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Major Article

Variation in temephos resistance in field populations of *Aedes aegypti* (Diptera: Culicidae) in the State of Sergipe, Northeast Brazil

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Abstract

Introduction: The organophosphate temephos was first used in Brazil in the 1960s for the control of *Aedes aegypti.* Because of its extensive and longstanding use worldwide, selection for mosquito populations resistant to the chemical has been observed not only in different regions of Brazil but also in several parts of the world. The objective of this study was to evaluate the susceptibility of *Ae. aegypti* to the organophosphate temephos, a larvicide used in vector control activities in Sergipe/Northeast Brazil. **Methods**: This study included mosquito populations from seven municipalities of Sergipe and was carried out from October 2010 to August 2011. Qualitative bioassays of diagnostic dose and dose responses were performed. The resistance ratio was calculated based on lethal concentrations for mosquitoes of the susceptible Rockefeller strain. **Results:** All populations were classified as resistant to temephos. The resistance ratio ranged from 22.2 to 297.9, the lowest being seen in Aracaju, a coastal area of the state, and the highest in Pinhão, a semi-arid region, 96.6km from Aracaju. **Conclusions:** High levels of temephos resistance were observed in the *Ae. aegypti* populations of Sergipe. The variation between regions indicates that there have been different regimes of insecticide use and also points to the potential of small cities to generate and spread insecticide resistance.

Keywords: *Aedes aegypti.* Temephos. Dengue. Resistance. Vector control.

INTRODUCTION

The intense and long-term use of chemical insecticides for vector control has selected for populations of resistant mosquitoes and reduced both the presence of susceptible individuals and the variability of field populations[1]. Chemical insecticides are still widely used in vector control programs, despite selection for resistant populations of *Aedes aegypti* and *Aedes albopictus*, which threatens the effectiveness of control programs[2].

The organophosphate temephos has been used in Brazil for the control of *Ae. aegypti* since 1967, but its use increased in the 1980s due to dengue epidemics[3]. Due to the continued use of temephos throughout the world, many populations of *Ae. aegypti* were reported to be resistant not only in Brazil[4-11], but in several other countries[12-15], as well. In 1999 and 2000, populations of *Ae. aegypti* from 67 municipalities in Brazil were evaluated and resistance to temephos was shown to be widely distributed[16-18], primarily in the Southeast and Northeast[10]. Subsequently, dose-response (DR) bioassays have been performed and have shown resistance ratio$_{95}$ (RR) ranging from 4.0 to 27.1[16].

Temephos-containing larvicides in emulsifiable concentrate preparations, diluted solutions, granules, and slow release formulations[19] are commercially available, and can be applied in different ways depending on the place and rate of a required application[1]. Temephos-based larvicides have many advantages over other classes of insecticides, such as a low odor, low cost, and low toxicity[15], and because they can be safely used in drinking water when the dosage do not exceed 56-112g/ha (5.6-11.2mg/m^2) or 1mg/l[20]. However, a disadvantage to these insecticides is related to the acetylcholinesterase binding sites. This enzyme hydrolyzes acetylcholine molecules, resulting in an exacerbation of nerve impulse transmission and results in the paralysis and death of a target insect[9], but this enzyme is also present in vertebrates[5,9]. In addition, its effectiveness depends on many factors, such as water turnover rate, as well as environmental factors, such as organic debris, temperature, and exposure to sunlight[19].

The false notion that temephos alone would be able to eliminate *Ae. aegypti* eventually led to negligence regarding container removal activities. In addition, its indiscriminate use

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and the low tolerance of the human population to the treatment of drinking water are factors attributed to the failure of vector control by this larvicide[21].

Thus, the use of temephos in Brazil has been suppressed since 2009, with the belief that it could reverse the spread of resistant populations[7]. As a consequence of the high degree of resistance, the Brazilian Ministry of Health initially promoted its replacement by products based on *Bacillus thuringiensis* (Bti) in regions with reported resistance[16]; these were subsequently replaced by growth inhibitory products, such as inhibitors of chitin synthesis and juvenile hormone analogs[22]. However, products used to replace temephos have the disadvantage of short residual effects[6], which necessitates periodic reapplication, and they do not cause immediate mortality in larvae, but rather mortality occurs at the pupal stage or during the emergence of an adult[9]. This latter fact makes it necessary to modify the method of estimating mosquito infestation, as the House Index may be overestimated in treated areas[23].

