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Acute oral, pulmonary and intravenous toxicity/pathogenicity testing of a new formulation of *Bacillus thuringiensis* var *israelensis* SH-14 in rats

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ABSTRACT

During the last decades, efforts are being made to develop microbial insecticides as biological control agents. *Bacillus thuringiensis* has been one of the most consistent and significant biopesticides for using on crops as an insecticidal spray. The aim of this study was to assess and to compare the pathogenicity of a new formulation of *B. thuringiensis* var *israelensis* SH-14 in rats through oral, intranasal and intravenous single dosing. Through 21 days after administration, clinical examinations were performed daily, and body weight gain was evaluated. Clearance was estimated by means of collection of feces or examination of lungs and blood, and infectivity was evaluated enumerating microorganisms from organs of *Bti* SH-14 treated animals sacrificed at intervals. Gross necropsy of animals was performed at interim or final sacrifice. There were no treatment-related mortalities, and no evidence of pathogenicity or treatment related toxicity, although in the intravenous study, the microorganism was capable of achieving persistence in organs after administration, and the *Bti* SH-14 treated animals developed skin ulcerations and hemorrhages at the injection site. It could be concluded that the tested microorganism was not toxic or pathogenic to rats via oral or intranasal route, although it was capable of achieving persistence in organs after intravenous administration, eliciting local effects at the injection site.

1. Introduction

Increased public concern of the potential adverse environmental effects associated with the heavy use of chemical pesticides has prompted the examination of alternative methods for pest control. Since the 1960s, biological insecticides such as insect-pathogenic microorganisms have received considerable attention as environmentally benign, highly desirable alternatives (Sudakin, 2003). The most widely used microbial insecticide to date is based on *Bacillus thuringiensis* (*Bt*). *Bt*, first described by Berliner in 1911, is a gram-positive, spore-forming bacterium living in the soil. It is known for its ability to produce crystalline, proteinaceous, delta-endotoxin during sporulation, which are highly toxic to a wide variety of important agricultural and health-related insect pests as well as other invertebrates (Sauka and Benintende, 2008). The OECD (Organization for Economic Cooperation and Development) predicts that the biopesticide may grow to 20% of the world's pesticide market by 2020 (Whalon and Wingerd, 2003).

Around 1976, a new *Bt* strain was discovered with high toxicity to mosquito larvae (Goldberg and Margalit, 1977) which was later identified and designated *Bt* var. *israelensis*, serotype H14 (de Barjac, 1978), since raised to subspecies status as *B. thuringiensis israelensis* (*Bti*). This Diptera-active strain has come to be used extensively for the control of larvae of pest and vector black flies and mosquitoes around the world, providing both medical and environmental benefits (Lacey et al., 2001; Roh et al., 2007).

Extensive laboratory studies required by the U.S. Environmental Protection Agency and regulatory authorities in other countries have shown that *Bt* insecticides are safe for vertebrates at doses several thousand-fold higher than it would be encountered when these insecticides are used in accordance with recommendations provided by their manufacturers (Siegel, 2001). Despite the remarkable safety record of *Bt* use, concerns about the taxonomic relationship of *Bt* to other bacilli that can cause any illness in humans, have raised questions about the safety of *Bt* insecticides.

LABIOFAM (Cuba) is developing a biolarvicide that possesses as active biological agent *Bti* serotype H14; this strain is registered in the Culture Collection catalog (IEBC) of the Pasteur Institute as 266/2 9-VII-98. With the aim of satisfy the current requirements on environmental care, the preservative used in the older formulation was change by a non-toxic and biodegradable preservative. With the objective of assessing and comparing the toxicity/pathogenicity of this new formulation of *Bti* SH-14 to rats through differ-

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ent routes, this product was administered by oral, intranasal and intravenous single dosing. The studies were performed taken into account the experimental design described in the Tier 1 of Toxicological Evaluation of Microbial Pest Control Agents that has the purpose of assessing the pathogenicity, infectivity/unusual persistence and toxicity of these products (U.S. EPA, 1996a). Pathogenicity can be defined as the ability to inflict injury and damage in the host after infection, while toxicity could be considered as the injury or damage in a host caused by a poison or toxin where infection by and/or replication or viability of the microorganism are not necessarily required (U.S. EPA, 1996a). Infectivity is the ability of a microorganism to cross or evade natural host barriers to infection, understanding as infection the disruption to the host caused by multiplication of the inoculum in tissue, toxin production, or both. When there is no evidence of infection but colony forming units (CFU) are recovered, then a different condition, persistence, has occurred (Siegel, 2001).

2. Materials and methods

Studies were accomplished according to the international principles of Good Laboratory Practice (U.S. EPA, 2000), and were approved by the CENPALAB Animal Ethics Committee.

The new formulation of *B. thuringiensis* var *israelensis* SH-14 was obtained from Grupo Empresarial de Producciones Biofarmacéuticas y Químicas (LABIOFAM) (Havana, Cuba) (Watery suspension, Batch No. 0709002). Inactivated product was obtained by means of autoclaving *Bti* SH-14 at 121 °C for 20 min.

