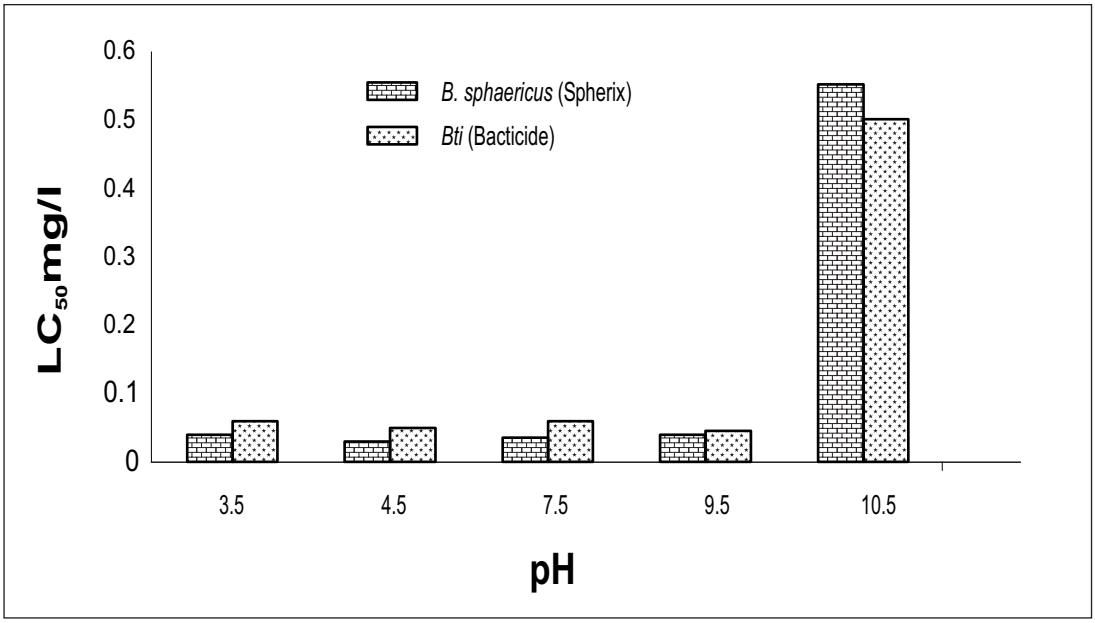

Fig. 7: Effect of water pH on the activity of biolarvicides

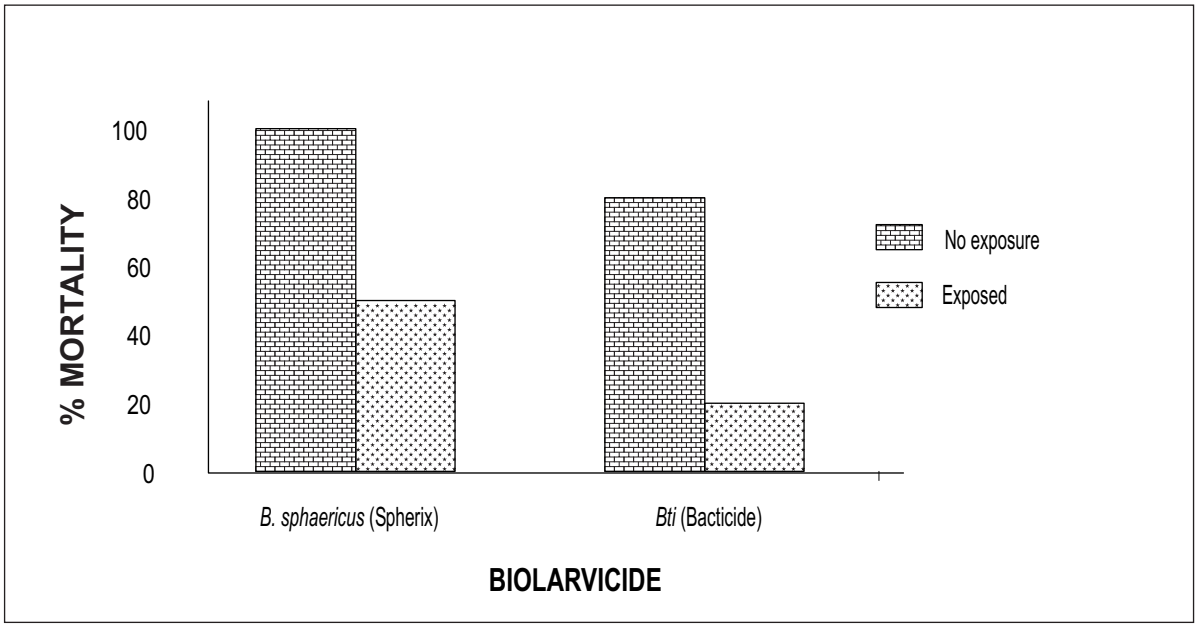

Fig. 8: Effect of sunlight on the activity of biolarvicides

programme, either independently as a larvicide or along with other biological control agents and natural predators of mosquito larvae[37], as these bacterial agents are highly specific in action against mosquitoes and are safe to other organisms. The studies carried out so far have shown that the formulations of *B. sphaericus* and *Bti*, are the larvicides of choice for the control of *Culex* and *Aedes* species, respectively. *B. sphaericus* formulations can be used for the control of culicine vectors of filariasis and Japanese encephalitis (JE) and also urban malaria vector *An. stephensi* in non potable waters, but they are not effective against *Aedes* species, while *Bti* formulations have broader spectrum of activity against vectors of malaria, filariasis, JE and dengue. *B. sphaericus* formulations have been shown to be very effective in the control of *Cx. quinquefasciatus* even in highly polluted water habitats, but continuous use of *B. sphaericus* results in the faster development of resistance in target mosquitoes. The efficacy and persitence of the larvicidal action of biolarvicides depend on various bioenvironmental factors and the type of formulation. Though, *Bti* formulations are very effective in the control of *Aedes* species, and have low potentiality for the development of resistance, their larvicidal action persists for a shorter duration against surface feeding *Anopheles* species and in polluted water habitats of *Cx. quinquefasciatus.* The utility of *Bti* formulations against malaria vectors have many limitations. The spore-crystal complex containing protein toxins is sensitive to sunlight (U-V light) and the spores of the bacilli, sediment rapidly from the larval feeding zone, thus limiting the duration of control. The spores probably do not germinate and produce fresh toxin-producing cells outside the protein rich larval cadaver.

