Monitoring of resistance to the pyrethroid cypermethrin in Brazilian *Aedes aegypti* (Diptera: Culicidae) populations collected between 2001 and 2003

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Resistance to cypermethrin of different Aedes aegypti *Brazilian populations, collected at two successive periods (2001 and 2002/2003), was monitored using the insecticide-coated bottles bioassay. Slight modifications were included in the method to discriminate between mortality and the knock down effect. Although this pyrethroid was recently started to be used in the country to control the dengue vector, a decrease in susceptibility was noted between both periods analyzed, particularly in the city of Rio de Janeiro. The results indicate that resistance is due at least in part to a target site alteration.*

Key words: *Aedes aegypti* - pyrethroid resistance - knock down effect

Dengue is presently one of the major Public Health problems at Brazil. Since the 1980's several epidemic bursts have occurred in the country, and it is now considered to be endemic. This is due, in part, to the resistance of dengue's main vector, *Aedes aegypti*, to insecticides used in its control.

The use of organophosphates, employed since 1967 throughout the country against *Ae. aegypti* larvae and adults, was intensified after the 1986 epidemics, that started at Rio de Janeiro and spread over several other regions (Lima et al. 2003, Braga et al. 2004). At that time, temephos was the only larvicide used by the Brazilian dengue control program. As a consequence of its massive use, in 1998 a decrease in the residual effect of this organophosphate was reported by field personnel. In order to face this problem, in 1999, the National Health Foundation (Funasa) implemented: (i) the coordination of an *Ae. aegypti* insecticide resistance monitoring program, focusing on those municipalities exhibiting high mosquito infestation or dengue incidence levels and (ii) the substitution of organophosphates for the pyrethroid cypermethrin in the control of *Ae. aegypti* adults, performed through space sprayings and residual perifocal applications (Funasa 1999). This was done to avoid placing selection pressure of different mosquito developmental stages with the same insecticide class, a measure that could, theoretically, improve the useful life of each compound.

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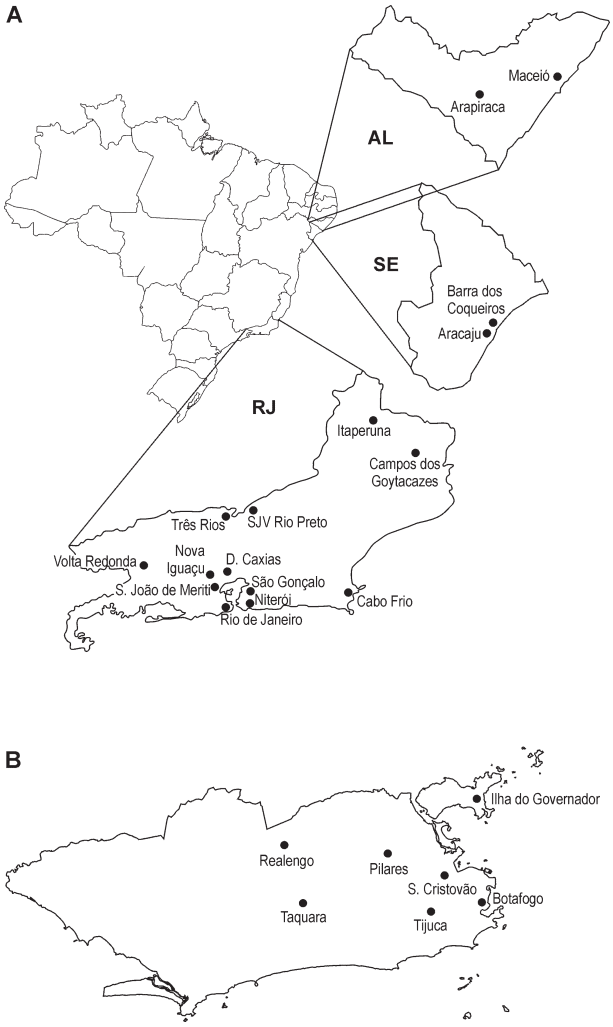
Organophosphates and pyrethroids act on different target sites in the insect's central nervous system, namely, Acetylcholinesterase and the voltage gated sodium channels, respectively. After linkage to pyrethroids, the sodium channels in the neurons are maintained for a longer length of time in their opened conformation, which results in a continuous nervous impulse that causes bursts of contractions, culminating with paralysis (Bloomquist 1996). Depending on the insecticide's dosage, this effect, known as the "knock down" mechanism, is reversible if contact with the insecticide is interrupted. Resistant individuals that have a *kdr* mutation exhibit the knock down effect but can recover from pyrethroid dosages that are lethal to susceptible insects (Milani 1954, Pauron et al. 1989). Sodium channels are also the target site for organochlorines, an insecticide class that has not been used in the Public Health at Brazil since the reintroduction of *Ae. aegypti* in 1967 (Franco 1976).

