Revista Brasileira de Entomologia 59 (2015) 1-6

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution
www.sbe.ufpr.br/

Medical and Veterinary Entomology

Isolation of *Bacillus thuringiensis* from the state of Amazonas, in Brazil, and screening against *Aedes aegypti* (Diptera, Culicidae)

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ARTICLE INFO

Article history:
Received 18 February 2014
Accepted 16 December 2014
Associate Editor: Mario A. Navarro Silva

Keywords:
Amazonian
Bioinsecticide
Dengue
Vector

ABSTRACT

We investigated the use of *Bacillus thuringiensis* isolated in the state of Amazonas, in Brazil, for the biological control of the dengue vector *Aedes aegypti*. From 25 soil samples collected in nine municipalities, 484 bacterial colonies were obtained, 57 (11.78%) of which were identified as *B. thuringiensis*. Six isolates, IBt-03, IBt-06, IBt-07, IBt-28, IBt-30, and BtAM-27 showed insecticidal activity, and only BtAM-27 presents the five genes investigated *cry4Aa, cry4Ba, cry10Aa, cry11Aa*, and *cry11Ba*. The IBt-07 and IBt- 28, with lower LC50 values, showed equal toxicity compared to the standards. The isolates of *B. thuringiensis* from Amazonas constitute potential new means of biological control for *A. aegypti*, because of their larvicidal activity and the possibility that they may also contain new combinations of toxins.

Introduction

The mosquito *Aedes aegypti* (Linnaeus, 1762) is of major epidemiological importance, as the main vector of several arboviruses affecting humans. This mosquito is the most important vector of dengue in the Americas and particularly in Brazil. Dengue and dengue hemorrhagic fever are a serious public-health problem worldwide, and are the second most important arboviral disease with respect to the number of infected people, affecting 50 to 100 million people in more than 100 countries each year, including Brazil (WHO, 2013).

The lack of a vaccine for dengue makes *A. aegypti* the main target for disease-control programs. In Brazil, although efforts to control dengue have been intensified through the use of multiple methods, vector indices remain high in the entire country (Brazil, 2013). Reduction of *A. aegypti* population levels is a challenge for public-health agencies, mainly due to the mosquito's resistance to pesticides, the main tool for vector control (Braga et al., 2004: Donalisio and Glasser, 2002; Funasa, 2000).

Therefore, biological control has been strongly encouraged, mainly through the use of the bacterium *Bacillus thuringiensis* Berliner (Eubacteriales, Bacillaceae), which, because of its history of success, is the main active ingredient in biopesticides worldwide (Alves, 1998; Bravo et al., 2011; CAB International Centre, 2010). The success of this bacterium as a biological control agent is due to its production of crystalline protein inclusions at the time of sporulation, which are toxic to many organisms, especially insects (Alves, 1998; Bravo, 1997; Glare and O'Callaghan, 2000; Höfte and Whiteley, 1989).

These proteins have molecular weights between 40 and 140 kDa and are encoded by different genes, named the *cry* gene (Alves, 1998; Bravo, 1997). The toxicity of an isolate is related to possession of different types of functional *cry* genes encoding Cry proteins. The presence of these genes serves as a parameter to estimate the effectiveness of a bacterial strain against a particular group of insects (Ben-Dov, 2014; Bravo et al., 2013; Frankenhuyzen, 2009, 2013). Isolates may have different combinations of toxic insecticides, and for mosquitoes the toxins *Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa*, and *Cry11Ba* show synergistic activity and have good effectiveness, in particular for *A. aegypti* control (Höfte and Whiteley, 1989; Park et al., 2011; Tokcaer et al., 2006).

In various regions of the world, studies of *B. thuringiensis* have sought new strains with different potentialities from those that are currently known for new *cry* genes with different insecticidal effects (Dias et al., 2002; Liang et al., 2011; Praça et al., 2004; Su et al., 2007; Tan et al., 2009). Brazil harbors diverse environments that are suitable for microorganism development, comprising natural sources for the isolation of new bacteria (Costa et al., 2010). In view of the in-

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0085-5626/
http://dx.doi.org/10.1016/j.rbe.2015.02.001

valuable biodiversity of the Amazon environment, isolation of *B. thuringiensis* strains from this region may be helpful in finding new isolates with combinations of *cry* gene toxins that are different from those currently known. Accordingly, this study aimed to isolate strains of *B. thuringiensis* from soils in the state of Amazonas, for use in the biological control of *A. aegypti.*

Material and methods

Soil sample collection

Strains of *B. thuringiensis* were isolated from soil samples collected in forest areas in nine municipalities of Amazonas: Careiro, Castanho, Coari, Iranduba, Itacoatiara, Manaus, Manaquiri, Presidente Figueiredo, Rio Preto da Eva, and São Gabriel da Cochoeira. In all sites, soils were collected in forest area.

