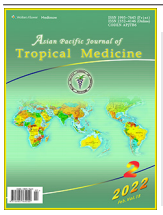

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Evaluation of Cuban *Bacillus thuringiensis* (Berliner, 1911) (Bacillales: Bacillacea) isolates with larvicidal activity against *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae)

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ABSTRACT

Objective: To evaluate 11 Cuban native *Bacillus* (*B.*) *thuringiensis* isolates in order to select one with the best larvicidal activity against *Aedes* (*Ae.*) *aegypti* and low cytotoxicity.

Methods: The *cry* and *cyt* genes of the isolates (A21, A51, L95, L910, M29, R84, R85, R87, R89, U81 and X48) were amplified by PCR. The influence of organic matter and NaCl on the larvicidal activity was tested by bioassays. Cytotoxicity was assayed on peritoneal macrophages of BALB/c mice.

Results: The *cyt1* (*Aa, Ab, Ba*), *cyt2*, *cry4aA*, *cry4Ba*, *cry11* (*Aa, Ba, Bb*) and *cry10* genes were identified in all native Cuban isolates. The larvicidal activity (LC_{90}) of seven isolates was affected by the presence of organic matter in the water, while A21, A51, L910, R84, U81 and X48 had better LC_{50}, LC_{90}, LC_{95} than the 266/2 9-Ⅶ-98 control strain. The LC_{50} of two isolates was affected by the presence of NaCl and A21, A51, R85 isolate had better larvicidal activity than the 266/2 9-Ⅶ-98 control strain. In terms of toxicity against macrophages, the extracts of nine isolates were less cytotoxic than the control strains.

Conclusions: Native isolate A21 had the main virulence factors against *Ae. aegypti* larvae, displayed a good larvicidal activity in presence of different factors related with *Ae. aegypti* breeding sites, and had low citotoxicity against macrophages. These results can contribute to the improvement of existing biological control strategies and the development of new biolarvicides.

KEYWORDS: Mosquitoes; Biological control agent; *Bacillus thuringiensis*; Bioassays; *Aedes aegypti*; *cry* and *cyt* genes

1. Introduction

Climate change, global warming, human activities, among other factors increase the abundance and worldwide geographical distribution of *Aedes* (*Ae.*) *aegypti* (Linnaeus, 1762) (Diptera: Culicidae)[1]. This mosquito is considered the principal vector that transmits Zika, dengue, chikungunya and yellow fever in the Americas; therefore, its control is of paramount importance to interrupt the transmission of these diseases[2].

Significance

Vector control strategies should be adapted to the local conditions, mainly in low- and middle-income countries which are most affected by dengue and climate change. This study provides an integral, objective and practical evaluation of *Bacillus thuringiensis* Cuban native isolates in order to select the best isolates for biolarvicide development. This kind of evaluation (based not only on the *cry* and *cyt* genes) emphasizes the importance of obtaining non-toxic isolates that maintain their high larvicidal activity against *Aedes aegypti* in presence of different factors associated with the breeding sites. It is a valuable tool for the development of new and safe biolarvicides, more adapted to *Aedes aegypti* breeding sites conditions.

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In this sense, the most effective method to reduce *Ae. aegypti* populations is the use of chemical insecticides aimed to control immature or adult insects[2]. However, the increase in insecticide resistance[3] requires alternative methods of control such as microbial insecticides[4]. The most widely used microbial biopesticides are derived from *Bacillus* (*B.*) *thuringiensis* (Berliner, 1911) (Bacillales: Bacillacea)[5].

Biolarvicides based on *B. thuringiensis* are specific to a limited number of insect species with no toxicity against humans or other organisms, and an effective tool for *Ae. aegypti* larval control[6]. The principal virulence factors of this bacterium (cry and cyt toxins) have a more distinct mode of action on mosquito larvae than chemical insecticide[6]. Nevertheless, the larvicidal activity of *B. thuringiensis* in field has a low persistence owing to the low stability of its toxins under field conditions[6,7]. In particular, the larvicidal activity of *B. thuringiensisis* is conditioned by several factors, namely organic enriched habitats, exposition to UV light, temperature increase, changes in pH, chlorination or bacterial degradation[8–10]. Thus, the continuous search of native isolates is a current need in order to generate biolarvicide formulations more adapted to the conditions of each region and provide a highly effective and low-cost product[11–13].