Due to the advantages that temephos presents and the need for the rotation of products with different active ingredients, it is expected that the use of temephos may once again be a possibility for the control of *Ae. aegypti* larvae. However, a reduction in resistance levels is slow and mediated only by the absence of selection pressures[24].

The objective of this study was to evaluate the susceptibility of populations of *Ae. aegypti* to temephos, a larvicide used intensively in vector control activities in Sergipe. This evaluation makes it possible to examine the anthropic interference in the environment through selective pressures imposed on mosquito populations, as well as to monitor the evolution of susceptibility to temephos after the interruption of its use in the State of Sergipe. Unlikely in other states, Bti has not been introduced in Sergipe, so the use of temephos continued until 2009, when its gradual replacement by diflubenzuron started. By virtue of being in a transition of larvicides, this study represents a baseline for surveying the evolution of susceptibility in the absence of selection pressures from organophosphates.

METHODS

Study area

This study was carried out in the State of Sergipe, covering municipalities of different geo-environmental parameters, such as topography, hydrography, climate, fauna, and flora. In addition, the State Department of Health was consulted to define the municipalities of interest in each area. In general, these were municipalities without information about house infestations or those with failed vector control activities. The municipalities were: Aracaju, Maruim, and Capela in the tropical humid area, Pinhão, Carira and Neópolis in Agrest, and Canindé de São Francisco in the semiarid area (**Figure 1**). In Aracaju, the sampled area was limited to one neighborhood, Porto Dantas. The largest City is Aracaju, the state capital, with 570,937 inhabitants in 2010, all living in the urban area. The smallest City is Pinhão with a population of 5,973 inhabitants, 55.7% of which live in the urban area. The replacement of temephos by diflubenzuron began gradually in 2009, until it covered all cities in the state. However, at the time of collection, temephos remained the larvicide of choice for larval control in all the areas surveyed.

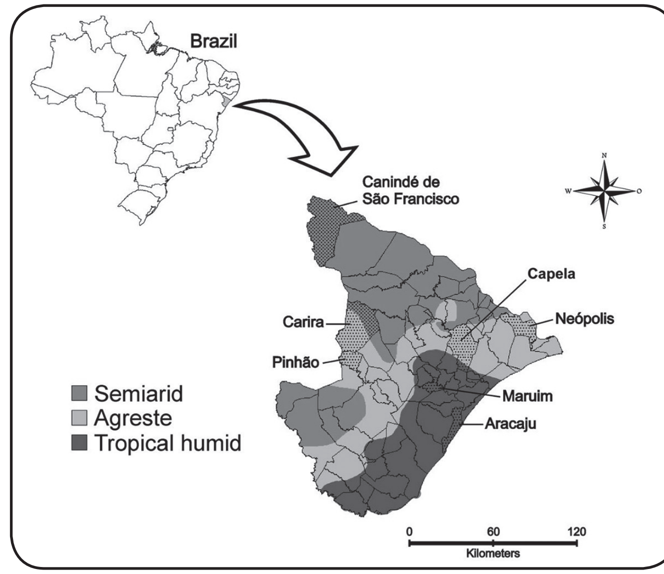

FIGURE 1: Map of the State of Sergipe (Brazil) showing the cities selected for the study.

Eggs collections

Eggs of *Ae. aegypti* were collected in each selected municipality at an interval of eight months from October 2010 to June 2011. A second collection was carried out in the municipality of Pinhão in August 2011. The eggs were collected by ovitraps with an infusion of grasses. The municipalities were small (<50,000 premises), and with the exception of Aracaju, their total urban area was divided into 100 quadrants, with one trap was installed in each quadrant. In the case of Aracaju, 30 ovitraps were installed in the Porto Dantas neighborhood, one per block. The traps remained on the premises for two consecutive weeks. Each week, the wooden strips were removed and the water of the trap discarded to eliminate larvae that could have hatched from oviposition. The strips collected in the field were taken to the Laboratory of Entomology and Tropical Parasitology [*Laboratório de Entomologia e Parasitologia Tropical* (LEPaT) of the *Universidade Federal de Sergipe*] where the eggs were counted and stored. Installation of the traps was carried out in each municipality in partnership with the State Department of Health and the Municipal Departments of Health, and with the support of the health care workers of each locality.