Soil was collected from depths up to 5cm, in shaded locations. The collection points were georeferenced. At each collection site, approximately 15 g of soil were collected with a wooden spatula and placed in 15 mL sterile Falcon tubes. The material was packed in a cooler and transported to the Laboratório de Malária e Dengue at the Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas, where they were labeled and stored.

Isolation of Bacillus thuringiensis

Procedures for *B. thuringiensis* isolation followed the method recommended by the World Health Organization, colony morphology criteria (WHO, 1985). It consists of mixing 1 g of soil in 10 mL of a saline solution. The samples were then serially diluted in the saline solution. A 1 mL aliquot of the solution was homogenized and subjected to heat shock at 80 °C for 12 minutes, and then placed in ice for 5 minutes. Next, 100 µL of the solution was transferred to Petri dishes containing nutrient agar and spread on the dish by means of a Drigalski spatula. The dishes were inverted and stored in a laboratory oven and grown at 28 °C for 48 hours.

Colonies that showed typical characteristics of *Bacillus* spp. were inoculated into nutrient broth containing penicillin G and placed in a rotating incubator at 28°C at 180 rpm for about 48 hours (Guaycurus, 1999). The colonies that grew were observed with a phase-contrast light microscope (1000× magnification), in order to determine the presence of parasporal inclusions (crystal protein) and morphological characterization.

The isolated strains were deposited in the entomopathogenic bacteria collection of the Laboratório de Malária e Dengue.

Preparation of bacterial culture for use in the bioassays

For the preparation of the culture containing bacterial spores/crystals, each isolate of *B. thuringiensis*, including the *B. thuringiensis israelensis* strain was inoculated into nutrient broth and allowed to grow at 28 °C and 180 rpm for 72 hours, until at least 90% sporulation was observed with the aid of a phase-contrast optical microscope. After this period, the bacteria count was performed on plates, and the suspension obtained was used for the preparation of suspensions of bioassays.

Bioassays with Aedes aegypti *larvae*

For the bioassays, *A. aegypti* third-instar larvae were obtained from colonies maintained at the insectary of the Laboratório de Malária e Dengue. The larvae were reared under controlled conditions, with a mean temperature of 28 ± 2 °C, relative humidity around 85%, and 12-hour photophase.

Selective and quantitative bioassays were carried out. Isolates that showed larvicidal activity against *A. aegypti* were identified by testing them with the final total bacterial culture. The aggressiveness of the isolates was calculated in virulence bioassays by means of estimates of the Median Lethal Concentration (LC_{50}).

Selective bioassays

The selective bioassays were carried out with all the isolates to verify insecticidal action of the same. For each isolate evaluated, four replicates of four plastic beakers containing 10 mL water and 10 third-instar larvae were prepared. To three beakers, 1 mL of the total *Bacillus* culture was added; one replicate had no bacteria inoculated, thus serving as a negative control. For comparison, an additional replicate containing the standard strain *B. thuringiensis israelensis* was used as a positive control.

The number of live and dead larvae in each beaker was recorded at intervals of 24 and 48 hours, and the percentage of larval mortality for each isolate was determined. If the mortality rate was greater than 50%, the isolate was selected for the quantitative bioassay. The bioassays were held at 28 ± 2 °C and 80% relative humidity.

Quantitative bioassays

The selected isolates, including the standard strain, were assessed in quantitative bioassays to estimate the $LC_{50.}$ Each strain was inoculated into 12 mL of nutrient broth in 50 mL Erlenmeyer flasks, and was then grown in a rotary incubator at 28 °C and agitation rates of 180 rpm, for five days.

