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Original Article

Isolates of *Bacillus thuringiensis* from Maranhão biomes with potential insecticidal action against *Aedes aegypti* larvae (Diptera, Culicidae)

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Abstract

Entomopathogenic agents are viable and effective options due to their selective action against insects but benign effects on humans and the environment. The most promising entomopathogens include subspecies of *Bacillus thuringiensis* (Bt), which are widely used for the biological control of insects, including mosquito vectors of human pathogens. The efficacy of *B. thuringiensis* toxicity has led to the search for new potentially toxic isolates in different regions of the world. Therefore, soil samples from the Amazon, Cerrado and Caatinga biomes of the state of Maranhão were evaluated for their potential larvicidal action against *Aedes aegypti*. The isolates with high toxicity to mosquito larvae, as detected by bioassays, were subjected to histological evaluation under a light microscope to identify the genes potentially responsible for the toxicity. Additionally, the toxic effects of these isolates on the intestinal epithelium were assessed. In the new *B. thuringiensis* isolates toxic to *A. aegypti* larvae, *cry* and *cyt* genes were amplified at different frequencies, with *cry4*, *cyt1*, *cry32*, *cry10* and *cry11* being the most frequent (33-55%) among those investigated. These genes encode specific proteins toxic to dipterans and may explain the severe morphological changes in the intestine of *A. aegypti* larvae caused by the toxins of the isolates.

Keywords: mosquito vectors, entomopathogenic bacterium, *cry* and *cyt* genes, histology.

Isolados de *Bacillus thuringiensis* de biomas maranhenses com potencial ação inseticida contra larvas de *Aedes aegypti* (Diptera, Culicidae)

Resumo

Os agentes entomopatógenos são alternativas viáveis e eficazes, devido à sua ação seletiva para insetos sendo inofensivos ao homem e ao meio ambiente. Dentre os entomopatógenos mais promissores, destacam-se as subespécies de *Bacillus thuringiensis* (Bt) amplamente utilizadas no controle biológico de insetos incluindo espécies de mosquitos vetores de

agentes patogênicos ao homem. A eficiência da toxicidade de Bt incentiva a prospecção de novos isolados em diversas regiões do mundo. Desta forma, em busca de novos isolados de *B. thuringiensis* potencialmente tóxicos, amostras de solo provenientes dos biomas Amazônia, Cerrado e Caatinga do estado do Maranhão foram avaliadas em relação ao seu potencial larvicida para *Aedes aegypti*. Os isolados que provocaram elevada toxicidade para larvas do mosquito, detectada por bioensaios, foram avaliados em relação aos potenciais genes responsáveis pela atividade tóxica, além da avaliação de efeitos tóxicos no epitélio intestinal através de análises histológicas em microscopia de luz. Os novos isolados de Bt tóxicos para larva de *A. aegypti* amplificaram frequências diferentes de genes *cry* e *cyt* sendo os mais frequentes (55-33%) os *cry4*, *cyt1*, *cry32*, *cry10* e *cry11* dentre os investigados. Esses genes codificam para proteínas tóxicas específicas para ordem Diptera, e podem explicar as severas alterações morfológicas provocadas pelas toxinas dos isolados observadas no intestino das larvas de *A. aegypti*.

Palavras-chave: insetos vetores, bacilos entomopatogênicos, genes *cry* e *cyt*, histopatologia.

1. Introduction

Aedes (*Stegomyia*) *aegypti* (L. 1762) is an important vector of the viruses that cause dengue fever, urban yellow fever, chikungunya and Zika fever. This mosquito is anthropophilic and domestic, with diurnal haematophagic activity (Consoli and Lourenço-de-Oliveira, 1994; Vasconcelos, 2015; Valle et al., 2016; Montagner et al., 2018), and its adaptive success is associated with favourable domestic or peridomestic conditions, such as the presence of humans and aquatic breeding sites; rapid development and eggs that are resistant to desiccation (Zara et al., 2016; Carvalho and Moreira, 2017; Bassani et al., 2019). These factors contribute to the difficulty in controlling this mosquito and thus to a global public health problem.

Currently, the *A. aegypti* mosquito is controlled by eliminating breeding sites or using chemical insecticides to target adults and/or larvae (Braga and Valle, 2007; Brasil, 2019). However, the frequent use of chemical insecticides selects for resistant mosquito populations, limiting the effectiveness of this strategy in shrinking the *A. aegypti* population. Therefore, it is necessary to search for alternative methods of reducing the population size of this vector, such as the use of entomopathogenic bacteria (Carvalho and Moreira, 2017; Moyes et al., 2017; Aponte et al., 2019).

The entomopathogenic bacterium *Bacillus thuringiensis* (Bt), Goldberg and Margalit (1977) is considered highly effective at controlling immature forms of mosquitoes since several isolates produce δ-endotoxin-containing protein crystals with insecticidal action against several insect orders, including Diptera (Van Frankenhuyzen, 2013; Soares-da-Silva et al., 2017; Soberón et al., 2018). *Bacillus thuringiensis* subsp. *kurstaki/B. thuringiensis* subsp. *aizawai* (Lepidoptera), *B. thuringiensis* subsp. *tenebrionis* (Coleoptera) and *B. thuringiensis* subsp. *israelensis* (Diptera) are examples of subspecies with specific action against different groups of insects (Seifinejad et al., 2008).