In Cuba, previous studies reported native isolates of *B. thuringiensis* with a high larvicidal activity against *Ae. aegypti*[14,15], as well as the influence of temperature and water chlorination on this activity[16]. In this context, the present study carry out the final evaluation of Cuban native isolates in order to select the better isolates for biolarvicide development based on: 1) the presence of *cry* and *cyt* genes; 2) the influence of organic matter and water salination on the larvicidal activity, and 3) the cytotoxicity on macrophage.

2. Materials and methods

2.1. Bacterial control strains, isolates and mosquitos

B. thuringiensis serotype H-14, IPS 82 from the International Entomopathogenic Bacillus Centre, Institute Pasteur; Paris, France and *B. thuringiensis* var. *israelensis* serotype H-14 266/2 9-Ⅶ-98 (strain isolated from the most extensive biolarvicides used in Cuba: Bactivec® Labiofam, Cuba) were used as control strains.

Native *B. thuringiensis* isolates: A21, A51, L95, L910, M29, R84, R85, R87, R89, U81, and X48 were isolated from soil samples of the Cuban archipelago[14,15]. These isolates belong to the entomopathogenic bacteria collection from the Biological Control Laboratory of the Tropical Medicine Institute “Pedro Kourí”, IPK, Cuba.

Ae. aegypti (Rockefeller strain), a laboratory susceptible strain of Caribbean origin colonized after the 1930s, was provided by the Center for Disease Control and Prevention (CDC) Laboratory in San Juan, Puerto Rico.

Mosquitoes were maintained on 10% sucrose solution at (26.0±0.5) ℃, 80%-85% relative humidity with a 12 h light/dark cycle. Female mosquitoes were given access to an anesthetized mouse and allowed to blood feed for 30 min weekly. The larvae were fed with finely powdered fish food (CENPALAB, Cuba)[17].

2.2. Detection of cry and cyt genes

To detect the *cry* and *cyt* genes a 12 h of *B. thuringiensis* culture (control strains and isolates) in a nutrient medium plate was used. A loopful of cells was transferred to 0.1 mL of H_2O and treated with lysozyme for 2 h at 37 ℃ to obtain DNA using the procedure described by Maxwell® 16 Tissue DNA Purification Kit (Promega, USA). The PCR mix consisted of 1× green buffer (Promega, USA), 2 mM $MgCl_2$; 0.2 mMdNTP; 0.5 μM each primer (forward and reverse, Table 1); 2.5 U Go taq Flexi DNA polymerase (Promega, USA); and 2 μL of template DNA for a final volume of 50 μL. and PCR was carried out in a Mastercycler personal Eppendorf AG, Germany, as follows: 2 min at 95 ℃; 30 cycles of 1 min at 95 ℃, 1 min annealing at 46 ℃ to 54 ℃ (according to each primer combination, Table 1), and 1 min at 72 ℃; and 5 min at 72 ℃. Fifteen μL of PCR product was electrophoresed on 2% agarose gel and run 250 V during 45 min.

2.3. Influence of organic matter and NaCl on the toxicity of B. thuringiensis native isolates

Bacterial isolates and control strains (*B. thuringiensis* IPS-82 and *B. thuringiensis* 266/2 9-Ⅶ-98) were grown in a fermentation medium consisting of sucrose (2 g/L), bacteriological peptone (2 g/L), yeast extract (1 g/L), and inorganic salts (12.5 mmol/L $MgSO_4$; 0.05 mmol/L $MnSO_4$; 1.2 mmol/L $FeSO_4$; 1.2 mM $ZnSO_4$; 25 mmol/L $CaCl_2$); and incubated at 30 ℃ and 150 rpm shaking, until sporulation was completed (48-72 h). Concentrations were expressed in mg/mL (dry weight).

Quantitative bioassays were conducted following the World Health Organization (WHO) protocol[21]. Twenty-five larvae (Ⅲ-Ⅳ instar) were placed into 120 mL cups with 100 mL of dechlorinate water. Five concentrations of bacterial formulation that cause mortalities between 10% and 90% were accepted for validating the bioassay in order to calculate the lethal concentrations (LC). Four replicates were performed for each concentration tested per bioassay. Each bioassay was repeated four times in independent assays. Larval mortality was recorded 24 h after treatment.

To detect the effect of organic matter and NaCl on larvicidal activity the biosassays were performed with: 300 mg of non contaminated powdered leaf litter in 100 mL of dechlorinate water and dechlorinated water with a NaCl concentration of 5 g/L, respectively. The biosassays performed only in dechlorinated water were used as control. Finally, the influence of organic matter and NaCl versus dechlorinate water on the larvicidal activity of the Cuban isolates was tested and compared.

