TOXICITY INTRAPERITONEAL AND INTRAGASTRIC ROUTE OF *BACILLUS THURINGIENSIS* AND *MELIA AZEDARACH* IN MICE

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ABSTRACT

The aim of this investigation was the assessment of toxicity of two new isolates of *Bacillus thuringiensis,* and the aqueous extract of *Melia azedarach* through *in vivo* assays in CF1 mice. *Bt* 1958-2, *Bt* 2014-2 and the *BTh Thuricide* 63 standard isolates were grown in liquid usual glicosed medium, and Cry proteins were purified by centrifugation on a sucrose gradient. The supernatant was autoclaved at 121º C, 15min. to maintain the exotoxins. Dehydrated leaves of *M. azedarach* were used to prepare a 10% aqueous extract. Mice were treated either orally or intraperitoneally with a whole bacterial suspension (1.10^{10} UFC/mL), a culture supernatant or purified crystal protein (50 µg/mL), and with the plant extract (50 µg/mL). The stomachs of the mice were collected and observed in stereomicroscopy, and the stomach contents were analyzed in 10% SDS-PAGE. Results showed that none of the oral treatments were toxic to mice, but intraperitoneal bacterial suspensions were lethal to the animals 6 - 24 hours after injection. In conclusion, the Cry proteins of the new *B. thuringiensis* isolates must be evaluated for their use as tools in the biotechnology field, since they do not show toxicity against mammals, intragastrically or peritoneally, just like the *M. azedarach* aqueous extract (10%), with those being indicated for the biological control of pest insects.

KEY WORDS: Bacterium, mammalian, *Meliaceae*, toxicology.

RESUMO

TOXICIDADE VIA INTRAGÁSTRICA E INTRAPERITONEAL DE *BACILLUS THURINGIENSIS* E *MELIA AZEDARACH,* EM CAMUNDONGOS. O objetivo deste estudo foi a avaliação da toxicidade de dois novos isolados de *Bacillus thuringiensis* e o extrato aquoso de *Melia azedarach*, através de ensaios *in vivo* em camundongos CF1. Os isolados *Bt* 1958-2, *Bt* 2014-2 e o isolado padrão BTH Thuricide 63 foram cultivados em meio usual glicosado, e as proteínas Cry foram purificadas por centrifugação em gradiente de sacarose. O sobrenadante foi tratado em autoclave a 121º C, 15 min para manter as exotoxinas. As folhas desidratadas de *M. azedarach* foram utilizadas para preparar um extrato aquoso a 10%. Camundongos foram tratados, via oral ou por via intraperitoneal, com a suspensão bacteriana (1.10^{10} UFC/mL), o sobrenadante de cultura ou a proteína do cristal purificada (50 µg/mL), e com o extrato da planta (50 µg/mL). Os estômagos dos ratos foram coletados e observados em estereomicroscópio e os conteúdos estomacais foram analisados em SDS-PAGE a 10%. Os resultados mostraram que nenhum dos tratamentos orais foram tóxicos para os camundongos, mas, via intraperitoneal, as suspensões bacterianas foram letais para os animais entre 6 e 24 horas após a injeção. Em conclusão, as proteínas Cry dos novos isolados de *B. thuringiensis* devem ser avaliadas para sua utilização como ferramenta no campo da biotecnologia, uma vez que elas não mostram toxicidade contra mamíferos, intragástrica ou intraperitoneal, assim como o extrato aquoso (10%) de *M. azedarach*, podendo ser indicado para o controle biológico de insetos-praga.

PALAVRAS-CHAVE: Bactéria, mamíferos, Meliaceae, toxicologia.

INTRODUCTION

The entomopathogen *Bacillus thuringiensis* (Berliner, 1911), a Gram-positive bacterium, is naturally found in the soil (Höfte, Whiteley, 1989). It is characterized by crystal production during sporulation, containing Cry proteins, encoded by the *cry* genes, with a wide division into classes and

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subclasses according to their insecticide activity (Höfte, Whiteley, 1989), and presently classified according to the percent identity between Cry protein sequences (Schenpf et al., 1998; Crickmore, 1998; Crickmore 2012). Besides the Cry proteins, known as d-endotoxins, *B. thuringiensis* isolates can synthesize other toxins, such as b-exotoxin, phospholipases, proteases, chitinases, (Schenpf et al., 1998; Rabinovitch et al., 1998; Vilas-Bôas et al., 2012), and enterotoxins (Zahner et al., 2005).