The success of this bacterium in biological control is due to its wide insecticidal spectrum as well as the susceptibility of the target insect to the bacillus, which causes effective larval toxicity. The onset of larval toxicity occurs immediately after ingestion of the bacterial crystals, which are later solubilized at the alkaline pH in the larval midgut, releasing the protoxins. These protoxins are processed into a toxic form by serine proteases, and in this active form, the toxins bind to the specific receptors of the intestinal epithelium. This binding enables the formation of pores in the cell membrane, causing an ion imbalance that consequently causes cell rupture and disintegration and culminates in the death of the insect (Glare and O'Callagham, 2000; Zhang et al., 2017; Soberón et al., 2018).

Isolates of Bt with action against Diptera produce crystals with δ-endotoxins from the Cry and Cyt families that are encoded by the *cry* and *cyt* genes during sporulation (Schnepf et al., 1998), and these toxins can act synergistically in larval toxicity (Crickmore et al., 1995; Ben-Dov, 2014). As described above, *B. thuringiensis* subsp. *israelensis* has insecticidal activity against the larvae of different mosquito species; thus, it is used worldwide to manufacture insecticidal formulations for vector control (Raymond et al., 2010; Bravo et al., 2011). Due to the combination of *cry* and *cyt* genes, the toxic activity of this subspecies can cause larval death 24 h after of exposure (Sun et al., 2013; El-Kersh et al., 2014; Soares-da-Silva et al., 2017).

The presence of *cry* and *cyt* genes can be used to predict the insecticidal activity of a novel isolate. Due to the complexity of the action of δ-endotoxins, it is also necessary to assess the frequency of the genes involved in larval mortality and to understand the mode of action of these proteins in larval intestinal cells (Cavados et al., 2004; Oliveira et al., 2009; Soberón et al., 2018).

Information on the toxic effects of this important bacterium may aid in the selection of new isolates for use in the field control of mosquito disease vectors, such as *A. aegypti*. The toxicity of each Bt isolate must also be assessed through in vivo assays based on the ability of the bacteria to interact with the intestinal epithelium of the susceptible host.

Thus, the present research investigated the presence of genes encoding mosquito-acting insecticidal proteins and compared the epithelial damage due to different toxin combinations with that caused by the standard Bti strain. Assessing the ability of different combinations of Cry and Cyt toxins to interact with the intestinal epithelium of susceptible hosts will allow new isolates for the control of mosquito transmitters of pathogens, such as *A. aegypti*, to be selected.

2. Material and Methods

2.1. Selection of Bacillus thuringiensis isolates

A total of nine Bt isolates that showed high toxicity to *A. aegypti* larvae, as previously determined in susceptibility bioassays (Lobo et al., 2018), were selected. The gene profiles of these isolates differed from those of the strains that are already used to control mosquitoes, such as *B. thuringiensis* subsp. *israelensis* (*cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa* and *cry11Ba*), according to Soares-da-Silva et al. (2017). The purpose of this study was to characterize isolates with new combinations of *cry* and *cyt* genes that are effective in the control of *A. aegypti* for future use in rotation with existing strains. The Bt isolates used in the present study were obtained from the Banco de Bacilos Entomopatogênicos do Maranhão (BBENMA) (Table 1).

2.2. DNA extraction from Bacillus thuringiensis isolates

To obtain DNA from the bacteria, colonies of the new Bt isolates were grown in Petri dishes containing nutrient agar (Kasvi) for 18 h at 28 °C. A colony of each isolate was resuspended in 1 mL of sterile water in microtubes (1.5 mL) that were then centrifuged for 1 min at 17,226 g and 25 °C. After centrifugation, the supernatant was discarded, and 100 µL of InstaGene™ Matrix (Bio-Rad) was added. The samples were subjected to two incubations and shaking in a vortex mixer (Vision Scientific): the first at 56 °C for 25 min with shaking for 10 sec and the second at 100 °C for 8 min with shaking for 10 sec. Finally, centrifugation was performed at 17,226 g for 2.5 min and 25 °C. Aliquots of the supernatant (100 µL) were collected from the samples and transferred to sterile microtubes to evaluate the quantity and quality of the DNA by using an L-Quant spectrophotometer (Loccus). DNA samples were stored at -18 °C for later use.

2.3. PCR for amplification of Dipteran-specific Bacillus thuringiensis genes

In this study, the isolates BtMA-401, BtMA-755 and BtMA-761 were characterized by polymerase chain reaction (PCR) with general primers to identify the families of genes encoding the mosquitocidal toxins: *cry1*, *cry4*, *cry10*, *cry11*, *cry32*, *cry24*, *cy44Aa*, *cyt1* and *cyt2* (Table 2).

Table 1. *Bacillus thuringiensis* isolates collected in biomes State of Maranhão with larvicida activity in *Aedes aegypti*.

Isolates	Biome	Substrate	Municipality/MA	Latitude (S) Longitude (W)
BtMA-179[1]	Amazônia	Solo	Santa Luzia	S 04°38'20.5" W 046°23'30.1"
BtMA-229[1]	Amazônia	Solo	Santa Inês	S 03°85'73.3" W 045°53'49.2"
BtMA-237[1]	Amazônia	Solo	Santa Inês	S 03°85'73.3" W 045°53'49.2"
BtMA-401[2]	Cerrado	Solo	Colinas	S 06°04'53.5" W 044°23'10.6"
BtMA-459[1]	Cerrado	Solo	São J. dos Patos	S 06°50'37.5" W 043°68'65.8"
BtMA-559[1]	Cerrado	Solo	Balsas	S 07°53'53.3" W 046°03'91.1"
BtMA-686[1]	Cerrado	Solo	Duque Barcelar	S 04°13'72.5" W 042°94'91.8"
BtMA-755[3]	Amazônia	Solo	São J. de Ribamar	S 02°27'56.5" W 044°11'43.2"
BtMA-761[3]	Amazônia	Solo	São J. de Ribamar	S 02°27'56.5" W 044°11'43.2"

[1]Soares-da-Silva et al. (2017); [2]Lobo et al. (2018); [3]Banco de Bacilos Entomopatogênicos do Maranhão (BBENMA).

