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LARVICIDAL ACTIVITY OF *Bacillus sphaericus* 2362 AGAINST *Anopheles nuneztovari*, *Anopheles darlingi* AND *Anopheles braziliensis* (DIPTERA, CULICIDAE)

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SUMMARY

In this present study, preliminary data was obtained regarding the mortality rate of the Amazonian anophelines, *Anopheles nuneztovari*, *Anopheles darlingi* and *Anopheles braziliensis* when subjected to treatment with *Bacillus sphaericus* strain 2362, the WHO standard strain. Initially, experiments were conducted to test the mortality rate of the three species of anopheline larvae. The third larval instar of *An. nuneztovari* and the second and third larval instars of *An. darlingi* proved to be the least susceptible. In other experiments, the same three mosquito species were tested with the standard strain 2362, *An. nuneztovari* was the least susceptible to this insect pathogen, while *An. braziliensis* was the most susceptible. This latter species showed a difference in the level of LC_{50} concentration, when compared to the former, of 2.4, 2.5 and 1.8 in readings taken 24, 48 and 72 hours after exposure to the bacillus.

KEYWORDS: Malaria; Biological control; Anopheline; *B. sphaericus*.

INTRODUCTION

The Amazon is considered to be a region of high malaria transmission. This is particularly so in areas recently cleared of forest, areas of colonization and mining, and some indigenous settlements. In Manaus, the capital of Amazonas, 587 people out of 100,000 inhabitants contracted malaria in 1995[20]. These figures are alarming because this is an area where vector control with chemical insecticides and treatment of the *Plasmodium* bearing patients are routinely carried out.

Important aspects of malaria were discussed at the Ministerial meeting on malaria in Amsterdam (1992)[22]. Emphasis was placed on the necessity of launching a search for more selective, alternative and/or complementary methods of vector control, while taking into account man's relationship with the environment.

The incidence of human malaria cases in the forest edges around Manaus is very high. *An. darlingi* is the main human malaria vector in Amazonia and most of Brazil[2,19,21]. Other species were also shown to be malaria vectors. *An. nuneztovari* has been found to be infected with *Plasmodium vivax* and *Plasmodium falciparum*[1,13,18] and *An. braziliensis* with *P. falciparum*[3].

Biological control is a method which uses biotic agents that are toxic, antagonostic or lethal to a target insect. Available literature cites several bacteria that, according to laboratory and field trials could be used to control the vector at breeding sites, however, few deal with *B. sphaericus* against anophelines.

In view of the increasing resistance of mosquitoes to chemical insecticides and the lack of new alternative and/or complimentary methods to control these insects, *Bacillus sphaericus* is being considered as a possible control measure against malaria vectors in Brazil.

LACEY & ORR (1994)[9] report differences in the mortality rate to *B. sphaericus* strain 2297 (H25) between *An. albimanus* and *An. quadrimaculatus*. Differences were also reported between *An. culicifacies* and *An. stephensis*[10]. In field trials, using a concentrated spray of the *B. sphaericus* 2362 against *An. gambiae*, to obtain complete population control, $1x10^2$ to $2x10^3$ viable spores were necessary in the feeding zone[12]. KARCH et al. (1991)[7] treated a natural breeding site with a 2362 strain showing larvicidal activity in high dosages against *An. stephensis*, *An. subpictus* and *An. culicifacies*.

This first report on the activity of *B. sphaericus* 2362 against *Anopheles* species in Amazonia constitutes the basis of this paper. The mortality rate of various larval instars and the dosage response line of *B. sphaericus* strain 2362 against larvae of *An. nuneztovari, An. darlingi and An. braziliensis* was studied under laboratory conditions.

