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Characterization of Enzymatic profiles of *Aedes aegypti* strains from the State of Rio Grande do Norte, Brazil

Caracterização de perfis enzimáticos de cepas do *Aedes aegypti* do Estado do Rio Grande do Norte, Brasil

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Abstract *This study was conducted in four strains of* Aedes aegypti *mosquitoes to evaluate the enzymatic activity profiles in the city of Mossoró, Rio Grande do Norte, and correlate them with biochemical mechanisms of resistance to insecticides. Mosquitos were used to quantify the following detoxification enzymes: Mixed-Function Oxidase (MFO), PNPA-esterase (PNPA-EST), and Acetylcholinesterase (AChE). The profiles were compared statistically with profiles from the Rockefeller strain, through the Kruskal-Wallis test and Dunn's multiple comparisons ($p < 0.05$). The 99 percentile of the values of enzyme activity from the reference strain was calculated for each enzyme, and the percentage of individuals above the 99 percentile was quantified. The enzyme activities were classified as "Unchanged" (< 15%), "Identified change" (> 15% and < 50%), and "Substantially changed" (> 50%). The statistical analysis revealed significant differences in the MFO and AChE profiles, which are fundamental in the determination of profiles of resistance to insecticides. Three populations were classified as "Substantially changed" for MFO. The altered enzymatic activity showed that the changes could have an important role in exposing resistance to insecticides.*

Key words Aedes aegypti, *Dengue fever, Resistance mechanisms, Insecticides*

Resumo *Este estudo foi realizado em quatro cepas de mosquitos* Aedes aegypti *da cidade de Mossoró, Rio Grande do Norte, com o intuito de avaliar os perfis de atividade enzimática e correlacioná-los com os mecanismos bioquímicos de resistência a inseticidas. Mosquitos foram utilizados para quantificar as seguintes enzimas de detoxificação: oxidase de Função Mista (MFO), PNPA-esterase (PNPA-EST) e acetilcolinesterase (AChE). Os perfis das populações foram comparados estatisticamente com os da cepa Rockefeller por meio do teste de Kruskal-Wallis e do de comparações múltiplas de Dunn ($p < 0,05$). O percentil 99 dos valores de atividade enzimática da cepa referência foi calculado para cada enzima, e o percentual de indivíduos acima desse valor foi quantificado. As atividades enzimáticas foram classificadas como "Inalterada" (< 15%), "Alteração Identificada" (> 15% e < 50%), e "Substancialmente Alterada" (> 50%). A análise estatística revelou diferenças significativas nos perfis de MFO e AChE, que são fundamentais na determinação de perfis de resistência a inseticidas. Três populações foram classificadas como "Substancialmente Alteradas" para MFO. Os níveis alterados de atividade enzimática demonstram que essa mudança pode desempenhar um importante papel na resistência a inseticidas.*

Palavras-chave Aedes aegypti, *Dengue, Mecanismos de resistência, Inseticidas*

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Introduction

The mosquito *Aedes aegypti* is the most important vector of diseases in urban areas due to its great ability to adapt to that environment's conditions. It is the vector for the transmission of four distinct serotypes of the dengue virus (DENV-1, 2, 3, 4), which is the etiologic agent of the dengue fever[1,2]. This disease is currently a major public-health problem, having become the most significant vector-borne viral disease worldwide; about 2.5 billion people are at risk of infection with the dengue virus, mainly in tropical countries where the environmental conditions favor the proliferation of *Aedes aegypti*[3]. In Brazil, the vector control began between 1902 and 1907. Currently, the only method of control or prevention of dengue fever is combating the vector mosquitoes, which is mainly achieved through the massive use of chemicals to control the development of adults and larvae, a practice of questionable efficiency. Traditional approaches to dengue prevention and control have been inherited from the vertical programs that targeted vector elimination in domestic habitats, with the use of larvicides and insecticides in addition to breeding site elimination as the core of Aedes control strategies. However, the main problem with the use of chemical pesticides is the development of resistance resulting in reduced efficiency of the products. Thus, resistance to insecticides poses a threat to the effectiveness of strategies to control mosquito populations and, moreover, infections with the dengue virus[4-6]. In many countries, chemical control has proved inadequate to prevent infestation by the mosquito vector. Many measures, including sanitation and trash removal, epidemiological, entomological, and virological vigilance, health education, and community participation has proven essential to decisively prevent outbreaks[7].

