DOI: 10.1590/1808-1657000132018

Toxicity of Cry2 proteins from *Bacillus thuringiensis* subsp. *thuringiensis* strain T01-328 against *Aedes aegypti* (Diptera: Culicidae)

Toxicidade das proteínas Cry2 do **Bacillus thuringiensis** *subsp.* **thuringiensis** *T01-328 contra* **Aedes aegypti** *(Diptera: Culicidae)*

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ABSTRACT: *Bacillus thuringiensis* subsp. *israelensis* has been used to control the *Aedes aegypti* (*Diptera: Culicidae*) mosquito larvae, the vector of virus diseases such as dengue, Chikungunya and Zika fever, which have become a major public health problem in Brazil and other tropical countries since the climate favors the proliferation and development of the transmitting vector. Because *B. thuringiensis* has shown potential for controlling insects of the Diptera order, this work aimed at testing the *Bacillus thuringiensis* subsp. *thuringiensis* strain T01-328 and its proteins Cry2Aa and Cry2Ab for control *A. aegypti* and at comparing the results to the *B. thuringiensis* subsp. *israelensis* specific dipteran strain. To this end, bioassays using spore-crystal of both strains, and Cry2Aa and Cry2Ab proteins from the heterologous expression in *Escherichia coli*, were performed against *A. aegypti* larvae. The results showed that the *B. thuringiensis thuringiensis* T01-328 has insecticidal activity against the larvae, but it is less toxic than *B. thuringiensis* subsp. *israelensis*. Cry2Aa and Cry2Ab proteins expressed heterologously were effective for controlling *A. aegypti* larvae. Therefore, the results indicate that the Cry2Aa and Cry2Ab proteins of the *B. thuringiensis thuringiensis* T01-328 can be used as an alternative to assist in the control of *A. aegypti*.

KEYWORDS: Cry proteins; biological control; dengue; Zika; mosquito.

RESUMO: *Bacillus thuringiensis* subsp. *israelensis* vem sendo empregada no controle do díptero *Aedes aegypti*, vetor do vírus causador de doenças como dengue, febre Chikungunya e Zika, que se tornou um dos grandes problemas de saúde pública no Brasil e em outros países de clima tropical, que favorece a proliferação e o desenvolvimento do transmissor. Em virtude do potencial de *B. thuringiensis* no controle de dípteros, a proposta deste trabalho foi testar as proteínas Cry2Aa e Cry2Ab da linhagem de *Bacillus thuringiensis* subsp. *thuringiensis* T01-328 no controle de *A. aegypti*, em comparação à linhagem díptero específica *B. thuringiensis* subsp. *israelensis*. Para tanto, foram realizados bioensaios com larvas de *A. aegypti* com o esporo-cristal de ambas as linhagens, bem como com as proteínas Cry2Aa e Cry2Ab com expressão heteróloga em *Escherichia coli*. A linhagem *B. thuringiensis thuringiensis* T01-328 apresentou atividade inseticida contra as larvas, porém foi menos tóxica que a *B. thuringiensis* subsp. *israelensis*. As proteínas Cry2Aa e Cry2Ab expressas de forma heteróloga foram eficazes no controle de *A. aegypti*. Os resultados obtidos sugerem as proteínas Cry2Aa e Cry2Ab da linhagem *B. thuringiensis thuringiensis* T01-328 como alternativas para contribuir no controle do *A. aegypti*.

PALAVRAS-CHAVE: proteínas Cry; controle biológico; dengue; Zika; mosquito.

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Received on: 02/01/2018. Accepted on: 08/31/2018

INTRODUCTION

Dengue is one of the major public health problems in tropical countries, and its incidence has increased dramatically around the world in recent decades. During the 2017 Epidemiological Week, 251,711 probable cases of dengue were registered in the country according to the Health Department (BRASIL, 2018), although the actual numbers of dengue cases are underestimated and in many cases incorrectly classified. It is estimated that 500 thousand people with severe dengue require hospitalization each year, a large proportion of children and about 2% of those affected die (WHO, 2017).

In 2014 and 2015, health officials also identified cases of Chikungunya and Zika fevers in Brazil, both caused by viruses that are carried and transmitted by *Aedes aegypti* mosquitoes (BRASIL, 2014). Furthermore, Zika fever, caused by the Zika virus (ZIKV), can be transmitted via mosquito bite, as well as sexually from human to human and vertically, from mother to fetus (D'ORTENZIO et al., 2016; MANSUY et al., 2016). Maternal transmission to the fetus presents a serious risk of causing fetal congenital anomalies (SCHULER-FACCINI et al., 2016).