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MATERIAL AND METHODS

Anopheline rearing

Adult females anopheline were collected around Manaus (AM), Jaciparaná (RO) and Maruanum, Macapá (AP). They were individualy isolated to ovulate. After emerging from the eggs, the larvae were transfered to enamel pans each containing 500 ml of potable water from an artesian well and 2ml of food. The previously prepared food, contained 1g of liver powder and 8g of fish flour, dissolved in 500 ml of water. The water in the enamel pans was changed every two days, until the larvae reached the appropriate larval developmental stage to be used in the bioassays, taking from three to five days. The insectary was maintained at a temperature of 26 ± 2ºC with relative humidity of between 80% to 90%. The standard methodology for maintaining anopheline larvae is consistent with methods of the Malaria Vector Laboratory (INPA)[15,16].

Bacillus sphaericus 2362 samples

The bacillus used was the WHO standard strain 2362 obtained from the Bacterial Entomopathogens Unit of the Pasteur Institute (France). The bacteria were grown in a nutritive broth in a rotating incubator (30±0.5ºC, 200 rpm) for 48 - 72 hours, centrifuged at 10,000 rpm for 20 minutes and afterwards the pellets were lyophilized for 12 to 15 hours.

Bioassays

The bioassays were carrried out at a mean temperature of 26±2ºC, using plastic cups containing 100 ml of drinking water, 20 anopheline larva, 1 ml of food for the larvae and a specific volume of bacillar suspension (mother-suspension) to attain the previously defined concentrations.The method used in the observation of mortality and survival of the bioassays was that described by DULMAGE et al. (1990)[4], with some modifications.

The mortality rate in the control group could not exceed 20% of the larval population as this was considered the demarcation point for acceptability of the bioassay.

Suspensions formulated for bioassays

Mother suspensions were formed by dissolving 50mg of lyophilized bacteria in 10 ml of drinking water. The test tube was shaken in vortex for two minutes to facilitate dissolution. Starting from the mother suspension, other suspensions were prepared for decimal dilution.

Bioassays for evaluating susceptibility of different instars

Bioassays with anopheline larvae that were in one of the four developing larval stages were carried out using the following bacillus concentrations: 5 ppm, $5x10^{-3}$ ppm and $5x10^{-6}$ ppm. In each concentration, 60 larvae were tested for each of the three species, totalling 180 for each instar not including the control treatment. The readings were made after 24, 48 and 72 hours of exposure to the bacillus. The first set of experiments aimed to evaluate the larvicidal activity of *B. sphaericus* 2362 strain against larvae in different instars of *An. nuneztovari* and *An. darlingi*.

Bioassays for calculating LC_{50} against *An. darlingi*, *An. nuneztovari* and *An. braziliensis*.

The methods used were those described by WHO (1985)[23], using anopheline larvae in the third instar with concentrations of *B. sphaericus* 2362 of 1ppm, 0.5 ppm, 0.25 ppm, 0.12 ppm, 0.06 ppm, 0.02 ppm, 0.01 ppm. Three trials were conducted for each concentration. Mortality rate readings were made at 24, 48 and 72 hs of exposure.

Corrected mortality calculations were made using Abbott's formula as modified by FINNEY (1981)[5]. The LC_{50} calculations of the target population in each reading were performed by the probit analysis using the POLO PC program[14]. There was a confidence limit of 95% in all tests.

RESULTS AND DISCUSSION

Larvicidal Activity Against Various Instars

The results of the tests carried out on the four larval instars, and using different concentrations of bacteria (5 ppm, $5x10^{-3}$ and $5x10^{-6}$ ppm) showed that the third larval instar of *An. nuneztovari* was the least susceptible. In all situations 100% mortality was observed at 48 h using the highest concentration (Fig.1). *Anopheles darlingi* showed the lowest mortality rate when in it's third larval instar. The second instar also showed low corrected mortality rates at the three concentrations used, especially at 48 hour exposure (Fig. 2).

