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AN ASSESSMENT OF THE BIOLOGICAL ACTIVITY OF *BACILLUS THURINGIENSIS* LFB-FIOCRUZ 907 IN *CHRYSOMYA MEGACEPHALA* (DIPTERA: CALLIPHORIDAE)

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ABSTRACT

Studies have shown that larvicides based on *Bacillus thuringiensis* yield good results against insects of medical and veterinary importance. The objective of the present study was to evaluate the susceptibility of *Chrysomya megacephala* to strains of *B. thuringiensis.* Two strains of *B. thuringiensis* were used; one of them, LFB-FIOCRUZ 907, was isolated from a soil sample from Rio de Janeiro city and the other one, LFB-FIOCRUZ 584, originated from IPS-82 standard powder and has been used as an international standard for *B. thuringiensis* subsp. *israelensis.* The biological activity of the bacterial strains against *C. megacephala* was evaluated by means of three individual bioassays. The assays were carried out in plastic cups which contained each 25 g of the diet (ground cattle meat) plus 25 first instar larvae. Five different concentrations of the *B. thuringiensis* biomass mixed into the diet were used. The tests of biological activity on *C. megacephala* demonstrated that LFB-FIOCRUZ 907 is active (LC_{50} = 14.3 mg/g), while strain LFB-FIOCRUZ 584 showed no activity at the concentrations used. Although the strain LFB-FIOCRUZ 907 showed activity, no changes in the larval and total developmental period (larvae to adults) were observed; for all concentrations the modal day for emergence was the ninth.

KEY WORDS: *Bacillus thuringiensis, Chrysomya megacephala,* vector control.

INTRODUCTION

Dipterous muscoids such as *Musca domestica, Stomoxys calcitrans, Haematobia irritans, Chrysomya albiceps* and *C. megacephala,* and others, are involved in the transmission of agents causing several human and animal diseases.

Several studies have been conducted with the objective of determining the biological activity of strains of *Bacillus thuringiensis* (*Bt*) against muscoid flies. According to Karamanlidou et al. (1991) *Bt* exhibited toxicity for laboratory populations of *Dacus oleae* (olive fly). Temeyer (1984) demonstrated the possibility of action of these bacteria against *Haematobia irritans,* while Wilton and Klowden (1985) indicated the toxic activity of *B. thuringiensis* subsp. *israelensis (Bti)* for *M. domestica* and *S. calcitrans* adults.

1This paper is part of the first author's M.Sc. dissertation.

Species of the genus *Chrysomya* cause concern from the epidemiological point of view due to their feeding habits, that may vary from excrement, garbage and decomposing meat to fresh food, their capacity of spreading out and their population density, especially, due to the fact that they may transport pathogenic agents (Furlanetto et al., 1984). The presence of nonenteropathogenic *Salmonella* and *Escherichia coli* has been already reported in *C. chloropyga*. The polyvirus of type III has also been isolated from *C. megacephala*; both dipterous species were collected in public fairs in the City of São Paulo (Furlanetto et al., 1984).

In Brazil, research on the activity of *Bt* against these muscoids flies is still incipient. This state of affairs has motivated this study, that has the objective of assessing the susceptibility of *C. megacephala* to treatment with δ-endotoxins of *Bt*.

MATERIALS AND METHODS

Bacterial strains

Two strains of the *B. thuringiensis* (*Bt*) were used; one of them, *B. thuringiensis* subsp. *israelensis* (*Bti*) IPS-82 (LFB-FIOCRUZ) is considered to be a standard by the Pasteur Institute of Paris, and it is currently used also as an active principle in commercial biological insecticides. The second strain was that of *Bt* (LFB-FIOCRUZ 907) isolated from a soil sample in the Laboratory of Bacterial Physiology (LFB) of the Department of Bacteriology at the Oswaldo Cruz Institute/FIOCRUZ.

For bioassays to assess the toxic activity, each bacterial sample was grown initially in a liquid medium to obtain sufficient amounts of biomass for conducting the tests.

Preparing the bacterial biomass

The samples of *Bt* were grown in Nutrient Broth (Bacto Nutrient Broth, Difco Laboratories) supplemented with 5 g/L of glucose and metals such as $MgSO_4.7H_2O$ – 0.02 g/L; $MnSO_4.7H_2O$ – 0.03 g/L; $ZnSO_4.7H_2O$ – 0.02 g/L; $FeSO_4.7H_2O$ – 0.02 g/L and $CaCl_2$ – 0.1 g/L, pH 7.0.

