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Molecular characterization of the gene profile of *Bacillus thuringiensis* Berliner isolated from Brazilian ecosystems and showing pathogenic activity against mosquito larvae of medical importance

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ABSTRACT

The occurrence of *Aedes aegypti*, *Culex quinquefasciatus*, and mosquitoes of the genus *Anopheles* potentiate the spread of several diseases, such as dengue, Zika, chikungunya, urban yellow fever, filariasis, and malaria, a situation currently existing in Brazil and in Latin America. Control of the disease vectors is the most effective tool for containing the transmission of the pathogens causing these diseases, and the bacterium *Bacillus thuringiensis* var. *israelensis* has been widely used and has shown efficacy over many years. However, new *B. thuringiensis* (*Bt*) strains with different gene combinations should be sought for use as an alternative to *Bti* and to prevent the resistant insects selected. Aiming to identify diversity in the *Bt* in different Brazilian ecosystems and to assess the pathogenicity of this bacterium to larvae of *Ae. aegypti, C. quinquefasciatus,* and *Anopheles darlingi*, *Bt* strains were obtained from the Amazon, Caatinga (semi-arid region), and Cerrado (Brazilian savanna) biomes and tested in pathogenicity bioassays in third-instar larvae of *Ae. aegypti* under controlled conditions in the laboratory. The isolates with larvicidal activity to larvae of *Ae. aegypti* were used in bioassays with the larvae of *C. quinquefasciatus* and *An. darlingi* and characterized according to the presence of 14 *cry* genes (*cry1*, *cry2*, *cry4*, *cry10*, *cry11*, *cry24*, *cry32*, *cry44Aa*, *cry1Ab*, *cry4Aa, cry4Ba*, *cry10Aa*, *cry11Aa*, *and cry11Ba*), six *cyt* genes (*cyt1*, *cyt2*, *cyt1Aa*, *cyt1Ab*, *cyt2Aa* and *cyt2Ba*), and the *chi* gene. Four hundred strains of *Bt* were isolated: 244 from insects, 85 from Amazon soil, and 71 from the Caatinga biome. These strains, in addition to the 153 strains isolated from Cerrado soil and obtained from the Entomopathogenic Bacillus Bank of Maranhão, were tested in bioassays with *Ae. aegypti* larvae. A total of 37 (6.7%) strains showed larvicidal activity, with positive amplification of the *cry*, *cyt,* and *chi* genes. The most frequently amplified genes were *cry4Aa* and *cry4Ba*, both occurring in 59.4% in these strains, followed by *cyt1Aa* and *cyt2Aa,* with 56.7% and 48% occurrence, respectively. Twelve (2.2%) strains that presented 100% mortality within 24 h were used in bioassays to estimate the median lethal concentration (LC_{50}) for *Ae. aegypti* larvae. Two strains (BtMA-690 and BtMA-1114) showed toxicity equal to that of the *Bti* standard strain, and the same LC_{50} value (0.003 mg/L) was recorded for the three bacteria after 48 h of exposure. Detection of the presence of the *Bt* strains that showed pathogenicity for mosquito larvae in the three biomes studied was possible. Therefore, these strains are promising for the control of insect vectors, particularly the BtMA-1114 strain, which presents a gene profile different from that of *Bti* but with the same toxic effect.

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1. Introduction

Diseases whose etiological agents are transmitted by mosquito vectors are among the major diseases affecting humans (WHO, 2017). Considering Latin America, among the species of greater epidemiological importance is *Aedes* (*Stegomyia*) *aegypti* (Diptera: Culicidae) (Linnaeus, 1762), it is the main vector of the Zika, dengue, chikungunya, and urban yellow fever viruses (Honório et al., 2015; Ebi and Nealon, 2016; Ferreira-de-Brito et al., 2016).

Culex quinquefasciatus Say, 1823 (Diptera: Culicidae) is another species of mosquitoes of importance to public health in the Americas. It is the vector of lymphatic filariasis, a disease of a chronic nature that mainly affects populations of low socioeconomic level (Brasil, 2016; Rebollo and Bockarie, 2017).

The species *Anopheles darlingi* Root 1926 is the main vector of malaria in America, mainly in the Amazon region, winch recorded more than 83% of the cases (Siqueira et al., 2017; Tadei et al., 2017).

There are epidemic cycles of these diseases in the Latin America, it is necessary to seek ways to control. Several obstacles to mosquito control exist, with resistance to chemical agents being noted as one of the main challenges to the current vector control program (Moyes et al., 2017; Seixas et al., 2017).

The use of the bacterium *Bacillus thuringiensis* (*Bt*) Berliner, 1915 is one of the biological control strategies that has been showing better efficacy (Bravo et al., 2011; Lacey et al., 2015). *Bacillus thuringiensis* var. *israelensis* (*Bti*) is a natural enemy of several species of mosquitoes of the genera *Culex* Linnaeus, 1758; *Aedes* Meigen, 1818; and *Anopheles* Meigen, 1818. It is the microbial agent more commonly used in the control of these insects, and its continued use for more than 30 years has not resulted in the evolution of resistance in mosquito populations treated in different regions of the world (Bravo et al., 2011; Stalinski et al., 2016).

The lack of resistance is attributed to the complex mechanism of action of *Bti*, which has toxins known as Cry toxins (4Aa, 4Ba, 11Aa, 11Ba, and 10Aa) that are capable of interacting with the intestinal epithelium of the mosquito larvae and also has cytolytic (Cyt) toxins that are less specific but facilitate the insertion of Cry toxins into the intestinal epithelium and may thus increase insecticidal activity (Bravo et al., 2007; Ben-Dov, 2014; Zhang et al., 2016) . The synergism between the Cry and Cyt toxins is fundamentally important for the efficacy of the bacterium (Frankenhuyzen, 2013; Zhang et al., 2016).

