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Detection of an Allele Conferring Resistance to *Bacillus sphaericus* Binary Toxin in *Culex quinquefasciatus* Populations by Molecular Screening▽

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The activity of the *Bacillus sphaericus* binary (Bin) toxin on *Culex quinquefasciatus* larvae depends on its specific binding to the Cqm1 receptor, a midgut membrane-bound α-glucosidase. A 19-nucleotide deletion in the *cqm1* gene (*cqm1*$_{REC}$) mediates high-level resistance to Bin toxin. Here, resistance in nontreated and *B. sphaericus*-treated field populations of *C. quinquefasciatus* was assessed through bioassays as well as a specific PCR assay designed to detect the *cqm1*$_{REC}$ allele in individual larvae. Resistance ratios at 90% lethal concentration, gathered through bioassays, were close to 1 and indicate that the selected populations had similar levels of susceptibility to *B. sphaericus*, comparable to that of a laboratory colony. A diagnostic PCR assay detected the *cqm1*$_{REC}$ allele in all populations investigated, and its frequency in two nontreated areas was 0.006 and 0.003, while the frequency in the *B. sphaericus*-treated population was significantly higher. Values of 0.053 and 0.055 were detected for two distinct sets of samples, and homozygote resistant larvae were found. Evaluation of Cqm1 expression in individual larvae through α-glucosidase assays corroborated the allelic frequency revealed by PCR. The data from this study indicate that the *cqm1*$_{REC}$ allele was present at a detectable frequency in nontreated populations, while the higher frequency in samples from the treated area is, perhaps, correlated with the exposure to *B. sphaericus*. This is the first report of the molecular detection of a biolarvicide resistance allele in mosquito populations, and it confirms that the PCR-based approach is suitable to track such alleles in target populations.

Bacillus sphaericus Neide is considered the most successful microbial larvicide to date for the control of mosquito species from the *Culex pipiens* (Diptera: Culicidae) complex (20). *B. sphaericus* biolarvicides commercially available are based on highly toxic strains characterized by their ability to express the binary (Bin) protoxin, a crystal protein produced in large amounts during sporulation (7). This heterodimer is formed by the BinA (42-kDa) and BinB (51-kDa) subunits that act in synergy to produce larvicidal activity upon *Culex* larvae (3, 23). The BinB subunit is responsible for the recognition and binding of the toxin to specific receptors on the midgut epithelium surface, while BinA is primarily responsible for the toxic effects, but first the crystal has to be ingested by the larvae and the protoxin must be processed into toxin by the midgut (7). The Bin toxin receptor in *C. pipiens* (Cpm1) and *Culex quinquefasciatus* (Cqm1) is a 60-kDa α-glucosidase attached to the epithelial cell membrane by a glycosylphosphatidylinositol anchor (9, 30, 31). The action of the Bin toxin on *Culex* larvae relies on its specific binding to those membrane-bound receptors (24). Disruption of the interaction between the toxin and the midgut is the major mechanism underlying resistance, and it has already been reported from different laboratory- or field-selected colonies (25, 26, 27, 33). Unrelated mechanisms may also be involved in resistance, but they have not been characterized so far (25, 27).

Molecular studies revealed that mutations in the genes *cpm1* and *cqm1*, which prevent the production of functional membrane-bound receptors, are the main reasons behind the lack of binding of the Bin toxin to the midgut epithelium. Four *cpm1*/*cqm1* resistance alleles were found in *Culex* populations of different origins. The *cpm1*$_{GEO}$ allele, detected in a *C. pipiens* laboratory-selected colony (GEO) from California, contains a nonsense mutation leading to the synthesis of a truncated protein lacking the glycosylphosphatidylinositol anchor and is therefore not able to localize to the apical membrane of the midgut epithelium (11, 38). A 19-nucleotide deletion in the gene *cqm1*, here named *cqm1*$_{REC}$, was associated with the resistance of a *C. quinquefasciatus* laboratory colony (CqRL1/2362) originating from the city of Recife (Brazil), and this mutation prevents the expression of Cqm1 on the midgut brush border membranes (28, 29, 30). Two alleles were found in the *C. pipiens* BP population from France (8). The *cpm1*$_{BP}$ allele had a single nucleotide mutation that prevented the expression of a membrane-bound protein, while the *cpm1*$_{BP}$*-del* allele involves transposon-mediated resistance and codes for a mutant membrane-bound protein that is unable to interact with the Bin toxin (10).