For each isolate, the number of bacteria in the total culture was estimated by the viable plate count procedure (Alves, 1998), and the number of crystal-spores/mL was determined and the concentration was standardized for 10^5 for LC_{50}. From this original concentration, 10 serial dilutions of the bacterial culture were prepared, and for each isolate, five logarithmically spaced doses were established, with the aim of obtaining a larval mortality rank. Five replicates were used for each isolate. The replicates were composed of 180 mL plastic cups containing 99 mL of distilled water, 20 third-instar larvae, and 1 mL of the bacillus concentration. For each bioassay, a control group consisting of a cup with larvae and no bacillus treatment was added. The bioassays were carried out in triplicate, on alternate days, and used 300 larvae per dose (Dulmage et al., 1990). *Bacillus thuringiensis israelensis* IPS-82 was used as the standard strain. The bioassays were monitored at intervals of 24 and 48 hours after application of the bacillus, and live and dead larvae were counted.

The median lethal concentration (LC_{50}) was estimated with the software POLO-PC (Leora Software, Berkeley, CA) and was calculated from the data for larval mortality at the different bioassay doses (Finney, 1981; Haddad, 1998).

Gene analysis

The presence of the genes *cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa*, and *cry11Ba* with insecticide activity against *A. aegypti* larvae was determined using the PCR technique. These genes are the most important in the encoding of specific toxins to *A. aegypti* and are present in the strain Bti IPS-82 used worldwide in vector control.

DNA was extracted from the *B. thuringiensis* strains with the method of boiling and consecutive freezing/thawing. DNA was obtained from an aliquot of bacterial culture grown on nutrient agar at 28 °C for 18 hours. (Guaycurus, 1999; Starnbach et al., 1989). The DNA samples were stored at –4 °C.

For each *B. thuringiensis* isolate, a PCR test was carried out with all the primers listed in Table 1. These *cry* genes were amplified with the following reagents and concentrations: 2.5 µL buffer solution (1X); 2.5 µL MgCl (25 mM); 2.5 µL dNTPs (2.5 mM); 1 µL of each primer, at a concentration of 5 pMol/µL; 0.3 µL Taq DNA polymerase (5 U); 2 µL DNA, and 13.2 µL sterile MilliQ H_2O, with a final volume of 25 µL.

The gene amplification reaction was performed in a Mastercycler Gradient thermocycler (Eppendorf). Amplification conditions were optimized to: initial denaturation at 94 °C for 30 s; 30 cycles at 94 °C for 30 s, for denaturation; 30 s at 48-52 °C, for primer annealing; 2 minutes at 72 °C, for polymerization, and a final step of 5 minutes at 72 °C, for extension.

PCR-generated DNA fragments were observed by electrophoresis on 1% agarose gel. After the amplification reaction, 5 µL were withdrawn from each sample and added to 2 µL loading buffer dye. Samples were applied to agarose gel and submitted to a 90-V electric field, in 1X TBE (Tris/borate/EDTA) at alkaline pH. The marker DNA ladder 1Kb (Invitrogen) was used as standard molecular weight. After electrophoresis, the gels were observed in a UV-light transilluminator and photographed with the TCL Documentation apparatus (Vilber Lourmat).

Table 1.
Sequences of oligonucleotide used in the PCR reaction of *Bacillus thuringiensis cry* genes with specific action against *Aedes aegypti*, their respective sequences, expected fragment size, and primer melting temperature.

Gene	Sequence 5' → 3'	Size (bp)	Primer melting temperature (°C)
cry4Aa[a]	5'-GGGTATGGCACTCAACCCCACTT	1,529	48
	3'-GCGTGACATACCCATTTCCAGGTCC		
cry4Ba[a]	5'- GAGAACACACCTAATCAACCAAT	1,951	52
	3'-GCGTGACATACCCATTTCCAGGTCC		
cry10Aa[b]	5'-ATTGTTGGAGTTAGTGCAGG	995	48
	3'-AATACTTTGGATGTGTCTTGAG		
cry11Aa[a]	5'-CCGAACCTACTATTGCGCCA	470	50
	3'-CTCCCTGCTAGGATTCCGTC		
cry11Ba[b]	5'-TACAGGATGGATAGGGAATGG	608	50
	3'-TAATACTGCCATCTGTTGCTTG		

[a] Primers designed by Ben Dov, Ben Gurion University of the Negev, Israel.
[b] Primers designed by Costa (2010).

Results

All the soil samples were positive for the presence of *B. thuringiensis*. From the 25 soil samples used for bacterial colony isolation, 484 colonies were obtained, of which 57 (11.8%) were identified as *B. thuringiensis* (Table 2).

