Bioefficacy of acrystalliferous *Bacillus sphaericus* M3 against field collected mosquito larvae

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Abstract: The present investigation was directed to determine the pathogenicity; bioefficacy and larvicidal activity of *Bacillus sphaericus* M3 against *Anopheline* and *Culicine* mosquitoes. *B. sphaericus* M3 showed high pathogenicity of 85.0% and 98.8% to *Anopheline* larvae, using vegetative cells or spores, respectively. In contrast, pathogenicity toward *Culicine* larvae was 45.0% and 48.8% for vegetative cells and spores, respectively. *Bacillus sphaericus* M3 possesses high larvicidal activity with LC_{50} values 6.11 x 10^3, 1.71 x 10^4 spores/ ml or 0.872, 24.4 mg/l against *Anopheline* & *Culicine* respectively.
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1. Introduction

Mosquitoes transmit some of the world's most life threatening and debilitating and viral diseases and dengue fever (principally *Aedes aegypti*). Alarmingly, these diseases are on the rise in many tropical and subtropical areas (Miller, 1992; Monath, 1994; Priest, 1992) including malaria (*Anopheles),* fialariasis (*Culex*, *Mansonia* and some *Anopheles* spp). Approaching to reducing the incidence of malaria have focused largely on controlling mosquito population with chemical insecticides (Priest, 1992) and by physical barrier methods (impregnated nets), or by using drugs to prevent infection with malarial parasites (*Plasmodium* spp.). Limited trials of a candidate malaria vaccine have received much attention, but it may be some time before this type of vaccine is adopted (Maurice, 1995). Likewise, various candidate dengue virus vaccines are being developed, but it is not known when an effective vaccine will be available (Monath, 1994; Brandt, 1990).

Biological control of mosquito larvae with naturally occurring bacteria that synthesize potent mosquitocidal toxin (Hofte and Whiteleym, 1989) has received much less attention, despite the fact these bacteria have been used safely in the field for many years (Priest, 1992). Commercial control of lepidopteron and coleopteron pests with entomopathogenic strains of *Bacillus thuringeinsis* (*Bt)* is now well accepted and its usefulness established. Moreover, control of the aquatic larvae of blackflies (the vector of the filarial parasite *Onchocerca volvulus*) with *B. thuringeinsis* subsp. *israelesis* (*Bti*) in West Africa has been hugely successful, eradicating onchocerciasis for many areas (Priest, 1992).

Realization of insecticidal resistance and environmental impact of spraying on a global basis has led to considerable resources being devoted to the search for biological control agents. However, greater success has been achieved with spore formers, especially species of genus *Bacillus*. In fact, four species of *Bacillus* viz., *Bacillus thuringiensis*, *Bacillus popillae*, *Bacillus lentimorbis* and *Bacillus moritai* account for nearly one half of all the trade name microbial products in existence (Ignoffo, 1981). Another species of spore formers evaluated as potential microbial pesticide is *Bacillus sphaericus*, which is effective against gnats and mosquitoes. Barbazan et al. (Barbazan et al., 1998) carried out studies at Maroua (North Cameroon), showed effective control of anophelines using *Bacillus sphaericus* strain 2362 larvicidal treatments at the rate of 10 g/m^2. Due to the gradual development of resistance against commercial products of *Bacillus sphaericus* (Abbott, 1925; Adak et al.,1995; Mc Gaughey and Beeman, 1988; Mc Gaughey, 1985; Tabashnik et al., 1990) in mosquitoes and instability of these products in environmental conditions, further research in this particular area is needed. Promising new formulations of the microbial larvicides *Bacillus sphaericus* (*Bs*) and *B. thuringiensis var. israelensis* (*Bti*) have recently been shown to give excellent control of the major vectors of malaria in Africa (Fillinger et al., 2003; Fillinger and Lindsay, 2006). Use of these biological control agents is better than chemical larvicides since they are very species specific, environmentally safe

(W.H.O., 1990) and appear not to induce resistance when used together (Mulla et al., 2003).

In view of the above limitations, it is desirable to screen potential pathogenic microbes for the potential insect control. The present investigation was, therefore, undertaken to test the pathogenicity & to determine the LC_{50} value of *Bacillus sphaericus* M3 against *Anopheline* and *Culicine* mosquitoes.