Aedes aegypti is present in all States and most cities in Brazil; it is present in approximately 98% of the municipalities in the State of Rio Grande do Norte. The municipality of Mossoro, in this context, presents high levels of infestation, and thus, is endemic for dengue fever. So far, control strategies has shown not very effective due to intense disease urbanization and limitation of the disease surveillance process itself[8]. According to data from the 2011 Epidemiological Surveillance bulletin from the Brazilian Ministry of Health, this municipality presented 1,652 confirmed dengue fever cases, which represents a 10 fold increase to the number of cases from 2010 (160 confirmed cases). Moreover, Mossoró was listed among the 72 municipalities in Brasil where insecticide resistance profiles were detected in mosquito populations according to the Technical Note No. 023 from the Brazilian Ministry of Health issued in 2009.

Chemical insecticides still have an important role in the control of vectors for disease transmission. The most widely used act on the central nervous system (CNS) of insects and are classified into four large groups according to their chemical nature: organochlorines, organophosphates, carbamates and pyrethroids. Insect resistance to these compounds stems mainly from a change in their metabolic sites of action or greater efficiency for detoxification[2,9-12].

The use of insecticides in Brazil since 1967 has been exposing the vector of dengue fever to intense selective pressure[9]. This study evaluated enzymatic profiles in different populations of the *A. aegypti* mosquito from the municipality of Mossoro, and correlated profiles alterations with insecticide resistance and the use of chemical insecticides in this municipality.

Materials and methods

Mosquitoes

Larvae and pupae of *Aedes aegypti* mosquitoes were collected from four previously chosen sites in the city of Mossoró located at 06°12'43" S, 37°20'39" W and 285 km from Natal, the capital of Rio Grande do Norte State in Brazil. (Figure 1). The collection sites corresponded to the neighborhoods named Santo Antonio (SA), Paredões (PA), Abolições (AB), and Bom Jardim (BJ), which are located in the northern area of the municipality, the region with the highest population density and greater epidemiological significance related to dengue fever. This region has the most new and recurrent cases, besides presenting the highest rates of infestation by the vector mosquito, according to the Epidemiological Surveillance. These neighborhoods are mainly urban areas and with low socio-economic conditions in this city. Data collected was directed by the density map of disease cases by neighborhoods, using a simple random probability sampling, reaching a minimum of 20 households per district. All samples were collected in June month, as suggested by Bessa Júnior et al.[8]. Specimens were collected and maintained between 25 and 30ºC, protected from light, and under controlled air relative

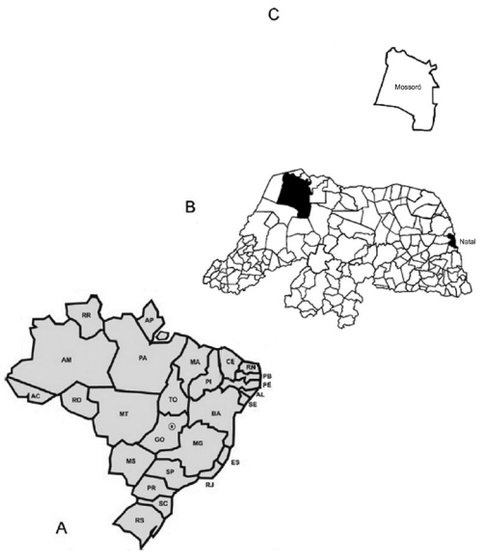

Figure 1. (A) Map of Brazil; (B) Map of the Rio Grande do Norte State; (C) Municipality of Mossoro where Aedes aegypti mosquitoes were collected for the enzymatic evaluations.

humidity. The samples used were derived from field population, being tested the F0 generation. Adult females, at up to one day old (0 to 24 h after emergence), not blood fed or exposed to chemical insecticides during the adult and the larval stage were used in the study. The choice of females was based on their feeding habits, which consequently leads to the transmission of the dengue virus. The separation of adult mosquitoes by sex was made considering the sexual dimorphism represented by the antennae. Additionally, the Rockefeller strain was used as internal control in all experiments because it is the reference internationally known to be susceptible to insecticides[9,10].