Colony rearing

Each population of *Ae. aegypti* was reared in the insectary of LEPaT in a climatized room with a temperature of 26 ± 2°C, at 60% ± 10% humidity, and with a photophase of 12h. Eggs were transferred to a tray (30cm × 20cm × 6cm) containing 1.0L of drinking water and crushed cat food. The total number of eggs reached around 2,000. After 24 hours, the larvae that hatched were transferred to other trays containing 1.5L of water and 100mg of cat food per 150 larvae. Each tray contained at most 600 larvae, and those that emerged constituted the adults of one cage. Upon reaching the pupal stage, they were transferred to cages (30cm × 30cm × 30cm), where adults emerged and were fed daily with a 10% sucrose solution. Females also fed on anesthetized rats for 30 minutes, three times a week. A cup containing water and oviposition egg paper was placed in each cage. During each blood meal, the cups were replaced and the oviposition egg papers were laid to dry at room temperature. After drying, the egg papers were dated and packaged in envelopes which were identified with the name of the population. Eggs of the F1 generation were used in the experiments. The Rockefeller strain (ROCKE) was used as a reference for insecticide susceptibility. The experiments were performed according to the standard protocol of the World Health Organization[25] for diagnostic dose (DD) and response dose (DR) bioassays.

Diagnostic dose and dose response bioassays

A stock solution of 1,000ppm was prepared using technical grade 97.4% temephos (Pestanal® Sigma-Aldrich). A new solution was prepared from the stock solution for each test. One milliliter of the insecticide solution at 0.012ppm of temephos, the standardized DD of this product for *Ae. aegypti*[13] was added to 224mL of water and shaken lightly to ensure a homogeneous test solution. Next, 20 late third or early forth instar larvae were added to the cup. Each bioassay consisted of 8 exposed and 4 controls cups. Mortality values were recorded after 24h exposure.

After confirming resistance in the DD bioassays, quantitative DR tests were conducted. Nine different concentrations of the insecticide were used for each population. These concentrations were also used in the experiments with 80 third stage larvae, distributed in four replicates per concentration, and in the control treatment that exposed 20 larvae to 0.5% ethanol. The tests were repeated three times on different days. All tests were performed concomitantly with exposure of the ROCKE strain at concentrations of 0.012ppm and 0.006ppm. Mortality values were recorded after 24h exposure.

Statistical analysis

The LC_{50} and LC_{99} (lethal concentration) were calculated using probit analyses (Polo-PC, LeOra Software, Berkeley, CA). RR_{50} and RR_{99} were obtained by dividing the results for each population by the equivalent Rockefeller values. RR values between 3 and 5 were classified as low, 5 to 10 as moderate, between 10 and 20 as medium, and above 20 as high resistance[6]. The ratio of LC_{99} to LC_{50} for each population was calculated in order to verify heterogeneity of resistance.

Ethical considerations

The colony of *Ae. aegypti* requires blood feeding on animals. This was carried out in the LEPaT insectary under the approval of the Ethics and Animal Research Committee of the Federal University of Sergipe (Protocol 12/07). The insectary follows the technical standards contained in the Parameters of Biosafety for Insect and Vector Infectivity of the Oswaldo Cruz Foundation[26].

RESULTS

The ovitraps revealed high degrees of infestation by *Ae. aegypti* in the State of Sergipe, reaching 91% of the total traps installed. In addition, Maruim and Capela municipalities, closest to Aracaju, had over 70% of positive traps and showed the greatest numbers of eggs (**Table 1**). Mortality ranged from 0% to 10% in DD experiments, classifying all populations as resistant to temephos. Neopolis had the population with the highest mortality [mean of 9.75% and standard deviation (SD) of 2.12] and Pinhão the lowest (mean of 0.12% and SD of 0.35%). Once populations were identified as resistant, DR bioassays were carried out and the LC_{50} and LC_{99} for each population were estimated. The Pinhão population had values that were very different compared to the other populations, even when compared to those near the same location. A second round of egg collection was performed for this population. The first round took place in November 2010 and the second in August 2011. The population from the second round of collections showed values even larger than those from the first round and had narrower confidence intervals (CI). The Porto Dantas populations showed the lowest LC_{50} and LC_{99} values, which

TABLE 1: Ovitrap positivity and number of eggs collected according to cities in Sergipe, from 2010 to 2011.