2. Material & Methods

Bacterial strain

The *Bacillus sphaericus* M3 is a acrystalliferous (crystal-minus) strain was grown in 'St. Julian' medium consisting of (g/l) glucose – 2.0; KH_2PO_4 – 3.0; tryptone – 5.0; yeast extract – 15.0; Benzyl Penicillin - 10 (µg/ml); Agar – 20.0; pH – 7.2-7.5 (St. Julian et al., 1963).

Mosquito larval culture

Field collected second, third and fourth instar larvae of *Anopheline* and *Culicine* mosquitoes were used for bioassay. Sampling of *Anopheline* and *Culicine* larvae was made during the rainy season from the infected water storage tank in and around Pantnagar and Haldwani in Nainital district of Uttarakhand ($29^0$55' N and $70^0$44'E; 400amsl) (Bisht et al., 1996). Larvae were identified by the courtesy of Dr. B. P. Shukla, O/I, Malaria Research Center, Haldwani in their laboratory. Healthy larvae were collected on the same day of two days prior to the conduction of experimental studies.

Seed inoculum preparation

Seed inoculum of *B. sphaericus* M3 was prepared for bioassay as per Collier (1957) active culture technique and modified by Halvorson (1957) by the resuspension of an inoculating loop of spores from a plate, in 10 ml sterile water, followed by a heat shock at 80°C for 30 min to eliminate vegetative cells. This preparation was then inoculated into 90 ml St Julian medium and grown at 28±2^0C and 200 rpm on gyratory shaker, with samples taken every 4 h to monitor growth phases, and bioassays of representative samples were carried out.

Mass spore production in roux bottle

One ml of the activated seed inoculum of *B. sphaericus* M3 consisting of vegetative cells (100%) was transferred to each 500 ml roux bottle containing St. Julian agar, tilted upward and downward for proper spreading of culture and incubated at 28±2^0C for 48 h for complete sporulation. The spore population of *B. sphaericus* M3 was determined by heat stable counts (HSC) and maintained 10^{10}- 10^{11} spore/ml. For insect feeding one ml spore culture from roux bottle was taken and diluted hundredfold. The undiluted culture consisted of spore in order of 10^{10}-10^{11} HSC/ml.

Preparation of test solution of standard "Spharix" powder

500 mg of Spharix, a carrier based formulation of *Bacillus sphaericus* B101 obtained from ICMR, Govt. of India, New Delhi, was placed in 10 ml sterile distilled water contained in 50 ml flask and thoroughly homogenized by shaking at 200 rpm for 15 minute. From this homogenate a stock solution was made in test tube by adding 1 ml of homogenate to 9 ml sterile distilled water and agitated on vortex for few seconds. The concentration in stock solution was 5 mg standard/ml (WHO, 1990).

Preliminary pathogenicity test

Preliminary pathogenicity test was carried out by taking 10 ml of spores and vegetative cells were suspended in 90 ml of water placed in 250 ml beaker, appropriate dilutions were made to achieve a population of spore 10^8-10^{10} and vegetative cells in the range of 10^4-10^7 cfu/ml. For each assay, equal number (20 each) of II, III and IV instar larvae of a susceptible *Anopheline* or *Culicine* colony were placed in 100 ml natural water contained in 250 ml beaker and different bacterial dilutions were added. Each dilution replicated four times with three controls C1-media; C2-Tap water and C3- natural water. At least five concentrations giving mortality between 2 and 98% were tested, and mortality was recorded after bioassays. After the treatment larvae were considered dead if they were unable to return to surface after being forced to the bottom. Mortality was counted after 24 h (Abbott, 1925; Bisht et al., 1996).

Bioefficacy & determination of LC_{50}

Bioefficacy of *B. sphaericus* M3 was performed in quadruplet in 100 ml natural water placed in 250 ml beakers containing equal number (20 each) of II, III, IV instar larvae of *Anopheline sp.* or *Culicine sp.* and incubated at 28+2°C. A small amount of yeast powder was spread on the surface of water as larval diet. Four dilutions of spores of *B. sphaericus* M3 were assayed and undiluted vegetative cells were used for comparing with undiluted spores. The numbers of surviving and dead larvae were counted at an interval of 24 h after inoculation and each treatment was replicated in quadruplets keeping three controls same as in the pathogenicity test (Ramoska and Hopkins, 1981) $LC_{50's}$ were determined using sporulating cultures from roux bottles. For obtaining maximum spore population in a treatment, undiluted cultures were

used subsequently 10- fold dilutions were prepared to achieve the various spore populations. Each test was replicated four times at six different dilutions in addition to the controls. Abbott's Formula (1925) determined the corrected percent mortality. The lethal concentration required to kill 50% of the test population was subjected to probit analysis with the help of log dose and probit mortality. The LC_{50} was determined by dose mortality regression line plotted on log probit paper and the 95% confidence (Litchfield and Wilcoxan, 1949).