Growth started with a pre-inoculum, where the strains were cultivated with the objective of adapting them to the environment, reducing the duration of the lag phase of bacterial growth. After inoculation in 125 mL Erlenmeyer flasks containing 50 mL of the medium, the flasks were incubated in a New Brunswick Scientific series 25D agitator, at 175 r.p.m. and 30°C during 6 hours.

Afterwards, 3 mL was transferred to 500 mL Erlenmeyers flasks containing 150 mL of the medium and incubated as previously described for a further 72-hour period.

Once sporulation had reached 90% of free spores, each culture was centrifuged (6000 *g*, 10°C). Afterwards, the biomasses were suspended in distilled water acidified with 0.7% propionic acid to pH 3.0 to keep the crystals active and then left to rest for an hour. All samples were submitted to a second centrifugation and then the biomasses were kept in an amber container with the pH adjusted with propionic acid to 5.0, so that they could be preserved in a refrigerator.

Determination of the dry weight

Samples of 0.5 g biomass were weighed. For each sample three replications were done which were kept for 24 hours in a vacuum incubator at 70°C under a negative pressure of 62 mm Hg. Thereafter, the dried biomasses were transferred to a desiccator and submitted to vacuum for an

hour, so that they reached the room temperature. They were then weighed on a analytical balance up to the fourth decimal case, to determine the mean weight of the dry biomass and its moisture content.

Counting the number of viable cells

A sample corresponding to 25 mg (dry weight) of the biomass was transferred to a 50 mL volumetric flask; the volume was completed with distilled water and the solution was homogenized for five minutes. This mother-suspension thus contained 0.5 mg/mL. For the quantification of viable spores, five milliliters of this solution was subjected for 12 minutes to 80°C in a water bath. After cooling to room temperature, decimal dilutions were prepared (1 mL of suspension in 9 mL of sterile saline) in sterilized tubes, down to $1:10^5$. Of the last three dilutions ($1:10^3$, $1:10^4$ and $1:10^5$), 0.1 mL was drawn for breeding by spreading on the surface of Nutrient Agar poured in Petri dishes. Three dishes were used for each dilution and incubation was for 24 hour at 33°C. The colonies were counted, the average determined and the number of spores/mg calculated.

Quantification of entomopathogenic activity in *Chrysomya megacephala*

The bioassays with *C. megacephala* were conducted as follows: recently emerged larvae were put into contact with the diet (minced beef) previously mixed with the larvicide; since they had not been fed previously, they were voracious. For each strain of *Bt* three bioassays were done, in which five different concentrations of the bioinsecticide preparation were used. The initial concentrations were chosen through simple randomization and the intervals between them were kept constant (=1.56 in relative numbers). The values obtained corresponded to the dry weight of biomass per 25 g diet. For each of them triplicates were used, adding up at the end to three bioassays, nine repetitions for each concentration. The tests were done in small plastic flasks containing 25 g diet and 25 first instar (L_1) larvae. The amount of diet used corresponded to 1 g diet per insect, this being the ratio necessary for the development of the larvae (Queiroz and Milward-de-Azevedo, 1991). The biomass was mixed into the diet and afterwards the insects were added as L_1. The flasks were then placed inside larger plastic containers containing vermiculite and sealed with a cotton screen. The third instar larvae (L_3) when about to pupate, left the diet spontaneously for the second container, where they became pupae. When this took place, the pupae where taken out of the recipient and placed in glass containers properly tagged with the date and the concentration of the biomass from which they had been removed; the containers were sealed with a cotton screen. Once the adults had emerged, they were counted and separated according to sex. The presence or absence of anomalies in these adults was recorded. From some of the dying larvae Gram stained smears were done, to check for the presence of vegetative forms of *Bacillus* (Fig. 1). The results obtained relate to the dry weight of biomass per weight unit of the diet (mg/g of diet).

Assessment of the biological activity (LC_{50}) was determined through log-probit analysis and the adjustment for control mortality through Abbott's formula.

The mean lengths of the larval periods were calculated for grouped data, the coefficient of variation and the significance of the differences determined by a t-Student test at the 5% level.

The bioassays with *C. megacephala* were done at the Biology and Insect Control Laboratory of the Department of Biology of the Oswaldo Cruz Institute/FIOCRUZ.

