Neotropical Biology and Conservation
2(2):80-83, may - august 2007

Comparison between highly toxic *Bacillus thuringiensis* serovar *israelensis* and *Bacillus sphaericus* strains against *Lutzomyia longipalpis* Lutz & Neiva 1912 (Diptera, Psychodidae, Phlebotominae) larvae

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Abstract

The effects of standardized concentrated spores and crystals suspensions of *Bacillus thuringiensis* serovar *israelensis* and *Bacillus sphaericus* against 3rd instar larvae of *Lutzomyia longipalpis* were recorded by means of qualitative bioassays. The experiments showed a significant difference on mortality and moulting delay between the entomopathogenic bacilli strains tested ($p < 0.001$). *B. thuringiensis* serovar *israelensis* LFB-Fiocruz 584 (a clone of IPS-82) was active against insect larvae assayed (62.5% ± 14.2 of mortality), however, no significant effect was observed with *B. sphaericus* LFB-Fiocruz 736 (a clone of strain 2362). The data reinforce the suggestion that *B. thuringiensis* serovar *israelensis* should be considered for further evaluation as a potential agent against *L. longipalpis* larvae.

Key words: *Bacillus*, *Lutzomyia longipalpis*, control, bioassays.

Introduction

The entomopathogenic bacteria are recognized as one of the most promising biological control agents among all other entomopathogenic organisms due to their mode of action and specificity against larvae of several insect Orders, such as Diptera, Lepidoptera, Coleoptera, Hymenoptera, Isoptera, and also some other invertebrates of the Phyla Nematoda and Mollusca (Crickmore, 2000; Priest, 2000; Rabinovitch *et al.*, 2000; Castilhos-Fortes *et al.*, 2002; Oliveira *et al.*, 2004).

Within this bacterial entomophatogenic group, there are some species of *Bacillus* and correlated genera, which present the most renowned entomopathogenic properties, attractive to many

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researchers and industrial companies (Vilas-Bôas and Lemos, 2004). Members of those genera are spore-forming aerobic Gram-positive bacteria although some of them are also able to grow in anaerobiosis. This group includes some of the most well-known entomopathogenic species: *Bacillus sphaericus* Neide 1904, *Bacillus thuringiensis* Berliner 1915, *Brevibacillus laterosporus* Laubach 1916, *Paenibacillus larvae* White 1906, *Paenibacillus lentimorbus* Dutky 1940 and *Paenibacillus popilliae* Dutky 1940 (Priest, 2000; Rabinovitch *et al*., 2000).

The strains *B. thuringiensis* serovar *israelensis* IPS-82 (Institute Pasteur, serotype H-14, **ISR**) and *B. sphaericus* (strain 2362, serotype H-5a, 5b) are used in several formulated bioinsecticides against Diptera vectors of tropical diseases, and have been systematically employed in biological control programs all over the world (Becker, 2000). These environmental strains have high larvicidal properties due to the production of protoxins, being innocuous to a wide range of non-target invertebrate and vertebrate organisms (Charles *et al.*, 2000; Rabinovitch *et al.*, 2000).

The sand fly *Lutzomyia longipalpis* Lutz & Neiva 1912 (Diptera, Psychodidae, Phlebotominae) is the vector of visceral leishmaniasis, a significant public health problem in the American Continent (Lainson and Rangel, 2003). Although sand fly vectors have been successfully controlled with chemical insecticides, development of insecticide resistance is always a risk and alternative strategies to control this insect have not always been successfully implemented. There is no effective control method for sand fly immature stages due to the limited knowledge of their ecology and breeding sites in nature, although it is well established that phlebotomine larvae act in decomposition processes that consume organic matter (Wermelinger *et al.*, 2000b). This environment provides conditions that are suitable for the development and long-term persistence of many entomopathogens, which could be used to control those insect larvae (Warburg, 1991; Warburg *et al.*, 1991; Wermelinger *et al.*, 2000a).

Several groups have developed experimental bioassays against phlebotomine larvae and/or adults over the years by (Barjac *et al.*, 1981; Pener and Wilamowski, 1996; Wahba *et al.*, 1999; Wermelinger *et al.*, 2000c). Those assays were based on the use of *Bacillus* spores and crystals preparations mixed to larva food, and offered to larvae. It is important to improve those methodologies, since their complexity, due to difficulties on insect handling, always represent a problem on execution of bioassays.

We investigated the effect of mosquitoes highly toxic larvicidal strains of *B. thuringiensis* serovar *israelensis* LFB-Fiocruz 584 (clone of IPS-82, Rabinovitch *et al.*, 2005) and *B. sphaericus* LFB-Fiocruz 736 (clone of strain 2362, Rabinovitch *et al.*, 2005) against *L. longipalpis* larvae, as a step toward their potential use in the biological control of this vector.

