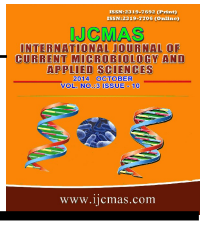
*International Journal of Current Microbiology and Applied Sciences*
***ISSN: 2319-7706*** **Volume 3 Number 10 (2014) pp. 885-892**
**http://www.ijcmas.com**




**Original Research Article**

# Does the dynamic of insecticide resistance affect the results of susceptibility tests?

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**Keywords**

Resistance monitoring tools, insecticide, dynamic, malaria vectors, Benin.

**A B S T R A C T**

Routine monitoring of insecticide resistance in the natural populations of vectors helps us to detect early resistance and improve effectiveness of operational control strategies. We investigated the influence of the dynamic of insecticide resistance on the results of susceptibility tests. Larvae and pupae of *Anopheles gambiae s.l.* mosquitoes were collected from breeding sites in Oueme department. WHO susceptibility tests were conducted on unfed females mosquitoes aged 2-5 days old with impregnated-papers with deltamethrin (0.05%) and bendiocarb (0.1%) in 2008 and in 2010 whereas CDC susceptibility tests were conducted with stock solutions of deltamethrin and bendiocarb (12.5µg per bottle) in 2008 and in 2010. *Anopheles gambiae s.l.* populations from Dangbo were susceptible to deltamethrin in 2008, but they were resistant to this product in 2010 according to both WHO and CDC methods. These *Anopheles gambiae s.l.* populations were susceptible to bendiocarb in 2008 and in 2010 according to both resistance monitoring tools. The current study clearly shows that even if each resistance monitoring tool has its own specificity, the dynamic of insecticide resistance does not affect the results of WHO and CDC susceptibility tests. Susceptibility tests can be assessed either with WHO method or with CDC method from one year to another with *Anopheles* mosquitoes from the same area or location under laboratory conditions.

## Introduction

In 2010, 78 countries reported that they were carrying out insecticide resistance monitoring (WHO, 2011).

Although public health uses account for only a very small fraction of overall insecticide quantities applied, many vector species of public health importance have already developed resistance to one or more insecticides. Development of resistance is a complex and dynamic process and depends upon many factors. Most commonly, when the frequency of resistant insects in a vector population increases, efficacy of the treatment decreases up to the point where the insecticide has to be replaced by another one. Increasing the dosages in an attempt to maintain efficacy is not a recommended

option because of environmental and safety concerns and increased cost of the insecticide. The resistance genes in the vector population may also be driven to even higher frequencies. Replacing an insecticide by a new one has important cost, logistic and sociological implications. In addition, a significant reduction of morbidity and mortality can be achieved only if the efficacy of vector control interventions is continuously maintained at a very high level (IRAC, 2011).

A recent study was carried out by Aïzoun *et al*. (2013a) to investigate the advantages and drawbacks of both WHO and CDC protocols for the determination of insecticide susceptibility in malaria vectors. Another recent study was carried out to investigate the shelf-life and the re-use of a WHO impregnated paper with insecticide under field conditions and of a CDC coated bottle or Wheaton coated bottle with insecticide under laboratory conditions (Aïzoun *et al.*, 2014a). The complementarities and the specificities of these two tools for the determination of insecticide susceptibility in malaria vectors were also recently investigated (Aïzoun and Azondekon, 2014). Thus, there is a need to investigate the influence of the dynamic of insecticide resistance on the results of susceptibility tests.

The aim of this study was to investigate the influence of the dynamic of insecticide resistance on the results of susceptibility tests.

## Materials and Methods

### Study area

The study area is located in the Republic of Benin (West Africa) and includes the department of Oueme in the South-Easthern Benin. The study was carried out in Dangbo district, a rural district of Oueme department. The choice of the study sites took into account the economic activities of populations, their usual protection practices against mosquito bites, indoor residual spraying (IRS) with bendiocarb recently implemented in Oueme department ended in 2010 and peasant practices to control farming pests. These factors have a direct impact on the development of insecticide resistance in the local mosquito vectors. Dangbo is in Oueme region characterized by a sub-equatorial type of climate with four seasons, two rainy seasons (March–July and September–November) and two dry seasons (December-March and August-September). The temperature ranges from 25 to 30°C with the annual mean rainfall between 900mm and 1,500 mm.