Biochemical Assays

Enzymatic dosing for Acetylcholinesterase (AChE and AChI), the first in the absence of the propoxur inhibitor, and the second in the presence of the inhibitor, PNPA-esterase (PNPA-EST), and Mixed-Function Oxidase (MFO), and total protein quantification were performed in ten female mosquitoes, in the adult stage, from each studied site, and from the Rockefeller strain. Each mosquito was homogenized in 300 µl of Milli-Q water and the homogenates distributed, in duplicates, in 96-well plates; each enzyme was analysed in a separate plate. The homogenates were centrifuged at 12,000 g for 60 seconds in a refrigerated centrifuge before aliquots were taken to dose PNPA-EST enzyme and total protein (Eppendorf Centrifuge 5430 R®). Absorbance was measured spectrophotometrically for each enzyme using the appropriate wavelengths (405 and 620 nm) in a microplate reader (Asys Expert Plus®). Standard curves were obtained for BSA (Bovine Serum Albumin) and Cytochrome C to correct the amounts of total protein and (MFO), respectively. Samples for which the standard deviation were equal or greater than 30% of the average measurement were discarded. The experiments and processing of absorbance values were based on the manual "Methodology for the Quantification of Enzyme Activity Related to Resistance to Insecticides in *Aedes aegypti*"[9].

Mixed-Function Oxidases (MFO)

The assay used measures the amount of heme groups in the mosquito, thus, allowing an indirect estimate of the cytochrome P450 enzyme activity, with which the heme group is mainly associated[10]. In this assay, performed in duplicate, the following was added to every 20 µl of homogenate: 60 µl of 90 mM potassium phosphate buffer (final pH adjusted to 7.2), 200 µl of Na acetate/TMBZ working solution (0.012g of 3,3,5,5 tetramethyl benzidine dihydrochloride in 6 ml of methanol and 18 ml of 250 mM sodium acetate buffer at pH 5.0), and 25 µl of 3% hydrogen peroxide (H_2O_2). The plates were incubated for 90 minutes at room temperature, protected from light, and subsequently read at 620 nm.

Acetylcholinesterase (AChE)

The dosing of acetylcholinesterase was performed in two separate 96-well plates, assigned to AChE and AChI, to estimate the total activity of acetylcholinesterase in the presence or absence of the *propoxur* inhibitor, respectively. The following was added on 25 µl of homogenates, to all plates and in duplicate: 145 µl of Triton/Na phosphate (5 ml of 100% Triton X-100 in 50 ml of 1M sodium phosphate buffer at pH 7.8 and 455 ml of distilled water), and 10 µl of DTNB/Na phosphate (10 mM DTNB in 100 mM sodium phosphate buffer at pH 7.0). In the AChE plates, 10 mM acetylcholine iodide in water was added to each well in the absence of propoxur. In the AChI plates, 10 mM acetylcholine iodide in wa-

ter was added in the presence of propoxur (6 µl of 0.1 M in acetone). All plates were incubated for an hour at room temperature, protected from light, and read at 405 nm.

PNPA Esterase (PNPA-EST)

The homogenates were centrifuged at 12,000 g for 60 seconds and 10 µl were taken in duplicate and placed in 96-well plates. The following was added to each well: 200 µl of PNPA/Na phosphate working solution prepared by adding 100 mM PNPA in acetonitrile (0.01815 g of PNPA in 1 ml of acetonitrile) to 24.75 ml 50 mM sodium phosphate buffer at pH 7.4 (50 ml of 1 M sodium phosphate buffer at pH 7.4 in 950 ml of distilled water). This assay aims to estimate the reaction kinetics, therefore, the absorbance variation represented by the amount of substrate consumed[9] was measured through nine readings at 405 nm, every 15 seconds.