The elucidation of mutations conferring resistance to the Bin toxin is essential for the development of molecular tools to detect alleles containing such mutations in mosquito populations. The bioassay is the most widely used method to evaluate insect susceptibility; however, when resistance alleles are re-

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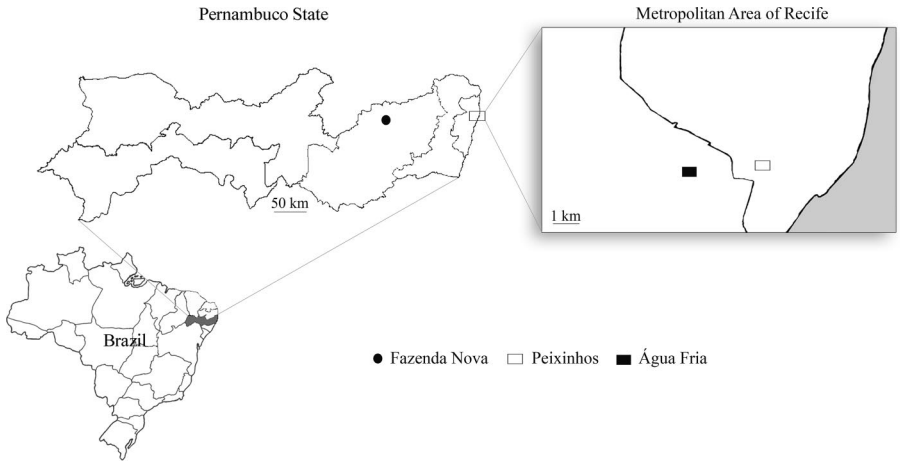

FIG. 1. *Culex quinquefasciatus* samples were collected in three areas of Pernambuco state, located in northeastern Brazil. Fazenda Nova is a nontreated rural district located 180 km from the RMA; both the nontreated area of Peixinhos and the *Bacillus sphaericus*-treated area of Água Fria are located in the RMA.

cessively inherited, heterozygote larvae cannot be identified and detection of resistant homozygotes is difficult. This is a critical issue since the evolution of resistance in populations subjected to selection pressure depends on the initial frequency of resistance alleles. The development of diagnostic PCR methods, or DNA-based kits, has opened new perspectives in the molecular monitoring of resistance (14, 18, 36).

The Recife Metropolitan Area (RMA), located in the state of Pernambuco in northeastern Brazil, has been subjected to a vector control program to reduce *C. quinquefasciatus* density in districts where filariasis is endemic (32). Among other control measures, *B. sphaericus*-based larvicides have been used, and one of the major concerns regarding their use as a microbial larvicide is monitoring the susceptibility of populations under selection. The $cqm1_{REC}$ allele was identified in a *C. quinquefasciatus* colony derived from the RMA (30), and it is believed that this mutation may be present and subjected to *B. sphaericus* selection in field populations. Here we report the development of a diagnostic PCR approach based on the detection of $cqm1_{REC}$ and its use to investigate the frequency of resistance in nontreated and treated *C. quinquefasciatus* populations.

