# Standardization of Bioassays for Monitoring Resistance to Insecticides in *Aedes aegypti*

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**Abstract**

Since the reintroduction of *Aedes aegypti* in Brazil in the 1980s, insecticide use for its control is routine. The chemical control efficacy is threatened by vectors developing resistance to insecticides. The World Health Organization, recognizing the impact of insecticide resistance in vector control programmes, proposed standardizing bioassays for detecting and monitoring resistance using a diagnostic dose method. As Brazil has a national programme for monitoring the resistance of *Ae. aegypti* populations to insecticides, this study was designed to compare diagnostic bioassays at WHO suggested concentrations and those estimated for local conditions. Populations were resistant to both temephos doses. But important differences were seen for fenitrothion and malathion, which could lead to under- or over-estimation of resistance respectively. These results and inclusion of a diagnostic dose bioassay standard for larvae are discussed.

**Keywords**: *Aedes aegypti*, monitoring resistance to insecticides, diagnostic dose bioassay.

## Introduction

Although the development of insecticide resistance in the vector *Aedes aegypti* has been documented worldwide[1,2]. Brazil routinely uses chemical control for larvae and adults during dengue epidemics[3]. The Dengue Control Programme is based on house-to-house visits for source reduction, education and foci treatment with larvicides every two months. During dengue transmission, in addition to larviciding, space spraying is recommended in the area in a radius of 100 metres around each dengue case. In São Paulo State, the programme has been adapted to restrict larvicide use to just dengue transmission or to situations where source reduction is not feasible. Both options may potentially loose efficacy as continuous use of insecticides may hamper any resistance management strategy[4].

Since 1960, the World Health Organization (WHO) has proposed standardization of bioassays for detecting insect resistance to insecticides[5]. With procedure standardization, results from different and distant areas can be compared. According to WHO guidelines[6,7,8,9], resistance can be detected and monitored by bioassays using two methods: diagnostic dose and estimating resistance ratio (RR). The dose is called "diagnostic" because it permits discrimination of insect response: those that die after exposure are labelled as susceptible

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and those who survive as resistant. The mortality rate in a dose-diagnostic assay indicates population status when tested on a significant number with sufficient replications. Davidson and Zahar[10] proposed a criterion for interpreting this kind of response classifying as "susceptible" insects presenting 98–100% mortality and "resistant" to mortality as below 80%. An intermediate level would be insects with 80–97% mortality. More recently, in 1998, there was a revision of this criteria for the last range of mortality, adding the classification of resistance strongly suspected in insects that present an average mortality between 80–95% in bioassays run in very good conditions and with a sample size bigger than 100 insects[11].

Establishing the diagnostic dose level is based on bioassays of a susceptible strain submitted to a range of insecticide doses (or concentrations for larvae) and the 99% lethal concentration is estimated. The 99% lethal concentration is then multiplied by a factor of 2, 3 or 4 to determine the diagnostic dose. The choice of the multiplication factor depends on the desired discrimination level. There is a WHO recommendation of double the lowest concentration that systematically provides 100% mortality in a range of susceptible strains to establish diagnostic dose and that field populations should be periodically submitted to this dose for resistance monitoring[6].

The WHO-suggested doses* or concentrations are based on tests performed in reference laboratories with the latest publication of reference doses in 1992[1]. The doses listed in that publication were obtained from several WHO collaborative centres, with a range of susceptible strains and some of them adopted the lowest 100% mortality concentration. It is also clear in this publication that the list of diagnostic dosages (concentrations) for various species of vectors should be considered as a guide and that they may be refined for each local situation whenever it is possible.

Besides the bioassays, biochemical assay techniques can detect resistance mechanisms and, along with molecular assays, should be part of a global approach for monitoring resistance. The biochemical and molecular assays can detect resistance mechanisms in individual insects and thus facilitate confirmation of survivors from bioassay tests.

The first indication of resistance to temephos was recorded in São Paulo State in 1991[12]. Reduced susceptibility to temephos was reported in a population from Goiás State in 1995[13]. A state programme for monitoring resistance of *Ae. aegypti* was started in São Paulo State in 1996 and was extended nationally to all states in 1999[14]. There is an annual evaluation of populations in areas with elevated dengue incidence which means the intense use of insecticides. Besides bioassays with diagnostic dose and resistance ratio for larvae, the National Monitoring Programme also performs biochemical assays in order to characterize resistance mechanisms. As there are many laboratories involved in the national programme, there was a need to standardize procedures so that results could be comparable. The aim of this study was to evaluate the diagnostic dose procedures and dose levels for larvae suggested by the WHO with diagnostic doses estimated at local level.