Some *Bt* strains also produce vegetative insecticidal proteins (Vips) produced in vegetative phase and chitinolytic toxins (Chi), the toxins chitinolytics are another group of toxins that may contribute to larval mosquitoes mortality by destroying the peritrophic matrix of insects (Sampson and Gooday, 1998; Djenane et al., 2017).

Although no records exist of resistance to *Bti*, the possibility of the select of resistant populations cannot be discounted. This bacterium is an important biological agent; therefore, the use of other strains with different combinations of *cry* and *cyt* genes is necessary as a form of management and prevention of resistance to *Bti* (Cánton et al., 2015; Peralta and Palma, 2017).

The diversity of the Cry toxins already found and described in the literature demonstrates the possibility of discovery of different combinations of *Bti* with different insecticidal potentials (Crickmore, 2017).

Several studies have sought to obtain more *B. thuringiensis* isolates with insecticidal potential for mosquitoes, which is done by isolating native strains from substrates such as soils from different ecosystems, dead insects, plants, and other sources. In addition, these strains are investigated at the molecular level by detecting the genes encoding the Cry and Cyt toxins present in the toxic crystal, which makes predictions of their insecticidal activity possible (Bravo et al., 1998; Jouzani et al., 2008; Costa et al., 2010; El-kersh et al., 2016).

The present study investigated the diversity of *Bt* strains isolated from soils of different Brazilian biomes and from dead insects, and showing larvicidal activity against mosquitoes *Ae aegypti, Cx quinquefasciatus* and *An darlingi* in the laboratory which are the main mosquitoes of medical importance in Latin America.; in addition, the gene profiles of the strains pathogenic.

2. Methods

2.1. Sampling and isolation of Bacillus thuringiensis

A total of 37 soil samples from two biomes (15 from the Caatinga biome and 22 from the Amazon biome) and 44 samples from dead insects were processed according to the World Health Organization (1985) protocol for the isolation of *Bt* strains.

The soil samples consisted of 10 g of soil, which was collected at a depth of 5 cm, placed in sterile flasks, and sent to the Laboratory of Medical Entomology (LABEM) at the Universidade Estadual do Maranhão − UEMA.

The insect samples consisted of 44 dead insects collected in the Cerrado biome. The insects were identified as belonging to the orders Coleoptera (22), Hymenoptera (15), and Hemiptera (07). All samples were collected in the state of Maranhão, Brazil, which contains the three biomes (SISBIO/59840; IBGE, 2017).

2.2. Morphological identification of Bacillus thuringiensis isolates

The strains were cultured in nutrient agar (peptic digest of animal tissue 5 g/L, sodium chloride 5 g/L, meat extract 1.5 g/L, and yeast extract 1.5 g/L pH 7.4 ± 2) containing penicillin G (100 mg/L) for 48 h; then, and viewed at 1000 x magnification under an Axio Scope A.1 (*Zeiss*) microscope by phase-contrast to detect the presence of crystal for differentiation from *Bacillus cereus*. The *Bt* strains were submitted the gram-staining test (Jung et al., 1998).

The strains the *Bt* were stored at 4 °C in filter-paper strips, impregnated with spore suspension, immersed in autoclaved distilled water, and stored at 4 °C in triplicate. The strains were individually identified with BtMA (*Bacillus thuringiensis* from Maranhão) followed by the number corresponding to the order of isolation and deposited in the Entomopathogenic Bacillus Bank of Maranhão (BBENMA), located in the municipality of Caxias, Maranhão, Brazil.

2.3. Selection of strains for mosquito pathogenicity assays

For preliminary screening of strain pathogenicity to *Ae. aegypti*, 553 strains of *Bt*, of which 400 were obtained in this study and 153 were isolated from Cerrado soil and kept at BBENMA, were used after preselection of the strains for larvicidal activity. The bioassay was performed in triplicate: three plastic cups containing 10 mL of drinking distilled water and 10 third-instar *Ae. aegypti* larvae were used, with 1 mL of the total bacterial culture being added to each. The negative control consisted of 10 larvae placed in a plastic cup with water, but without inoculation with the bacterium; for the positive control, *Bti* T04001 lyophilized was used under the same conditions. The bioassays were conducted at the LABEM, UEMA, with a temperature of 26 ± 2 °C, relative humidity of 80% and photoperiod of 12 h light followed by 12 h dark (12L:12D) (WHO, 2005).

The strains that reached 100% mortality in up to 48 h, along with the *Bti* standard strain, were grown in NYSM medium incubated at 28 °C for 5 days for complete sporulation and release of the crystal proteins. After, the samples were centrifuged at 1700x*g* for 15 min at 4 °C, and the pellet was recovered and transferred to Falcon tubes with 10 mL of autoclaved distilled water and 0.01% Triton® X- 100. The spores were then counted using a Neubauer chamber in an Axio Scope A.1 (*Zeiss*) phase-contrast optical microscope (Alves and Moraes, 1998). The strains were tested again for *Ae. aegypti* larvae at the standard concentration of 1.5×10^7 spores/mL under the same abovementioned conditions.

Strains that killed 100% of the larvae in 48 h were cultured in

600 mL of nutrient yeast extract salt medium (NYSM) (Yousten, 1984) for 5 days at 28 °C at 180 rpm. The culture obtained was centrifuged at 10.000 x g for 30 min at 4 °C, washed with autoclaved distilled water, frozen, and lyophilized for approximately 16 h (Santos et al., 2012).

2.4. Pathogenicity bioassays in Culex quinquefasciatus and Anopheles darlingi larvae

Strains that killed 100% of the *Ae. aegypti* were selectively tested at a concentration of 10 mg/L against *C. quinquefasciatus* and *An. darlingi* larvae. The larvae of the F1 generation, obtained from adults collected in the field and eggs reared in the laboratory (SISBIO/21264-3). Collection was carried out in the city of Caxias, Maranhão for *C. quinquefasciatus* and in Manaus, Amazonas for *An. darlingi*. The bioassays followed the same conditions of the tests described above for *Ae. aegypti*.