The highest percentage of isolates obtained from a single soil sample was observed in sample 23, from Urucu, municipality of Coari (33.3%), followed by samples 7 and 19 (28.8%) from the municipalities of Manaquiri and Rio Preto da Eva, respectively. The determination of crystal protein morphology revealed the predominance of *B. thuringiensis* with only one crystal protein (87.7%), while the remaining (12.3%) strains had more than one crystal per bacterium. More than 96.5% of the isolates showed rounded (oval

Table 2.
Bacillus thuringiensis isolates obtained from soil samples from several municipalities in the state of Amazonas.

Municipality	Site coordinates		BC (N)	Isolate
	Latitude (S)	Longitude (W)		
1. Careiro Castanho	04°07'54.2"	60°44'56.3"	24 (2)	IBt-01; 02
2. Coari	04°49'27.0"	65°14'54.0"	4 (1)	IBt-03
3. Coari	04°49'52.1"	65°15'31.5"	14 (1)	IBt-04
4. Iranduba	03°11'05.5"	60°26'27.0"	17 (1)	IBt-05
5. Itacoatiara	03°08'06.7"	58°25'34.2"	18 (3)	IBt-06-08
6. Manaquiri	04°07'18.1"	60°44'08.5"	14 (2)	IBt-09; 10
7. Manaquiri	04°05'59.5"	60°42'22.9"	7 (2)	IBt-11; 12
8. Manaquiri	04°03'43.8"	60°39'20.4"	28 (2)	IBt-13; 14
9. Manaus	02°43'06.3"	60°02'49.8"	19 (3)	IBt-15-17
10. Manaus	02°53'28.2"	65°02'05.3"	20 (1)	IBt-18
11. Manaus	02°37'49.3"	60°02'20.0"	26 (2)	IBt-19; 20
12. Manaus	02°48'17.6"	60°02'10.3"	32 (2)	IBt-21; 22
13. Manaus	03°03'09.4"	59°53'34.7"	19 (2)	IBt-23; 24
14. Presidente Figueiredo	02°10'45.7"	60°00'34.3"	23 (1)	IBt-25
15. Presidente Figueiredo	02°27'10.8"	60°01'28.5"	23 (1)	IBt-26
16. Presidente Figueiredo	02°05'42.6"	59°59'52.9"	25 (1)	IBt-27
17. Rio Preto da Eva	02°42'16.1"	59°43'09.6"	10 (1)	IBt-28
18. Rio Preto da Eva	02°44'11.3"	59°46'51.6"	29 (3)	IBt-29-31
19. Rio Preto da Eva	02°40'34.5"	59°42'54.6"	7 (2)	IBt-32; 33
20. São G. da Cachoeira	00°07'20.1"	67°04'54.9"	24 (1)	IBt-34
21. São G. da Cachoeira	00°06'53.2"	67°05'28.6"	24 (4)	IBt-35-38
22. São G. da Cachoeira	00°07'21.3"	67°04'39.1"	31 (7)	IBt-39-45
23. Urucu (Coari)	04°33'59.5"	65°19'19.8"	18 (6)	IBt-46-51
24. Urucu (Coari)	04°53'21.2"	65°18'52.3"	17 (3)	IBt-52-54
25. Urucu (Coari)	04°53'10.2"	65°20'56.0"	21 (3)	IBt-55-57
TOTAL			484 (57)	

BC, Number of bacterial colonies; N, Number of colonies of *Bacillus thuringiensis*.

and cuboid) crystals, and only 3.5% showed bipyramidal (diamond-shaped) crystals.

Of 57 isolates assayed against *A. aegypti*, five (8.8%) showed larvicidal activity: IBt-03, from Coari; IBt-06 and IBt-07, both from Itacoatiara, and IBt-28 and IBt-30 from Rio Preto da Eva. During the bioassays, four isolates caused 100% larval mortality after 24 hours of application. Isolate IBt-30 showed only 83.3% larva mortality at the same assessment time, and 86.6% after 48 hours.

In the genetic analysis of the strains to assess the presence of dipteran-specific genes, of all the isolates obtained and subjected to PCR, only BtAM-27 amplified the five genes tested. Fragments with 1529 bp were obtained for the *cry4Aa* gene, 951 bp for *cry4Ba*, 995 bp for *cry10Aa*, 470bp for *cry11Aa*, and 608 bp for *cry11Ba*. These fragment sizes were expected for the genes analyzed, and the result was equivalent to that observed for the standard strain of *B. thuringiensis* var. *israelensis*. No DNA fragment amplification for these genes was observed for the other strains tested (Figs. 1-3).