MATERIALS AND METHODS

Mosquito laboratory colonies. Three colonies were used in this study: CqSF is a *C. quinquefasciatus* colony susceptible to *B. sphaericus* which was established from egg rafts collected in districts located in the RMA; the CqRL1/2362 colony was derived from the CqSF colony by laboratory selection with *B. sphaericus* strain 2362 and displays a high level of resistance (>100,000-fold) to this entomopathogen (29); RecL is an *Aedes aegypti* colony established from egg samples collected in the RMA. All colonies have been maintained in the insectarium of the Centro de Pesquisas Aggeu Magalhães (CPqAM-FIOCRUZ), Brazil, for more than 5 years. Larvae were reared in dechlorinated tap water and fed with cat biscuits. The adults were fed on 10% sucrose solution, and females were also fed with chicken blood. Insects were maintained at 26 ± 1°C, 70% humidity, and a photoperiod of 12 h of light and 12 h of darkness.

***Culex quinquefasciatus* field populations.** Three field populations from the state of Pernambuco (Brazil), two of them from a nontreated area and one from a *B. sphaericus*-treated area, were investigated (Fig. 1). The nontreated areas were Fazenda Nova, a rural district located 180 km from Recife, and Peixinhos, located in the RMA. The treated area of Água Fria is located in the RMA, 3 km away from Peixinhos, and has been subjected to the Filariasis Control Program, carried out by the municipality's Department of Health (32). The vector control subprogram includes the utilization, since 2003, of *B. sphaericus*-based larvicides in *Culex* breeding sites mapped in that area. Previous analysis of Água Fria larva susceptibility to *B. sphaericus* did not indicate the development of resistance (32) (Table 1). Samples in Peixinhos and Água Fria consisted of egg rafts collected using oviposition traps (4) randomly placed in households within the two neigh-

TABLE 1. Toxicity of *Bacillus sphaericus* strain 2362 (standard powder SPH88) against *Culex quinquefasciatus* fourth-instar larvae from susceptible laboratory colony (CqSF), field populations of Fazenda Nova (FN) and Peixinhos (PX), and a *B. sphaericus*-treated population of Água Fria (AF)

Colony[a]	No. of treatments	No. of larvae	LC_{50}[b]		LC_{90}[b]	
			Mean (95% confidence interval)	RR[c]	Mean (95% confidence interval)	RR[c]
CqSF	0	2,580	0.003 (0.002–0.004)	1.0	0.038 (0.021–0.114)	1.0
FN	0	1,080	0.011 (0.005–0.028)	3.7	0.022 (0.015–0.072)	0.6
PX	0	1,020	0.012 (0.009–0.017)	4.0	0.025 (0.020–0.036)	0.6
AF	13[d]	960	0.008 (0.004–0.013)	2.7	0.020 (0.015–0.036)	0.5
	24	1,140	0.026 (0.015–0.030)	8.6	0.044 (0.035–0.067)	1.2

[a] Field sampling was performed in December 2007 (FN), May 2007 (PX), May 2005 (AF, 13 treatments), and April 2007 (AF, 24 treatments).
[b] Lethal concentrations (mg/liter) for 50% (LC_{50}) or 90% (LC_{90}) of larvae treated after 48 h.
[c] RR between the LC toward the field population studied and the LC toward the CqSF colony.
[d] Data for sampling after 13 treatments were obtained from the work of Silva-Filha et al. (32).

borhoods. In Fazenda Nova, large samples of egg rafts and larvae were directly collected from breeding sites. Between 50 and 300 egg rafts per population were used to establish subcolonies that were maintained under laboratory conditions, as described above. Larvae from F1 or F2 progenies were evaluated through bioassays, and fourth-instar larva samples were stored at −70°C for diagnostic PCR and enzymatic analysis, as described in the following sections.

Bioassays. *C. quinquefasciatus* susceptibility to *B. sphaericus* was analyzed through bioassays using early fourth-instar larvae, according to standard procedures (41). Larvae were exposed to serial dilutions of spore crystal lyophilized standard powder from *B. sphaericus* 2362 (SPH88; Pasteur Institute, France), for 48 h. Cups held 20 larvae in 100 ml of bacterial suspensions in water, and three replicates were performed for the six concentrations tested per bioassay. A control group, tested on water only, was run in each experiment, and the bioassay was repeated at least three times. The mean lethal concentrations for 50% (LC_{50}) and 90% (LC_{90}) of exposed larvae during 48 h were estimated through probit analysis using the program SPSS 10.0 for Windows. Resistance ratios (RR) for each population analyzed were calculated by comparing their LC values to that of the susceptible CqSF colony, used as a reference.