2.5. Bioassays for estimating the lethal concentration (LC_{50}) and (LC_{90}) for Aedes aegypti larvae

These bioassays were performed with the 12 strains that showed 100% mortality in 24 h for *Ae. aegypti*. Toxicity bioassays were performed in the laboratory with *Ae. aegypti* due to the ease of obtaining this species.

For each strain, six concentrations (0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg/L) were initially tested, from which more concentrations (0.09–0.001 mg/L) were established to obtain a mortality of 100% and 5% for each strain.

Each concentration was tested in three replicates, was performed in the different days. Each replicate used five plastic cups containing a final volume of 150 mL and 20 third-instar larvae, and the amount corresponded to each concentration of the isolates. For each bioassay, a negative control group was added, which consisted of a cup with larvae and without inoculation with the bacillus. The bioassays were monitored at intervals of 24, 48, and 72 h after the application of the bacillus, with dead larvae being counted at each interval.

To compare the larvicidal activity of the isolates, *Bti* T04001 was used. The bioassay was performed under the same conditions described for the other isolates and In accordance with the recommendations Guidelines For Laboratory And Field Testing Of Mosquito Larvicides (WHO, 2005).

2.6. Lethal concentration (LC_{50}) and (LC_{90}) and statistical analyses

The mortality data obtained in the toxicity bioassays were submitted to Probit analysis at $p = 0.05$ (Finney, 1971), with the statistical software POLO PLUS (LeOra Software, 2003) being used to estimate the LC_{50} and LC_{90}.

The toxicity of each isolate was compared to that of the standard strain *Bti* T04001 using Student's *t*-test when the data were parametric or the Mann-Whitney test for non-normal data; the level of significance was set as 5% ($\alpha = 0.05$). The statistical program used was BioEstat 5.0 (summer) for Windows (Ayres et al., 2007).

2.7. Molecular characterization

The strains of *Bt* with larvicidal activity were investigated for the presence of the *cry* (14), *cyt* (6), and *chi* genes encoding toxins active against mosquito larvae using polymerase chain reaction (PCR). Eleven universal primers—*cry1*, *cry2*, *cry4*, *cry10*, *cry11*, *cry24*, *cry32*, *cry44*, *cyt1*, *cyt2* and *chi*—and 10 specific primers— *cry1Ab*, *cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa*, *cry11Ba*, *cyt1Aa*, *cyt1Ab*, *cyt2Aa* and *cyt2Ba*—were used for gene amplification (Table 1). The following strains were used as positive controls: *B. thuringiensis* var. *aizawai* (XenTari-WDG; *cry1*, *cry1Ab*), *B. thuringiensis* var. *kurstaki* (Dipel WP; *cry2*), *B. thuringiensis sotto* (T03 001; *cry24*), *B. thuringiensis* var. *yunnanensis* (T20 001; *cry32*), *B. thuringiensis entomocidus* (T06a 001; *cry44*) and *B. thuringiensis* var. *israelensis* (T04 001) for the remaining genes, provided by the Laboratory of Bacterial Genetics and Applied Biotechnology (LGBBA) of the School of Agrarian and Veterinary Sciences, São Paulo State University, Universidade Estadual Paulista − FCAV/UNESP Jaboticabal).

Table 1
lists the studied *B. thuringiensis* genes with the respective primers used and the expected fragment sizes from PCR as well as the annealing temperature.

Gene	Sequence of primres (5′- 3′)	TF of pb	TM
cry1[1]	CTGGATTTACAGGTGGGGATAT (f) TGAGTCGCTTCGCATATTTGACT (r)	543–594	52
cry1Ab[2]	AAGCAAGGGTTATTACATTACG (f) CCAATACTAAGATCAGAGGG (r)	~550	56
cry2[3]	GTTATTCTTAATGCAGATGAATGGG (f) CGGATAAAATAATCTGGGAAATAGT (r)	689–701	52
cry4[3]	GCATATGATGTAGCGAAACAAGCC (f) GCGTGACATACCCATTTCCAGGTCC (r)	439–459	52
cry4Aa[4]	GAACTGGGTATGGCACTCAAC (f) CTCACAACGATTAGACCCTTC (r)	777	50
cry4Ba[4]	GCGAGGTTTCCCATGTCTAC (f) GTTGTAGGGTGGAATTGTTATC (r)	347	52
cry10[3]	TCAATGCTCCATCCAATG (f) CTTGTATAGGCCTTCCTCCG (r)	348	51
cry10Aa[4]	ATTGTTGGAGTTAGTGCAGG (f) AATACTTTGGATGTGTCTTGAG (r)	995	50
cry11[1]	TTAGAAGATACGCCAGATCAAGC (f) CATTTGTACTTGAAGTTGTAATCCC (r)	305	51
cry11Aa[4]	AGGATGGATAGGAAACGGAAG (f) CCGTATTCCAGCAGGTAAGC (r)	470	50
cry11Ba[4]	TACAGGATGGATAGGGAATGG (f) TAATACTGCCATCTGTTGCTTG (r)	608	52
cry24[3]	TTATCAATGTTAAGGGATGC (f) ACTGGATCTGTGTATATTTTCCTAG (r)	304	48
cry32[3]	TGGTCGGGAGAGAATGGATGGA (f) ATGTTTGCGACACCATTTTC (r)	676–677	54
cry44Aa[5]	CATTACACGGGGTGCGTTAT (f) CCGCACTTACATGTGTCCAA (r)	444	60
cyt1[3]	CCTCAATCAACAGCAAGGGTTATT (f) TGCAAACAGGACATTGTATGTGTAATT (r)	477–480	52
cyt1Aa[4]	AACTCAAACGAATAACCAAG (f) TGTTCCTTTACTGCTGATAC (r)	300	53
cyt1Ab[4]	AAGCAAGGGTTATTACATTACG (f) CCAATACTAAGATCAGAGGG (r)	698	54
cyt2[3]	ATTACAAATTGCAAATGGTATTCC (f) TTTCAACATCCACAGTAATTTCAAATGC (r)	355–356	52
cyt2Aa[4]	GCATTAGGAAGACCATTTG (f) AAGGCTAAGAGTTGATATCG (r)	361	53
cyt2Ba[6]	CAGGAACTCTTAATCAAAGTGTAAT (f) CATCTACTTGAGGTTCTAAATTTGT (r)	177	50
chi[7]	ATGGTCATGAGGTCTC (f) CTATTTCGCTAATGACG (r)	2027	45