*WHO diagnostic concentration (DC) is estimated from a range of susceptible strains (and not only one or two) as the double of the concentration that systematically kills 100 % of test specimens of all the reference susceptible strains. As such, DC is NOT a tool for early detection of resistance. Priority has been given by WHO to easy use and reliability and not to sensitivity.

Any National Programme has the entire freedom to adapt locally WHO proposed DC based on local evidence, what Brazil did well. However, this does not mean that what is good for Brazil should be adopted without previous testing by other countries, including neighbouring ones. - Editor

## Methodology

The first procedure for standardizing bioassays was to estimate lethal concentrations for the *Rockefeller* and *Bora Bora* susceptible reference strains using temephos solutions. The susceptible strains were kindly provided by the Centers for Disease Control and Prevention in Puerto Rico and the Institut de Recherche pour le Developpement in Montpellier, respectively. Lethal concentrations were also estimated for four field populations of *Ae. aegypti*.

Larvae were exposed to a range of eight different insecticide concentrations[6,8]. Results, expressed in the number of dead specimens per dose, were submitted to statistical treatment and analysed with the software Polo-PC[15]; 50%, 95% and 99% lethal concentrations were estimated ($LC_{50}$ $LC_{95}$ and $LC_{99}$). Three complete tests were performed for each insecticide and each mosquito population. The number of exposed larvae per dose was 80, repeated four times. Three different temephos solutions were used: two sent by WHO, Geneva, and one prepared with technical grade (BASF 94.6%) at the Superintendência de Controle de Endemias laboratory in Marília. Resistance ratios (RR) were then estimated for field populations and results from the three solutions compared.

Lethal concentrations for fenitrothion and malathion were also estimated for the Rockefeller strain using technical grade fenitrothion (SUMITOMO Co 93%) and technical grade malathion (CHEMINOVA AGRO A/S 95%). The local diagnostic dose was estimated from susceptible strain by doubling the $LC_{99}$ lethal concentration.

Sixteen field populations were assayed using the WHO[1] and local diagnostic doses for fenitrothion, malathion and temephos. Bioassay results were expressed in mortality percentage and populations classification according to Davidson and Zahar[10] were compared.

## Results

The comparison of results using different solutions of temephos was part of a collaborative study coordinated by Dr Pierre Guillet from WHO, Geneva. The two solutions provided by WHO were different. One was supplied ready for use (coded AS) and the other a bottled insecticide deposit ready for local suspension by adding alcohol (coded D). The locally prepared solution was made with technical grade provided by BASF (coded Sucen).

Table 1 shows that independent of the solution used and the susceptible strain, the resistance ratio for each field population was quite similar. Results are presented in increasing order of field population RR.

***Table 1.*** *Resistance ratio estimation according to susceptible Ae. aegypti strain and insecticide solution*

| Solution/Field population | RR $LC_{95}$ Rockefeller | | | RR $LC_{95}$ Bora Bora | | |
|---|---|---|---|---|---|---|
| | D | AS | Sucen | D | AS | Sucen |
| São José Rio Preto | 2.0 | 2.1 | 2.4 | 2.1 | 1.9 | 1.9 |
| Barretos | 2.6 | 2.1 | 2.2 | 2.3 | 2.1 | 1.9 |
| Recife | 4.4 | 4.1 | 3.8 | 5.1 | 3.8 | 3.1 |
| Aracaju | 5.2 | 4.9 | 5.0 | 5.0 | 5.0 | 4.3 |
| Itabaiana | 8.3 | 7.9 | 7.4 | 7.2 | 7.1 | 6.9 |

Rockefeller – Susceptible reference strain
Bora Bora – Susceptible reference strain
D – Solution prepared adding alcohol in deposit insecticide
AS – Solution mailed and kept in cold chain, ready to use
Sucen: Solution prepared for the Brazilian National Programme for Monitoring Resistance of *Aedes aegypti* using technical grade temephos

Table 2 shows the 99% lethal concentrations ($LC_{99}$) estimated for the Rockefeller strain with temephos, fenitrothion and malathion, local diagnostic concentration (double of $LC_{99}$), and WHO suggested concentrations[1].