The results showed that to standardize the bioassays, third instar larvae must be used. These results are in accordance with observations made by KARCH (1984)[6] who tested the sensitivity of the last three

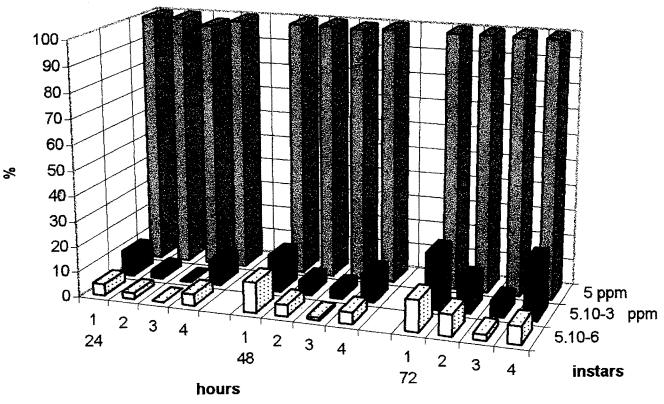

Fig. 1 - Corrected mortality rates of *B. sphaericus* against 3rd. larvae of *An. nuneztovari*, using 3 concentrations.

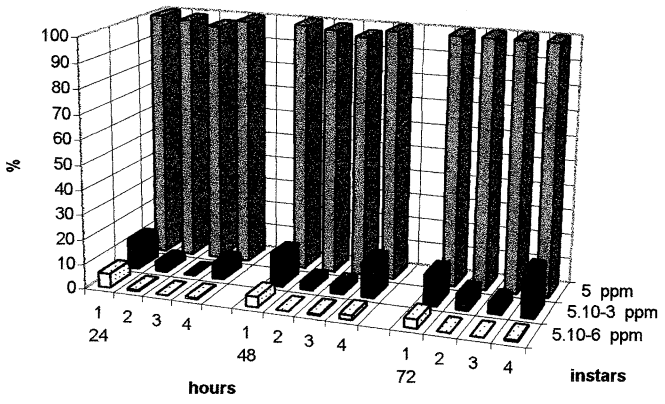

Fig. 2 - Corrected mortality rates of *B. sphaericus* 2362 against 3rd. larvae of *An. darlingi*, using 3 concentrations.

larval instars of *An. stephensi* to *B. sphaericus* 1593-4. His results showed that third and fourth larval instars were less affected than were earlier instars. MULLA (1990)[11] reported that first larval instars were difficult to handle, which might have caused high mortality rates due to the handling procedures. The fourth instar larvae that feed very little or have ceased to feed are less susceptible, since their ingestion of the toxin is minimal during this short period[11].

Mortality Rate of *Anopheles* Species Mosquitoes When Subjected to *B. sphaericus* 2362

Figure 3 represents the dosage response line of *B. sphaericus* 2362 against third instar larvae of *An. nuneztovari*, *An. darlingi* and *An. braziliensis* at 24, 48 and 72 hs of exposure. These results were used to calculate the LC_{50} values presented in Table 1. Considering the mortality rates of *An. nuneztovari, An. darlingi* and *An. braziliensis* to *B. sphaericus* 2362, the results of the different concentration tests (1 ppm to 0.1 ppm) showed that the first species is only a little less sensitive than the second, with values very close to LC_{50} (0.471 ppm and 0.457 ppm respectively). *Anopheles braziliensis* is the species that showed the greatest mortality rate when exposed to the bacillus for 24, 48 and 72 hs, as can be seen in Table 1. In the

TABLE 1
Values of the mean lethal concentration (LC_{50}) of *B. sphaericus* 2362 against *Anopheles* species

Species	Time		
	24 hours	48 hours	72 hours
	LC_{50} (ppm)		
An. braziliensis	0.310	0.182	0.133
An. darlingi	0.735	0.457	0.249
An. nuneztovari	0.895	0.471	0.225