Table 2. Lists of genes and primers used for gene amplification *cry* e *cyt* of *Bacillus thuringiensis* in PCR.

Genes	Sequence 5'-3'	Product size	TM (°C)	Reference
cry1	F - CTGGATTTACAGGTGGGGATAT R - TGAGTCGCTTCGCATATTTGACT	543-594	52	Bravo et al. (1998)
cry4	F - GCATATGATGTAGCGAAACAAGCC R - GCGTGACATACCCATTTCCAGGTC	439-459	50	Jouzani et al. (2008)
cry10	F - TCAATGCTCCATCCAATG R - CTTGTATAGGCCTTCCTCCG	~348	51	Jouzani et al. (2008)
cry11	F - TTAGAAGATACGCCAGATCAAGC R - CATTTGTACTTGAAGTTGTAATCCC	~305	51	Bravo et al. (1998)
cry24	F - TTATCAATGTTAAGGGATGC R - ACTGGATCTGTGTATATTTTCCTAG	~304	52	Ibarra et al. (2003)
cry32	F - TGGTCGGGAGAGAATGGATGGA R - ATGTTTGCGACACCATTTTC	676-677	48	Ibarra et al. (2003)
cry44Aa	F - CATTACACGGGGTGCGTTAT R - CCGCACTTACATGTGTCCAA	~444	60	Vidal-Quist et al. (2009)
cyt1	F - CCTCAATCAACAGCAAGGGTTATT R -TGCAAACAGGACATTGTATGTGTAATT	477-480	52	Ibarra et al. (2003)
cyt2	F - ATTACAAATTGCAAATGGTATTCC R -TTTCAACATCCACAGTAATTTCAAATGC	355-356	56	Ibarra et al. (2003)

The other isolates were previously characterized by Soares-da-Silva et al. (2017). The following DNA samples from Bt subspecies were used as positive controls in the reactions: *B. thuringiensis* subsp. *azawai*-Bta Xentari® WDG (*cry1*), *B. thuringiensis* subsp. *israelensis*-Bti T14 001 and BtMA-750 (*cry4*, *cry10*, *cry11*, *cyt1* and *cyt2*), *B. thuringiensis* subsp. *sotto*-T03 001 (*cry24*), *B. thuringiensis* subsp. *yunnanensis*-Bty T20 001 (*cry32*) and *B. thuringiensis* subsp. *entomocidus*-T06a001 (*cry44Aa*).

The PCRs were performed using the following reagents: 2.5 µL of GoTaq® Flexi DNA polymerase buffer, 2.5 µL of dNTPs, 0.5 µL of MgCl2, 0.5 µL of each primer, 0.1 µL of GoTaq® DNA polymerase (Promega) at approximately 50 ng/µL and enough water (Milli-Q, Millipore) to reach a final volume of 12.5 µL. Amplifications were performed in a Gencycler-G96G thermocycler (Biosystems) with the following program: an initial denaturation step at 94 °C for 5 min, followed by 35 denaturation cycles at 94 °C for 1 min, annealing (*cry32* – 48 °C; *cry4* – 50 °C; *cry10* and *cry11* – 51 °C; *cry1*, *cry24* and *cyt1* – 52 °C; *cyt2* – 56 °C; *cry44Aa* – 60 °C), extension at 72 °C for 1 min and a final extension step at 72 °C for 7 min.

The amplified products were stained with a mix of 2X Blue/Orange Loading Dye (Promega), loading buffer and 1X GelRed Nucleic Acid Gel Stain (Biotium) dye and were electrophoretically separated on 1% agarose gels. The sizes of the generated fragments were compared to the DNA Ladder® 1-Kb molecular weight marker (Promega), and the electrophoresis gels were visualized using the L-PIX TOUCH imaging system (Loccus Biotechnology).

2.4. Histological analysis

The morphological changes in intestinal epithelial cells of *A. aegypti* larvae caused by Bt isolates were evaluated according to Oliveira et al. (2009), with modifications. The assays were performed using groups of 10 third-instar larvae from a colony kept at an average temperature of 26 ± 2 °C, a relative humidity of 85% and a photophase of 12 h in the Medical Entomology Laboratory. The larvae were treated with 5 mg/L crystal/spore suspensions obtained from sporulated cultures of each bacillus isolate in containers (50 mL) holding 10 mL of distilled water. A replicate treated with *B. thuringiensis* subsp. *israelensis* T14 001 was used as a positive control. The bioassays were performed in triplicate and maintained at 26 ± 2 °C and a relative humidity of 80%.

Morphological changes in the larval intestine were examined 6 h and 24 h after exposure to the bacterial solution. At each time point, live larvae were collected individually using Pasteur pipettes, placed in sterile microtubes (1.5 mL) containing 4% paraformaldehyde fixative and stored at room temperature. For histological analysis, mainly live larvae were used, with the exception of strains that showed 100% mortality in 24 h.

The material was first incubated in 70% alcohol and maintained in an automatic tissue processor for histological examinations (OMA DM40) for 8 h. The samples were then serially dehydrated in ethanol (from 70% to 100%), cleared in xylol and embedded in paraffin at 56-58 °C (Leica *EG* 1120). Histological sections (4 mm) were obtained using a microtome (Zeiss HYRAX M55) stained with haematoxylin and eosin (HE) and mounted with Entellan. The samples were observed and analysed under a light microscope at magnifications of 40X and 100X (Leica LAS EZ).