Diagnostic PCR. For DNA isolation, individual fourth-instar larvae were homogenized in DNAzol (Invitrogen, Carlsbad, CA), as recommended by the manufacturer, followed by precipitation with ethanol and resuspension in Tris-EDTA buffer. PCR was performed using specific primers flanking the 19-nucleotide deletion in the $cqm1_{REC}$ allele (5′ primer, Cpq2F, 5′-CGA GAA TTC ATG CAG GAC TTC AAA GAG-3′; 3′ primer, Cpq1R, 5′-GCA CTG CAG GGA AGT GGT GGA AGG TAC-3′). The reactions were carried out with the Platinum *Taq* DNA polymerase (Invitrogen) for 35 cycles with an annealing temperature of 55°C, using a Biometra thermocycler (Goettingen, Germany). Amplification products were separated in electrophoresis on 2.5% agarose gels. Each assay included no-DNA samples and *A. aegypti* DNA as negative controls. Selected PCR products were subjected to automatic sequencing to confirm the identity of the fragments. The statistical analysis to compare the proportions of $cqm1_{REC}$ found in field populations was performed through the chi-square test with significance at 5%, using the program R-2.6.2 (www.r-project.org).

α-Glucosidase (EC 3.2.1.20) assays. The α-glucosidase assay was performed essentially as described previously (30). Individual larvae were homogenized in phosphate-buffered saline buffer and solubilized in sodium dodecyl sulfate-polyacrylamide sample buffer, lacking 2-mercaptoethanol, followed by electrophoresis on 8% acrylamide gels. The gels were then incubated three times for 15 min in a 2.5% Triton X-100 aqueous solution, followed by incubation for 20 min at 37°C with a 100 mM sodium citrate-phosphate buffer, pH 6.5, containing 2 mM 4-methylumbelliferyl-α-D-glucoside (Sigma, St. Louis, MO), under gentle agitation. Catalytic bands, including the Cqm1 α-glucosidase receptor, were visualized under UV light. Samples of 150 to 500 larvae from each population were analyzed.

RESULTS

Selected field populations of *C. quinquefasciatus* never exposed to *B. sphaericus*, or samples from a treated area, were first compared through bioassays to assess their susceptibilities to this bacterium. The LC_{50}s derived from the nontreated populations of Fazenda Nova and Peixinhos were similar, producing resistance ratios (RRs) for both populations, compared to that of the susceptible laboratory colony CqSF, of equal to or near 4. The RRs at LC_{90} for these populations were less than 1, indicating that natural variations in susceptibility can be found in field populations that have not been exposed to *B. sphaericus* (Table 1). An initial evaluation of the treated population of Água Fria, performed in a previous study, after 13 rounds of *B. sphaericus* treatment produced an RR value at LC_{50} of 2.7 (32). Here, the evaluation performed after 24 treatments indicated an RR at LC_{50} close to 9 (Table 1). Nevertheless, these larvae had RR values at LC_{90} comparable to those of the CqSF larvae at both times analyzed, indicating that there were no statistical differences in susceptibility between the two populations, according to this parameter.