Total protein

Dosing of total protein in each mosquito is necessary to correct the values of enzymatic activity related to metabolic resistance and the weight of each individual insect. Duplicates of 10 µl homogenates were taken after centrifugation and plated in 96-well plates; 300 µl of the Bradford reagent 1:5 diluted was added to each well. The plates were read at 620 nm 3 to 5 minutes after the addition of the reagent.

Controls

The controls were performed in all experiments and divided into "blank" and "positive" controls. They did not receive homogenates but received the specific reagent for each enzyme and were used to adjust the absorbance values of the wells containing homogenates. There were only wells corresponding to blank samples in the AChE and PNPA-EST tests, whereas in the MFO test there were blanks and controls. In the (MFO) assay, the three blank control wells received 80 µl of potassium phosphate buffer, and the three positive control wells received 60 µl of potassium phosphate buffer and 20 µl of cytochrome C solution (0.01 mg/ml in 250 mM sodium acetate pH 5.0). In the (AChE and AChI) assay, the six blank control wells received 25 µl of water and the other common reagents. In the PNPA-EST assay, the six blank control wells received the PNPA/Na phosphate reagent and 10 µl of water. In the total protein assay, the three blank wells received the Bradford reagent and 10 µl of water; the three positive control wells received 10 µl of 1 µg/µl BSA solution in addition to the Bradford reagent.

Data Analysis

The absorbance values obtained in the duplicate samples were corrected in relation to the volume of homogenates, and the values for the enzymatic activities related to metabolic resistance were corrected by the total protein dosing measurements. The level of significance was set to p-value < 0.05. The medians of the enzymatic activity values from each population of *A. aegypti* were compared with the values from the Rockefeller reference strain using the Kruskal -Wallis and Tukey's nonparametric tests (p-value < 0.05). The comparison between the obtained values for the pool of populations and reference strain was performed using the Mann-Whitney test (nonparametric T-test) at p-value < 0.05.

The calculation of the 99 percentile of the Rockefeller strain values in each enzyme was used to qualify the changes detected in the enzymatic activity in the studied populations. The activities were classified as "Unchanged", "Identified change" and "Substantially changed" if the percentages of individuals in each population over the 99 percentile were $< 15\%$, between 15% and 50%, and $> 50\%$, respectively. The multiple comparisons test of Dunn's was used to analyze the variations in the medians from the populations, thus, determining which populations varied significantly among themselves. The GraphPadPrism software, version 5.00 for Windows, San Diego California, USA was used for the analyses and generation of graphs[10,11].

Results

According to the Kruskal-Wallis test, the comparative analyses between the studied populations showed that the median activity of all enzymes, with the exception of PNPA-EST, differed significantly (p-value < 0.05). Additionally, all populations presented changes in enzyme activity according to the classification generated by the 99 percentile analysis, with the exception of PNPA EST. Table 1 shows the results from the individuals evaluated in each test and the median and percentage of individuals with activity above the 99 percentile of the reference strain values.

Moreover, a comparison between the enzymatic profiles of pooled samples from the studied sites, and thus, mimicking a single population named "Mossoró", and the reference strain Rockefeller, named "Rock" was performed. All tests, with the exception of PNPA-EST, showed statistical significance in this analysis; some at p-value < 0.05, as shown in Figure 2.

Mixed-Function Oxidase (MFO)

The median level of enzymatic activity observed in the BJ population differed significantly from the "Rock" reference strain (p-value < 0.05). No statistical significance was observed in PA and AB populations (p-value > 0.05). The difference was significant (p-value < 0.05) in the comparison between the pooled populations and reference strain (Figure 2). The SA population presented an "Unchanged" enzymatic profile. All other populations presented the "Substantially changed" profile and a number greater than 50% of individuals were placed above the 99 percentile of the reference strain; among these, 80% of individuals from the PA population showed important enzymatic activity changes (Table 1).

Acetylcholinesterase (AChE)

The population from PA was the only that showed differences in medians with statistical significance compared to the Rockfeller strain (p-

Table 1. Quantification of enzyme activity for Acetylcholinesterase (AChE), Esterase PNPA (PNPA-ESP), and Mixed-Function Oxidase (MFO) in populations of *Aedes aegypti* from Mossoró in Rio Grande do Norte State, Brazil.