Table 1. Primers used in the *cry* and *cyt* gene detection.

Primer pair	Sequence	Anneal temp (℃)	Genes	Product size (bp)	References
Cb-1	CCTCAATCAACAGCAAGGGTTATT (d) TGCAAACAGGACATTGTATGTGTAATT(r)	52	*cyt1Aa* *cyt1Ab* *cyt1Ba*	477 480 477	[18]
Cb-2	ATTACAAATTGCAAATGGTATTCC (d) TTTCAACATCCACAGTAATTTCAAATGC(r)	50	*cyt2Aa* *cyt2Ba* *cyt2Bb* *cyt2Ca*	356 355 355 355	[18]
Cb-4	TCAAAGATCATTTCAAAATTACATG (d) CGGCTTGATCTATGTCATAATCTGT (r)	54	*cry4aA*	459	[18]
Cb-5	CGTTTTCAAGACCTAATAATATAATACC (d) CGGCTTGATCTATGTCATAATCTGT (r)	54	*cry4Bb*	321	[18]
Cb-6	CGCTTACAGGATGGATAGG (d) GCTGAAACGGCACGAATATAATA (r)	50	*cry11Aa* *cry11Ba* *cry11Bb*	342 343 352	[18]
Cb-7	TCAATGCTCCATCCAATG (d) CTTGTATAGGCCTTCCTCCG (r)	51	*cry10*	348	[18]
Cb-9	ATATGAAATATTCAATGCTC(d) ATAAATTCAAGTGCCAAGTA (r)	46	*cry10A*	550	[19]
Cb-11	TAGAAGATACGCCAGATCAAGC (d) CATTTGTACTTGAAGTTGTAATCCC (r)	51	*cry11A* *cry11B*	305 305	[20]
Cb-12	AACCCCTCAATCAACAGCAAGG (d) GGTACACAATACATAACGCCACC (r)	51	*cyt1Aa* *cyt1Ab*	522 525	[20]

d and r: direct and reverse primers, respectively.

2.4. Macrophage cytotoxicity assay

The spore-crystal mixtures of native isolates and control strains (*B. thuringiensis* IPS- 82 and *B. thuringiensis* 266/2 9-Ⅶ-98) were re-suspended in 50 mM Na_2CO_3 for 1 h at 37 ℃. After that, the supernatants were centrifuged at 13 000 × *g* during 10 min at 4 ℃. Then the clarified supernatants were passed through a 0.45 μm membrane filter, and the pH was adjusted to 8.0.

The filtered supernatant was used directly (aqueous extract) or diluted in alcohol at 80% (hydroalcoholic extract). Both solutions were kept standing for 7 days at 4 ℃ with occasional manual shaking (3 times a day for 1 minute). Subsequently, the solvent from the samples was evaporated in a Concentrator Plus (Eppendorf, Germany) during 4 h. The supernatant was removed and the pellet was re-suspended in dimethylsulfoxide (DMSO; BDH, England), until a final concentration of 20 mg/mL was obtained. In parallel, a control with culture medium was included.

Peritoneal macrophages for cytotoxic assays were collected from healthy female BALB/c mice as follows: twelve animals were euthanized by cervical dislocation and macrophages were obtained by lavage with 5 mL of RPMI-1640 medium (Sigma, USA) into the peritoneal cavity.

The median cytotoxic concentration (CC_{50}) of the extracts on macrophages was determined. Peritoneal macrophages in RPMI-1640 medium supplemented with antibiotics (penicillin 200 UI, streptomycin 200 μg/mL) were seeded in 96-well V-bottom plates at a concentration of 3×10^5 cells/well and incubated for 2 h at 37 ℃ in 5% CO_2 to obtain a monolayer culture. The non-adherent cells were removed by washes with phosphate-buffered saline solution (PBS).

Then, in each well, 50 μL of medium with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, USA) and antibiotics (penicillin 200 UI, streptomycin 200 μg/mL) were added, into the wells of column 2 and 7, additional 48 μL of medium were dispensed and 2 μL of tested extracts and two-fold serial dilutions down each lane were carried out to give final concentrations from 12.5 to 200 μg/mL. Thereafter, the treated macrophages were incubated at 37 ℃ in an atmosphere of 5% CO_2. After 72 h of incubation 15 μL of a solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) was added to each well. After incubating for 4 h, at the same conditions, the formazan crystals were dissolved in DMSO (100 μL per well). Absorbance was measured at 560 and 630 nm as the reference wave length[22] and lineal concentration response curves were constructed. Evaluations were performed in triplicate in independent assays.