The identification of the $cqm1_{REC}$ variation provided the tools for the development of an approach to monitor the selection of resistance associated with this allele. A diagnostic PCR procedure was devised which consisted of the amplification of a DNA fragment encompassing the deleted segment. This assay was first performed using larvae from the *B. sphaericus*-susceptible (CqSF) and -resistant (CqRL1/2362) colonies, consisting of individuals homozygous for the *cqm1* and $cqm1_{REC}$ alleles, respectively, to evaluate its reliability. Using a single pair of primers, two distinct fragments, of 208 and 189 bp, were amplified from the susceptible and the resistant alleles of the *cqm1* gene, respectively (Fig. 2, lanes 1 and 2). Single fragments of the expected sizes were amplified from DNA samples from each reference colony, and sequencing of selected samples confirmed their identity as products of the *cqm1* gene. Amplification of both fragments was observed in heterozygote larvae, obtained from the cross between susceptible and resistant individuals from these colonies (Fig. 2, lane 3), and no fragments were amplified in samples with *A. aegypti* DNA or those without DNA, used as negative controls (Fig. 2, lanes 4 and 5).

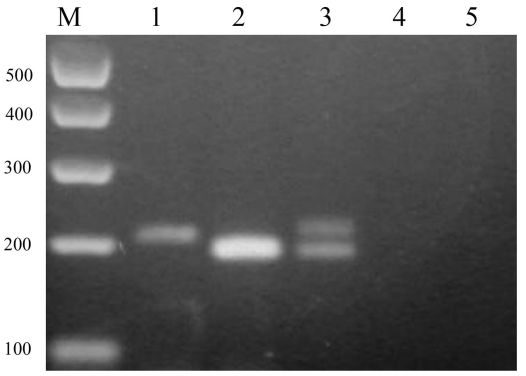

FIG. 2. DNA fragments amplified from the *Culex quinquefasciatus* *cqm1* and $cqm1_{REC}$ alleles of susceptible and *Bacillus sphaericus*-resistant larvae, respectively. The diagnostic PCR produces distinct profiles for susceptible homozygote, resistant homozygote, and susceptible heterozygote larvae (lanes 1, 2, and 3, respectively). No fragments were amplified from the samples with *Aedes aegypti* DNA or without DNA (lanes 4 and 5, respectively). On the left, molecular size (M) markers are shown in base pairs.

A second approach to evaluate the susceptibility of individual *C. quinquefasciatus* larvae to *B. sphaericus* was performed using an in-gel α-glucosidase assay. Susceptible larvae from the CqSF colony showed a reproducible profile of four catalytic bands, and the Cqm1 α-glucosidase migrated with an apparent molecular mass of 83 kDa, under semidenaturing conditions (Fig. 3, S). Resistant larvae from the CqRL1/2362 colony, which are homozygotes for the $cqm1_{REC}$ allele, were characterized by the lack of the catalytic band corresponding to Cqm1 (Fig. 3, R). Analysis of individual larva samples, using different body parts for both PCR and α-glucosidase assays, confirmed the correlation between genotype and phenotype (data not shown).

Both the PCR and the α-glucosidase assays were then used to investigate individual larvae from the populations tested with the bioassays. Using PCR, the $cqm1_{REC}$ resistance allele was detected in samples from all field populations analyzed. In Fazenda Nova, an area without a previous history of *B. sphaericus* utilization, three larvae out of 504 analyzed were het-

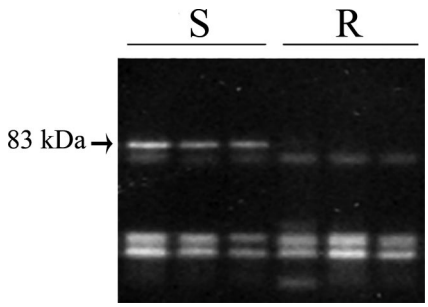

FIG. 3. Analysis of α-glucosidases from susceptible (S) and *Bacillus sphaericus*-resistant (R) *Culex quinquefasciatus* larvae. In-gel assays were performed using crude extracts from individual larvae. Samples were separated by 8% semidenaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and submitted to enzymatic detection. Catalytic bands were visualized under a UV transilluminator, and the arrow indicates the 83-kDa band corresponding to the Cqm1 α-glucosidase.

erozygotes for $cqm1_{REC}$ and its frequency was 0.0029 (Table 2). The frequency in the nontreated population of Peixinhos was 0.0061 based on the detection of six heterozygote individuals among 492 larvae analyzed. This frequency, although not significantly different from that observed in the Fazenda Nova sample, showed a twofold increase (Table 2). All larvae tested from the two nontreated populations displayed expression of Cqm1, as observed by the in-gel α-glucosidase assay, and this finding was in agreement with the PCR screening data with no homozygotes for the $cqm1_{REC}$ allele (Table 2).