Legend (f) = forward; (r) = reverse; Pb = base pair. [1] Bravo et al. (1998), [2] Fatoretto et al. (2007); [3] Jouzani et al. (2008), [4] Costa et al. (2010) , [5] Vidal-Quist and Castañera (2009), [6] Costa et al. (2014), [7] Lin and Xiong (2004). Legend: TF = Fragment size, pb = pairs of base and TM = melting temperature.

2.8. Total DNA extraction and amplification reaction of mosquito-specific genes

Total DNA extraction from the strains was performed using the InstaGene Matrix DNA extraction kit (Bio-Rad, USA), according to the manufacturer's recommendations. One colony the each isolate was grown for approximately 12 h in nutrient agar was transferred to a microtube containing 1 mL of sterile Milli-Q water; this sample was then centrifuged for 1 min at 12,000 rpm and 20 °C. The supernatant was discarded; then, 200 µL of InstaGene Matrix (Bio-Rad) was added, and the material was incubated in a water bath at 56 °C for 25 min, after vortexed thoroughly for 10 s and then incubated at 100 °C for 8 min. The sample was again vortexed at moderate speed for 10 s and centrifuged at 20 °C for 2.5 min. The DNA which were stored in a freezer at −20 °C until the time of use.

PCR reactions were performed at a final volume of 25 µL with: 1X GoTaq® Flexi DNA Polymerase buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.2 pmol/µL of each primer (Invitrogen), 1U of GoTaq® DNA Polymerase enzyme (Promega), and 50 ng of DNA. The PCR was performed for all genes mentioned above; the reaction was performed in a Gencycler-G96G thermocycler (Biosystems). The general amplification conditions were programmed according to the following specifications: 94 °C for 5 min; followed by 30 cycles at 94 °C for 30 s, with the annealing temperature optimized according to each primer (Table 1), and final extension at 72 °C for 5 min. For amplification of the *chi* (chitinase) gene, the program was as follows: 5 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 45 °C, and 1.5 min at 72 °C; and 10 min at 72 °C for a final extension (Costa et al., 2010).

Amplification products were analyzed by electrophoresis in a 1,5% agarose gel with current applied at 90 V in 1X TBE (Tris/Borate/EDTA) buffer with alkaline pH and photographed in an L-PIX device (Loccus Biotechnology).

3. Results

3.1. Isolation of Bacillus thuringiensis

Four-hundred strains of *Bt* were obtained, 244 from insects, 85 from the Amazon soil, and 71 from the Caatinga soil. The Amazon soil was the substrate with the highest isolation rates, with 72.7% of the samples presenting *Bt*, followed by the Caatinga soil with 53.3% and then dead insects with 43.2%.

However, the highest mean occurrence of *Bt* per sample was observed for dead insects, with 5.5 strains, whereas for soil samples, the rates were 4.7 for the Caatinga and 3.9 for the Amazon.

In the insects, Hymenoptera was the group with the highest number of *Bt* strains, with 193 strains and a mean of 12.9 for sample, a value three times higher than that found for the other orders, Coleoptera and Hemiptera, which had 100 and 21 strains and means of 4.5 and 3, respectively.

3.2. Selection bioassays of Bacillus thuringiensis strains pathogenic for mosquitoes

From the total of 553 strains of *Bt* selectively tested against *Ae. aegypti*, 37 (6.7%) showed pathogenicity, 12 of which killed 100% of the larvae in 24 h (Table 2). The remaining 25 strains showing pathogenicity reached 100% mortality after 48 h.

Among the 37 pathogenic strains, approximately one-half (47.7%) were obtained from the Cerrado soil. The rate of strains from this biome that are active against mosquitoes was 11.11% of the total number of strains tested, three times higher than that obtained for the Caatinga biome, for which the lowest rate (3.5%) was found.

3.3. Molecular characterization

In all 37 strains that showed pathogenic activity, positive amplification occurred for the genes tested using the universal and specific primers (Table 3).

The PCR reactions showed positive amplification of all genes studied; however, variation was observed in the number of *cry* and *cyt* genes per strain. Twelve different gene combinations were observed, with three strains (BtMA-37, BtMA-626, and BtMA-215) containing a single gene, whereas five strains (BtMA-676, BtMA- 684, BtMA-685, BtMA-688, and BtMA-690) amplified fragments with the expected size for 15 genes, thus confirming the presence of six families of genes active against Diptera (*cry4*, *cry10*, *cry11*, *cyt1, cyt2,* and *chi*) and the presence of nine genes specific to mosquitos. This profile was similar to the one obtained with the *Bti*-T4001 standard strain, for which positive amplification was also observed for the nine genes encoding toxins active against mosquitoes and for the *chi* gene. The BtMA −679 and 687 strains, in addition to the nine *Bti* genes, showed amplification for the *cry32* gene (Table 3).