The extracts from native isolates with CC_{50} higher than the CC_{50} obtained with the controls strain used in the study (IPS-82 and 266/2 9-Ⅶ-98) were considered non-cytotoxic.

2.5. Statistical analysis

In all bioassays *Ae. aegypti* larval mortality data were used to calculate the lethal concentrations for 50%, 90% and 95% of exposed individuals (LC_{50}, LC_{90} and LC_{95} respectively) through log probit analysis[23] using the program SPSS 21. The means of larval mortality caused by each isolate and the control strains against *Ae. aegypti* were calculated. Once the lethal doses were calculated, the LC_{95}/LC_{50} ratio was performed to determine how many times it is necessary to increase the LC_{50} in order to obtain higher mortality. A lower ratio is indicative of better formulation efficiency[24].

To detect the effect of organic matter and NaCl on larvicidal activity data analysis was performed by *t*-Student test using the statistical package SPSS 21. In all cases, statistically significant differences were identified at $P<0.05$ level.

In macrophage cytotoxicity assay the medium cytotoxic concentration (CC_{50}) was obtained from linear dose-response. Results are expressed as median and 95% confidence intervals (*CI*) of three independent replicates. The statistical differences between CC_{50} of the control and isolates extracts were determined using Kruskal-Wallis with Statistica for Windows Program (Version 13.1, StatSoft, Inc 2016), considering statistical differences as $P<0.05$.

2.6. Ethical approval

All the experimental procedures involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition, which was approved by the Ethics Committee (CEI-IPK 21-16), Havana, Cuba.

3. Results

3.1. Detection of cry and cyt genes

The specific cry and cyt type primers were used to detect *cry* and *cyt* genes in the isolates by PCR analyses, *cry11*-type, *cry4*-type, *cyt1*-type, and *cyt2*-type genes were found in all native isolates (Table 2). The presence of *cyt1* (*Aa, Ab, Ba*), *cry11* (*Aa, Ba, Bb*) and *cry10* were detected in all isolates. On the other hand, we could detected other *cyt1* genes (*Aa, Ab*) in 10 isolates (90.1%). The presence of *cry10Aa* gene was only detected in two isolates, L910 and M29 (18.2%). In 10 isolates, a band of 305 bp was obtained as a result of the amplification with cb-11 primer (*cry11 A, B*).

3.2. Influence of organic matter on the toxicity of B. thuringiensisnative isolates

In the performed bioassays, the control mortality was lower than 5.0%. The LC_{90} of A21, A51, L95, L910, M29, R85 and X48 isolates were affected ($P<0.05$) (Table 3) by the presence of organic matter in the water comparing with those exposed to declorinated water. A21, A51, L910, R84, U81 and X48 isolates exhibited better larvicidal activity (LC_{50}, LC_{90} and LC_{95}) than the 266/2 9-Ⅶ-98 strain in presence of organic matter as shown in Table 3. A51 isolate had lower LC_{90} than IPS-82 control strain. Efficiency for R85 in presence of organic matter was 9.0, which was the most affected isolate (Table 3).

3.3. Influence of NaCl on the toxicity of B. thuringiensisnative isolates

The LC_{50} of R84 and X48 were affected by the presence of NaCl. (Table 3). The larvicidal activity (LC_{50}, LC_{90}, LC_{95}) of A21, A51, R85 and U81 isolates were significant better (all $P<0.05$). A51 and U81 isolates had lower LC_{90} than IPS-82 strain with presence of NaCL (both $P<0.05$) (Table 3).

In summary, A21, A51 and U81 isolates exhibited better larvicidal activity than 266/2 9-Ⅶ-98 strain in presence of organic matter and NaCl.

3.4. Macrophage cytotoxicity assay

The aqueous extracts of: A21, L95, L910, M29, R84, R85 and U81 isolates, as well as the hydroalcoholics of: A21, L95, L910, M29, R84, R87, R89 and U81 isolates did not show cytotoxicity given at 200 μg/mL (Table 4). On the contrary, both aqueous and hydroalcoholic extracts of X48 isolate showed CC_{50} values significantly lower ($P<0.05$) than the strains used as control and therefore they were considered cytotoxic.

Table 2. Detection of *cry* and *cyt* genes in *Bacillus thuringiensis* native isolates and control strains.