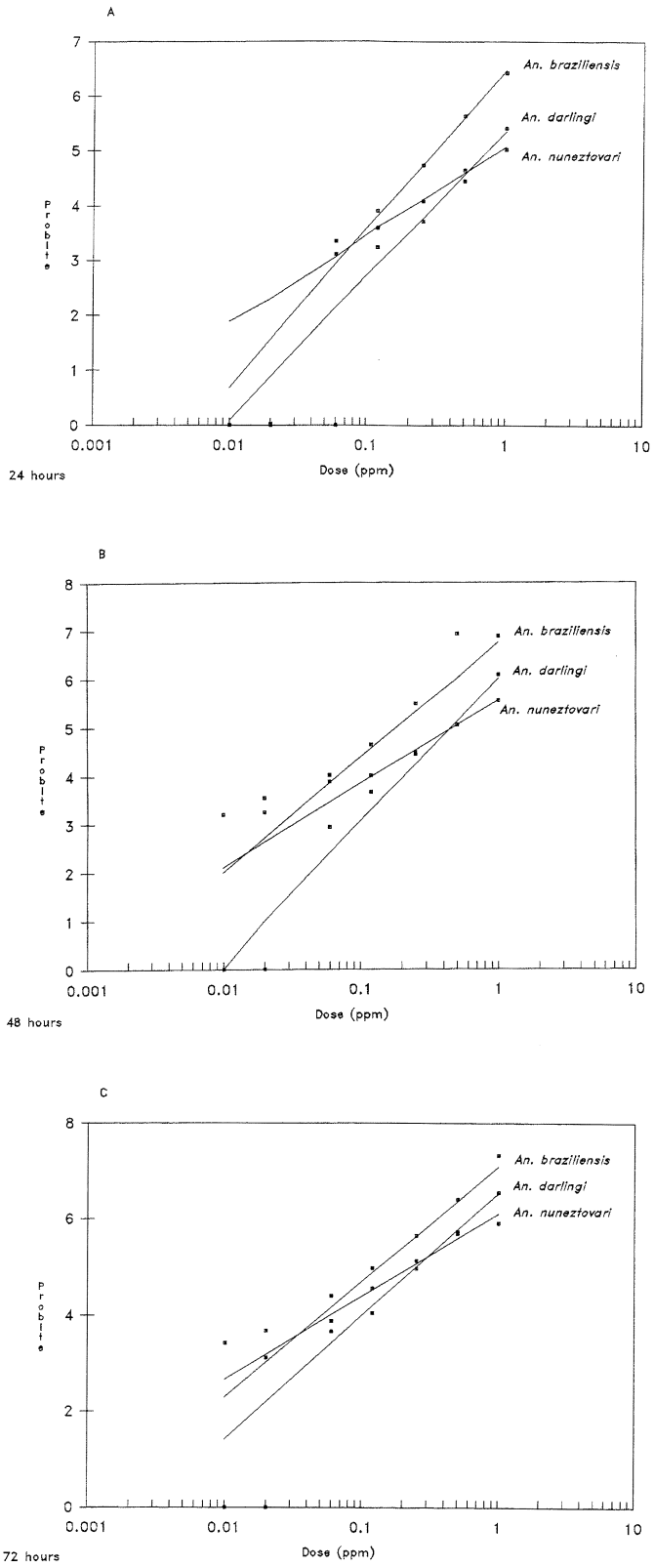

Fig. 3 - Dosage response line of *Bacillus sphaericus* 2362 instar larvae of *An. nuneztovari* and *An. darlingi* at 24 (A), 48 (B) and 72 (C) hours.

case of the other two anopheline species, very close values of LC_{50} in the three readings were observed. For 24 and 48 h, the LC_{50} values for *An. darlingi* are slightly lower than those calculated for *An. nuneztovari*. In the 72-h exposure, the reverse is seen: *An. nuneztovari* is slighty more susceptible than *An. darlingi* to *B. sphaericus* 2362.

The probit analysis showed that *An. darlingi* became more sensitive than *An. nuneztovari* to the bacillus when the concentration was higher than 0.12 ppm, causing a reversal in mortality rate. A similar reversal in mortality rate occurs when comparing *An. braziliensis* and *An. nuneztovari*. In comparing the sensitivity of *An. braziliensis* and *An. darlingi*, the results showed that the bacillus caused a higher mortality rate in the first species at all concentrations and exposure times (Fig. 3).