The d-endotoxin is specific to insects, while the b-exotoxin, also called thuringiensin, does not have host specificity, it is thermostable and toxic to vertebrates (Sebesta et al., 1981; Gohar; Perchat, 2001). That toxin is analogous with ATP, being identified as an inhibitor of the rRNA synthesis (Mackedonski; Hadjiolov, 1972), resulting in dispersion and decreased number of chromosomes as well, double and micronuclei, tetraploid cells, among other effects of in vitro assays with *Alium cepa* (Sharma; Sahu, 1977).

Besides the use of that entomopathogen, plants with insecticidal properties have been assessed for pest control. The Chinaberry tree, *Melia azedarach* (Linnaeus) is a meliacea causing insecticidal activity against different pest, affecting behavioral and feeding changes, and mortality (Carpinella et al., 2003). For these reasons, *M. azedarach* extracts, together with *B. thuringiensis* isolates, have become a promising with respect to biological control, since some isolates of that bacterium constitute commercial insecticides, with registration in the USA since 1961 (Siegel, 2001, Capalbo et al., 2005).

Besides those formulae, the current biotechnological research aims at the genetic alteration of plants using *B. thuringiensis cry* genes; transgenic corn, cotton, and potato are already on the market (Shelton et al., 2002; Schroder et al., 2007; James 2011). Related data by O'Callaghan et al. (2005) show no negative effects of those transgenic plants on beneficial insects, such as polinizers and natural enemies. But the growing concern regarding the use of these biopesticides or transgenic plants has to do with non-target organisms, such as the vertebrates (Vasquez-Padrón et al., 2000).

In the health field, the works of Prasad; Shethna (1975) suggest that *B. thuringiensis* proteins have anti-tumor activity in the Yoshida sarcoma in rats, in addition to enhancing the immune reaction in lambs. Yamashita et al. (2000) also showed a cytotoxic effect on leukemia cells in in vitro assays. As for *M. azedarach*, traditional medicine uses a leaf extract from this meliacea as a diurectic, and it has emmenagogue properties (Keshri et al., 2003).

Additionally, with the appearance of new *B. thuringiensis* isolates and extracts of *M. azedarach*, it is necessary to study their effects on non-target organisms, such as mammals. Thus, this work focused on the in vivo effects of two new isolates of *B. thuringiensis*, the *BTh Thuricide* 63 standard isolate and the *M. azedarach* aqueous extract on mice.

MATERIAL AND METHODS

Bacillus thuringiensis **isolates**

Bacillus isolation has been adapted according to the method described by the World Health Organization (WHO, 1985). *Bt* 1958-2 and *Bt* 2014-2 isolates come from the "Banco de Bactérias do Laboratório de Microbiologia e Toxicologia da Universidade do Vale do Rio dos Sinos", and as the standard, isolate from the BTh Thuricide 63 was used, corresponding to the Thuricide® product, provided by the International Entomopathogenic Bacillus Centre, from the Pasteur Institute, Paris. Bacterial growth was carried out in UG liquid medium (Barjac; Lecadet, 1976). The isolates used in this paper showed the presence of the genes that code the Cry1 and the Cry2 proteins in Bt 1958-2, and the Cry3 protein in Bt 2014-2 (Pinto; Fiuza, 2003).

Quantification of cells and spores was carried out in a Neubauer Chamber and optical microscopy, and set by dilution to 1.10^{10} UFC/mL. The supernatant was autoclaved at 121º C for 15 min, as described by Perani et al. (1998), since that is a procedure which preserves the b-exotoxins if present in the culture medium.

Bacillus thuringiensis **Cry proteins**

B. thuringiensis was grown in UG liquid medium (30º C, 180 rpm), until 90% cell lysis was observed in phase-contrast microscopy. Culture remains were centrifuged (5,000 rpm, 15 minutes), and the supernatant was kept for experiments, as described above.

Purification of Cry proteins present in the pellet was carried out by ultracentrifugation on a sucrose gradient (67 to 79% g/w), bands were collected, and crystal proteins were solubilized in alkaline (pH 10) buffer, according to Fiuza (1995). Protein quantification was done according to Bradford (1976), and protein crystal solutions adjusted to 50 µg protein per mL with PBS buffer pH 7.4.

Melia azedarach **extract**

The leaves of *M. azedarach* were used after going through a drying process at 40º C in a greenhouse, with air circulation for 48h; the leaves (10 g) were then crushed and diluted in sterile distilled water (100 mL) which resulted in a 10% crude extract

(Brunherotto; Vendramim, 2001). The dosage of total plant proteins was done according to Bradford (1976) and solutions were adjusted to 50 µg protein/mL, with PBS buffer, pH 7.4.