City	Date	Traps	Egg frequency	Total of eggs collected
		n	%	
Maruim	Feb/11	100	91.0	21,896
Carira	Oct/10	100	80.0	6,161
Capela	Mar/11	100	77.0	7,140
Aracaju (Porto Dantas)	Mar/10	30	56.0	1,540
Canindé de São Francisco	Jun/10	100	51.0	2,302
Pinhão	Nov/10	100	39.0	2,367
Neópolis	Mar/10	100	28.0	2,801

TABLE 2: Lethal concentrations for of temephos of for Rockefeller and field populations of *Aedes aegypti* according to cities in Sergipe in 2011.

Population	Estimated value (mg/L)	Confidence interval (95%) low – upper (mg/L)	LC_{99}/LC_{50}
Rocke			
LC_{50}	0.002	0.002 – 0.003	4.5
LC_{99}	0.009	0.007 – 0.012	
Porto Dantas			
LC_{50}	0.034	0.032 – 0.037	5.9
LC_{99}	0.200	0.170 – 0.250	
Neópolis			
LC_{50}	0.074	0.071 – 0.078	3.7
LC_{99}	0.26	0.24 – 0.28	
Canindé			
LC_{50}	0.08	0.06 – 0.10	10.8
LC_{99}	0.86	0.60 – 1.45	
Maruim			
LC_{50}	0.09	0.08 – 0.10	8.6
LC_{99}	0.77	0.60 – 1.08	
Carira			
LC_{50}	0.08	0.06 – 0.10	16.8
LC_{99}	1.34	0.81 – 3.04	
Capela			
LC_{50}	0.11	0.09 – 0.13	7.2
LC_{99}	0.79	0.53 – 1.56	
Pinhão 1			
LC_{50}	0.31	0.27 – 0.36	6.1
LC_{99}	1.89	1.26 – 3.72	
Pinhão2			
LC_{50}	0.45	0.41 – 0.48	6.0
LC_{99}	2.68	2.30 – 3.20	

LC_{50}: lethal concentrations 50; **LC_{99}:** lethal concentrations 99.

were 0.03mg/L and 0.20mg/L, respectively. The LC_{99} was 3.7 to 16.8 times greater than the LC_{50} (**Table 2**).

All populations showed RR_{99} greater then 20, and thus, all municipalities were classified as highly resistant to temephos (**Table 3**).

TABLE 3: Resistance ratio of *Aedes aegypti* populations to temephos according to cities in Sergipe 2011.

Population	RR_{50}	RR_{99}
Rocke	1	1
Porto Dantas	17	22.2
Neópolis	35	28.9
Canindé	40	95.6
Maruim	45	85.6
Carira	40	148.9
Capela	55	87.8
Pinhão 1	155	210.0
Pinhão 2	225	297.8

RR_{50}: resistance ratio 50; **RR_{99}**: resistance ratio 99.

DISCUSSION

Sergipe is a state highly infested by *Ae. aegypti*, which can be seen by the high ovitrap positivity index in all municipalities studied. Cities nearest to the capital Aracaju showed more than 70% ovitrap positivity and had the highest number of eggs collected. Maruim, 30km from of the capital, had the highest number of eggs, 21,896, and Canindé de São Francisco, 213km from the capital, had the lowest number of eggs, 2,302 eggs. This difference in infestation level could be related to the demographic density of the cities, as cities near the capital had a high number of inhabitants per km^2 and most of these cities are interconnected. Crowded places often have a wide availability of containers with conditions suited to the development of mosquitoes and are devoid of geographical barriers. Densely populated regions tend to present with a greater proliferation and spread of *Ae. aegypti*[27,28].

High levels of infestation by *Ae. aegypti* have consequently led to high transmission levels of dengue and other arboviruses[29]. Brazil reported 1,500,535 dengue cases in 2016[30], epidemics of Chikungunya and Zika[31], and 10,867 suspected cases of microcephaly related to the Zika virus[30]. The State of Sergipe reported 270 newborn babies with microcephaly in 2016[32].

The main strategies of the former National Dengue Control Program, current National Arbovirus Control Program, and the Zika Zero campaign for arbovirus control are vector elimination[33] by container removal, raising awareness, and insecticide application. Although it was in use in most Brazilian cities until 2010[6], temephos resistance was spread widely throughout the country[16-18].