These tropical diseases are mainly controlled by combating its transmitting vector. One of the methods used is the chemical control, which consists of using chemical insecticides to kill the mosquitoes. However, the rapid increase of mosquito resistance to various chemical insecticides containing one or few active ingredients that promote selection insects resistant and the growing public concern about environmental pollution have resulted in the development of alternatives for controlling the mosquitoes, such as using the biological agent *Bacillus thuringiensis* subsp. *israelensis* (Bti). In addition, *A. aegypti* ranks the first in the list of Diptera species with the highest number of resistance cases worldwide (ARTHROPOD PESTICIDE RESISTANCE DATABASE, 2018).

Bacillus thuringiensis subsp. *israelensis* is highly relevant in the fight against the dengue vector, since it acts as an efficient biolarvicide integrated in the *A. aegypti* Control Program (PCA), conducted in several municipalities affected by the disease (BRAGA et al., 2004). In Rio de Janeiro, Ceará, and Rio Grande do Norte, it was recommended to replace the Temephos (organophosphorus) insecticide with biolarvicides based on *B. thuringiensis* subsp. *israelensis*, considered one of the most effective biological control agents against culicids (BRASIL, 2000). The larvicidal action of *B. thuringiensis* subsp. *israelensis* is achieved by the expression of the Cry4Aa, Cry4Ba, Cry11Aa, Cyt1Aa, Cry10Aa and Cyt2Ba proteins, whose synergistic action reduces the probability of resistance selection. Despite the lack of reports on the emergence of *A. aegypti* resistance to bioinsecticides based on this subspecies in the field, it is important to search proteins with alternative action mode that can be used for controlling the mosquito (BEN-DOV, 2014). Furthermore, it is also essential to study other *B. thuringiensis* subspecies pathogenic to *A. aegypti* that could represent a promising source of control agents for eventual use in the future.

Thus, the present work aimed at investigating the bioinsecticidal potential of the *B. thuringiensis thuringiensis* strain T01-328 to control the *A. aegypti* vector. This strain genome is sequenced in the GenBank with the ARXZ020000000 accession number, so that the promising genes with bioinsecticidal activity against dipterans were identified (VARANI et al., 2013).

MATERIALS AND METHODS

Dipterans

The *A. aegypti* eggs were kindly provided by the Malaria and Dengue Laboratory of the National Research Institute of Amazonas, in Manaus, Amazonas, Brazil. After egg hatching, the larvae were maintained in water and kept in air-conditioned (Bio-Oxygen Demand) B.O.D. incubator at 25 ± 2ºC, 70% ± 10% relative humidity, and 14 h light: 10 h dark photoperiod. The larvae were fed with three-pellet feed for reptiles (Reptolife).

Bacterial spore-crystal bioassays with *Bacillus thuringiensis*

Bacillus thuringiensis subsp. *thuringiensis* strain T01-328 and *Bacillus thuringiensis* subsp. *israelensis* T14001 (positive dipteran control) were cultured in solid nutrient agar medium (3.0 g/L meat extract, 5.0 g/L gelatin peptone, 15.0 g/L agar and pH 6.8) (GORDON et al., 1982) and incubated at 30ºC for 5 days, as to allow complete sporulation and crystal release. After this period, all bacterial contents were retrieved from the culture using a platinum loop and transferred into tubes containing 10 mL of sterile distilled water and 0.05% Tween (adhesive spreader). The obtained spore-crystal suspension was homogenized by vortexing, and the suspended material was diluted, so that the spores were counted using the Neubauer Chamber placed on the optical microscope stage (ALVES; MORAES, 1998). The strains were tested at five different concentrations (3.0×10^9, 1.5×10^9, 0.75×10^9, 0.38×10^9 and 0.19×10^9 spore-crystal/mL). Twenty 3rd-instar larvae were placed in plastic cups containing 200 mL of deionized water (negative control) and water plus the spore-crystal suspension of the studied strains (five treatments) with three replicates per treatment. The bioassays were kept in B.O.D. incubator at 25 ± 2ºC and 12 h light: 12 h dark photoperiod. In data analysis, mortalities corrected according to Abbott's formula, from different replicates, were grouped for analysis (WHO, 2005). The LC_{50} was determined using the probit analysis of the POLO-PC statistic software (LeOra Software, Berkeley, California, United States).