Strains	AChE(% activity)			PNPA-EST(Δabs/mg ptn/min)			MFO (nmolescit/mg ptn)		
	N[a]	Median[b]	p99[c]	N	Median	p99	N	Median	p99
Rockefeller	48	11.10	19.71	40	3.15	15.41	38	7.99	16.28
	N	**Median**	**% >p99[d]**	**N**	**Median**	**% >p99**	**N**	**Median**	**% >p99**
Santo Antônio	39	10.95	13.00	40	3.3	10.00	40	13.61	10.00
Paredões	40	19.05	40.00	39	1.5	0.00	40	28.36	80.00*
Abolições	40	18	30.00	40	2.75	10.00	40	35.69	70.00*
Bom Jardim	40	18.75	40.00	40	4.15	0.00	40	39.05	75.00*

[a] Number of tested mosquitoes. [b] Median for each enzymatic activity. [c] 99 percentile for the Rockefeller reference strain.
[d] Percentage of mosquitoes with enzymatic activity over the 99 percentile for the Rockefeller strain. * > 50% of mosquitões over the 99 percentile (Enzimatic activity "Substantially change").
The Kruskal-Wallis test and multiple comparisons test of Dunn's were used to establish comparisons between the medians of the populations studied and the reference strain and to analyze the variations between the median of the population, respectively.

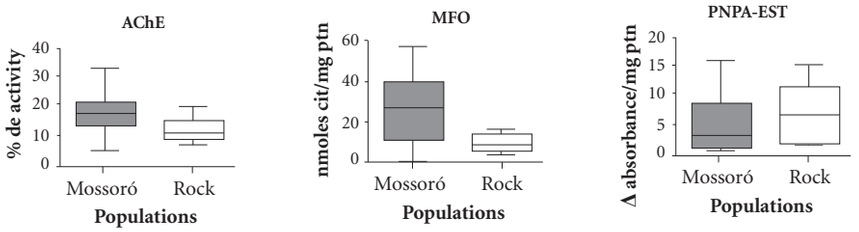

Figure 2. Graphic representation, *box-plot* type, of enzymatic activity in populations of *Aedes aegypti* from Mossoró (four study site pooled samples) and Rockefeller (Rock). Acetylcholinesterase, Mixed-Function Oxidase, and Esterase PNAPA are represented, respectively.

value < 0.05). AB and BJ was not differed statistically (p-value > 0.05). Three populations (PA, AB, and BJ) were categorized with "Identified change" profile because they placed between the 15% and 50% of individuals above the 99 percentile of the reference strain. The SA population presented an unchanged enzymatic profile (Table 1).

PNPA Esterase (PNPA-EST)

No statistical significant difference was observed in the medians of PNPA-EST enzymatic activities between the tested and reference strains (p-value > 0.05). None of the samples (among the studied populations and sites) showed changes in enzyme activity ("Unchanged" profile) when compared with the 99 percentile of the reference strain (all presented less than 15% of individuals with activity above the p99) (Table 1).

Discussion

According to the Epidemiological Surveillance bulletin from the Municipality and Department of Public Health of the Rio Grande do Norte State, the organophosphate Temephos was used in large scale during the last decade. During the past six years, only in the years of 2008 (in which Cipemetrine adulticid pyrethroids were administered and Deltamethrin) and 2010 (in which Malathion GT 96% adulticid organophosphate was used) emergency treatment with chemical adulticids were used.

The MFO are commonly involved in resistance to pyrethroids, and organophosphates insecticides to a lesser degree[13-16]. Studies by Paeporn et al.[17] found increased levels of MFO in populations of *Aedes aegypti* from from Thailand that had been selected through resistance to deltamethrin and permethrin, which are pyrethroid insecticides; the former is routinely used in vector control in Mossoró. Rodríguez et al.[18] described that MFOs were important enzymes in the detection of resistance to organophosphates in specimens from Latin America. In our study, changes in the enzymatic activity of MFO were observed; in fact, this enzyme showed the greatest profile changes in three of the studied populations rated as "Substantially changed" according to the 99 percentile of the reference strain (> 50%). The fact that both pyrethroids and organophosphates have been used during routine activities for vector control in the municipality under study could probably be the cause for the selection of resistant populations. In addition, Polson et al.[10] still considers the participation of Temephos in the changing the MFO profiles.