Future prospects for the use of biolarvicide formulations against malaria vectors will depend on the enhanced activity and ability of protein toxins to persist in the feeding zone of anopheline larvae, for a longer duration, their protection from sunlight (U-V light) and low-cost of production. Development of controlled release and surface floating formulations and a combination of genetic manipulation approaches such as high level expression of toxin combination[38,39] or encapsulation of toxin in a living organism, which would retain the toxin on the water surface and also act as phagostimulant[40], might provide the solution for effective and sustainable control of surface feeding *Anoph-*

eles species[38–43]. However, the release of transgenic insecticidal organisms as larval food into the breeding habitat needs careful monitoring as they might prove to be counter productive[44].

Existing *Bti* formulations are, however, highly effective against *Aedes* mosquitoes. However, further improvement, particularly to extend their long-term effect and to enhance control, will accelerate this process further. Tablet and granule formulations of *Bti* have been developed which can be used by individuals and community particularly to control container breeding *Ae. aegypti*.

Though, *B. sphaericus* is highly effective against larvae of *Culex* species, even in highly polluted waters and its apparent longer impact on larval populations would, however, reduce the number of applications needed for satisfactory control of *Cx. quinquefasciatus,* the potential for the development of resistance in *Cx. quinquefasicatus* to *B. sphaericus* had limited its role in vector control. Also *B. sphaericus* has no activity against *Aedes* spp. However, development of genetically engineered recombinant strains by cloning of toxin genes of *Bti* and *B. sphaericus* might help in broader spectrum of activity and in delaying the development of resistance by the synergistic effect of their toxins[2,38,39]. *B. sphaericus,* however, can also be used in rotation with *Bti* to delay the development of resistance in target mosquitoes.

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