Obtaining the Cry2Aa and Cry2Ab proteins

Escherichia coli BL21 (DE3) clones containing the *cry2Aa* and *cry2Ab* gene of the *B. thuringiensis thuringiensis* strain T01-328 cloned in the pET-Sumo vector (Invitrogen, Carlsbad, California, United States) were cultured in Luria Bertani medium (LB) with kanamycin (50 µg/mL). An isolated colony was transferred to liquid LB medium supplemented with kanamycin (50 µg/mL) and multiplied under constant stirring at 250 rpm and 37ºC for 16 h (Eppendorf / New Brunswick Scientific Innova 4340 Illuminated Refrigerated Incubator Shaker). When the cultures prepared from the pre-inoculum reached optical density $(O.D)_{600}$ 0.6, they were induced to express the proteins by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were maintained at 200 rpm and 22ºC for 16 h (Eppendorf / New Brunswick Scientific Innova 4340 Illuminated Refrigerated Incubator Shaker). Subsequently, they were centrifuged at 17,400 g for 20 min, and the cells were resuspended in extraction buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole) plus 3 mg/mL lysozyme and 0.01 mg/mL deoxyribonuclease (DNAse), and kept at 200 rpm and 37ºC, for 30 min (Eppendorf / New Brunswick Scientific Innova 4340 Illuminated Refrigerated Incubator Shaker). The samples were sonicated at 60 W for 60 s, twice, with a 10-second interval. Finally, the lysates were centrifuged at 17,400 g and 4ºC for 30 min, and the supernatants were stored at -20ºC until further use. The same culture and extraction procedures were performed starting from *E. coli* BL21 (DE3) colony without the expression vector.

The protein expression was verified on 12% sodium dodecyl sulfate polyacrylamide electrophoresis gel (SDS-PAGE), according to LAEMMLI (1970). The concentration of the Cry2Aa and Cry2Ab proteins in each extract was obtained by SDS-PAGE densitometry using serum bovine albumin as a standard in the ImageQuant TL 8.1 software (GE Healthcare Bio-Sciences, AB, Uppsala, Sweden).

Bioassays with the Cry2Aa and Cry2Ab proteins against *Aedes aegypti*

The Cry2Aa and Cry2Ab proteins and the Cry2Aa + Cry2Ab protein mixture were distributed in cups at the concentrations of 10, 20, 40, 50 and 60 µg/mL. Two controls were used: deionized water and *E. coli* lysate from a culture without induction of heterologous expression. The treatments were performed in triplicate with 20 larvae per replicate. Mortality rate was evaluated after the larvae were exposed to the treatment for 24 hours, and only completely inert larvae were counted as dead. The bioassays were kept in B.O.D. incubator at 25 ± 2ºC, and 12 h light: 12 h dark photoperiod. The LC_{50} and LC_{90} were estimated by the probit analysis of the POLO-PC software (LeOra Software, Berkeley, California, United States). The interaction between the proteins was analyzed at 1:1 protein ratio according to TABASHNIK (1992). The synergism factor (SF) was given by the expected and observed LC ratio.

RESULTS

Spore/crystal bioassays with *Bacillus thuringiensis* subsp. *thuringiensis* strain T01-328 and *B. thuringiensis israelensis*

Bioassays with the spore/crystal mixture were performed to evaluate the insecticidal activity of *B. thuringiensis thuringiensis* strain T01-328 against *A. aegypti* larvae (Table 1). The LC_{50} 9.1 x 10^8 spore-crystal/mL obtained in the trials confirmed larva susceptibility to *B. thuringiensis thuringiensis*. However, *B. thuringiensis thuringiensis* strain T01-328 was less effective for controlling Diptera *B. thuringiensis israelensis* compared to the reference *B. thuringiensis israelensis* subspecies that caused 100% mortality at all concentrations indicating a LC_{50} less than 0.1875 x 10^9.

Bioassay with Cry2Aa and Cry2Ab proteins

The Cry2Aa and Cry2Ab heterologous expression was confirmed by visualizing the approximately 70 kDa bands for both in the SDS-PAGE (Fig. 1).

Bioassays with Cry2Aa and Cry2Ab proteins against *A. aegypti* larvae indicated that these proteins might be a promising alternative for mosquito control (Table 2). The equivalent

Table 1. Susceptibility of *Aedes aegypti* larvae to spore/crystal protein from *Bacillus thuringiensis* strains.