Strains/isolates	*cry* and *cyt* genes detected
IPS-82	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry10Aa, cry11(A, B)*
266/2 9-Ⅶ-98	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry10, cry11 (A, B)*
A21	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10*
A51	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry11 (A,B)*
L95	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry11 (A, B)*
L910	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry10Aa, cry11 (A, B)*
M29	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry10Aa, cry11 (A, B)*
R84	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry11 (A, B)*
R85	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry11 (A, B)*
R87	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry11 (A, B)*
R89	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry11 (A, B)*
U81	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11spe (Aa, Ba, Bb), cry10, cry11 (A, B)*
X48	*cyt1 (Aa, Ab, Ba), cyt2, cry4aA, cry4Ba, cry11 (Aa, Ba, Bb), cry10, cry11 (A, B)*

Table 3. Lethal concentration (LC) of *Bacillus thuringiensis* isolates and control strains against *Aedes aegypti* larvae after 24 h exposure obtained from probit analysis (mg/L).

Isolates/strains	Variable	Dechlorinated water	NaCl	Organic matter
A21	LC_{50} (CL)	0.004 (0.003-0.004)	0.003[b] (0.005-0.008)	0.013[ab] (0.011-0.014)
	LC_{90} (CL)	0.013 (0.010-0.016)	0.010[b] (0.010-0.024)	0.033[ab] (0.023-0.046)
	LC_{95} (CL)	0.018 (0.014-0.024)	0.016[b] (0.012-0.029)	0.045[ab] (0.035-0.065)
	Efficiency	4.8	4.5	3.5
A51	LC_{50} (CL)	0.002 (0.001-0.002)	0.002[b] (0.002-0.002)	0.003[ab] (0.003-0.004)
	LC_{90} (CL)	0.005 (0.004-0.006)	0.005[b] (0.004-0.005)	0.007[abc] (0.006-0.010)
	LC_{95} (CL)	0.006 (0.005-0.008)	0.006[b] (0.005-0.007)	0.009[ab] (0.007-0.014)
	Efficiency	4.1	3.4	3.0
L95	LC_{50}(CL)	0.099 (0.083-0.117)	0.147 (0.070-0.086)	0.136 (0.114-0.166)
	LC_{90} (CL)	0.217 (0.173-0.309)	0.269 (0.175-0.244)	0.362[a] (0.272-0.563)
	LC_{95}(CL)	0.272 (0.231-0.325)	0.266 (0.224-0.331)	0.479[a] (0.343-0.808)
	Efficiency	2.8	1.8	3.5
L910	LC_{50} (CL)	0.012 (0.009-0.011)	0.012[b] (0.010-0.016)	0.015[ab] (0.001-0.002)
	LC_{90} (CL)	0.019 (0.018-0.022)	0.024[b] (0.019-0.045)	0.034[ab] (0.003-0.005)
	LC_{95} (CL)	0.023 (0.021-0.027)	0.030 (0.022-0.055)	0.043[ab] (0.033-0.062)
	Efficiency	2.2	2.4	3