Studies carried out by the National Network of *Aedes aegypti* Resistance Surveillance [*Rede Nacional de Monitoramento da Resistência de Aedes aegypti a Inseticidas* (Rede MoReNAa)], using mosquitos from Aracaju in 2001, assigned a RR_{99} of 6.1, suggesting medium resistance to temephos[3]. Seven years later, in 2008, a RR_{99} of 19.83 was found[34]. The network only monitored three of the 75 cities that form the state; therefore, the spread and intensity of resistance throughout the state was not known. Our results show a small increase in the RR_{99} in Aracaju (22.2), and we reclassified the status for the city as high (>20). We also observed high resistance in small cities not monitored by the Ministry of Health network.

Mosquito populations in all Sergipe cities showed high resistance to temephos, with RR_{99} values ranging from 22.2 (Aracaju) to 297.9 (Pinhão). Thus, it is likely that Aracaju does not represent an objective picture of the actual resistance status of the state. The use of Aracaju as a sentinel city may have obfuscated the spread of the resistance, delaying the replacement of temephos and leading to an intensification of temephos resistance in the region. Variation in mosquito resistance in closed cities has been seen in other areas in the country[4,7,8]. Because of the large variability in the level of resistance, it is likely that sentinel sites cover an area smaller than expected, considering the values in Sergipe, and this should be taken into account in resistance surveillance plans. In addition, the RR_{99} values found in Pinhão were very high in relation to those in the other cities, including an increase in the level of resistance over a short period. This fact suggests that larvicides could have been used in different ways in the cities in terms of frequency, amount, and range. This variation in practices is a matter of concern, as the same practices that may have resulted in temephos resistance in these cities can also select for resistance to the products now replacing this organophosphate.

The LC_{99} values for the majority of the populations were more than six times the LC_{50}, showing that the Sergipe populations of *Ae. aegypti* are heterogeneous in relation to resistance. Studies carried out in 2001 using populations from different areas of Rio de Janeiro, Alagoas, and Sergipe (including Aracaju) showed differences between the values of LC_{90} and LC_{50} lower than 2.7. The same year, the difference in the Aracaju sample was 2.2[3], while values of 5.9 were seen in this study. Even though our sample was limited to one neighborhood and we used LC_{99} for the calculation, it is likely that the population of *Ae. aegypti* became less homogenous over time.

The high levels of resistance observed in *Ae. aegypti* populations in Sergipe, as well as in other areas in the country[5-8], may be related to how temephos is used in each locality. It may be used at different intensities and frequencies, and the mosquito populations could be under different selection pressures. In addition, different resistance mechanisms could be involved[14]. Resistance is a genetically inherited trait and selection is not uniform[3,35]. Resistance can be associated with high level of esterase enzymes, which act to decrease the amount of active insecticide in the target[15]. This kind of resistance mechanism was observed in Thailand[36,37], Malaysia[38], Cuba[35], Porto Rico[14], and Colombia[13]. It is interesting to consider that variation in the resistance levels observed in our bioassays did not imply a genetic differentiation, as subsequent studies using the same samples showed low genetic structuring and close relationships among populations[39].

Container size was a determining factor for larvicideuse in each area. In areas where the majority of containers were small or disposable, removal activities predominate over the use of insecticides. On the other hand, in places with disruptions in water supply, non-disposable containers for water storage predominate and larvicides may be widely applied. Variation in the size of the containers or the presence of large containers could result in a mis-estimation for the amount of product to use, due to either inability or negligence. In the city of Aracaju, up to 82% of the breeding places were containers for water storage[34], and this percentage may be higher in cities with water supply problems and for semiarid areas, as well.

It is necessary to understand the factors involved in the evolution of insecticide resistance in order to improve resistance management plans and integrated vector control[40]. The variation in resistance levels observed in *Ae. aegypti* populations in the small State of Sergipe highlight the need for an effective program of resistance surveillance, and also indicates that the potential of small cities to generate and spread insecticide resistance may have been underestimated. The fragility of the vector control programs in small cities with low levels of financial support and reduced operational capability to perform technical routine activities, after the decentralization of public health services, may represent a determining factor in this process[41].

Many natural products have been assessed with the goal of discovering new insecticide products with lower costs, lower toxicity, and better cross-acceptance between the population and public health workers[6]. Taking into account that the mechanisms of action for these products are not yet known, the possibility of cross-resistance should be considered. Therefore, effective bioassays of promising new molecules should also be conducted on resistant mosquito populations. In addition, further surveillance of temephos resistance in the absence of selection pressures to this insecticide should be emphasized by surveillance programs.

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Conflict of interest

The authors declare that there is no conflict of interest.

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