Treatment	LC_{50} (spore-crystal/mL) (CI Min. – Max.)[a]	Chi-square	b ± (SE)[b]
B. thuringiensis thuringiensis strain T01-328	9.1 x 10^8 (4.2 x 10^8 — 1.2 x 10^9)	0.7	1.8 ± 0.5
B. thuringiensis subsp. *israelensis*	< 0.1875 x 10^9	ND[c]	ND

[a](CI Min. – Max.): confidence interval (95%CI); [b]b ± (SE): slope and standard error; [c]undetermined.

toxicity of the Cry2Aa and Cry2Ab proteins used to control *A. aegypti* larvae is verified by the overlapping confidence interval of the obtained results. The Cry2Aa + Cry2Ab toxin mixture was slightly less toxic than expected, as the expected LC_{50} was below the limit of the observed LC_{50} confidence interval, thus suggesting an antagonistic tendency among proteins. However, the expected LC_{90} is within the observed LC_{90} confidence interval for *A. aegypti* larvae. The antagonism observed between the Cry2Aa and Cry2Ab proteins was weak at LC_{50} and disappeared at LC_{90}.

DISCUSSION

Creating and maintaining *B. thuringiensis* collections is important for the characterization of new δ-endotoxins that are effective against several insect orders.

The studied *B. thuringiensis thuringiensis* strain T01-328 has genes with insecticidal activity against Lepidoptera and Coleoptera, which is important for agriculture, and showed toxic activity against Dipteran. The Cry2Aa and Cry2Ab proteins responsible for this activity exhibit 87% structural conservation between themselves (CRICKMORE, 2016).

Likewise, ARMENGOL et al. (2006) tested positive isolates carrying the *cry2*, *cry4* and *cry11* genes against *Culex quinquefasciatus* mosquitoes and reported higher efficiencies for *B. thuringiensis* subsp. *israelensis* positive control than the isolates carrying the *cry2* only and *cry2/cry11* genes.

In another study, COSTA et al. (2010) performed bioassays to compare the LC_{50} of isolates selected for the *cry4Aa*, *cry4Ba*, *cry11Aa*, *cry11Ba*, *cyt1Aa*, *cyt1Ab*, *cyt2Aa* and *chi* genes and *B. thuringiensis* subsp. *israelensis*. After 24 h, these authors reported that the treatment containing 1.5 x 10^7 spores-crystal/mL of *B. thuringiensis* subsp. *israelensis* caused 100% larval mortality, whereas the mortality rate of the other isolates ranged from 0 to 100% due to the presence of genes. In addition, isolates carrying the *cry4Ba* and *chi* genes had mortality rates varying from 0 to 56%, while the isolates carrying the *cry4Aa*, *cry4Ab* and *cry11* genes and one or more of the *cyt1Ab*, *cyt2Aa* and *chi* genes resulted in 100% mortality rate.

Similar to this work, this difference can be explained by the number of genes acting on these isolates since *B. thuringiensis thuringiensis* strain T01-328 has only two insecticidal genes, *cry2Aa* and *cry2Ab*, in its genome, whereas *B. thuringiensis* subsp. *israelensis* strain carries eight genes, *cry4Aa*, *cry4Ba*, *cry11Aa*, *cry11Ba*, *cyt1Aa*, *cyt1Ab*, *cyt2Aa*, and *chi*. The proteins present in the *B. thuringiensis* subsp. *israelensis* (Cry4Ba, Cry11A, and Cyt) have synergistic effect, increasing subspecies efficiency on vector control (FERNÁNDEZ-LUNA et al., 2010). The synergistic action of these toxins reduces the likelihood of developing resistance against them (BECKER, 2000; REGIS et al., 2001).