M29	LC_{50} (CL)	0.066 (0.058-0.074)	0.091[b] (0.077-0.012)	0.360[ab] (0.320-0.399)
	LC_{90} (CL)	0.136 (0.114-0.175)	0.204[b] (0.156-0.318)	0.710[ab] (0.616-0.878)
	LC_{95} (CL)	0.168 (0.137-0.225)	0.256 (0.187-0.434)	0.860[ab] (0.724-1.126)
	Efficiency	2.6	2.8	2.4
R84	LC_{50} (CL)	0.010 (0.009-0.104)	0.018[ab] (0.017-0.020)	0.008[ab] (0.008-0.009)
	LC_{90}(CL)	0.019 (0.017-0.022)	0.033[b] (0.029-0.038)	0.018[ab] (0.016-0.021)
	LC_{95}(CL)	0.026 (0.023-0.030)	0.039 (0.034-0.046)	0.023[b] (0.020-0.027)
	Efficiency	2.7	2.1	3
R85	LC_{50}(CL)	0.008 (0.007-0.009)	0.005[ab] (0.004-0.005)	0.053[ab] (0.033-0.139)
	LC_{90} (CL)	0.017 (0.015-0.022)	0.010[ab] (0.009-0.011)	0.296[ab] (0.119-2.560)
	LC_{95} (CL)	0.022 (0.018-0.028)	0.012[ab] (0.011-0.014)	0.480[ab] (0.169-5.900)
	Efficiency	2.8	2.7	9.0
R87	LC_{50} (LC)	0.029 (0.026-0.032)	0.006[ab](0.005-0.006)	0.033 (0.030-0.037)
	LC_{90} (CL)	0.078 (0.067-0.095)	0.015[ab] (0.013-0.017)	0.077[b] (0.067-0.092)
	LC_{95} (CL)	0.104 (0.087-0.131)	0.02[a] (0.017-0.024)	0.097[b] (0.082-0.120)
	Efficiency	3.6	3.5	3
R89	LC_{50} (CL)	0.072 (0.064-0.079)	0.010[ab] (0.010-0.012)	0.080[ab] (0.068-0.095)
	LC_{90} (CL)	0.173 (0.150-0.208)	0.034[ab] (0.030-0.041)	0.187[b] (0.148-0.266)
	LC_{95} (CL)	0.222 (0.187-0.278)	0.047[ab] (0.040-0.059)	0.238[b] (0.181-0.361)
	Efficiency	3.1	4.3	2.9
U81	LC_{50} (CL)	0.005 (0.004-0.005)	0.001[ab] (0.001-0.002)	0.004[b] (0.004-0.004)
	LC_{90} (CL)	0.009 (0.008-0.010)	0.003[ab] (0.002-0.004)	0.007[bc] (0.007-0.008)
	LC_{95} (CL)	0.011 (0.010-0.013)	0.003[ab] (0.003-0.004)	0.009[b] (0.008-0.010)
	Efficiency	2.4	2.7	2.3
X48	LC_{50} (CL)	0.002 (0.002-0.003)	0.027[ab] (0.023-0.034)	0.005[ab] (0.004-0.009)
	LC_{90} (CL)	0.006 (0.004-0.006)	0.063[ab] (0.048-0.100)	0.012[ab] (0.007-0.056)
	LC_{95} (CL)	0.009 (0.008-0.011)	0.081[ab] (0.058-0.139)	0.016[ab] (0.009-0.010)
	Efficiency	4.4	2.9	3.2
IPS-82	LC_{50} (CL)	0.002 (0.002-0.003)	0.002 (0.002-0.002)	0.006[a] (0.004-0.008)
	LC_{90} (CL)	0.006 (0.005-0.007)	0.005 (0.005-0.006)	0.011[a] (0.008-0.029)
	LC_{95} (CL)	0.009 (0.007-0.012)	0.007 (0.006-0.009)	0.013[a] (0.009-0.044)
	Efficiency	3.3	3.4	2.2
266/2 9-Ⅶ-98	LC_{50} (CL)	0.008 (0.008-0.010)	0.009[a] (0.008-0.001)	0.024 (0.019-0.025)
	LC_{90} (CL)	0.020 (0.018-0.023)	0.020 (0.018-0.023)	0.042[a] (0.064-0.094)
	LC_{95} (CL)	0.025 (0.022-0.029)	0.025 (0.022-0.029)	0.106[a] (0.086-0.144)
	Efficiency	3.3	2.8	4.4