The *cry4Aa* and *cry4Ba* genes had a higher occurrence rate in the strains (59.4%), followed by the *cyt1Aa* and *cyt2Aa* genes, which were present in 56.7% and 48% of the strains, respectively. Next, in decreasing order of frequency, were *cry10Aa* (45%), *cyt1Ab* (43.24%), *cry11Aa* and *cyt2Ba* (37.8%), *chi* (35.3%), *cry11Ba* (32.4%), *cry32* (27%), *cry1* (8.1%), and *cry44Aa* (2.7%). No positive amplification was found for the *cry1Ab*, *cry2,* and *cry24 genes*.

3.4. Bioassays for estimating the lethal concentration (LC_{50}) and (LC_{90})

The toxicity bioassays were performed with the 12 strains of *Bt* that achieved 100% mortality after 24 h. Based on the obtained values of LC_{50} and LC_{90} and the respective confidence intervals, four toxicity groups were formed. The most toxic strains were BtMA-690, BtMA-1114, and the *Bti* T4001 standard strain, followed by the group composed of BtMA-679, BtMA-687, and BtMA-688, and then the strains BtMA-37, BtMA- 681, BtMA-684, BtMA-685, BtMA-689, and 703. The lowest toxicity was observed for the BtMA-691 strain, which showed the highest values for LC_{50} and LC_{90} (Table 4).

The quantitative bioassays showed that two strains (BtMA-690 and BtMA-1114) had similar performance to the *Bti* standard strain, for which no significant difference between the LC_{50} values was observed in the three evaluation periods (ANOVA: $F = 16$, $p = 0.06$). In the 24-h evaluation, BtMA-690 and *Bti* obtained the same LC_{50} value of 0.003 mg/L; however, the LC_{90} was 0.009 mg/L and 0.014 mg/L, respectively. For BtMA-1114, the LC_{50} was 0.004 mg/L, and the LC_{90} was 0.008 mg/L (Table 4).

In the period (48 h), BtMA-690, BtMA-1114, and the *Bti* T4001 obtained the same value of LC_{50} (0.003 mg/L), but the LC_{90} was 0.009 mg/L for the two isolates and 0.011 mg/L for the standard strain. At the evaluation after 72 h, the LC_{50} value was 0.001 mg/L for *Bti* and 0.002 and 0.003 mg/L for BtMA-690 and BtMA-1114, respectively, whereas the LC_{90} was 0.005 mg/L for BtMA-690 and 0.006 mg/L for the other two strains (Table 4).

Based on the LC_{50} values obtained at the three evaluation periods, the less toxic isolates (BtMA-37 and BtMA-691) showed LC_{50} values approximately 20 times higher than the BtMA-690, BtMA-1114, and *Bti* standard strains, whereas the groups BtMA-679, BtMA-687, and BtMA-688 were five times less toxic than *Bti*.

4. Discussion

The bacterium *Bt* is found in all environments, but soil has been the most used source of isolation (Polanczyk et al., 2004; Gobatto et al., 2010; El-Kersh et al., 2016; Reyaz et al., 2017). In the present work, in which soil samples and insects were analyzed, the insects were the substrates with the highest number of *B. thuringiensis* per sample, and the presence of the bacterium was detected in 56% of the insect samples used.

The presence of *Bt* in insects is generally high (Gobatto et al., 2010; Pinto et al., 2003; Assaeedi et al., 2011).These bacteria develop with these organisms, thus making them a natural source of the pathogen and making it possible to find new strains of this bacterium both in dead and live insects (Berhnard et al., 1997; Abulreesh et al., 2012 Abulreesh et al., 2012).

In the present work, detection of the bacterium was possible in three orders of insects, with Hymenoptera being the most promising, for which was found in 66% of the samples, corresponding to more than twice the percentage occurrence for the orders Coleoptera with 31.8% and Hemiptera with 28.5%.

Other studies have shown different results for *Bt* occurrence rates in insects, such as the order Coleoptera with 60% (Hernandez et al., 2005), whereas for Hymenoptera, 40% of the bacterial colonies obtained from two species of this order of insects were identified as *Bt*

Table 2
Bacillus thuringiensis isolates with 100% larvicidal activity for *Aedes aegypti* larvae at 24 and 48 h in the laboratory conditions according to the origin of the isolation substrate in municipality the isolated.

Biome	substrate	Larvicidal activity		Municipality	Latitude (S) Longitude (W)
		24 h	48 h		
Amazônia					
BtMA- 37	solo	100	–	Viana	S 03°13′12.3” W 045°08′88.7”
BtMA-179	solo		100	Santa Luzia	S 04°38′20.5” W 046°23′30.1”
BtMA-215	solo		100	Bela Vista	S 03°75′60.3“W 045°22′62.9”
BtMA-229	solo		100	Santa Inês	S 03°85′73.3“W 045°53′49.2”
BtMA-233	solo		100	Santa Inês	S 03°85′73.3“W 045°53′49.2”
BtMA-237	solo		100	Santa Inês	S 03°85′73.3“W 045°53′49.2”
BtMA-241	solo		100	Santa Inês	S 03°85′73.3“W 045°53′49.2”
Cerrado					
BtMA-459	solo		100	São J. dos Patos	S 06°50′37.5” W 043°68′65.8”
BtMA-527	solo		100	Benedito Leite	S07°22′55.5” W 044°55′97.2”
BtMA-559	solo		100	Balsas	S 07°53′53.3“W 046°03′91.1”
BtMA-626	solo		100	Coelho Neto	S 04°25′31.0” W 043°01′38.9”
BtMA-676	solo		100	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
BtMA-679	solo	100	–	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
BtMA-681	solo	100	–	Duque Barcelar	S 04°13′72.5“W 042°94′91.8”
BtMA-682	solo		100	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
BtMA-684	solo	100	–	Duque Barcelar	S 04°13′72.5“W 042°94′91.8”
BtMA-685	solo	100	–	Duque Barcelar	S 04°13′72.5“W 042°94′91.8”
BtMA-686	solo		100	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
BtMA-687	solo	100	–	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
BtMA-688	solo	100	–	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
BtMA-689	solo	100	–	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
BtMA-690	solo	100	–	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
BtMA-691	solo	100	–	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
BtMA-694	solo		100	Duque Barcelar	S 04°13′72.5” W 042°94′91.8”
Insetos					N° da Amostra
BtMA-1054	Hymenoptera		100	Mirador	1
BtMA-1061	Hymenoptera		100	Mirador	1
BtMA-1107	Coleoptera		100	Mirador	2
BtMA-1108	Coleoptera		100	Mirador	2
BtMA-1109	Coleoptera		100	Mirador	2
BtMA-1114	Coleoptera	100	–	Mirador	3
BtMA-1115	Coleoptera		100	Mirador	3
BtMA-1116	Coleoptera		100	Mirador	3
BtMA-1119	Coleoptera		100	Mirador	3
BtMA-1120	Coleoptera		100	Mirador	3
BtMA-1134	Hymenoptera		100	Mirador	4
BtMA-1147	Hymenoptera		100	Mirador	5
Caatinga					
BtMA −703	solo	100		Santa Quitéria	S 03°48′34.6“W 042°55′59.8”

