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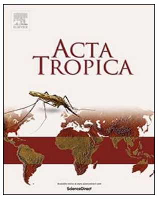

Molecular characterization of the gene profile of *Bacillus thuringiensis* Berliner isolated from Brazilian ecosystems and showing pathogenic activity against mosquito larvae of medical importance

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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