3. Results

3.1. Gene profile of Bacillus thuringiensis isolates

The investigated *cry* and *cyt* genes were detected in all of the new Bt isolates, some of which showed amplification of a single gene, while others showed a combination of two or more genes (Table 3 and Figure 1). The *cry24* and *cry44Aa* genes, characteristic of *B. thuringiensis* subs. *sotto* and *B. thuringiensis* subs. *entomocidus*, respectively, were not identified in any of the evaluated Bt isolates (Table 3).

The most frequent genes were *cry32* and *cyt1*, which were present in 56% of the new Bt isolates, followed by the *cry11* and *cyt2* genes (44%) and the *cry4* gene (33%). The *cry1* and *cry10* genes were the least frequent, with each found in only a single isolate: BtMA-459 and BtMA-755, respectively (Table 3 and Figure 2).

Among the isolates investigated, the isolate with the most genes was BtMA-459, with the amplification of five genes: three *cry* genes (*cry1*, *cry4* and *cry32*) and two *cyt* genes (*cyt* 1 and cry 2). The isolates BtMA-401 and BtMA-559 each harboured only one gene (*cry32* and *cry11*, respectively), while the others showed combinations of two or more toxic genes (Table 3 and Figure 1).

Table 3. *Bacillus thuringiensis* isolates and *cry* and *cyt* genes detected in PCR assays.

Isolates	Genes
Bta*	*cry1*
Bti*	*cry4*, *cry10*, *cry11*, *cyt1* and *cyt2*
Bt sotto*	*cry24*
Bty*	*cry32*
Bt entomocidus*	*cry44Aa*
BtMA-750	*cry4*, *cry10*, *cry11*, *cyt1* and *cyt2*
BtMA-179[1]	*cr4*, *cry11* and *cyt1*
BtMA-229[1]	*cy4*, *cry11* and *cyt2*
BtMA-237[1]	*cry32* and *cyt1*
BtMA-401[2]	*cry32*
BtMA-459[1]	*cry1*, *cry4*, *cry32*, *cyt1* and *cyt2*
BtMA-559[1]	*cry11*
BtMA-686[1]	*cry11* and *cyt2*
BtMA-755[3]	*cry10*, *cry32*, *cyt1* and *cyt2*
BtMA-761[3]	*cry32* and *cyt1*

Legend: positive controls. *Bta = *Bacillus thuringiensis* subsp. *Aizawai*; Bti = *Bacillus thuringiensis* subsp. *israelensis*; Bt sotto = *B. thuringiensis* subsp. *sotto*; Bty = *Bacillus thuringiensis* subsp. *yunnanensis*; Bt entomocidus = *B. thuringiensis* subsp. *entomocidus*; BtMA = *Bacillus thuringiensis* of Maranhão. [1]Soares-da-Silva et al. (2017); [2]Lobo et al. (2018); [3]Banco de Bacilos Entomopatogênicos do Maranhão.

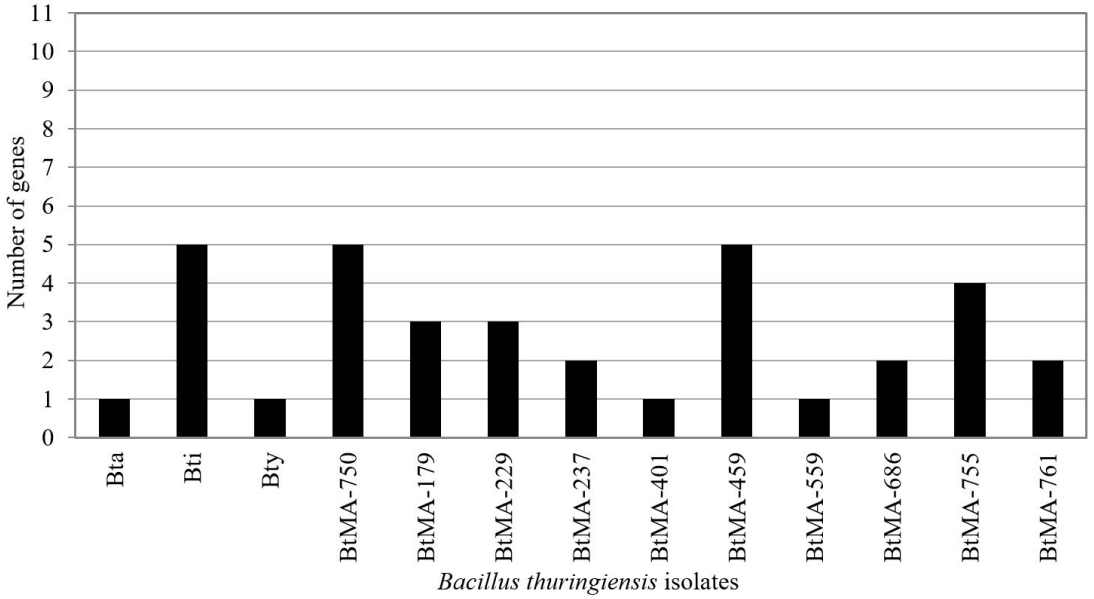

Figure 1. Genes profiles the 10 new isolates of *Bt* from Maranhão. Legend: *Bacillus thuringiensis* subsp. *aizawai* (Bta), *Bacillus thuringiensis* subsp. *israelensis* T14001 (Bti), *Bacillus thuringiensis* subsp. *Bacillus thuringiensis* subsp. *yunnanensis* (Bty) and BtMA *Bacillus thuringiensis* of Maranhão.