In spite of the negative results for gene presence, the isolates were subjected to quantitative bioassays to estimate LC_{50}. The isolates showed good toxicity levels (Table 3).

The IBt-07 and IBt-28 isolates presented increased efficiency in the control of larvae of *A. aegypti*, with the lowest LC_{50} values, 0.0011 and 0.0014 × 10^5 respectively, equal to the value of 0.007 x 10^5 found here for the standard strain Bti IPS-82. The isolate IBt-06 showed the least performance, since it had the highest LC_{50} value, 2.7 × 10^5. Note that although the others have submitted values higher than the median lethal concentration, these showed toxicity for larvae, with LC_{50} ranging between 0.26 and 0.59 × 10^5 (Table 3).

Table 3.
Mean Lethal Concentration (LC_{50}) of *Bacillus thuringiensis* isolates against *Aedes aegypti* larvae recorded after 48 hours of application.

Isolate	Total *Bacillus* culture (UFC/mL)	CL_{50} (CI 95%)
IBt-03	7.76 × 10^6	0.59 (NE) × 10^5
IBt-06	1.12 × 10^7	2.7 (0.8-0.46) × 10^5
IBt-07	7.16 × 10^4	0.0011 (0.006-0.0021) × 10^5
IBt-28	3.92 × 10^4	0.0014 (0.0011-0.0019) × 10^5
IBt-30	5.81 × 10^6	0.61 (0.44-0.89) × 10^5
BtAM-27	8.80 × 10^8	0.26 (0.16-0.40) × 10^5
Bti IPS-82	6.71 × 10^7	0.007 (0.006-0.009) × 10^5

NE, not estimated by the program POLO-PC; CI, Confidence interval.

Discussion

Since the discovery of its potential and effectiveness as a biological-control agent, *B. thuringiensis* has been isolated in various parts of the world (Bravo et al., 2011; Jung et al., 1998; Liang et al., 2011; Paris et al., 2011). In Brazil, *Bt* has been isolated from a wide variety of substrates (Dias et al., 2002; Polanczyk et al., 2004; Silva et al., 2002; Souza-Filho, 2007). In this study, *Bt* was isolated from soil and was present in all samples, although, in different proportions ranging from 4 to 33%, with a mean of 11.8%. A similar index was obtained in a study in China, which also used soil samples from tropical forests (Su et al., 2007), and of the 683 samples analyzed, *Bt* was found in 90 of them (13.2%). However, Polanczyk et al. (2004) analyzed 85 soil samples from other types of environments and found 772 bacterial colonies with 293 (37.95%) corresponding to *Bt*.

The *Bt* index seems to be quite different in various regions of the world. Several factors are associated with *Bt* variation, including climate conditions and the properties of soil, a complex environment where many factors may affect the diversity and density of the microbial population (Polanczyk et al., 2004). However, few studies of *Bt* isolation have also characterized the properties of the soil, which makes it difficult to more thoroughly compare factors that may be directly associated with the presence of this bacterium.

About 9% of the isolates obtained in this study showed larvicidal activity against *A. aegypti*, a relatively high value compared to other studies. Praça et al. (2004) tested 300 isolates of *Bt* against larvae of *A. aegypti* and *Culex quinquefasciatus* and found that only two – less than 1% – showed activity. Relatively few strains of *Bt* with larvicidal activity against insects of public-health importance, particularly *A. aegypti*, have been discovered (Costa et al., 2010; Dias et al., 2002; Frankenhuyzen, 2013; Gobatto et al., 2010; Praça et al., 2004; Souza-Filho, 2007). Dias et al. (2002) described the activity of strains of *Bt* with toxic action in larvae of *A. aegypti* for only 1.9% of isolates. Souza-Filho (2007) also studied strains of *Bt* from the Amazon basin and found a high percentage of lineages with toxic action (21.7%).