3. Results and Discussion

Thus realizing the above merits, the present investigation was undertaken to evaluate a novel, acrystalliferous *B. sphaericus* M3 for pathogenicity to mosquito larvae. Preliminary pathogenicity tests of *B. sphaericus* M3 against *Anopheline* and *Culicine* mosquitoes were conducted during the month of July - September 1996.

The mortality data indicated that spores as well as vegetative cells of M3 were highly pathogenic recording 98.8% and 85.0% mortality respectively to *Anopheline* as compared to *Culicine* showing 48.8% and 45.0% mortality. Mortality ranged from 0 – 7.5% for three controls (Figure 2 (a-d) & Table 1). The data also indicated that a cell population in the range of 10^6 –10^8 cfu/ml to achieved higher mortality.

Table 1. Pathogenicity of *Bacillus sphaericus* M3 against larvae* of *Anopheline* and *Culicine* mosquitoes after 24h at temperature 28±2ºC

Stage of test organism	cfu/ ml	Target mosquitoes	Percent Mortality
Spores	10^8–10^{10}	*Anopheline*	98.8
		Culicine	48.8
VC	10^4-10^7	*Anopheline*	85.0
		Culicine	45.0
C_1	ND	*Anopheline*	0.0
		Culicine	5.0
C_2	ND	*Anopheline*	0.0
		Culicine	3.5
C_3	ND	*Anopheline*	3.8
		Culicine	7.5

VC - Vegetative cells; C1 - Normal water Control; C2 - Distilled water control; C3 - Growth Media control; * - Mixture of equal number (20 each) of II, III & IV instars larvae; ND - Not Detected

On a comparative basis it is concluded that spores were more effective than the vegetative cells. The results obtained from the above preliminary trial demonstrated that *B. sphaericus* M3 spores had significantly higher mosquitocidal activity against *Anopheline* followed by the vegetative cells. These results confirm to the findings of several workers using crystalliferous and noncrystalliferous *B. sphaericus* M3 (Davidson and Yousten, 1990; Davidson, 1984; de Berjac, 1990). It is also reported that the strains of *B. sphaericus* in which parasporal inclusions were not observed, some larvicidal activity was detected which could be due to toxin being localized in cell wall (Meyers and Yousten, 1980) or cytoplasm (Davidson, 1982).

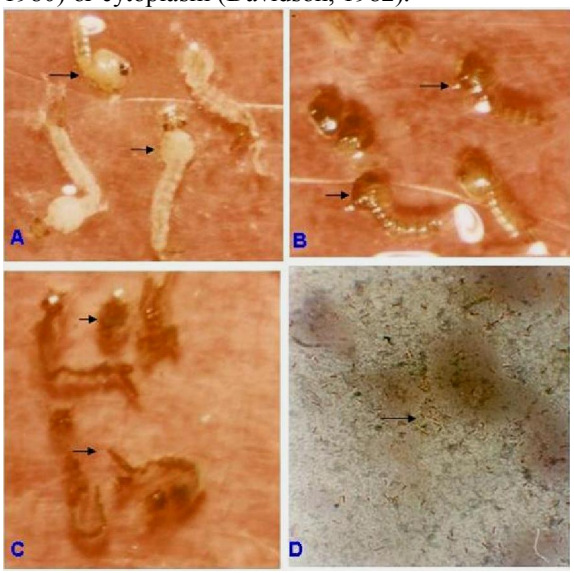

Figure 1. A) Healthy mosquito larvae, B) after 24 hrs of treatment, C) after 48 hrs of treatment, and D) presence of vegetative cells of *B. Sphaericus* M3 in the haemolymph of mosquito larvae

Based on the preliminary trail established the pathogenicity of *B. sphaericus* M3, it was thought worthwhile to examine the period of exposure with a view to achieve maximum mortality of mosquitoes under study.