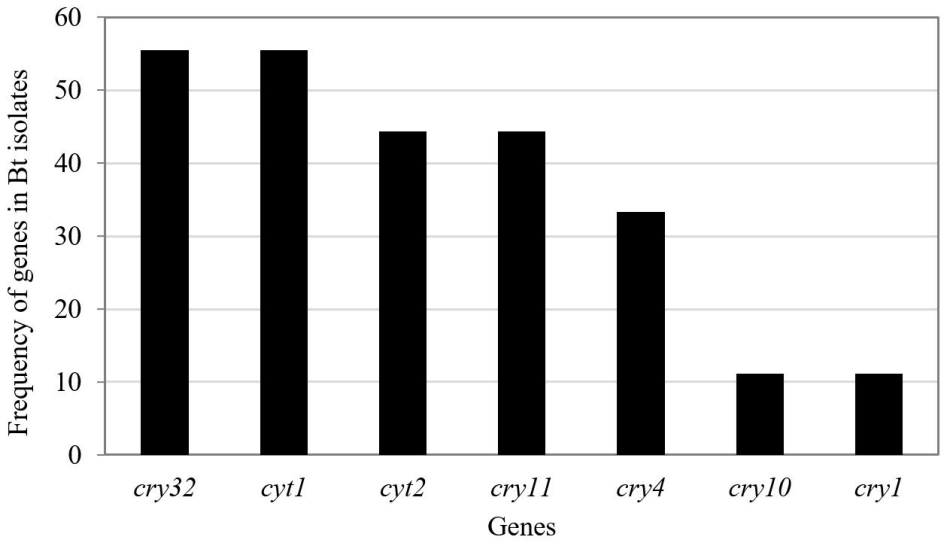

Figure 2. Frequency of the *cry* and *cyt* genes present in the new 10 isolates of Bt from Maranhão.

3.2. Alterations in the intestinal epithelium of Aedes aegypti larvae

The nine *B. thuringiensis* isolates evaluated in this study were toxic to *A. aegypti* larvae and caused cytopathologic damage to the intestinal epithelium after treatment with 5 mg/L crystal/spore suspensions. This result was similar to that observed for the positive control, in which the larvae treated with *B. thuringiensis* subsp. *israelensis* presented significant damage to intestinal cells. After this exposure period, the larvae presented structural disorganization of the epithelium, extravasation of the vesicles in the space between the lumen and the epithelium and the presence of digestive vacuoles (Figure 3).

The larvae treated with the Bt isolates displayed a series of toxic effects on the epithelium after 6 h of exposure to the isolates. The microvilli of the intestinal cells of the larvae treated with Bt were irregular and partially destroyed (Figure 4F), and dissociation of the intestinal cells (Figure 4A, B, F, G and H) and evidence of cell degeneration were observed (Figure 4C, D, E and I).

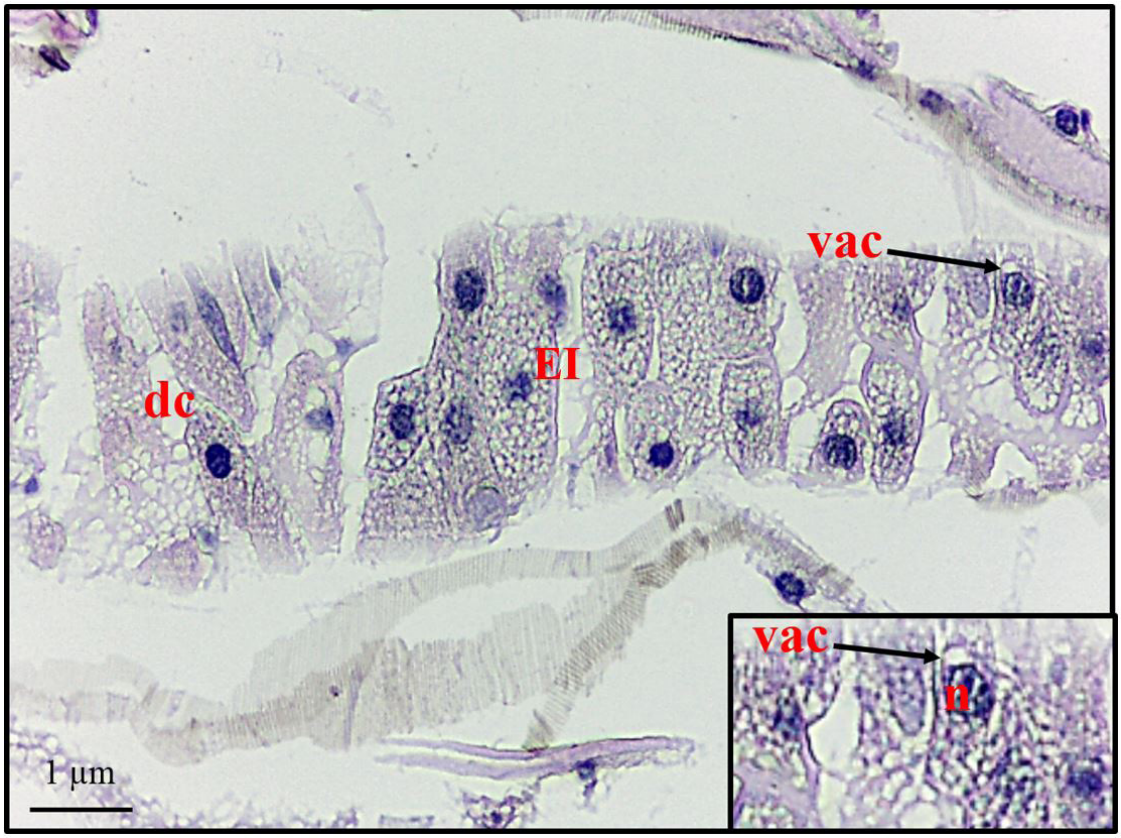

Figure 3. Intestinal cells of larvae of third instar of *Aedes aegypti*. Positive control: after 6h of exposure to the of *Bacillus thuringiensis* subsp. *israelensis*. EI = intestinal epithelium; n = nucleus; vac = vacuole; dc = degenerated cells.