SDS-PAGE

The proteins of *M. azedarach* and *B. thuringiensis* (50 µg protein/mL) were analyzed for the proteic profile in poliacrilamid gel at 10%, following Laemmli (1970). Protein bands were compared to the molecular weight marker (Invitrogen®) using Kodak Digital Science 1D program.

Mice

"The experiments were conducted in the period between the years 2003/2004 and followed the Guidelines for Research on animals and theNational Institutes of Health (NIH) and Brazilian College of Animal Experimentation."

Adult male mice (CF1 strain), 80 - 100 days old, came from the "Universidade do Vale do Rio dos Sinos" biotery. The animals were maintained at 21° C, subjected to 12-h light/dark cycles, grouped in acrylic boxes, and allowed free access to water and food (Purina® special food for mice).

In vivo assay - intragastric route

For these assays, the mice were individually kept in acrylic boxes, and divided into 12 groups of 5 individuals: 200 µL was orally administered to each mouse, containing either 50 mg of *B. thuringiensis* crystal proteins or *M. azedarach* aqueous extract, or 1 x 10^{10} CFU of *B. thuringiensis*. In all animals the treatments were carried out through gavage for 0, 12 and 24 hours, at cumulative doses, according to (Vázquez-Padrón et al., 2000). The collection of the total amount of feces from each animal was carried out at 24 and 48 hours after treatment application (HAT). The animals were sacrificed at 48 HAT (hours after treatment), and their stomach content analyzed in a 10% SDS-PAGE (Laemmli, 1970). The stomachs were observed under stereomicroscopy, with 40x magnification, according to a method adapted by Marroni et al. (1994).

In vivo assay - intraperitoneal route

The animals were kept in 3 groups (triplicate experiments) of 5 individuals in acrylic boxes. Treatments were the same as for the oral toxicity assays, although the administration in those treatments was carried out with intraperitoneal injections (200 µL). After treatment application, the animals were monitored for 72 hours, according to Ghazaleh et al. (1992).

RESULTS

Effects of the bacterial and plant extract on the intragastric route in mice

The mice treated intragastrically with *B. thuringiensis* and *M. azedarach* did not show any symptoms such as shaking, convulsions, diarrhea, lethargy, salivation and hair loss (Siegel, 1997), when compared with the control.

Data referring to the protein profile of *B. thuringiensis*, *M. azedarach* and BTH Thuricide used in the experiments are shown in Figure 1.

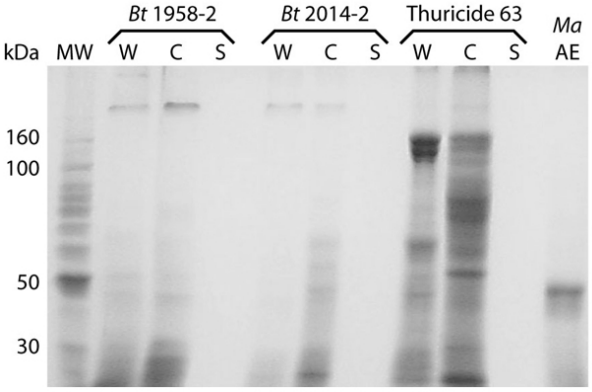

Fig. 1 - *Bacillus thuringiensis* and *Melia azedarach* protein profiles in 10% SDS-PAGE.
MW: molecular weigh markers; W: whole culture suspension; C: purified crystal proteins; S: supernatant; *Ma* AE: *M. azedarach* aqueous extract.

The protein profile of the stomach content and feces from mice treated and not treated with *B. thuringiensis* and *M. azedarach* toxins, assessed in 10% SDS-PAGE, reveal bands of different sizes (Table 1).

Table 1 - Protein profile of stomach contents and feces of CF1 mice treated intragastrically with toxins from *Bacillus thuringiensis* and *Melia azedarach*.

Treatment	Protein profile (kDa)*		
	Stomach content	Feces 24 HAT**	Feces 48 HAT
T_1	98 a 29	127 a 30	108 a 41
T_2	90 a 40	123 a 41	119 a 39
T_3	97 a 38	100 a 24	124 a 29
T_4	100 a 31	143 a 35	137 a 42
T_5	90 a 37	158 a 63	182 a 63
T_6	95 a 40	81 a 36	146 a 33
T_7	170 a 31	214 a 30	157 a 34
T_8	91 a 16	196 a 14	78 a 16
T_9	Ø	Ø	Ø
T_{10}	97 a 38	135 a 29	124 a 42
T_{11}	153 a 32	144 a 35	178 a 53
T_{12}	115 a 28	98 a 30	132 a 34

T_1: suspension *Bt* 1958-2; T_2: supernatant *Bt* 1958-2; T_3: Cry proteins *Bt* 1958-2; T_4: suspension *Bt* 2014-2; T_5: supernatant *Bt* 2014-2; T_6: Cry proteins *Bt* 2014-2; T_7: aqueous extract *M. azedarach*; T_8: PBS pH 7.4; T_9: water; T_{10}: suspension *BTh Thuricide* 63; T_{11}: supernatant *BTh Thuricide* 63; T_{12}: Cry proteins *BTh Thuricide* 63. *Molecular weight proteins in kiloDaltons; **hours after treatment; Ø showed no bands.