Bioefficacy of *Bacillus sphaericus* M3 in term of exposure time is presented in Table 2 showed that a considerably higher spore population (10^9 – 10^{11} cfu/ml) of strain R3 resulted in 100 and 82.8% mortality against *Anopheline* and *Culicine* respectively with an exposure time of three days. Even the vegetative cells with a low population of 10^4 – 10^6 cfu/ml were found to be also effective at the end of similar period of exposure as evident from mortality data of 96.3 & 62.3% for *Anopheline* and *Culicine* respectively. Recent literature has revealed that the three mosquitocidal toxin mtx1, mtx2 and mtx3 which are expressed during vegetative growth of *B. sphaericus* are wide spread among various strains, including those with low, moderate and high toxicity (Liu et al., 1993). These two toxins do not display any similarity to each other to crystal protoxin or any other insecticidal proteins (Thanabalu et al., 1991; Thanabalu et al., 1992). The

reported mtx toxin may also be exists in the vegetative cells of *B. sphaericus* M3 which might be responsible for high mortality. There was none or very low mortality (8.8%) in controls. These results also indicated that there was a progressive increase in mortality with the time (1 to 3 day) to both *Anopheline* and *Culicine* mosquitoes in exposure to either spores or vegetative cells. (Table 2)

Table 2. Bioefficacy of *Bacillus sphaericus* M3 against larvae* of *Anopheline* and *Culicine* mosquitoes at different period of exposure.

Stage of test organism	Target mosquitoes	Cell population (cfu/ml)	Percent Mortality Exposure Time (days)**		
			1	2	3
Spores	*Anopheline*	1.96×10^9	91.3	98.8	100.0
	Culicine	1.58×10^{11}	18.8	38.8	82.8
VC	*Anopheline*	4.6×10^6	71.3	85.0	96.3
	Culicine	4.25×10^4	25.0	40.5	62.3
Control	*Anopheline*	ND	0.0	0.0	0.0
	Culicine	ND	5.0	7.5	8.8

VC- Vegetative cells; ND - Not Detected; * - Mixture of equal number (20 each) of II, III & IV instars larvae; ** - Indicative of feeding time of spores or vegetative cells

Toxicity of the *B. sphaericus* M3 was compared with the Standard Spherix powder against *Anopheline* and *Culicine* (Table 3). *B. sphaericus* M3 possesses higher larvicidal activity with LC_{50} values 6.11×10^3, 1.71×10^4 spores/ml or 0.872, 24.41 mg/l against *Anopheline* & *Culicine* respectively as that of Standard Spharix powder.

Table 3. Toxicity of *Bacillus sphaericus* M3 & Standard Spharix powder against larvae* of *Anopheline* and *Culicine* mosquitoes after 3 days of exposure at temperature 28± 2ºC

MI	TM	Heterogeneity d. f.	Heterogeneity X^2	Regression equation	LC_{50}	Fiducial Limit
BS	*A*	4	11.4	Y=5.6227 +0.4135x	6.1×10^3	0.914×10^{-2} – 0.11×10^{-6}
	C	4	9.0	Y=3.655+ 1.329x	1.7×10^4	0.47×10^{-4} – 0.32×10^{-6}
SSP	*A*	4	16.0	Y=4.6539 +0.6382x	5.9×10^{-3}	0.59×10^{-3} – 0.55×10^{-6}
	C	4	6.5	Y=4.9635 +0.2474x	4.6×10^{-3}	0.19×10^{2} – 0.51×10^{-6}

Y = Probit Kill; X = Log (concentration x 10^6)

MI – Microbial Insecticide; TM- Targeted Mosquito; BS - *Bacillus sphaericus* M3; SSP - Standard Spharix Powder; A- *Anopheline*; C – *Culicine;* d. f. - Degree of freedom; * - Mixture of equal number (20 each) of II, III & IV instars larvae

The results support the hypothesis that the implementation of large-scale application of this test strain can be applied successfully in extended floodplain areas either as liquid or as a carrier based formulation by hand, which leads to a reduction in larval abundance in the natural habitats and could be an additional tool in an IVM programme.

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