***Table 2.*** *Lethal concentration estimation in bioassays with susceptible Rockefeller strain*

| Insecticide | Lethal concentration 99% (mg/l) (f.l.)* | Diagnostic concentrations | |
|---|---|---|---|
| | | Sucen** | WHO*** |
| Fenitrothion | 0.0050 (0.0047–0.0051) | 0.0100 | 0.0200 |
| Malathion | 0.0900 (0.0852–0.1080) | 0.200 | 0.1250 |
| Temephos | 0.0040 (0.0038–0.0042) | 0.0080 | 0.0120 |

*Fiducial limits
** Estimated at local conditions ($LC_{99}$ multiplied by 2)
***WHO: suggested in 1992[1]

The WHO-suggested temephos concentration is three times the locally estimated $LC_{99}$. For fenitrothion it is four times the local $LC_{99}$, and for malathion it is only 1.4 times the local $LC_{99}$.

At this point of the study, the Rockefeller strain was chosen as the susceptible reference because it is the one used in the Brazilian National Resistance Monitoring Programme. Also, it has been used in several susceptibility studies of *Ae. aegypti*[16,17,18,19] and would therefore make comparisons easier.

Table 3 shows bioassay results using diagnostic concentrations for field populations of *Ae. aegypti*. The data are sorted out in decreasing mortality percentage. Comparing temephos results, three populations classified as resistant (<80% mortality) according to Davidson Zahar criteria[10], using the WHO concentration (Aracaju, Arapiraca and Itabaiana) had the same classification as the local concentration. In eight populations classified as susceptible (>98% mortality) using the WHO concentration, three were classified as susceptible while the other five presented values between 80% and 97% mortality. Only one population with decreased susceptibility (80–95% mortality) with WHO concentration had the same response with the local concentration (Barra dos Coqueiros); the other four presented as resistant (<80% mortality) with the local concentration (Jaboatão dos Guararapes, Maceio, Recife, and Santos).

The WHO-suggested concentration for fenitrothion seems to be unable to provide discriminated response as almost all populations presented high mortality; 13 of the 16 populations were classified as susceptible while with local diagnostic dose there was discrimination. Only three were classified as susceptible (Campinas, Marilia and Presidente Prudente); seven had decreased susceptibility, and five were classified as resistant. There was only one population presenting the same range of decreased mortality (between 80-95%) with both concentrations, Arapiraca, which would be quoted with resistance strongly suspected.

In contrast, the WHO-suggested concentration for malathion is low and almost all populations showed low susceptibility; only two from 16 populations were classified as susceptible (Bauru and Presidente Prudente). These two had the same classification with the local concentration; they have been known as susceptible field populations since 1998[20]. The Araçatuba population was the only one of five populations that showed decreased susceptibility with both concentrations. None of the nine populations classified as resistant with WHO concentration had the same response with local concentration. In seven populations the mortality was between 80% and 95% (resistance strongly suspected), one presented mortality of 95% (Aracaju) and one was susceptible (Barretos) with local concentration. Eight showed decreased mortality and one was susceptible.

***Table 3.*** *Average mortality percentage in bioassays with diagnostic concentrations Origin of Brazilian Ae. aegypti populations*