Material and Methods

The lyophilized bacterial strains are stocked as spores at the *Coleção de Culturas do Gênero Bacillus e Gêneros Correlatos* - CCGB, Rio de Janeiro, RJ, Brazil (Rabinovitch *et al*., 2005). *Bacillus* strains were germinated in Nutrient Broth-Difco and then inoculated into Nutrient Agar-Difco medium slants supplied with metals (Mg^{2+} [1 mg%] and Mn^{2+} [1 mg%]) - ANM (Rabinovitch *et al.*, 1975) and incubated at 30 °C ± 0.5 °C, for 72 h to reach a high sporulation level (> 95%) and to produce a large amount of protoxins particles. To ensure that strains reached desirable sporulation levels, before bioassays, bacterial growth was directly monitored by fresh-slide examinations (1,000´) under phase-contrast microscopy.

Concentrated suspensions of spores and protoxins were prepared by scrapping-off grown bacteria from surface of ANM medium and vigorously vortexing in sterile distilled water. Then, suspensions were standardized at an optical density of 0.5 at 600 nm with a spectrophotometer (Beckman, DU 640). This is expected to contain high suspensions doses of protoxins, which were used on qualitative bioassays.

Aliquots of 1.5 mL from each standard suspension were submitted to heat shock treatment (80 °C for 10 min and ice for 5 min) to eliminate the **vegetative cells** and 1 mL of each was immediately diluted down to 1:10,000. One hundred microliters from each diluted suspension were inoculated on Petri dishes containing Count Plate Agar (Difco), and incubated (30 °C ± 0.5 °C) for 30-48 h to determine the number of colony forming units per milliliter (CFU/mL).

The determination of colony forming units per milliliter results showed an average of 6.35 ± 0.20 log CFU/mL to *B. thurigiensis* serovar *israelensis* and 6.45 ± 0.07 log CFU/mL to *B. sphaericus*.

Experimental F1 *L. longipalpis* larvae were obtained from a laboratory colony originated from wild-type adults from Gruta da Lapinha, Lagoa Santa, Minas Gerais, Brazil (19°33.705'S, 043°57.478'W, elevation 682 m).

The qualitative bioassay methodology used was adapted from Wermelinger *et al.* (2000c) with the following modifications: Assays were conducted on conical expanded polystyrene vessels (50 mL of capacity) with four replicas, each one containing 10 (14 ± 2 day-old) 3rd instar larvae of *L. longipalpis*. Half a milliliter of standard spores/crystals suspensions were mechanically mixed and homogenized with 1 g of previously sterilized and grinded commercial aquarium fish food (GoldFish Colour™). Humidity excess was allowed to dry at 55 °C for 12 h to avoid quickly fungi proliferation, and toxicity of *B. thurigiensis* serovar *israelensis* and *B. sphaericus* spores/crystals was checked and confirmed by exposure of 20 3rd instar *Aedes aegypti* and *Culex quinquefasciatus* larvae, respectively, to 5 mg/

150 mL of the spore/crystal food, which, in these conditions, resulted on 100% of mortality. *B. thurigiensis* serovar *israelensis* and *B. sphaericus* spores/crystals food was offered to *L. longipalpis* larvae inside vessels partially fulfilled with a 15 mm layer of plaster of Paris, used as surface for larval foraging. The larval food was replaced every 48 h to avoid fungi proliferation, and from the 8th day on diet was substituted for a new one without spores/crystals. Negative controls consisted of four vessels containing larvae exposed to diet without *Bacillus*. The vessels were covered with Parafilm® M to avoid larval escape and left, inside Tupperware® boxes, in a Biological Oxygen Demand (B.O.D.) incubator at 25 °C ± 0.5 °C with relative humidity at 80%. Larval moulting and mortality were recorded every 48 h until adult emergence. Each assay was performed at least three times on different weeks and only assays with mortality below 20% in the negative control were considered.

Mortality data corresponds to the 16th day after the beginning of the assay and were statistically analyzed by GraphPad InStat Software v. 2.04a using ANOVA and Tukey's test to determine the effect of *B. thurigiensis* serovar *israelensis* and *B. sphaericus* on mortality and moulting of *L. longipalpis* larvae.