(Pinto et al., 2003).

The Caatinga biome showed a higher mean number of strains per samples compared to the Amazon soil when was isolated from the soil of the two biomes. The Caatinga, located in the northeastern region of Brazil, had already been reported as the region with the greatest abundance of *Bt* in the country (Silva et al., 2012), for which the presence of the bacteria was detected in 16.9% of the samples (Silva et al., 2002).

The number of strains of *Bt* per sample in the Amazon biome found in this study corroborates previous findings, in which even lower mean values 0.48 and 2.28 strains per sample were observed (Pereira et al., 2013; Soares-da-Silva et al., 2015). In contrast, *Bt* occurred in approximately 70% of the soil samples of this biome used for isolation, which demonstrates the wide distribution of this bacterium in this environment.

The persistence of *Bt* spores in the soil involves different factors, with the soil chemical constituents being suggested as one of the main factors affecting this persistence (Polanczyk et al., 2004). The number *Bt* of strains obtained from the different biomes varied considerably, the presence of this bacterium in all types of environments indicates that *Bt* must undergo intense selective pressure and, to survive, has developed different ways of resisting natural enemies (Habib and Andrade, 1998). This is confirmed by the pathogenicity of *Bt* to different groups of insects (Frankenhuyzen, 2009; 2013).

About the pathogenicity of *Bt* strains active against mosquitoes, mortality, in general, is low compared to other groups of insects (Armengol et al., 2006; Gobatto et al., 2010). In the present study, 6.7% of the tested strains showed pathogenic activity against larvae of the three species, confirming the low occurrence of the mosquito-specific strains.

These studies carried out in Brazil with native strains of *Bt* active against mosquito larvae also showed low occurrence of these strains, rate below 2% of *Bt* active against mosquitoes (Dias et al., 2002; Praça et al., 2004; Ootani et al., 2011). In Saudi Arabia, 33.8% of the native strains isolated from distinct parts in that country showing pathogenicity for *Anopheles gambiae,* an important vector of African malaria (Elkersh et al., 2016).

Studies performed on Amazon soil showed similar rates to that found in the present study, 8.7%and 2% of strains were active against *Ae. aegypti*, Pereira et al. (2013) and Soares-da-Silva et al. (2015), respectively.

The variation in the number of native strains of *Bt* with larvicidal activity against mosquitoes observed for the different biomes can be explained by the different profiles of the insecticidal toxin-producing

Table 3
Gene profile of the 37 isolates of *Bacillus thuringiensis* active against *Aedes aegypti* larvae obtained from the three soil e insects Brazilian biomes.

	Isolates																			
Genes	*cy 1*	*cry 1Ab*	*cry 2*	*cry 4*	*cy4Aa*	*cry4Ba*	*cry 10*	*cry10Aa*	*cry 11*	*cry11Aa*	*cry11Ba*	*cyt1*	*cyt1Aa*	*cyt1Ab*	*cyt2*	*cyt2Aa*	*cyt2Ba*	*chi*	*cry 24*	*cry 32*	*cry 44Aa*
Bt israelensis				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
Bt azawai	+	+																			
Bt kustaki		+																			
Bt sotto																			+		
Bt.entomocidus																					+
Bt.yuannanensis																				+	
Amazônia																					
BtMA-37					+																
BtMA-179				+		+			+			+									
BtMA-215																				+	
BtMA-229				+		+			+						+	+					
BtMA-233									+						+	+	+	+		+	+
BtMA-237													+	+						+	
Cerrado																					
BtMA-241							+						+		+	+				+	
BtMA-459	+			+		+						+			+	+				+	
BtMA-527							+		+				+	+				+			
BtMA 626														+							
BtMA-559									+							+		+			
BtMA-676				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
BtMA-679				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	
BtMA-681				+		+	+	+	+	+		+	+		+	+	+				
BtMA-682				+	+				+				+	+	+	+					
BtMA-684				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
BtMA-685				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
BtMA-686									+						+	+		+			
BtMA-687				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	
BtMA-688				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
BtMA-689				+	+	+	+	+	+	+		+	+	+	+	+	+			+	
BtMA-690				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
BtMA-691				+		+		+	+		+	+		+	+	+	+	+			
BtMA-694				+	+	+	+	+		+	+	+	+	+	+	+	+	+			
Insetos																					
BtMA-1054					+				+						+						
BtMA-1061				+					+				+		+						
BtMA-1107	+				+			+	+												
BtMA-1108				+	+		+	+		+					+					+	
BtMA-1109	+			+	+				+												
BtMA-1114				+	+	+	+		+	+		+	+		+					+	
BtMA-1115					+	+						+	+								
BtMA-1116					+	+							+								
BtMA-1119					+	+						+	+								
BtMA-1120					+	+							+								
BtMA-1134				+			+														
BtMA-1147				+		+															
Caatinga																					
BtMA-703				+	+	+	+	+	+	+	+	+	+	+	+	+	+				

Legend: + = presence and − = absence.