Screening of the treated area from Água Fria revealed a different scenario, and the $cqm1_{REC}$ frequency was significantly higher than those found in nontreated populations. In samples collected after 13 treatments with *B. sphaericus*, the frequency was 0.053, and within 499 larvae tested both heterozygote and homozygote larvae for $cqm1_{REC}$ were found (Table 2). After 24 treatments, 16 heterozygotes and one homozygote larva for this allele were detected within 162 individuals and the frequency remained high (Table 2). Sequencing of selected fragments of both sizes confirmed their identity and assured us of the reliability of the PCR assay. With respect to the Cqm1 expression, larvae lacking expression of Cqm1 were detected in both evaluations performed and confirmed the presence of resistant larvae in that population, as indicated by the allelic frequency (Table 2).

DISCUSSION

The detection of the $cqm1_{REC}$ allele within *C. quinquefasciatus* populations from the state of Pernambuco, Brazil, constitutes the first report of an allele conferring resistance to biolarvicides in mosquito field populations, assessed through a DNA screening procedure. This assay showed that the same resistance-inducing allele is present in populations without a previous history of *B. sphaericus* exposure, Fazenda Nova and Peixinhos, although at this stage it is possible that the slightly higher $cqm1_{REC}$ frequency detected in Peixinhos could result from indirect exposure to *B. sphaericus*, favored by the proximity with the treated area of Água Fria. The PCR-based approach also demonstrated that the frequency of the $cqm1_{REC}$ allele in the treated area of Água Fria was higher than that in the nontreated populations. This was further corroborated by the presence in Água Fria of individual resistant larvae, homozygotes for the $cqm1_{REC}$ allele and not expressing the Cqm1 α-glucosidade.

Resistance genes of lepidopteran and coleopteran populations exposed to *Bacillus thuringiensis* spraying or to Bt-transgenic crops have been investigated, and the frequency ranges from 10^{-5} to 10^{-1} (1, 2, 5, 6, 14, 16, 19, 35, 36). Global monitoring data on resistance have shown that the frequency of resistance alleles has not increased in pest insects from Bt crop areas, and it is likely that the high-dose refuge strategy has contributed to delaying resistance (21, 34). Most studies used the F2 screen to estimate the frequency of resistance alleles, which is a method based on the bioassay of F2 neonates obtained from isofemale lineages. Recently the first DNA screening for mutants of the cadherin gene associated with Bt cotton resistance of the pink bollworm *Pectinophora gossypiela* (Lepidoptera: Gelechiidae) was performed, but those alleles were not present in a sample of 5,571 insects from Arizona (36). A similar screening of a retrotransposon insertion in the cadherin gene (*HevCaLP*) associated with *B. thuringiensis* resistance of the lepidopteran *Heliothis virescens* did not detect this insertion among 7,000 individuals, and its frequency was estimated to be 7×10^{-5} (13, 14). The $cqm1_{REC}$ allele analyzed in this study was successfully detected through PCR screening in a *C. quinquefasciatus* population without a record of *B. sphaericus* exposure and geographically isolated from those populations that have been exposed, with a frequency of 3×10^{-3}. This finding is in agreement with the previous evidence that genes for resistance to bacterial toxins can be present at detectable frequencies in nontreated populations (12). For instance, the

TABLE 2. Frequencies of $cqm1/cqm1_{REC}$ alleles and expression of the Cqm1 receptor in *Culex quinquefasciatus* larvae from nontreated populations of Fazenda Nova (FN) and Peixinhos (PX) and the *Bacillus sphaericus*-treated population of Água Fria (AF)[a]