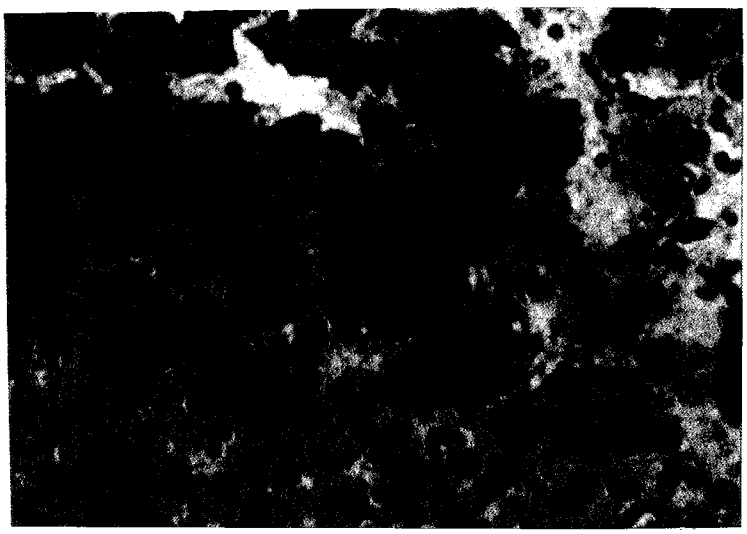

Fig. 1. Vegetative forms of *Bacillus thuringiensis* LFB-FIOCRUZ 907 observed inside a dead larva of *Chrysomya megacephala*, using a light microscope with ×1000 magnification.

RESULTS

Assessment of the entomopathogenic activity

Dry weight and number of viable cells: Degree of moisture and number of viable spores of the biomass used in the experiments were determined, with the LFB-FIOCRUZ 584 strain having 77.1% of moisture and 2.42×10^7 spores/mg and the LFB-FIOCRUZ 907 strain 75.6% of moisture and 1.81×10^7 spores/mg.

Entomopathogenic activity in Chrysomya megacephala: Tables 1 and 2 present the results of the bioassays for the assessment of biological activity against *C. megacephala.* They reveal a positive response for LFB-FIOCRUZ 907 (LC_{50} = 14.3 mg/g) and a negative response for LFB-FIOCRUZ 584, due to the low percentage mortality observed and the inconsistency of correlation between concentration and percentage mortality.

Another facet of the activity of *Bt* LFB-FIOCRUZ 907 was the reduction in the average rate of emergence of winged individuals of *C. megacephala* in a fashion corresponding to the concentrations tested. The LC_{50} calculated for the entire developmental period from larva to adult was of 6.1 mg/g (Table 3).

Despite the activity of the LFB-FIOCRUZ 907 strain, its influence on the mean larval developmental time of the *C. megacephala* was negligible, since the differences regarding this parameter between the concentrations tested were not significant at the 5 percent level (t-Student test) (Table 4). This applied also to the total developmental time, that was the same for all concentrations, using the ninth day as the criterion of emergence.

TABLE 1
Lethal activity of *Bacillus thuringiensis* LFB-FIOCRUZ 584 against *Chrysomya megacephala* larvae under laboratory conditions

Dose	Larvae (n)		Mortality (%)	
(mg/25 g diet)	Pre-Treatment	Dead	Real	Adjusted (*)
55	75	11	14.7	8.6
86	75	12	16.0	10.0
134	75	10	13.3	7.1
209	75	15	20.0	14.3
326	75	13	17.3	11.4
Control	75	5	6.7	0

The results represent an average of three bioassays. (*) = corrected for control mortality.

TABLE 2
Lethal activity of *Bacillus thuringiensis* LFB-FIOCRUZ 907 against *Chrysomya megacephala* larvae under laboratory conditions

Dose	Larvae (n)		Mortality (%)	
(mg/25 g diet)	Pre-Treatment	Dead	Real	Adjusted (*)
55	75	22	29.3	20.9
86	75	23	30.7	22.4
134	75	20	26.7	17.9
209	75	25	33.3	25.4
326	75	50	66.7	62.7
Control	75	8	10.7	0

The results represent an average of three bioassays. (*) = corrected for control mortality.

TABLE 3
The effect of *Bacillus thuringiensis* LFB-FIOCRUZ 907 fed to larvae of *Chrysomya megacephala* on emergence of winged adults under laboratory conditions

Dose	Larvae	Adults	Mortality (%)	
(mg/25 g diet)	Pre-Treatment (n)	(n)	Real	Adjusted (*)
55	75	38	49.3	29.6
86	75	39	48.0	27.8
134	75	38	49.3	29.6
209	75	27	64.0	50.0
326	75	5	93.3	90.7
Control	75	54	28.0	0

The results represent an average of three bioassays. (*) = corrected for control mortality.