Table 4
Lethal Concentrations LC_{50} and LC_{90} in mg/L at the 24-, 48-, and 72-hour evaluations for *Bacillus thuringiensis* isolates pathogenic to mosquitos.

Isolados	LC_{50}(IC 95%)	LC_{90}(IC 95%)	Slope ± SE	$\chi 2$ (GL = 3)
24 h				
BtMA-37	0.063 (0.056–0.073)	0.158 (0.114–0.380)	3.184 (0.323)	6. 3
BtMA-679	0.015 (0.012–0.019)	0.042 (0.029–0.082)	2.904(0.143)	14.5
BtMA-681	–	–	–	–
BtMA-684	0.078 (0.065–0.120)	0.161 (0.110–0.415)	4.047 (0.881)	2.4
BtMA-685	0.032 (0.021–0.038)	0.049 (0.040–0.103)	6.608 (0.428)	32.2
BtMA-687	0.017 (0.014–0.019)	0.052 (0.040–0.076)	2.603 (0.138)	5.1
BtMA-688	0.012 (0.011–0.013)	0.030 (0.027–0.034)	3.205 (0.148)	1.2
BtMA-689	0.049 (0.030–0.166)	0.319 (0.113–6.440)	1.579 (0.153)	7.7
BtMA-690	0.003 (0.002–0.003)	0.009 (0.007–0.011)	2.763 (0.137)	5.2
BtMA-691	0.437 (0.252–1.290)	3.997 (1.338–35.925)	1.334 (0.230)	0.5
BtMA-703	0.038 (0.029–0.044)	0.057 (0.047–0.130)	7.208 (0.549)	24.1
BtMA −1114	0.004 (0.003–0.005)	0.008 (0.006 − 0.018)	4.827 (0.271)	30.6
Bti t4001	0.003 (0.002–0.003)	0.014 (0.011–0.017)	2.010 (0.075)	12.5
48 h				
BtMA-37*	0.0474 (0.038–0.053)	0.1 27 (0.098–0.240)	2.994 (0.319)	5.0
BtMA-679	0.011 (0.011–0.012)	0.027 (0.025–0.030)	3.408 (0.154)	1.1
BtMA-681*	0.044(0.039–0.050)	0.072 (0.059–0.117)	5.968 (0.596)	6.5
BtMA-684	0.053 (0.049–0.058)	0.114 (0.093–0.157)	3.828 (0.466)	2.3
BtMA-685	0.015 (0.009–0.025)	0.057 (0.031–0.204)	2.163 (0.111)	19.5
BtMA-687*	0.015 (0.008–0.029)	0.046 (0.025–0.709)	2.593 (0.140)	48.8
BtMA-688*	0.009 (0.008–0.010)	0.022 (0.020–0.025)	3.276 (0.168)	1.8
BtMA-689*	0.040 (0.029–0.109)	0.131 (0.066–3.681)	2.480 (0.371)	5.9
BtMA-690	0.003(0.002–0.004)	0.009 (0.006–0.016)	2.650 (0.122)	20.0
BtMA-691*	–	–	–	–
BtMA-703*	0.036 (0.033–0.038)	0.051 (0.047–0.059)	8.287 (0.633)	5.1
BtMA-1114	0.003(0.0026–0.0034)	0.006 (0.005–0.008)	4.006 (0.206)	6.7
Bti t4001	0.003 (0.0021–0.0033)	0.011 (0.009–0.015)	2.055 (0.093)	3.8
72 h				
BtMA-37	0.023(0.006–0.033)	0.104 (0.085–0.196)	1.945 (0.542)	1.2
BtMA-679	0.008 (0.007–0.008)	0.018(0.016–0.019)	3.622 (0.173)	2.3
BtMA-681	0.034 (0.030–0.037)	0.072(0.061–0.096)	3.880 (0.351)	3.01
BtMA-684	0.044 (0.035–0.049)	0.064 (0.056–0.101)	7.963 (0.693)	37.2
BtMA-685	0.011 (0.009–0.013)	0.051 (0.036–0.081)	1.901 (0.091)	4.05
BtMA-687	0.011 (0.008–0.014)	0.031 (0.021–0.069)	2.799 (0.138)	21.7
BtMA-688	0.008 (0.007–0.009)	0.018 (0.016–0.023)	3.587 (0.211)	3.3
BtMA-689	0.033(0.023–0.069)	0.207 (0.089–1.638)	1.605 (0.138)	7.4
BtMA-690	0.002 (0.001–0.002)	0.005 (0.004–0.006)	3.009 (0.150)	0.6
BtMA-691	1.487 (0.440–39.74)	548.28 (25.29- NE)	0.499 (0.119)	0.4
BtMA-703	0.034 (0.027–0.038)	0.049 (0.043–0.068)	8.282 (0.695)	12.5
BtMA-1114	0.003 (0.002–0.004)	0.006 (0.005–0.001)	4.048 (0.206)	28.9
Bti T4001	0.001 (0.001–0.002)	0.006 (0.005–0.008)	1.924 (0.090)	2.7

Legend: LC_{50} and LC_{90} = Lethal Concentration; CI, Confidence interval. NE = NE, not estimated.
* Strains that showed a difference in toxicity for T Student. The control showed mortality ≤ 5%.

cry and *cyt* genes (Bravo et al., 1998; Armengol et al., 2006; Abulreesh et al., 2012; Reyaz et al., 2017).

In the isolates used in the present study, the variation of the gene profile of naturally occurring strains active against mosquitos showed different combinations of the genes encoding insecticidal proteins (Bravo et al., 1998; Ibarra et al., 2003; Jouzani et al., 2008; González et al., 2011; El-kersh et al., 2016). Gene profile variation was also observed in *Bt* isolates native to Colombia in a study of selection of strains in *C. quinquefasciatus* and Lepidopteran larvae (Armengol et al., 2006).