Intense production of digestive intestinal vesicles was also observed in the larvae after 6 h of action of BtMA-179 (Figure 4A), in addition to a large number of intestinal cells with large vacuoles (Figure 4H).

The damage caused by new isolates was much greater after 24 h of exposure to 5 mg/L crystal/spore suspensions, causing the death of the treated larvae. The larvae exposed to isolates BtMA-237, (B) BtMA-229, (C) BtMA-459, and (D) BtMA-179 showed disorganized, loose and disrupted intestinal cells with the presence of digestive vesicles trapped in the space between the lumen and epithelial cells (Figure 5A and B). At this point in the evaluation, the microvilli of the cells were completely destroyed (Figure 5C and D).

4. Discussion

Different profiles of toxic *cry* and *cyt* genes were detected among the evaluated Bt isolates, with a minimum of one and a maximum of five of the nine classes of the analysed genes. This diversity of profiles could be attributed to the high frequency of genetic information exchange between Bt bacteria, enabling variation in the genes within a single isolate (Bravo et al., 1998).

These new bacterial gene combinations can give rise to isolates with gene compositions specific to different groups of insects, and such a broad spectrum of action against different groups may be the reason why Bt is successful in insect control (Ben-Dov et al., 1997; Ben-Dov, 2014; Bravo et al., 2011). In addition, the genetic diversity of the new Bt isolates between the two biomes where they were collected (Amazonia and Cerrado) corroborated the results of other studies (Baig and Mehnaz, 2010; Mahalakshmi et al., 2012; Shishir et al., 2014) that showed genetic diversity in *cry* and *cyt* toxins between samples of isolates collected from different ecosystems. None of the Bt isolates harboured all of the investigated *cry* and *cyt* genes, but this result is perhaps expected since none of the known Bt species or subspecies presents all of the potential genes. However, it should be emphasized that some isolates may present high toxicity to susceptible insects even with a synthetic composition of *cry-cyt* genes (Costa et al., 2010).

The number of genes in a single isolate does not appear to be the factor determining the immediate death of the susceptible insect per se, but it may affect the toxic activity of bacteria when they encounter optimal conditions in the gut of the insect. This is exemplified by comparing the BtMA-401 and BtMA-559 isolates, each of which contained a single gene, and the BtMA-459 isolate, in which 5 genes were amplified, as all of these