The number of native *Bt* strains with power equal to or higher than the standard strain is generally low (Frankenhuyzen, 2009; 2013). When comparing the LC_{50} values obtained with the five isolates from the Amazon, only IBt-07 and IBt-28 strains show toxicity equal to the

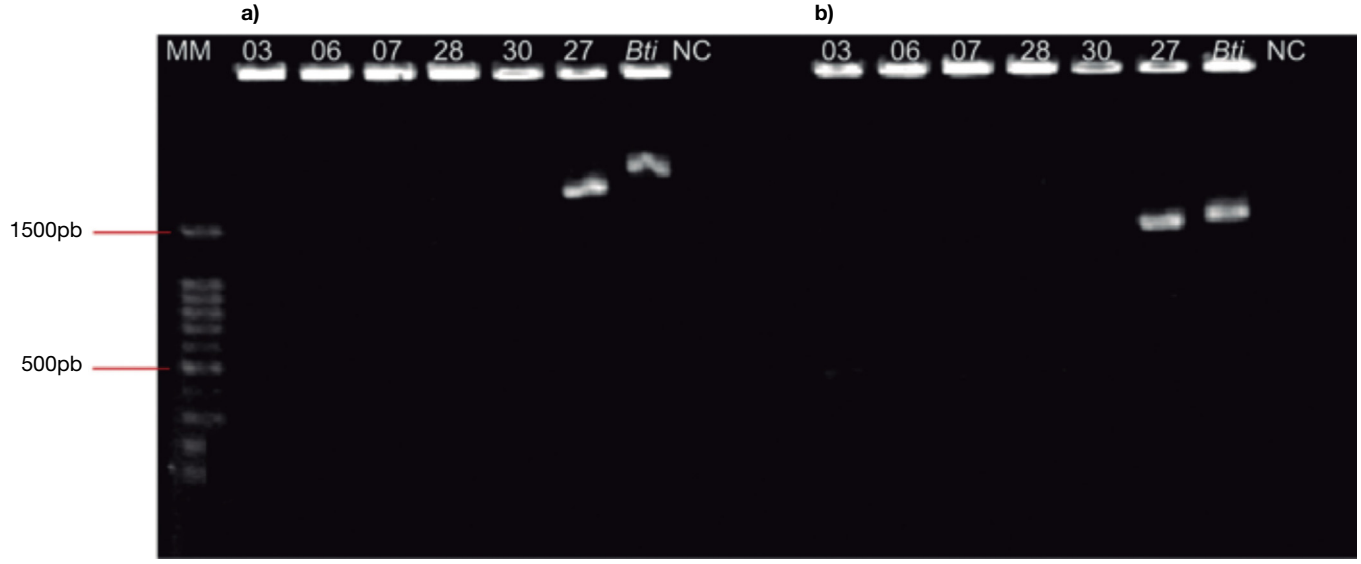

Figure 1. Amplification products of gen (A) *cry4Ba* and (B) *cry4Aa* of dipteran-specific genes isolated from *Bacillus thuringiensis* from the state of Amazonas, Brazil.