Bacillus thuringiensis subsp. *israelensis* results in this study are similar to the mortality of 100% for *A. aegypti* larvae reported by LEE et al. (2005) and ESPINDOLA et al. (2008). LIMA et al. (2005) used the *Bacillus thuringiensis* subsp. *israelensis* to control the 3rd instar larvae in containers made of different materials, under natural conditions in Rio de Janeiro,

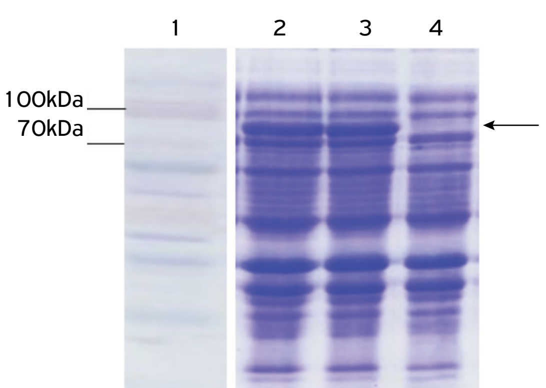

Figure 1. Sodium dodecyl sulfate polyacrylamide electrophoresis gel (SDS-PAGE) 12% of the heterologous expression of the Cry2A proteins in the channels: (1) Molecular Mass Marker (kDa) "Spectra™ Multicolor Broad Range ProteinLadder" (Fermentas); (2) Cry2Aa protein; (3) Cry2Ab protein

Table 2. Susceptibility of *Aedes aegypti* larvae to Cry2Aa and Cry2Ab proteins from *Bacillus thuringiensis* subsp. *thuringiensis* strain T01-328.

Proteins	LC_{50} (μg/mL) (CI Min. – Max.)[a]		SF	LC_{90} (μg/ml) (CI Min. – Max.)[a]		SF	b ± (SE)[b]	χ^2
	Observed	Expected		Observed	Expected			
Cry2Aa	52.6			133.0			3.2 ± 0.849	1.8
	(40.9 – 105.1)			(77.5 – 713.5)				
Cry2Ab	35.8			47.7			10.3 ± 1.4	4.7
	(30.7 – 40.9)			(41.6 – 67.6)				
Cry2Aa-Cry2Ab	51.3	42.6	0.8	63.8	70.2	1.1	13.5 ± 2.0	5.7
	(47.1 – 56.1)			(57.8 – 82.5)				

[a](CI Min. – Max.): confidence interval (95%CI); [b]b ± (SE): slope and standard error; SF: synergism factor.

Brazil, and reported mortality rates up to 70% for asbestos. *B. thuringiensis* subsp. *israelensis* bacterium is highly relevant in the fight against the vector of dengue viruses, because it acts as an efficient biolarvicide integrated in the scope of the PCA, conducted in several municipalities affected by the disease (BRAGA et al., 2004). The larvicide Bt-horus SC (*B. thuringiensis* subsp. *israelensis* - active principle) developed by Embrapa Genetic Researches and Biotechnology and Bthek Biotechnology was applied in São Sebastião (Distrito Federal, Brazil) successfully (MONNERAT et al., 2012). Furthermore, the research for new isolates and proteins with larvicidal toxicity against mosquito species vector of several diseases is a constant in biological control and aims to increase the efficacy of bioinsecticides (LOBO et al., 2018; SOARES-DA-SILVA et al., 2017; EL-KERSH, et al., 2016).

A previous study on the diversity of *B. thuringiensis* isolates conducted a search of the *cyt1*, *cyt2*, *cry2*, *cry4A*, *cry4*, *cry10*, *cry11*, *cry17*, *cry19*, *cry24*, *cry25*, *cry27*, *cry29*, *cry30*, *cry32*, *cry39* and *cry40* genes by polymerase chain reaction (PCR) and found four isolates (LBIT315, LBIT320, LBIT348, and IB604) more efficient than *B. thuringiensis* subsp. *israelensis* and one isolate (147-8906) slightly less toxic against *A. aegypti* larvae than *B. thuringiensis* subsp. *israelensis* positive control (IBARRA et al., 2010). However, the 147-8906 isolate, which carries the *cry11*, *cyt1*, *cyt2* and *cry30* genes, is highlighted, because it differs from *B. thuringiensis* subsp. *israelensis* control gene composition and maintains the insecticidal activity, despite having a lower number of genes. The use of toxins different from those present in the *B. thuringiensis* subsp. *israelensis* based on bioinsecticides can increase efficiency and prolong field use.