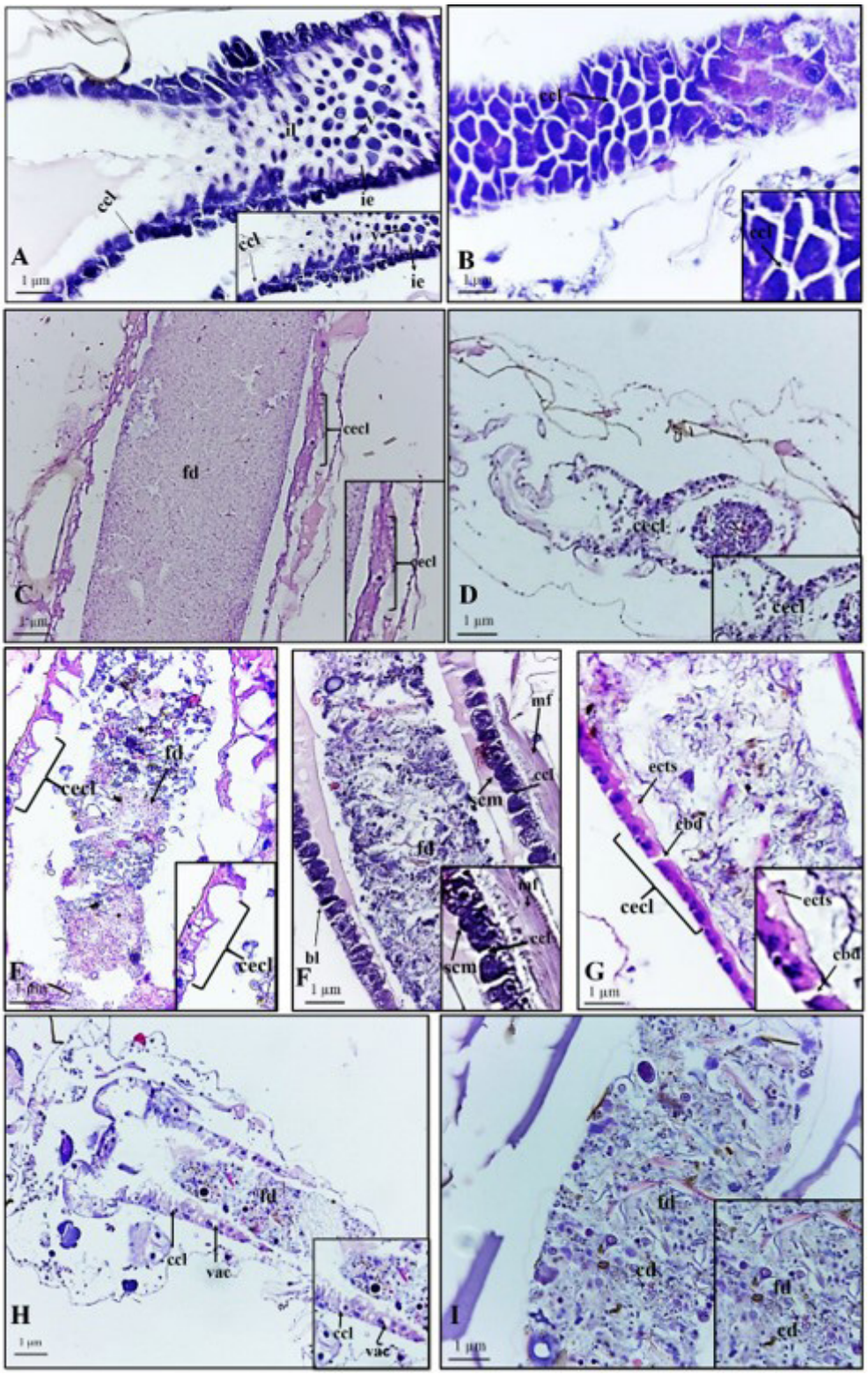

Figure 4. Intestinal cells of larvae of third instar *Aedes aegypti* after 6h of exposure to the new isolates of *Bacillus thuringiensis*. Legend: (A) BtMA-179; (B) BtMA-237; (C) BtMA-401; (D) BtMA-459; (E) BtMA-750; (F) BtMA-686; (G) BtMA-559; (H) BtMA-755; (I) BtMA-229. il = intestinal lumen; ie = intestinal epithelium; ects = ectoperitrophic space; mf = muscular fibers; bl = basal lamin; ccl = cell clearance; v = vesicle; fd = food; cecl = cell clutter; cd = cell degeneration; cbd = cell breakdown; scm = smooth cells without microvilli; vac = vacuole inside the cell around the nucleus.

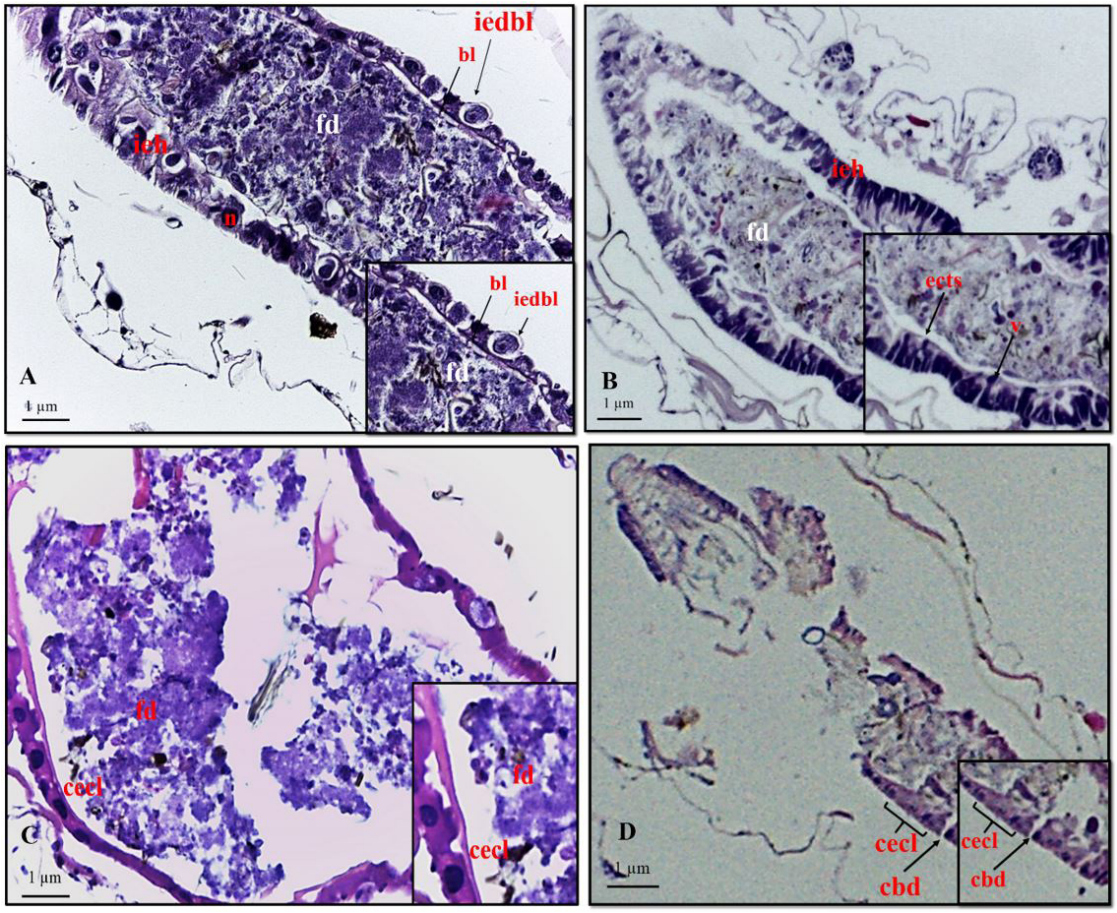

Figure 5. Intestinal cells of the larvae of the third stage *Aedes aegypti* after 24 hours of the toxic action of the new isolates of *Bacillus thuringiensis*. (A) BtMA-237; (B) BtMA-229; (C) BtMA-459; (D) BtMA-179. ieh = intestinal epithelium of hidgut; bl = basal lamin; ects = ectoperitrophic space; n = epitelial cell nucleus; fd = food; iedbl = intestinal epithelial cells detaching from basal lamin; v = vesicle; cecl = cell clutter; cbd = cell breakdown.