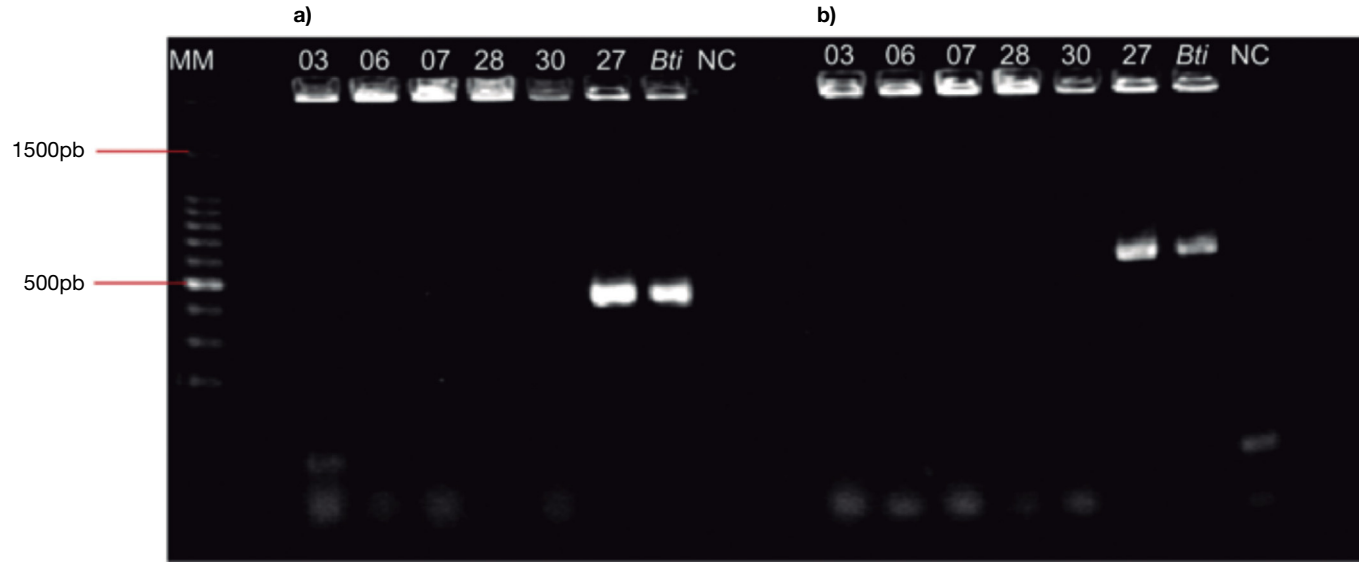

Figure 2. Amplification products of gen (A) *cry10Aa* and (B) *cry11A* of dipteran-specific genes isolated from *Bacillus thuringiensis* from the state of Amazonas, Brazil.

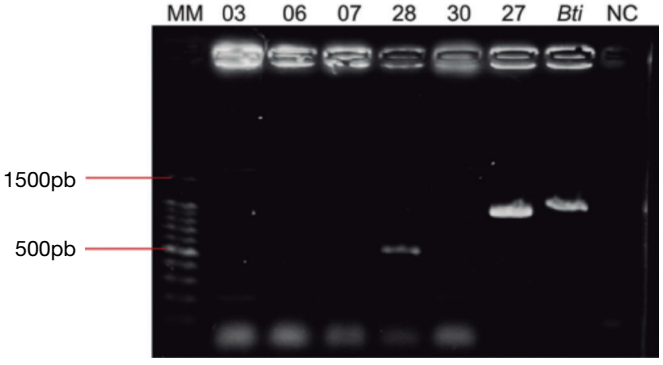

Figure 3. Amplification products of gen *cry11Ba* of dipteran-specific genes isolated from *Bacillus thuringiensis* from the state of Amazonas, Brazil. Legend: MM: marker 1kb-sized DNA ladder; 03: IBt-03; 06: IBt-06; 07: IBt-07; 28: IBt-28; 30: IBt-30; 27: BtAM-27; 82: Bti IPS-82 (positive control); NC: negative control.

standard strain. Costa et al. (2010) also found isolates of *Bt* highly toxic to the larvae of *A. aegypti*, with potential insecticide activity similar to that of the standard strain *B. thuringiensis* var. *israelensis*.

Molecular characterization has been used as a parameter to describe the potential of isolates or even new genes/toxins, increasing knowledge of the activity of *Bt* against insect pests from different orders (Bravo et al., 2013; Liang et al., 2011; Park et al., 2011). Here, only the strain BtAM-27 was similar to the result obtained with the standard strain IPS-82 and revealed the presence of genes that encode the toxins Cry4Aa, Cry4Ba, Cry11Aa, Cry10Aa, and Cry11Ba. These toxins have been shown to be associated with toxicity against *A. aegypti* (Crickmore et al., 1995). BtAM-27 showed good results in the toxicity test, so it can be seen that the presence of the toxins have been reported to have a specific effect on *A. aegypti*. The action of this combination of toxins to control insect larvae of the order Diptera, specifically mosquitoes, is well elucidated in the literature showing high toxicity for this group of insects, especially in synergy with cytolytic toxins (Ben-Dov, 2014; Promdonkoy et al., 2005).

In addition to the crystal toxins, Bt contains other virulence factors such as β-exotoxins, α-exotoxins, hemolysins, enterotoxins, chitinases, and phospholipases (de Maagd et al., 2003; Fernández-Luna et al., 2010; Höfte and Whiteley, 1989). However, the descriptions of these factors vary, and the precise contribution of each remains unknown, hindering the determination of the real toxic spectrum of an isolate that produces more than one type of toxin (Pinto et al., 2003).

The use of strains with a variety of toxins in a single crystal, such as the isolate BtAM-27 evaluated in the present study, has great potential. In addition to the strain BtAM-27, the results observed for the other five isolates for which the toxin genes are not described indicate the likelihood of other genes or toxins with potential activity against *A. aegypti*. To explore this possibility, molecular investigations of other genes should be expanded.

Acknowledgements

The authors thank the team of the Laboratory of Biology and Rearing of Insects (LBCI) and Genetics of Bacteria and Applied Biotechnology, UNESP, Jaboticabal. FAPEAM and CNPq are thanked for the financial support.

Conflicts of interest

The authors declare no conflicts of interest.

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