Table 2 - Mice mortality 72h after intragastric or intraperitoneal injection of *B. thuringiensis* or *M. azedarach* aqueous extracts.

Sample		Dose	Mortality (%)	
			Oral route	Intraperitoneous route
Bt 1558-2	Whole culture	10^{10} CFU	0	80.0
	Supernatant	50 µg	0	0
	Crystal proteins	50 µg	0	0
Bt 2014-2	Whole culture	10^{10} CFU	0	46.6
	Supernatant	50 µg	0	0
	Crystal proteins	50 µg	0	0
Bth Thuricide 63	Whole culture	10^{10} CFU	0	73.3
	Supernatant	50 µg	0	0
	Crystal proteins	50 µg	0	0
M. azedarach	Aqueous extract	50 µg	0	0

The microscopic examination of the stomach of mice treated with *B. thuringiensis* suspensions, supernatants, Cry proteins, and *M. azedarach* extract did not show any damage due to the treatments when compared with the control (data not shown).

Evaluations of SDS-PAGE (date not shown) of feces of mice treated and non treated with *Bacillus thuringiensis* and *Melia azedarach*, showed a profile of bands with variations, which cannot distinguish a correlation between treatments.

Effect of the bacterial and plant samples on the intraperitoneal route in mice

Results of the intraperitoneal administrations in the animals showed that when a suspension of *Bt* 1958-2, at a dose of 1 x 10^{10} UFC, was administered, there was 80% mortality in a period of 6 to 24 HAT. For *Bt* 2014-2, the mortality was only 46.6%, while 73.3% mortality was observed for *BTh Thuricide* 63 (Table 2). As for the other treatments, no animal died until the end of the assessments for 72 HAT.

The results of mortality intraperitoneal rout were compared through Anova, at a 5% probability rate [F (8.55) = 11.78; $p < 0.001$], as evidence of significant differences. Dunnett's and Fisher's tests (5% probability) confirmed there was a difference between the treatments with *Bt* 1958-2, *Bt* 2014-2 and *BTh Thuricide* 63 suspensions (whole culture) and the other ones, in which there was no mortality. These data point to the fact that the bacterial suspension isolates were toxic to mice, intraperitoneally and under the conditions in which the treatments were carried out.

DISCUSSION

In the literature reviewed, no data referring to the *in vivo* action of *B. thuringiensis* and *M. azedarach* in the stomach of mice or others mammals was found. Follow-up analyses of symptoms from chemical substances in animals showed no change in the stomach mucosa of CF1 mice when treated with *B. thuringiensis* and *M. azedarach*, when compared with the control.

Similar results were noticed by Bishop et al. (1999) after oral application of 5 x 10^{10} spores/day of *B. thuringiensis thuringiensis* and *B. thuringiensis israelensis* to mice. According to this investigation, those authors showed there was no significant difference in the body weight of treated and non-treated animals. Our results are in agreement with those of Siegel (2001), in which mice were orally treated with 10^{9} spores/day, for 730 days, without damage.

The protein profile data of the stomach content and feces, assessed in 10% SDS-PAGE (Table 1), suggest that *B. thuringiensis* and *M. azedarach* proteins are degraded by the mammals' digestive system. This protein degradation could also be associated with bacterial cells killed by the pH of the mammal's stomach, since Vilas-Bôas et al. (1998) showed that the germination and the viability of *B. thuringiensis* are inhibited in acidic conditions, below 5.0 pH (pH is around 3.15 in mammals) (Vidal et al., 2004). For the Chinaberry extracts, results also suggest a degradation and/or inactivation of the protein content.

On the other hand, Méndez et al. (2002) reported that *M. azedarach* leaves were toxic, orally, to bovines, showing effects such as dry and bloody feces, muscle tremors and hypothermia. In the swine the ingestion of mature fruit of the bakain tree effected changes in their nervous, muscular, and digestive functions (Timm; Riet-Correa, 1997). Despite the conditions under which those experiments were carried out, the aqueous extract of *M. azedarach* leaves has not shown toxicity against mice.