In order to obtain values that can indicate the anopheline mortality rate differences when exposed to *B. sphaericus* 2362, the relative mortality rate index was calculated by relative susceptibility (RS):

$$RS = \frac{(LC_{50}) \text{ anopheline standard}}{(LC_{50}) \text{ anopheline x}}$$

In the present, study *An. nuneztovari* was chosen as the standard because the highest LC_{50} values for 24 and 48 hs were obtained in bioassays with this mosquito. The relative susceptibility values (LC_{50} standard / LC_{50} anopheline) of *B. sphaericus* 2362, in relation to *An. darlingi* and *An. braziliensis*, considering *An. nuneztovari* as standard are shown in Table 2. The data in this table show that the values are much lower for *An. darlingi* in relation to *An. braziliensis*. For the 48-hr reading of *An. darlingi*, the susceptibility of the two species is very close (RS=1.30), with a reversal occurring in the 72-h reading (RS=0.90). The high mortality rate of *An. braziliensis* remains evident, especially in the 24-hs reading, where it was 189% more sensitive than *An. nuneztovari*.

TABLE 2
The relative activity values of *B. sphaericus* 2362 against *Anopheles* species

Species	Reading (Hours)	Relative activity
An. darlingi	24	1.22
	48	1.03
	72	0.90
An.braziliensis	24	2.89
	48	2.58
	72	1.69

Therefore, it can be expected that different mosquitoes will have a different susceptibility to *B. sphaericus* toxins. This fact has been showed by LACEY & SINGER (1982)[8], who found different LC_{50} values for *An. albimanus* and *An. quadrimaculatus*. Testing *B. sphaericus* strains 2013-4, they obtained LC_{50} values of 0.0187 and 0.0527 ppm, respectively. Using *B. sphaericus* strains 2013-6, the LC_{50} values were 0.0168 and 0.0558 ppm.

Little information exists regarding susceptibility in the laboratory of anopheline species to *B. sphaericus* strain 2362.

CONCLUSIONS

Bacillus sphaericus 2362 strain proved to be effective against all larval instars of *An. nuneztovari*, *An. darlingi* and *An. braziliensis*. It was shown that the third instar larvae of *An. nuneztovari* are less susceptible than the larvae in other instars. In *An. darlingi*, the second and third instars were equally less susceptible. When comparing susceptibility of the three *Anopheles* species to *B. sphaericus* 2362, *An. braziliensis* is the most susceptible, followed by *An. darlingi* and *An. nuneztovari*.

In field trials, it has been observed that *An. nuneztovari* and *An. darlingi* and, to a lesser extent, *An. braziliensis*, may coexist in breeding sites[18,20]. These ecological facts are relevant when considering the possibility of using *B. sphaericus* for the biological control of these three anopheline species. The LC_{50} values reported here show that, in habitats supporting the coexistence of all three species, a program of *B. sphaericus* application will affect even the species with the lowest susceptibly, *An. nuneztovari*.

RESUMO

Atividade larvicida do *Bacillus sphaericus 2362* contra *Anopheles nuneztovari, Anopheles darlingi* e *Anopheles braziliensis* (Diptera, Culicidae)

Foram obtidos neste trabalho, os primeiros dados relativos a sensibilidade de anofelinos vetores da malária na Amazônia – *An. nuneztovari*, *An. darlingi* e *An. braziliensis*, a *B. sphaericus* 2362 estirpe padrão da OMS. Inicialmente, foram realizados bioensaios para verificar a susceptibilidade dos estádios larvais. Para as duas primeiras espécies, os dados evidenciaram que o terceiro estádio é o menos sensível e que, para *An. darlingi*, além deste, o segundo estádio mostrou baixa mortalidade. Na continuidade, a estirpe padrão foi testada contra as espécies de *Anopheles* e verificou-se que *An. nuneztovari* foi a menos sensível a estirpe 2362 quando comparada as outras espécies, mostrando reduções nas concentrações da CL_{50}, em relação à primeira variando de 2,4 a 1,8, nas leituras 24, 48 e 72 horas de exposição ao bacilo.

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