We report on the monitoring of *Ae. aegypti* resistance to cypermethrin in municipalities of three Brazilian states: Sergipe (SE) and Alagoas (AL), located at Northeast Brazil and Rio de Janeiro (RJ), at the Southeast (Figure). Bioassays were performed with insecticide-coated bottles, as described by Brogdon and McAllister (1998).

We first calibrated the bottles with different dosages of cypermethrin, by testing mosquitoes from the Rockefeller strain. This lineage is employed worldwide as an insecticide susceptible reference. According to the original methodology (Brogdon & McAllister 1998), the criterion for mortality was that mosquitoes were not able to fly or to right themselves when the bottle is gently rotated. The same methodology recommends registers of mortality at regular intervals of 10-15 min until the last mosquito dies or up to 1-3 h.

In order to discriminate between mortality and the knock down effect, during calibration of the assay, an additional procedure was included: after all the mosqui-



Map of Brazil - A: Alagoas (AL), Sergipe (SE), and Rio de Janeiro (RJ) states; B: Rio de Janeiro municipality and the localization of the districts used in the present study.

toes had fallen to the bottom of the bottles, they were transferred to recovery cages, free of cypermethrin, and observed again, 24 h later. With this procedure, we verified that 2 µg cypermethrin/bottle was the minimal amount that "killed" 100% of the Rockefeller mosquitoes. This was accomplished after 60 min exposure. However, 24 h later, approximately 10% of the Rockefeller mosquitoes had recovered, and recovery could not be ascribed to resistance, since this is a reference susceptible strain.

In the course of these assays, we observed that several mosquitoes moved erratically. Some of them jumped, others turned upside down and executed a series of rotations of their bodies with the aid of the wings. According to Brogdon and McAllister (1998) these would be dead mosquitoes. We suspected that more consistent results could be achieved if the mortality criterion was slightly modified when applied to pyrethroid insecticides. It should be noted that the lowest cypermethrin dosage that "kills" 100% of Rockefeller mosquitoes, even after 24 h in the recovery cages, was 8 µg/bottle, four times the dose we would have chosen if the assay was interrupted after 60 min exposure.

When the mortality criterion used was the complete absence of movements, we confirmed that 8 µg cypermethrin/bottle is the lowest amount that kills 100% Rockefeller mosquitoes. This was attained after 30 min exposure, a period considered as the resistance threshold (Brogdon & McAllister 1998). In this case, as stated above, no recovery was observed after 24 h in the absence of insecticide.

In order to monitor *Ae. aegypti* resistance to cypermethrin, mosquito eggs were collected between January and September 2001 and between December 2002 and August 2003. Collection of eggs was performed according to the Resistance Monitoring Program recommendations, by means of ovitraps installed in several non-adjacent districts in each municipality. Egg collections of each municipality were analyzed after being pooled, with the exception of the city of Rio de Janeiro, where evaluation of different districts was done separately. In the majority of the cases, bioassays were performed with F1 or F2 female mosquitoes, 1-2 days after adult emergence. On average, three to four tests were performed for each locality. Each test consisted of three bottles coated with 8 µg cypermethrin in 1 ml acetone and one control bottle where 1 ml acetone was added. Fifteen females were exposed to each bottle. In all cases we used cypermethrin 250CE from Vectocell (São Paulo, Brazil, lot 030132) supplied by Funasa.