The search for gene-carrier isolates that are efficient to control dipterans such as *B. thuringiensis thuringiensis* strain T01-328 is important for controlling the vectors. The *cry4A*, *cry4B*, *cyt2*, *cry10* and *cry11* genes are commonly sought in *B. thuringiensis* isolates in order to control dipterans. EL-KERSH et al. (2014) searched for insecticidal genes in isolates from Saudi Arabia, including *cry2A* class genes, but *cry2* class genes were not found. Some isolates analyzed by these authors were more efficient to control *Aedes caspius* and *Culex pipiens* than *B. thuringiensis* subsp. *israelensis* positive control. The tests with these isolates against *A. caspius* resulted in LC_{50} ranging from 0.6 μg/mL (the most efficient) to 83 μg/mL (the least efficient). Therefore, *B. thuringiensis thuringiensis* strain T01-328 efficiency is within the interval of these isolates and the Cry2Aa + Cry2Ab mixture had LC_{50} of 17 μg/mL against *A. aegypti* larvae (LIMA, 2009).

Previous studies have been performed with the Cry2Aa and Cry11A proteins of *Bacillus thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis*, respectively, expressed by baculoviruses (LIMA, 2009). The results showed that Cry2Aa had no activity against 2nd instar larvae of *A. aegypti*, whereas Cry11A had LC_{50} of 53.3 ng/mL and the *B. thuringiensis israelensis* standard subspecies had an even higher toxicity, LC_{50} of 2.2 ng/mL. These results differ from those found in this work, because the Cry2Aa and Cry2Ab proteins of the T01-328 isolate were equally efficient to control *A. aegypti* larvae. These differences found between the two studies may stem from differences between mosquito populations, amino acid composition, and expression and preparation of toxins.

A study on the diversity of *cry2* genes in *B. thuringiensis* isolates from China found 322 isolates that were *cry2* gene carriers, while *cry2Aa* and *cry2Ab* were the most abundant (LIANG et al., 2011). In addition, toxicity studies against *A. aegypti* larvae showed that the Rpp39 isolate carrying the *cry2Aa* gene was the least toxic (LC_{50} > 100 μg/mL), whereas the Ywc5-4 (LC_{50} 23.42 μg/mL) isolates carrying the *cry2Aa* gene, JF19-2 (LC_{50} 2.541 μg/mL) carrying *cry2Ag*, Bts carrying *cry2Aa/cry2Ab* (LC_{50} 17.65 μg/mL) were the most efficient to control *A. aegypti*.

The Cry2Aa protein also exhibits toxicity against *Culex pipiens* larvae, demonstrating synergistic interaction when mixed with Cry4B protein (ZGHAL et al., 2006). In addition to the high insecticidal potential of Cry2Aa and Cry2Ab proteins against *A. aegypti* larvae, this may be an indication that the Cry2Aa/Cry2Ab mixture with other insecticidal toxins as Cry11 and Cry4 may potentiate the control of this vector.

The ability of gene transfer in *B. thuringiensis* allows using recombinant proteins with better characteristics/traits to improve the activity, yield, and stability, expressing several genes of toxins of this bacterium, generating new active products. Thus, genetic modifications to build hybrid proteins obtained in Bt subspecies are possible from conjugation, transformation, and recombination (CÉRON, 2004).

Although *B. thuringiensis* subsp. *israelensis* is effective to control mosquitoes and it is a World Health Organization (WHO)-recommended larvicide for use in potable water to control *A. aegypti* larvae, replacing chemicals, the diversity found in isolates may benefit vector control. The search for new isolates and proteins with insecticidal activity is a constant in the field of biological control and aims at increasing the insecticidal activity of the products, generating alternatives for resistance management and reaching insects of agronomic importance or vectors of pathogens that are not susceptible to the existing resources. The study of the Cry2Aa and Cry2Ab proteins of the *B. thuringiensis thuringiensis* strain T01-328 is relevant, because it characterizes this strain and these toxins as promising for controlling *A. aegypti,* the vector of important diseases such as Zika, dengue, and Chikungunya.

CONCLUSIONS

The spore-crystal suspension of *B. thuringiensis thuringiensis* strain T01-328 is toxic to *A. aegypti* larvae; however, it

showed lower insecticidal potential than the *B. thuringiensis* subsp. *israelensis*. Futhermore, Cry2Aa and Cry2Ab proteins have insecticidal activity against the vector *A. aegypti* and an additive mode of action. It is, therefore, recommended to search these genes in isolates that aim at controlling the *A. aegypti* larvae.

ACKNOLEDGEMENTS

To the São Paulo State Foundation for the Research Support (FAPESP) — Process #2016/03108-5 — and to Dr. Wanderli Pedro Tadei researcher from Malaria and Dengue Laboratory of the National Research Institute of Amazonas (INPA), for the supply of eggs of *A. aegypti*.

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