Results

A significant difference was noted for mortality *L. longipalpis* larvae exposed to *B. thuringiensis* serovar *israelensis* when compared to *B. sphaericus* and negative control ($F_{3,36} = 71.1$, $p < 0.001$). The average mortality of *L. longipalpis* larvae when exposed to *B. thuringiensis* serovar *israelensis* were 62.5% ± 14.2, to *B. sphaericus* strain 2362 were 20.0% ± 6.0 and negative control were 12.5% ± 11.4.

Larvae exposed to *B. sphaericus*, however, did not differ significantly from control ones. A significant difference ($F_{3,86} = 76.7$, $p < 0.001$) was also reported for moulting time (lasting from 3rd instar until adult emergence) of larvae exposed to *B. thuringiensis* serovar *israelensis* (26.1 ± 2.7 days), compared with the others. Larvae exposed to *B. sphaericus* and negative control had a moulting time period of 19.2 ± 1.9 days and 19.9 ± 1.8 days, respectively, which did not differ significantly from each other.

Discussion

Barjac *et al.* (1981) reported an important time-dependent mortality on *L. longipalpis* and *Phlebotomus papatasi* when treated with *B. thurigiensis* serovar *israelensis* and recorded a moulting inhibition effect on challenged larvae. These authors hypothesized that such effects could be caused by a sub-lethal dose of *B. thurigiensis* serovar *israelensis* protoxins, or simply by an inhibition of the feeding process. Wahba *et al.* (1999) also reported moulting delay over *P. papatasi* immature stages and adults treated with *B. thurigiensis* serovar *israelensis*. Sub-lethal doses of *B. sphaericus* were claimed to be responsible for the delayed mortality as well as a wide range of morphological alteration on adults of *Culex quinquefasciatus* (Lacey *et al.*, 1987; Mulla *et al.*, 1991). We did not record any apparent loss of feeding interest neither any morphological change on *L. longipalpis* specimens treated with *B. thurigiensis* serovar *israelensis* or *B. sphaericus*, in agreement with Wermelinger *et al*. (2000b) who worked with *B. thurigiensis* serovar *israelensis* against the same phlebotomine species. Although it was impossible to systematically measure amounts of food ingested by each larva, the moulting delay in *L. longipalpis* larvae exposed to *B. thurigiensis* serovar *israelensis* was probably not related to a loss of larvae feeding, as judged by the decrease of food amount inside the vessels. This observation agreed with Wermelinger *et al.* (2000b) and contradicted the hypothesis proposed by Barjac *et al.* (1981). The moulting delay here observed cannot be charged to sub-lethal doses of protoxin suspensions, since our qualitative bioassays supplied extremely high quantities of protoxins. Moulting delay and larval mortality of treated specimens drastically affected the number of adults emerging at the end of the biossays. Although *B. sphaericus* (strain 2362) is reported to be effective over some phlebotomines species, especially *P. papatasi* (Pener and Wilamowski, 1996; Robert *et al.*, 1997), in our work no significant *B. sphaericus* effect was noted on *L. longipalpis,* in agreement to Wermelinger *et al.* (2000a). Even though only qualitative assays are presented here, Wermelinger *et al.* (2000b) reported lethal concentration (LC) dose of *B. thurigiensis* serovar *israelensis* against *L. longipalpis* immature stages equivalent to 5.7mg/g (LC_{50}) and 96.17mg/g (LC_{90}), respectively.

The main problem in using *B. thurigiensis* serovar *israelensis* and other entomopathogens as biological agents, against phlebotomines is the difficulty of finding immature larvae under natural conditions, which precludes targeting sandfly breeding sites (Alexander and Maroli, 2003; Feliciangeli, 2004). Nevertheless, in our bioassays conditions, *L. longipalpis* larvae were susceptible to *B. thurigiensis* serovar *israelensis* protoxins, presenting more than 50% mortality and exhibiting a moulting delay when compared to negative controls. According to Oliveira *et al.* (1998), for qualitative bioassays, a *Bacillus* strain which reach mortality over 50% can be considered as a potential agent for biological control. These data suggests that *Bacillus thuringiensis* serovar *israelensis* (LFB-FIOCRUZ 584) could be considered for further evaluations as a potential biological control agent against *L. longipalpis*.

Acknowledgments

We would like to express our gratitude to the staff at NEPO, LGCM and LFB, especially to Jeane Quintanilha Chaves and Anderson Miyoshi. To Patricia Ce-

lestino from Ohio Wesleyan University, U.S., Vanessa Araújo (FAFAR/UFMG) and Rodrigo Redondo (ICB/UFMG) for critical readings, suggestions. This study was financially supported by PAPES III-Fiocruz, CNPq and CAPES.

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Submitted on: 2007/01/19
Accepted on: 2007/05/04