isolates caused severe damage to the intestinal epithelium of *A. aegypti* larvae and led to larval death after 6 h of toxic activity. These results were similar to those observed for Bti T14001 and BtMA-750, which contain seven and five genes that are toxic to mosquito larvae, respectively (Rey et al., 1998; Cavados et al., 2004; Soares-da-Silva et al., 2017). BtMA-401, despite harbouring only one gene, did not present the standard genes found in mosquito-acting strains when analysed for its protein content by the sodium dodecyl sulphate-polyacrylamide-based discontinuous gel (SDS-PAGE) method, showing a profile similar to that of Bti, which was verified by Lobo et al. (2018). There are a large number of proteins within the Cry and Cyt families, many of which have similar sizes, which may explain the similar protein profiles among Bt isolates.

The specificity of, quantity of and synergy between the toxic genes in each isolate, together with the susceptibility of the larvae and the highly alkaline pH of the intestine, which favours the solubilization and release of protein crystals (Habib, 1983; Boonserm et al., 2005; Lacey, 2007), are important factors determining the effectiveness of bacterial toxicity against larvae. The quantity of genes in Bt isolates was also analysed by other authors, such as Ejiofor and Johnson (2002), Baig and Mehnaz (2010), Khojand et al. (2013) and Soares-da-Silva et al. (2015), who reported the lack of a correlation between the number of amplified genes and the intensity of the toxic activity in the mosquito gut.

The lack of amplification of the *cry24* and *cry44Aa* genes in all of the Bt isolates can be explained by the different frequencies of Diptera-specific *cry* genes in native Bt isolates (Jouzani et al., 2017). Although these genes show specific action against mosquitoes, they are uncommon in new isolates with activity against mosquitoes (Ejiofor and Johnson, 2002; Soares-da-Silva et al., 2017).

The δ-endotoxins are classified into two families of proteins, namely, Cry and Cyt, that can frequently be produced by Bt subspecies. The combination of Cry and Cyt in the same subspecies is frequent in strains with action against Diptera. The Cry family of proteins comprises 74 types that are encoded by 770 different *cry* genes, whereas the Cyt family is grouped into three types that

are encoded by 38 *cyt* genes (Crickmore, 2017). Among the nine genes evaluated in this study, including seven in the *cry* family (1, 4, 10, 11, 24, 32 and 44) and two in the *cyt* family (1 and 2), the most frequent were *cry4*, *cry11*, *cry32*, *cyt1* and *cyt2*, which were expressed in 33% to 56% of the new Bt isolates. This result corroborates those of other studies (Khojand et al., 2013; El-Kersh et al., 2014) and is important since these genes identified in the new Bt isolates are toxic only to dipterans and can potentially act in synergy (Nazarian et al., 2009; Jouzani et al., 2017; Soares-da-Silva et al., 2017; Lobo et al., 2018) to enable the high activity observed against *A. aegypti* larvae.

The *cry1* and *cry10* genes were less frequent, which was consistent with the results of Konecka et al. (2012), Mahalakshmi et al. (2012), Nemappa et al. (2012), Shishir et al. (2014), Constanski et al. (2015) and Soares da Silva et al. (2017) but divergent from those of Jouzani et al. (2008), Salekjalali et al. (2012), Khojand et al. (2013) and El-Kersh et al. (2014), who found these genes to be more frequent in Bt isolates. The low frequency of *cry1* genes in the evaluated isolates does not necessarily represent a limitation since these genes are toxic not only to dipterans but also to insects in Lepidoptera or Coleoptera (Silva et al., 2012; Jouzani et al., 2017).

The dissociation of the intestinal cells from each other, the irregularity of the microvilli and even the cell degeneration in the *A. aegypti* larval intestine after 6 h and 24 h prove the highly toxic action of the evaluated Bt isolates. This result is consistent with data from Rey et al. (1998) and Cavados et al. (2004) that identified these characteristics as determinants of the toxic action of Bt species and the subsequent death of the susceptible host. However, after 6 h of exposure, we still observed the initial toxic action of the BtMA-179 isolate in the intestine of *A. aegypti* larvae, and the high production of digestive vesicles observed suggests an attempt at defence by the insect against the bacterial action (Oliveira et al., 2009).

The alterations due to the toxic activity of the new Bt isolates from Maranhão were more extensive after 24 h, with the intestinal epithelium showing loose and disrupted cells. Isolate BtMA-459 caused complete disintegration of the intestinal epithelium of *A. aegypti* larvae. This result was similar to that observed by Charles and Barjac (1981) and Lahkim-Tsror et al. (1983) for *B. thuringiensis* subsp. *israelensis*, demonstrating that the new isolates have the potential for future use in the control of *A. aegypti* larvae.

The probable synergy between the *cry* and *cyt* genes and their specificity against dipterans caused serious damage to the larval intestinal epithelium. However, isolates for which only one gene was detected caused epithelial damage comparable to that caused by isolates with a combination of *cry* and *cyt* genes, which may have been due to the presence of unknown genes. This finding may prompt a search for new gene profiles that differ from those currently known to enable mosquito toxicity.

The new isolates of *B. thuringiensis* from Maranhão soil are promising for the biological control of *A. aegypti* larvae, which can be evidenced by changes in the intestinal epithelium of larvae caused by Cry and Cyt toxins, making these isolates highly toxic and enabling them to be safely used as a biotechnological tool for the manufacture of biological larvicides with different combinations of toxins from those currently used.

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