LC_{50}: lethal concentration 50, LC_{90}: lethal concentration 90, LC_{95}: lethal concentration 95, CL: 95% confidence limits, Efficiency: LC_{95}/LC_{50}. [a]: LC values with statistical differences ($P<0.05$) by *t*-Student test with respect to a control bioassay (dechlorinate water bioassays at 25 ℃). [b]: LC values with statistical differences ($P<0.05$) by *t*-Student test with respect to the obtained results with 266/2 9-Ⅶ-98 strain. [c]: LC values with statistical differences ($P<0.05$) by *t*-Student test with respect to the obtained results with IPS-82 strain.

Table 4. Cytotoxicity of aqueous and hydroalcoholic extracts of *Bacillus thuringiensis* native isolates and control strains on peritoneal macrophages.

Extracts	CC_{50} (95% *CI*) μg/mL
Control-aqueous extract	> 200
-hydroalcoholic extract	> 200
A21-aqueous extract	> 200
-hydroalcoholic extract	> 200
A51-aqueous extract	137.8 (136.5-139.2)[e]
-hydroalcoholic extract	195.9 (191.4-200.4)
L95-aqueous extract	> 200
-hydroalcoholic extract	> 200
L910-aqueous extract	> 200
-hydroalcoholic extract	> 200
M29-aqueous extract	> 200
-hydroalcoholic extract	> 200
R84-aqueous extract	> 200
-hydroalcoholic extract	> 200
R85-aqueous extract	> 200
-hydroalcoholic extract	147.5 (143.6-151.4)[f]
R87-aqueous extract	134.0 (127.6-140.4)[d,e]
-hydroalcoholic extract	> 200
R89-aqueous extract	152.4 (139.7-165.2)[f]
-hydroalcoholic extract	> 200
U81-aqueous extract	> 200
-hydroalcoholic extract	> 200
X48-aqueous extract	97.3 (92.8-101.9)[b]
-hydroalcoholic extract	69.4 (68.7-70.0)[a]
IPS-82-aqueous extract	130.1 (127.3-132.8)[d]
-hydroalcoholic extract	106.5 (103.8-109.3)[b,c]
266/2 9-Ⅶ-98-aqueous extract	116.9 (111.0-122.8)[c]
-hydroalcoholic extract	116.4 (114.9-118.0)[c]

CC_{50}: median cytotoxic concentration, 95% *CI*: 95% confidence intervals. Statistical analysis carried out using Kruskal Wallis test. Different letters means significantly different values (P<0.05).

4. Discussion

B. thuringiensis exhibits high toxicity for diptera larvae[12,13]. The breeding sites treated with this bacterium attract *Ae. aegypti* female and act as lethal ovitraps[25,26]. However, the efficacy of the products based on this bacterium is affected by environmental conditions[8–10,27]. For this reason, the evaluation of native strains is an important step for developing biolarvicides adapted to our natural conditions. Cuban *B. thuringiensis* isolates, collected from soils samples of different environments[14,15] and evaluated in this and others studies[16,28], exhibited some differences that permitted a correct selection.

The genetic studies of native isolates allowed corroborating the presence of the main virulence factors against *Ae. aegypti* detected in *B. thuringiensis*[6,18,29,30]. The *cry10* gene (primer cb-7) was identified in 11 isolates, while the amplification of *cry10Aa* was only obtained in the control strain IPS-82 and in M29 and R84 isolates. According to the literature reviewed, the *cry10Aa* genetic variant active against Diptera has been described for the *cry10* gene[6,29–31]. However, the differences in *cry10* gene amplification with different primers, suggest the possibility of other genetic variants. The *cry* and *cyt* genes detected confirm the proteins patterns previously reports for these isolates[14,15].

The high larvicidal activity of the *B. thuringiensis* delta-endotoxins against mosquitos is attributed to complex interactions between their proteins[32]. The combinations: cry4Aa and cry4Ba[32], cry4Aa and cry11Aa, cry4Ba and cry11Aa, cry10Aa and cyt2Ba[32], cry10Aa with cyt1Aa[6,29,32], cyt2Ba with cry4Aa, cyt1Aa and cry11Aa[32]; are synergistic against *Ae. aegypti* larvae. The detection of different *cry* and *cyt* genes in all isolates allowed us to suggest the presence of these protein combinations, which would justify the high larvicidal activity previously reported[14,15].

The use of isolates with cry and cyt active proteins against Diptera would delay the development of resistance, taking into account that cyt proteins act as additional receptors for cry proteins and potentiate their activity[32,33]. Field and laboratory resistance to *B. thuringiensis* were reported in *Culex quinquesfasciatus* and *Culex pipiens* larvae[34,35]. However, only insignificant levels of resistance were attained against *Ae. aegypti* in laboratory conditions. In both genera of Diptera, resistance behaves unstable, and in absence of selection pressure it reverts to 50% after three generations[32,34,35]. Therefore, the detection of *cyt1A*, *B* and *cyt2* genes in all native isolates can predict low resistance in the field to future products based on these isolates.

It is good for us to have native *B. thuringiensis* isolates with excellent combinations of *cry* and *cyt* genes. However, the influence of different factors, like temperature increase, presence of chlorine, salt and organic matter, over *B. thuringiensis* larvicidal activity is another highlight to be considered.

According to a study carried out in 2019[16], some Cuban *B. thuringiensis* native isolates (A21, A51, L910, R85, and X48) maintained a good larvicidal activity against *Ae. aegypti* in presence of temperature increase (25-35 ℃) and chlorine. Nevertheless, the correct selection of native isolates implies the evaluation of other factors, such as salt and organic matter, to determine their influence on the larvicidal activity of these isolates.