| Insecticide | Temephos | | Fenitrothion | | Malathion | |
|---|---|---|---|---|---|---|
| Diagnostic concentration | WHO 0.012 mg/l | Sucen 0.008 mg/l | WHO 0.020 mg/l | Sucen 0.010 mg/l | WHO 0.125 mg/l | Sucen 0.200 mg/l |
| P. Prudente (SP) | 100.0 | 100.0 | 100.0 | 100.0 | 99.2 | 100.0 |
| Marilia (SP) | 99.8 | 100.0 | 100.0 | 100.0 | 91.3 | 99.8 |
| Bauru (SP) | 100.0 | 100.0 | 99.8 | 93.7 | 98.4 | 98.8 |
| Campinas (SP) | 100.0 | 91.0 | 100.0 | 99.5 | 86.0 | 99.3 |
| Araçatuba (SP) | 100.0 | 97.0 | 100.0 | 92.3 | 85.0 | 96.4 |
| Barretos (SP) | 100.0 | 94.0 | 100.0 | 93.8 | 75.2 | 98.5 |
| S.J.R. Preto (SP) | 100.0 | 95.5 | 100.0 | 96.6 | 85.6 | 97.4 |
| Rib. Preto (SP) | 99.2 | 87.5 | 100.0 | 94.1 | 83.4 | 98.8 |
| Santos (SP) | 80.5 | 79.0 | 99.8 | 75.3 | 52.6 | 86.2 |
| J. Guararapes (PE) | 84.5 | 15.0 | 98.7 | 85.1 | 54.1 | 85.7 |
| Maceio (AL) | 89.6 | 22.0 | 99.0 | 83.7 | 23.0 | 90.9 |
| Recife (PE) | 90.0 | 52.0 | 97.0 | 78.8 | 54.7 | 93.2 |
| B. Coqueiros (SE) | 94.3 | 82.5 | 100.0 | 74.2 | 63.4 | 93.0 |
| Arapiraca (AL) | 62.9 | 17.0 | 97.4 | 89.8 | 61.5 | 93.0 |
| Aracaju (SE) | 64.1 | 32.0 | 98.7 | 41.3 | 43.9 | 95.4 |
| Itabaiana (SE) | 65.3 | 12.5 | 97.6 | 40.0 | 55.4 | 80.7 |

*WHO suggested diagnostic concentrations[1]; Sucen: insecticide solutions prepared at local laboratory*

## Discussion and Conclusion

The bioassay laboratory procedures using different susceptible strains and different insecticide solutions for estimating resistance ratios are very well standardized and independent of insecticide solution and susceptible reference strain. The results can be compared with other laboratories using the same WHO-suggested procedure.

The response comparison in bioassays with different diagnostic concentrations shows that the WHO-suggested concentration for fenitrothion (0.2 mg/l), if used under local conditions, would have little chance of discriminating resistant field populations as it is four times the $LC_{99}$ of the susceptible strain. In fact, there is a high probability of underestimating resistance.

It is the opposite for malathion. As the WHO-suggested concentration is low at 0.125 mg/l, there is a high probability of over-estimating resistance.

The WHO-suggested concentration for temephos (0.012 mg/l) is about 50% higher than the local concentration. In the same way as fenitrothion, but to a lower degree, it could underestimate resistance, discriminating resistant populations but not discriminating a decrease in susceptibility.

As the procedure using double the local estimated $LC_{99}$ was able to discriminate resistance as well as the WHO-suggested concentrations, plus the advantage of discriminating decreased susceptibility, this procedure should be adopted in monitoring programmes because the key to resistance management is detecting it as early as possible.

It is important to make it clear that the adoption of a diagnostic concentration is always a compromise between sensitivity and specificity. A reduction in concentration might mean an increase in sensitivity with the risk of picking up non-resistant strains, or the opposite. A possible way to confirm if the classification of a field population (as susceptible or resistant) is correct is to confront it with the results of biochemical and molecular assays. If those results are consistent with the classification given on bioassays, the classification might be corrected.

Sucen laboratory introduced the WHO diagnostic concentration method as a quality control routine along with using four control and eight exposure replications for field populations, and adding two control and four exposure replications for the Rockefeller strain using half the dose concentration used for field populations. The test is considered "valid" if the susceptible strain mortality is higher than 98% as it should be for the $LC_{99}$ concentration. This procedure helps to confirm whether the concentration that the field population is exposed to is really double the $LC_{99}$ and also checks solution and bioassay procedures. The Brazilian National Programme has recommended incorporating this quality control measure since 2000[21].

Great care should be taken in interpreting results. As we propose using double $LC_{99}$, response discrimination should be well understood. A decrease in susceptibility is important information in vector control operations. But laboratory results should only be used as a guide or indication for field efficacy tests and monitoring. The results of monitoring assays with field strains should be interpreted over time and in conjunction with information from the field (selection pressure for resistance). This is an important strategy for avoiding field failure, which occurs when resistance has already spread in field populations.

It is important to consider that monitoring resistance is not solely based on bioassays with diagnostic dose. Besides them, it is important to rely also on biochemical and molecular assays in order to detect resistance mechanisms and their evolution over time, always interpreting results together with the information of insecticide use, as an expression of selection pressure of resistance.

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