Regarding the Cry proteins, the isolates used in this paper showed the presence of the genes that code the Cry1 and the Cry2 proteins in *Bt* 1958-2, and the Cry3 protein in *Bt* 2014-2 (Pinto; Fiuza, 2003). As such, data by Betz et al. (2000) reported that, in simulation models of human gastrointestinal conditions,

Cry1, Cry2 and Cry3 proteins were degraded in 30 seconds after in vitro assays, resulting in 2 kDa proteins. In this sense, a similar fact could have occurred in the mice's digestive system since the Cry proteins did not show toxicity against those animals, in vivo.

These results indicate that purified Cry proteins of *Bt* 1958-2 and *Bt* 2014-2 isolates can be promising for the biologic control of pests because they were intraperitoneally atoxic to CF1 mice. Nevertheless, Vasquez-Padrón et al. (2000) and Moreno-Fierros et al. (2000) reported that Balb/c mice showed a high production of IgA antibodies, followed by IgG and IgM, after oral, rectal and intraperitoneal administration of Cry1Ac protein, thus showing an effective immune reaction against those animals.

Taking into consideration the toxicity of the *B. thuringiensis* bacterial suspensions to mice, it can be suggested that it is associated with the presence of nonspecific virulence factors of that enthomopathogen, such as chitinases, proteases (Schenpf et al., 1998), phospholipases and enterotoxins, which correspond to those produced by *B. cereus* (Lereclus et al., 1996). Those authors showed that *B. thuringiensis* isolates have the *plc*A gene that synthesizes phospholipase C, and that it is temporarily regulated by the transition activator PlcR. This activator also regulates the expression of those other extracellular virulence factors in *B. thuringiensis* (Agaisse et al., 1999).

Another toxin that can be associated with animal mortality is the thuringiensin which corresponds to the β-exotoxin that is toxic to mammals. This toxin is thermostable and resistant to the sterilization process at 121º C for 15min, as described by Perani et al. (1998). But the presence of that toxin in the new isolates in this investigation was not confirmed in SDS-PAGE (10%) analyses, since the mice treated with the autoclaved supernatant did not die, or its concentration was too low in the supernatants.

As for the production of thuringiensin, Hernandéz et al. (2003) report that 79% of *B. thuringiensis thuringiensis* isolates produce that toxin, followed by 20% for *B. thuringiensis kenyae*, and 13% for *B. thuringiensis kurstaki*. In this paper the *BTh Thuricide* 63 isolate used as a pattern corresponds to the active ingredient of the commercial product Thuricide®, which was banned due to b-exotoxin production (Siegel, 2001). But that author reports that 18 human beings have ingested 1.000 mg of Thuricide® for 5 days and showed no intoxication effects. In that context, the doses applied in laboratory assays with vertebrates and invertebrates are much higher than those used in field applications.

At present, there is great concern regarding the effects of those genetically modified plants with *B. thuringiensis* genes on consumers, despite the fact that Azevedo; Araújo (2003) showed the absence of any toxic, mutagenic, teratogenic or clastogenic effects of transgenics. Betz et al. (2000) also reported that Cry proteins of *B. thuringiensis* were not toxic in direct contact, seeing that the target-animals exposure was extremely low, and the presence of those proteins in plant tissues also occurred at low concentrations.

Thus, it can be said that *Bt* 1958-2 and *Bt* 2014-2 bacterial isolates are toxic to CF1 mice only when a cell and spore suspension is inoculated intraperitoneally. But that is not a natural route used for the referred entomopathogen. This mortality may be associated with other virulence factors present in different strains of *B. thuringiensis* such as the VIP proteins, hemolysins, exotoxins and enterotoxins (Vilas-Bôas et al., 2012). Despite that, McClintock et al. (1995) report that mice mortality by *B. thuringiensis kurstaki* is associated with the vegetative stage of bacterial growth or the presporulation of bacterial cells, but not with the insecticidal crystal proteins.

Corrêa et al. (2012) tested Cyt and Cry proteins in human breast cancers cells MCF-7, whereas no toxic effect was observed for Cry toxins activated with trypsin. In this case, the Cyt2Ba protein was toxic to the cells when tested at 20 mg/mL.

That being so, the Cry proteins of the new *B. thuringiensis* isolates must be evaluated for their use as tools in the biotechnology field, since they do not show toxicity against mammals, intragastrically or peritoneally, just like the *M. azedarach* aqueous extract, with those being indicated for the biological control of pest insects.

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