The Cerrado biome showed the largest number of strains with combinations of mosquito-specific toxin-encoding genes, including *cry4, cry11*, *cry10*, *cyt1,* and *cyt2*, similar to the standard strain, and the only Caatinga isolate with larvicidal activity also has the same *Bti* genes. On the other hand, this gene profile was not observed for the Amazon strains.

The high frequency of mosquito-specific *cry* genes presents in 97.2% of isolates with larvicidal activity demonstrates the importance of this class of genes in the pathogenicity of *Bt* for this insect group. Among the mosquito-active *cry* genes described in the literature, the genes of the *cry4* and *cry11* stand out due to their larvicidal potential. In the present study, it was found that the *cry4Aa* and *cry4Ba* genes were the most frequent, presents in more than half (59.4%) of the strains. Higher frequency of *cry4Ba* genes in isolates with larvicidal activity against *Ae. aegypti* (Costa et al., 2010; Campanini et al., 2012).

In the present study, the frequency of *cyt* genes was lower than that of *cry*, the *cyt1Aa* gene was the most frequent. The presence of this gene is often detected in strains active against mosquito larvae (Costa et al., 2010; El-kersh et al., 2016).

This gene plays a key role in the activity of *Bt* against mosquitoes, as the Cyt1Aa proteins act directly in the insertion of the Cry toxins into the intestinal epithelium of the larvae, which can increase the toxicity of the strains where they are found (Pérez et al., 2005; Elleuch et al., 2015b). The toxin *cyt1Aa* may hinder selection by mosquito populations for resistance (Pérez et al., 2005).

The synergism between Cry and Cyt proteins presents greater toxicity than the use of Cry proteins alone or the combination between two or more Cry (Crickmore et al., 1995; Xu et al., 2014; Elleuch et al., 2015b). In the present study, the presence of the combination of *cry/cyt* genes was observed in 78% of isolates with pathogenic activity, and the results showed that the most toxic strains obtained in this study contain different combinations of *cry* and *cyt* genes.

The bioassays with *Ae. aegypti* help relate the toxicity information of each strain to the molecular identification of the genes that may be directly involved in the larvicidal activity in mosquitoes, this is the culicid species with the highest number of toxins with larvicidal activity already described (Frankenhuyzen, 2009; 2013).

In the present study, two strains (BtMA-690 and BtMA-1114) were found to have similar toxicity to the standard strain *Bti*-T4001, with equal LC $_{50}$ values in at least one of the three mortality evaluations.

Notably, the two isolates with the highest toxicity were obtained from different substrates BtMA-690 from a soil sample and BtMA-1114 from Coleoptera insects- both collected in a Cerrado area. These data are important for obtaining the diversity of toxic strains.

Considering the gene profile of the most toxic strains, all genes present in *Bti* were detected in BtMA-690, whereas BtMA-1114 presented positive amplification for the genes *cry4*, *cry11*, *cry10*, *cyt1*, and *cyt2*, but did not show amplification for the *cry11Ba* and *cyt1Ab* genes; in addition, the *cry32* gene was also identified in this strain.

Cry32 insecticidal proteins have three different genes *cry32Ba*, *cry32Ca,* and *cry32 Da* encoding toxins active against *Ae. aegypti* (Frankenhuyzen, 2009). The presence of the *cry32* gene is an indication that for the BtMA-1114 strain, indicate the diversity of the gene profile of the pathogenic strains for mosquitoes obtained in this study.

Several studies show the variation of the gene profile and toxicity of *Bt* González et al. (2011), while studying strains native to Cuba, observed that for three of these, the LC_{50} values were better than that of the standard strain, and two strains contained the same gene profile as *Bti*, whereas the other strain showed different plasmid and protein profiles. This was also observed in two strains—BLB355 from Portugal and BLB196 from Saudi Arabia—that presented larvicidal activity against *Ae. aegypti*, but the presence of *Bti* genes was not detected in these strains (Elleuch et al., 2015a).

On the other hand, other studies have reported that strains with a *Bti*-like gene profile are the most effective (Costa et al., 2010; Santos et al., 2012). In the present study, the BtMA-690 isolate presented a gene profile and a toxicity similar to those of the standard strain; the same profile was detected in seven other strains, but for those, the insecticidal activity was lower than that of the standard.

However, despite the BtMA-679 and BtMA-687 strains not showing the same degree of toxicity as *Bti*, the genes present in the standard strain, as well as the *cry32* gene, were found in these strains. Thus, as with BtMA-1114, these strains are promising for preventing the emergence of resistance to the combination of *Bti* Cry/Cyt toxins, as the use of new strains with gene profiles different from those of the strains already being used is a way to avoid selection of resistant mosquito populations (Peralta and Palma, 2017).

In addition to the combination of the *cry/cyt* genes found in the strains of this study, the presence of the chitinase gene was also observed in 35% of the strains. The presence of the insecticidal proteins Cry/Cyt combined with chitinase contributes to the overall toxicity of the strains because chitinases have the potential to destroy the peritrophic matrix of larvae, thus facilitating the contact between δ-endotoxins and their receptors in the intestinal epithelium (Sampson and Gooday, 1998; Juárez-Hernández et al., 2015). The presence of these toxins in strains active against mosquitos was also described in other Brazilian strains (Costa et al., 2010).

The present study provides evidence of the diversity the *Bt* with activity against larvae of *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. darlingi* isolated from soils and insects different biomes Brazilians. The Cerrado biome showed more promise for obtaining strains with higher toxicity for mosquitoes (BtMA-1114 and BtMA-690).The data are promising for control mosquitoes of medical importance, since *Bt* is an effective component in the control of these insects, which are currently considered a public health problem worldwide.

Conflict of interest

The authors declare that there is no conflict of interest.

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