Sprague Dawley rats were obtained from CENPALAB (Havana, Cuba).

- Oral: 21 rats/sex; 7 weeks old; average body weight: 169.3 g (females), 182.9 g (males).
- Intranasal: 24 rats/sex; 9 weeks old; average body weight: 220.1 g (females), 275.3 g (males).
- Intravenous: 21 rats/sex; 7 weeks old; average body weight: 168.0 g (females), 196.6 g (males).

Animals in all studies were randomly housed three per cage. Water and feed (EMO 1002, ALYco®, CENPALAB) were sterilized by autoclaving and were available *ad libitum*. Autoclaving was made at 120 °C for 60 min for water and at 120 °C for 20 min for feed. Room environment was 25–28 °C, 75–85% relative humidity, and 12 h photoperiod.

Treated rats in the acute oral toxicity/pathogenicity assay received 2 mL of test substance containing 3.4×10^8 CFU by intragastric administration (gavage), while treated animals in acute pulmonary toxicity/pathogenicity study were inoculated with 0.3 mL of test substance, containing 3.6×10^8 CFU through intranasal instillation, and treated animals in acute intravenous toxicity/pathogenicity study were inoculated with 0.1 mL of test substance, containing 3×10^7 CFU through intravenous injection (tail vein). An untreated control group and a control group receiving inactivated *Bti* SH-14, of three animals per sex each, were established for each study.

The observation period was 21 days after dosing. A careful clinical examination was made daily. Observations included evaluation of skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behavior pattern. Particular attention was directed to observation of tremors, convulsions, diarrhea, lethargy, salivation, sleep and coma. Weights of individual animals were determined shortly before *Bti* SH-14 was administered, weekly thereafter, and at interim or final sacrifice.

For evaluating infectivity, *Bti* was enumerated from kidney, brain, liver, spleen, lungs, caecum content, blood, and representative lymph nodes of three treated animals per sex, sacrificed 2 and 4 days after, and at one week intervals after dosing, taking one gram or 1 mL of sample from each organ. One gram of feces and lungs, and 1 mL of blood were collected from test animals soon after dosing and at 2, 4, 7, 14 and 21 days, and examined for the presence of *Bti* to estimate clearance after oral, pulmonary, or intravenous administration, respectively (for pulmonary clearance analysis, three treated animals per sex were also sacrificed at 3 h post-administration). Presence of *Bti* in organs samples of the untreated group was also tested at final sacrifice. Quantification of *Bti* (viable count) was performed by means of culture of serial dilutions of samples in Tryptone Soja medium (BioCen, Cuba). Count of colonies was made 3 days after, and this result was multiplied by the milliliter factor (5).

At interim or final sacrifice, the animals were bled through a femoral vein and applied a cervical dislocation maneuver. Gross examination of the external body surface, orifices, cranial, thoracic and abdominal cavities and all organs was conducted.

Body weight values were statistically analyzed using the Statistical Package Scientific System 11.5.1 (Statistical Package Scientific System, SPSS for Windows, Copyright SPSS Inc., 2002). Normality of the data was assessed by means of the Kolmogorov–Smirnov test. The statistical significance of differences between treated and untreated control group for each assay were determined using Student's t test. Statistical significance was assessed at the $p < 0.05$ level.

3. Results

3.1. Oral study

The survival in the assay was 100%. During the study, alterations of animal clinical signs were not observed, maintaining the normal behavior of the species (OECD, 2000). The detection of excretion of the product in the feces was significant 3 h after the administration of the microorganism, not being isolated in only one sample (see Table 1). On day 2, all animal's samples were positive for the isolation of *Bti*, although in a lower magnitude than the 3 h determination. In the subsequent days, significant isolation was not obtained. With regard to the study of the infectivity, the result of the product count in organs of the animals of intermediate sacrifices, showed scarce isolation of *Bti* in the rats along the study, being the liver the organ with a more significant isolation obtained in terms of positive isolation (66.7% of samples showing positive results on day 2) (see Table 2). Animals of the treated and untreated control group tested for infectivity on day 21 were negative for the presence of *Bti*. Macroscopic alterations of the observed organs were not detected.

3.2. Intranasal study

The survival in the assay was 97.6%, because of the death of a male of Treated group during the instillation of the test substance. During the study, alterations of the clinical signs of the animals were not observed, and the behavior of the animals was normal for the species (OECD, 2000). The detection of *Bti* of the product in the lungs was significant 3 h after the administration of the microorganism, with a 100% of samples *Bti* positive (see Table 1). In the subsequent days significant isolation was not obtained. With regard to the study of the infectivity, the result of the product count in the organs of the animals showed the highest isolation of *Bti* in the rats on day 2 of the assay (see Table 3), mainly in the caecum content (100% of 6 analyzed samples) and mediastinic ganglion (2 of 6 samples). In the subsequent days significant isolation was obtained only on day 7 at the mediastinic ganglion (83.3%