Mortality registers were performed at 10 min intervals up to 120 min. At that point, all mosquitoes, "dead" and alive, were transferred to recovery cages and observed 24 h later. Mortality rates obtained after 30 min (the resistance threshold of cypermethrin in our conditions) and 120 min exposure and after 24 h of recovery are shown.

Tables I and II show localities that were evaluated only at 2001 or at 2002/2003, respectively, while Table III depicts localities that were monitored during both periods. Populations were classified according to the WHO criteria, which consider mortality above 98% and below 80% representative of susceptible and resistant populations, respectively. Intermediate values are indicative of an incipient altered susceptible status and point to the need of surveillance of the population (Davidson & Zahar 1973).

In the 2001 evaluation, no population analyzed presented mortality levels compatible with true susceptibility. Seven out of 16 localities exhibited mortality levels lower than 80% in at least one time point analyzed (Tables I and III). In the majority of the cases this was attained at the 24 h evaluation, indicating recovery of mosquitoes. In spite of this, the general picture that arose that year indicated just a slight alteration of the pyrethroid susceptibility.

The same was not true during the 2002/2003 monitoring. From the 15 populations evaluated, only two, Arapiraca (AL) and São José do Vale do Rio Preto (RJ) exhibited just an incipient alteration at all the time points considered for analysis. The remainder populations showed mortality levels compatible with resistance (lower

TABLE I

Mortality levels of *Aedes aegypti* populations (expressed as percentage) from different Brazilian municipalities, collected between January and September, 2001, recorded after exposure, during 2 h, to cypermethrin impregnated bottles (8 µg/bottle). Afterwards, the mosquitoes were transferred to recovery cages and mortality was registered again 24 h later

State	Municipality	District	F	Assays	30 min (Exposure)	120 min (Exposure)	24 h (Recovery)
AL	Maceió		F2	3	77.7 ± 4.1	81.4 ± 10.5	62.0 ± 11.4
SE	Aracaju		F2	3	95.2 ± 4.2	90.5 ± 5.6	95.7 ± 3.2
	Barra dos Coqueiros		F2	2	95.4 ± 1.6	88.9 ± 3.1	92.7 ± 0.5
RJ	Duque de Caxias		F3	3	81.6 ± 6.4	85.4 ± 4.9	65.1 ± 19.2
	Niterói		F2/F3	3	86.7 ± 1.7	83.9 ± 4.8	73.3 ± 13.6
	São Gonçalo		F2	3	94.6 ± 1.0	95.7 ± 3.5	90.2 ± 13.1
	São João de Meriti		F2	3	81.7 ± 4.8	86.2 ± 4.4	81.5 ± 17.8
	Rio de Janeiro	Pilares	F2	4	77.2 ± 5.2	81.7 ±7.3	78.4 ± 10.5
		Realengo	F2/F3	4	91.7 ± 6.2	91.4 ±1.9	85.4 ± 15.5
		São Cristóvão	F2	3	83.7 ± 10.8	83.3 ±10.0	81.9 ± 19.4

F indicates the generation used in the bioassays; AL: Alagoas; SE: Sergipe; RJ: Rio de Janeiro

TABLE II

Mortality levels of *Aedes aegypti* populations (expressed as percentage) from different municipalities of the state of Rio de Janeiro, collected between December, 2002 and August, 2003, recorded after exposure, during 2 h, to cypermethrin impregnated bottles (8 µg/bottle). Afterwards, the mosquitoes were transferred to recovery cages and mortality was registered again 24 h later

State	Municipality	District	F	Assays	30 min (Exposure)	120 min (Exposure)	24 h (Recovery)
Cabo Frio			F1	4	80.0 ± 5.7	80.0 ± 5.4	66.1 ± 14.2
Itaperuna			F2	3	69.6 ± 19.9	76.3 ± 12.6	54.8 ± 9.0
SJV Rio Preto			F2	3	93.7 ± 2.3	89.1 ± 6.0	94.8 ± 9.0
Três Rios			F2	4	76.3 ± 9.3	80.0 ± 2.2	43.0 ± 14.8
Volta Redonda			F2	4	81.4 ± 4.2	85.4 ± 5.6	77.9 ± 6.1
Rio de Janeiro		Botafogo	F2	4	57.8 ± 7.7	64.4 ± 16.7	51.1 ± 23.0
		Freguesia	F2	4	74.4 ± 9.3	80.6 ± 1.1	78.3 ± 3.3
		Taquara	F2	3	60.8 ± 3.4	65.5 ± 12.5	48.7 ± 8.1
		Tijuca	F2	3	88.1 ± 1.3	80.7 ± 1.3	64.4 ± 4.4

F indicates the generation used in the bioassays.