Together with detoxification enzymes, AChE belongs to another enzyme class with fundamental importance in the determination of insecticide resistance profiles in mosquitoes. AChE is responsible for the degradation of acetylcholine (ACh) where the primary site of action of organophosphorus insecticides is located[2]; structural changes in this site resulted in the development of resistance in many insect vectors[19]. In the present study, this enzyme presented the "Identified change" profile (> 15% and < 50%) for three of the studied populations. Moreover, this enzyme showed the greatest significance in the statistical analysis (p-value < 0.05) in the comparison between the pooled populations (Mossoró) and the Rock strain. The Mossoró population presented proven resistance to Temephos, which is an organophosphorus insecticide and acts directly on the acetylcholinesterase sites, according to the Technical Note no. 023 from the Brazilian Ministry of Health in 2009.

The PNPA-EST were related to resistance to organophosphates and pyretroids in other studies in populations of *Aedes aegypti* in Brazil. This resistance is conferred by target site insensitivity and/or increased metabolic detoxification[20]. However, the PNPA-EST in Mossoró was not showed resistance, which corroborates the results reported by Polson et al.[10] with populations from Trinidad and Tobago.

In this study, locations that showed the biggest changes were the neighborhoods PA (with abnormal AChE) and BJ (with changes to MFO). The neighborhood that showed the greatest susceptibility was SA, getting to be quite similar to the reference strain (Rock) for AChE and MFO. All neighborhoods received the intervention of chemical control strategy, though the dynamics of this intervention was not uniform for all, thanks to peculiar characteristics (accessibility, treatment of water used by the population, intellectual level of the population and others) of each neighborhood. In the neighborhood SA, whose enzymatic activity showed the lowest rates of changings, the implementation of any control strategy is complicated by the difficulty of accessing certain areas of this neighborhood, which makes possible the existence of certain populations that have not undergone the influence of insecticides and therefore acquired character of susceptibility. Despite that bioassays represent tests of reference to assess resistance to insectici-

des in mosquito populations, they do not provide definite information about these profiles in these populations because many variables are involved. The distribution of enzymes is heterogenous in most field strains and through biochemical assays, individuals which may show altered activity are quickly detected. It is important to be able to identify populations which are incipiently altered because this makes it possible for intervention efforts to be taken before resistance spreads throughout the entire population[10]. However, the available data point to a probable correlation between enzymatic profiles and mechanisms related to the development of resistance to insecticides.

Conclusion

The detection of populations of *Aedes aegypti* mosquitoes in the city of Mossoró-RN with altered enzymatic activity, and the frequent use of chemical insecticides for vector control suggest enzymatic resistance in dengue's vector. This study may provide information that may assist the authorities responsible for controlling vectors to create a workable strategy to optimize vector control in different neighbourhoods in the city bringing economic and health care benefits to the population by avoiding the unregulated use of chemical insecticides. However, the organization and support from public authorities are required in order to make this strategy a standard procedure.

Hence, biochemical assays should be combined with vector control programs to monitor and prevent the generation of resistance to insecticides in mosquito populations. The implementation of this strategy in the city of Mossoró-RN alone can not be a viable alternative to control the successive dengue epidemics that have affected this population, but is an important indicator of the development of resistance in vectors, which, if tackled, could help to improve the quality of public health.

Collaborations

RFF Nunes: Processing of samples, conducting resistance tests, data interpretation, writing and submission the manuscript. MA Souza: Processing of samples, conducting resistance tests. JC Oliveira: Processing of samples, conducting diagnostic tests, data interpretation. RFO Grangeiro: Access to research subjects and release and supervision to obtain the samples listed. MJM Marinho: Technical support specialist, reading articles related to the theme and preparation of the manuscript. WO Pereira: Guidance and supervision during the research and writing and submission the manuscript.

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