Biolarvicides based on *B. thuringiensis* var. *israelensis* show low activity in organically enriched habitats[8–10]. Rydzanicz *et al* demonstrated that the optimum larvicidal effect of *B. thuringiensis* can be achieved in breeding habitats with limited organic content[9]. In this study, the larvicidal activity was significantly affected by organic matter in seven of 11 native isolates. This decrease may be associated to diversification of the food source of the *Ae. aegypti* larvae by the organic matter and consequently, they ingest a lower concentration of toxins, spores and vegetative cells. Additionally, the lamellar envelope of the toxic crystal of our isolates may interact powerfully with organic matter particles, leading to a major decrease of larvicidal activity[8]. On the other hand, the cyt proteins detected in these isolates may be bind irreversibly to the organic matter present in the medium and thus preventing their synergistic effect with cry proteins. This inhibitory effect was previously reported by Tetreau *et al* in 2012[8]. Notwithstanding, the larvicidal activity of six isolates was better than 266/2 9-Ⅶ-98 control strain.

The larvicidal activity of four native isolates increased significantly in presence of NaCl, which could be associated to the specific characteristics of each isolate. In some *B. thuringiensis* strains the NaCl increases the sporulation process and delta-

endotoxins production[36], leading to a major larvicidal activity. On the other hand, water salinity may lead to osmotic stress in *Ae. aegypti* larvae, which will increase the feeding needs and to compensate it, they will consume more *B. thuringiensis* toxins. Dawson *et al*, in 2019, did not obtain a reduction in the larvicidal activity of *B. thuringiensis* in presence of Na^+ and Cl^-[10]. However, other study, such as Jude *et al*, reported a significant reduction in the larvicidal activity of *B. thuringiensis* against *Ae. aegypti* in the presence of NaCl[37].

A high larvicidal activity against *Ae. aegypti* is very important in the selection of native isolates but a low cytotoxicity is essential in order to obtain safe candidates for biolarvicide development. Macrophages are essential effectors of the immune system response against microorganisms. The ability of some species of the *Bacillus* genus such as *B. cereus* (a species phylogenetically close to *B. thuringiensis*) to eliminate macrophage cells explains the persistence and dissemination of virulent strains in mammals[38]. The lower cytotoxicity against macrophages obtained with the extracts of 10 isolates is the first step that suggests safety in their use in future formulations. In this and previous studies, the X48 isolate showed a high larvicidal against *Ae. aegypti*[14,16]. This isolate has a principal virulence factors against *Ae. aegypti* larvae, but it was more cytotoxic against peritoneal macrophages than 266/2 9-Ⅶ-98 and IPS- 82 strains. This result allows us to preclude it for biolarvicide development.

According to our results, the U81 isolate kept a high larvicidal activity in presence of organic matter and NaCl, and it was less cytotoxic against peritoneal macrophages than 266/2 9-Ⅶ-98 and IPS-82 strains. Nevertheless, its activity was significantly affected by temperature increase and chlorine presence[16]. Taking into account the average of temperature increase in Cuba[39] and that chlorine is one of the most commonly used domestic water disinfectants in the world[40], we analyzed the larvicidal activity obtained with others isolates.

In this sense, A51 isolate had a better larvicidal activity based on the results obtained in this and in preceding studies[16], although the presence of beta exotoxins[28] excluded it as a candidate for biolarvicide development.

On the other hand, the results obtained in this and others studies[14–16,28] allow us to recommend A21 isolate as an active ingredient of biolarvicides. Its high larvicidal activity in presence of different factors related with *Ae. aegypti* breeding sites, their mains virulence factors against *Ae. aegypti* larvae and its low citotoxicity against macrophages are important points for this selection.

The results obtained from the evaluation and selection of native strains more adapted to *Ae. aegypti* breeding sites conditions can contribute to the improvement of existing biological control strategies and the development of new biolarvicides. Further investigations should be done with Cuban native isolates aiming to sequence the complete genome, to evaluate its larvicidal residual activity, and to carry out metabolomic studies; in order to clarify or improve the high larvicidal activity described.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Authors' contribution

AGR: Conceptualization, methodology data curation, formal analysis, investigation, writing-original draft, writing-review & editing, final approval of the version to be published; CECM, CRC: Formal analysis, investigation, writing-review & editing, final approval of the version to be published; ACI, ZMD: Formal analysis, investigation, writing- review & editing. final approval of the version to be published; LMF: Methodology data curation, formal analysis, investigation, writing-review & editing, final approval of the version to be published; HMHA: Resources, supervision, formal analysis, investigation, writing-review & editing, final approval of the version to be published.

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