TABLE III

Monitoring of cypermethrin resistance through adult bioassays, of *Aedes aegypti* populations collected in 2001 and in 2002/2003. Mortality levels (expressed as percentage) were recorded after 30 and 120 min exposure to impregnated bottles (8 µg/bottle). Mosquitoes were then transferred to recovery cages and mortality was registered again 24 h later

			2001					2002/2003				
State	Municipality	District	F	N	30 min (Exposure)	120 min (Exposure)	24 h (Recovery)	F	N	30 min (Exposure)	120 min (Exposure)	24 h (Recovery)
AL	ARA		F2	3	96.6 ± 1.6	95.5 ± 1.0	87.2 ± 6.3	F2	5	92.4 ± 6.4	90.2 ± 5.6	87.5 ± 10.7
RJ	CGO		F2	3	92.8 ± 3.4	95.7 ± 1.2	97.2 ± 3.5	F2	4	90 ± 4.3	93.9 ± 1.1	**73.3 ± 16.1**
	NIG		F3	3	89.4 ± 2.5	91.6 ± 6.6	**75.8 ± 12.8**	F2	7	82.5 ± 4.1	88.9 ± 6.5	**59.0 ± 16.6**
	RJ	Jamr	F1/F2	2	90.7 ± 8.4	88.3 ± 9.8	89.0 ± 6.0	F2	4	**58.9 ± 8.6**	**70.0 ± 4.3**	**65.0 ± 13.9**
		Penha	F2	5	**79.3 ± 8.1**	82.3 ± 4.4	**78.3 ± 4.4**	F2	4	**50.6 ± 6.4**	**63.3 ± 9.3**	**51.1 ± 9.4**
		Rmir	F2	4	85.6 ± 3.2	85.2 ± 1.9	**79.1 ± 19.4**	F2	3	**65.8 ± 7.0**	**73.4 ± 4.4**	**57.3 ± 12.5**

F indicates the generation used in the bioassays; N: shows the number of assays performed for each population; ARA: Arapiraca; CGO: Campos dos Goytacazes; NIG: Nova Iguaçu; RJ: Rio de Janeiro; Jamr: Jardim América; Rmir: Rocha Miranda; numbers in bold indicate mortality levels compatible with resistance status.

than 80%) in at least one of the time points analyzed. Six localities exhibited resistance throughout the assay, all of them at the state of RJ and five corresponding to districts from the city of Rio de Janeiro (Tables II and III). All of the localities from the state of Rio de Janeiro evaluated at both periods demonstrated lower mortality levels in 2003 (Table III). This was true not only during exposure of mosquitoes to the pyrethroid but also after recovery.

There was a remarkable fraction of insects that recovered after 24 h in some localities. The rate of recovering mosquitoes was greater when data from 120 min and 24 h were compared, instead of 30 min - 24 h. In particular, roughly 30% recovery of mosquitoes from Itaperuna (RJ) and Nova Iguaçu (RJ) was noted. An even higher proportion of recovery was noted with mosquitoes from Três Rios (RJ): 46% of knocked down mosquitoes (after 120 min) recovered 24 h later.

Our results indicate that, despite the recent use of pyrethroids to control *Ae. aegypti* in the country, alterations of the vector susceptibility to this class of insecticides is developing rather quickly. Although metabolic resistance can not be discarded, cypermethrin altered response could be at lest partially derived from target site mutations. We are presently evaluating the activity profile of different enzymes implicated in metabolic resistance of these populations and sequencing the sodium channel gene domain where *kdr* mutation is located, in order to identify alterations in Brazilian *Ae. aegypti* populations that can be correlated with pyrethroid resistance.

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