Colony	No. of treatments	No. of larvae with *cqm1* genotype				$cqm1_{REC}$ frequency	No. of larvae and Cqm1 expression status		
		Total	*cqm1/cqm1*	$cqm1/cqm1_{REC}$	$cqm1_{REC}/cqm1_{REC}$		Total	Positive	Negative
FN	0	504	501	3	0	0.0029	507	507	0
PX	0	492	486	6	0	0.0061	374	374	0
AF	13	499	452	41	6	0.053	325	323	2
	24	162	145	16	1	0.055	162	161	1

[a] Genotypes for the *cqm1* gene and the Cqm1 expression profile were determined by diagnostic PCR and in-gel α-glucosidase assays, respectively.

frequency of an allele conferring resistance to Bt poplars in *Chrysomela tremulae* (Coleoptera: Chrysomelidae) populations that have been not exposed to widespread use of *B. thuringiensis* ranged from 0.011 to 0.0037 (15, 37). A recent work also detected mutations associated with malathion resistance in specimens of the fly *Lucilia cuprina* (Diptera: Calliphoridae), collected between 1930 and 1949, before the introduction of this insecticide in 1950 (17). The $cqm1_{REC}$ frequencies in Água Fria samples collected after 13 and 24 treatments were similar, despite additional treatments performed on the second sample and the fact that the bioassay data showed that the RR values at LC_{50} shifted from 2.7 to 8.4 between the two samples. In this particular case, the RR values do not seem to ensure the increase of selection pressure or indicate resistance, since a previous study of Água Fria has shown similar variations in this parameter (2.7, 7.3, and 4.7), despite the progressively higher numbers of treatments performed (32). It is then possible that the selection pressure has not actually increased during the interval between the collection of the 13- and 24-treatment samples, perhaps due to other factors which can also influence the efficiency of this process, such as the coverage of treatment cycles, updating of new breeding sites, mosquito density, and climatic and other environmental factors. Nevertheless, our approach was not designed to directly evaluate the effect of *B. sphaericus* treatment upon the frequency of the $cqm1_{REC}$ allele, since samples of the Água Fria population before and after treatment were not available. At this stage it is not possible to infer if the higher frequency observed for the $cqm1_{REC}$ allele in the treated population is a response to treatment or a reflection of natural variations between different mosquito populations.

Novel approaches are needed to provide information for the management of resistance to bacterial toxins. The knowledge of the initial frequency of resistance alleles in field populations, particularly for those that are inherited recessively, is essential to establish strategies to delay resistance (14, 36, 37). In such cases, heterozygous individuals that emerge gradually in the population under selection cannot be identified by bioassays and resistance allele frequency can increase without being detected. Bioassays are further limited in that they demand large sample size and maintenance of F1 or F2 progenies in the laboratory and assays should be performed under specific conditions to provide reliable data. In the context of *Culex* resistance to *B. sphaericus*, the improvement in DNA screening is needed to track other resistance alleles already described (10, 11), since the PCR used in the study is specific for the $cqm1_{REC}$ allele. The α-glucosidade in-gel assay can also be useful to detect individuals not expressing Cqm1 whose resistance can be associated with alleles other than $cqm1_{REC}$, although this assay is able to detect only homozygous resistant larvae. Both methodologies, despite some limitations, proved to be suitable to screen for *B. sphaericus* resistance, and its association with diagnostic bioassays can increase the ability to detect the frequency of resistant individuals.

Bin toxin constitutes an important resource from the arsenal of environmentally safe insecticidal molecules available. Selection of resistance can be largely minimized through the coordinated use of Bin toxin with other insecticidal proteins, in mixtures or in rotation, in the scope of integrated control programs (22, 39, 40). Monitoring of population susceptibility, particularly focusing on the frequency of resistance alleles, is essential, and this study demonstrates the effectiveness of molecular tools to determine such frequency in *Culex* natural populations.

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