TABLE 4
Length of the larval stage (in days) of *Chrysomya megacephala*, when submitted to different concentrations of the *Bacillus thuringiensis* LFB-FIOCRUZ 907, under laboratory conditions

Dose (mg/25 g diet)	x ± s	Variation interval (days)	Variation coefficient (%)
55	4.72 ± 0.78	4—8	16.53
86	4.63 ± 0.62	4—8	13.44
134	4.71 ± 0.70	4—8	14.86
209	4.70 ± 0.68	4—8	14.42
326	4.65 ± 0.53	4—8	11.40
Control	4.59 ± 0.43	4—7	9.35

x = average of grouped data. s = standard deviation.

DISCUSSION

With respect to toxicity, the strain of *Bt* LFB-FIOCRUZ 907 used in this study was toxic to larvae of *C. megacephala* only at high doses (LC_{50} = 14.3 mg/g). This was consistent with the results obtained by Vankova (1981), who showed that *Bti* had little effect on larvae and pupae of *M. domestica*. The same was the case with the lineage of *B. thuringiensis* subsp. *kurstaki*, found in the commercial product Dipel. However, when the author used a strain of *Btt* (H-1), producing the β-exotoxin, good results (LC_{50} = 65 mg/kg), were obtained with inhibition of the development of larvae of this species in animal excrements. Van der Geest and De Barjac (1982) investigated the effect of *Bti* on *Glossina pallidipes* and showed that the action of this bacterium for the tsetse fly is noticeably different from its action on mosquitoes and simuliids, that is, the δ-endotoxin is not the cause of mortality; mortality is apparently caused by the penetration of the bacteria through the intestine wall to the coelimic cavity, reaching the haemolymph. However, Temeyer (1984), found good results (LC_{50} = 50 μg/g) with larvae of *H. irritans* when using preparations containing only purified crystals of *Bti*, although obtaining mortality only for the larval stage in which the insects were fed. Another study conducted by Wilton and Klowden (1985), using crystals of *Bti* indicated that, at the doses administered to adults of *M. domestica* and *Chrysoperla carnea* (1.2 μg and 2.8 μg/g, respectively), there was no reaction to the insecticide. However, for the doses used for *S. calcitrans* (2.5–2.6 μg/g) the authors recorded a reasonable proportion of dead insects. Singh et al. (1986) showed that a dosage of 4 μg/mL of *Bti*, despite not having a detectable effect at the level of the nerve extremities of *M. domestica*, caused severe damage to the muscles of the insect's body wall, showing with the passing of time a myotoxic effect, leading to the complete loss of this tissue's integrity. Karamanlidou et al. (1991) studied the activity of freshly isolated *Bt*, for larvae of *D. oleae*, obtaining higher levels of mortality (80 percent), when using relatively pure mixtures of crystals and spores, than when these were used separately.

The results obtained by these authors demonstrate biological activity, although this was manifest only at high concentrations, confirming our results obtained with the strain of *Bt* LFB-FIOCRUZ 907.

When the LC_{50} obtained with the strain LFB-FIOCRUZ 907 for larvae of *C. megacephala* (14.3 mg/g) is correlated with the mortality obtained for adults of this same species, it may be observed that the latter needed a lower concentration (6.1 mg/g) to obtain the same effect. This may result from the fact that the death of the winged forms is not due to the action of the

bacterial toxin but, in fact, from the likely germination of bacterial spores in the haemolymph of these flies (Fig. 1).

The difference between the results of biological activity of the strain LFB-FIOCRUZ 584, which is a *Bti*, serovar H-14, and of the LFB-FIOCRUZ 907, characterized as likely to be of the same subspecies (Cavados, unpublished findings), led us to the conjecture that two strains of the same subspecies do not necessarily have similar entomopathogenic activities against the same target insect; this is in accordance with the study of De Barjac and Frachon (1990). These authors showed that strains of the same subspecies could present toxicity for different orders of insects, as for instance *B. thuringiensis* subsp. *morrisoni,* that has samples toxic to Lepidoptera, Coleoptera and Diptera. On the other hand, it should be mentioned that strain 907 has some noteworthy difference at the molecular level in the peptides of the glycoprotein, causing it to present toxicity for larvae *C. megacephala,* since the toxicity of the strain LFB-FIOCRUZ 584 is considered negligible for this species of fly.

Although the results presented in this paper are limited to one subspecies of the *Bacillus,* more detailed studies will be conducted with the purpose of better assessing the toxicity of *Bt* for muscoid flies, not the least because there are other variables that deserve investigation. For instance, conduct assays with a larger number of strains of entomopathogenic species of *Bacillus* naturally found in the environment, in the same fashion as with purified toxins of *Bt* strains from different sources; and, lastly, experiments with δ-endotoxins activated by the hydrolysis of proteases of the trypsin and chymotrypsin type.

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