### Mosquito sampling

*Anopheles gambiae s.l.* mosquitoes were collected during the rainy seasons (March-July and September-November 2008 and 2010) across Dangbo district selected in South-Easthern Benin. Larvae and pupae were collected from breeding sites and kept in separated labeled bottles. The samples were reared to adults in the CREC (Centre de Recherche Entomologique de Cotonou, Benin) insectary. *Anopheles gambiae s.l* Kisumu, a reference susceptible strain, was used as a control for the bioassay tests. Susceptibility tests were done simultaneously following WHO and CDC protocols on unfed female mosquitoes aged 2–5 days old, reared from the larval and pupal collections. Each *An. gambiae s.l.* sample was separated into two batches: batch 1 was used for susceptibility tests following the WHO protocol and batch 2 for CDC susceptibility tests. All susceptibility tests were conducted in the laboratory of CREC at 25+/−2°C and 70 to 80% relative humidity.

**Testing insecticide susceptibility**
**WHO protocol**

The principle of the WHO bioassay is to expose insects to a given dose of insecticide for a given time to assess susceptibility or resistance. The standard WHO discriminating dosages are twice the experimentally derived 100% lethal concentration (LC100 value) of a reference susceptible strain (WHO, 1998). In this study, two insecticides were tested: deltamethrin (0.05%) and bendiocarb (0.1%). The choice of bendiocarb was justified by its use for Indoor Residual Spraying (IRS) campaign under the financial support of the PMI (President's Malaria Initiative) to control *Anopheles gambiae s.l.* populations from Oueme department in South-Easthern Benin (2008-2010). We used deltamethrin, because it is the insecticide used on PermaNets that are distributed free by the NMCP in the swampy areas of Oueme (2008-2010).

An aspirator was used to introduce 20 to 25 unfed female mosquitoes aged 2–5 days old from batch 1 into five WHO holding tubes (four tests and one control) that contained untreated papers. They were then gently blown into the exposure tubes containing the insecticide impregnated papers. After one-hour of exposure, mosquitoes were transferred back into holding tubes and provided with cotton wool moistened with a 10% honey solution. The number of mosquitoes "knocked down" at 60 minutes and mortalities at 24 hours post treatment were recorded following the WHO protocol (WHO, 1998).

**CDC protocol**

The principle of CDC bottle bioassay is to determine the time it takes an insecticide to penetrate an arthropod, traverse its intervening tissues, get to the target site, and act on that site relatively to a susceptible control. Anything that prevents or delays the compound from achieving its objective of killing the arthropods contributes to resistance.

Diagnostic doses that were applied in the current study were the doses recommended by CDC (Brogdon and Chan, 2010). These doses were checked on the *An. gambiae* Kisumu susceptible reference strain before being applied to field populations. For *An. gambiae s.l.*, the diagnostic dose of 12.5 µg per bottle for both deltamethrin and bendiocarb was used for a diagnostic exposure time of 30 minutes.

The solutions were prepared and the bottles coated according to the CDC protocol (Brogdon and Chan, 2010). Fifteen to 20 unfed female mosquitoes aged 2–5 days old from batch 2 were introduced into four Wheaton bottles of 250 ml each coated with insecticide and one bottle coated with acetone only as the control. The number of dead or alive mosquitoes was monitored at different time intervals (10, 20, 30, 40, 50, 60 minutes) in 2008 and (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 minutes) in 2010. This allowed us to determine the total percent mortality (Y axis) against time (X axis) for all replicates using a linear scale.

**Statistical analysis**

The resistance status of mosquito samples from batch 1 was determined according to the latest WHO criteria (WHO, 2013) as follows:

– Mortality rates between 98%-100% indicate full susceptibility
– Mortality rates between 90%-97% require further investigation

– Mortality rates < 90%, the population is considered resistant to the tested insecticides.

The resistance status of mosquito samples from batch 2 was determined according to the CDC criteria (Brogdon and McAllister, 1998; Brogdon and Chan, 2010). The susceptibility thresholds at the diagnostic time of 30 minutes for pyrethroids and carbamates are:

– Mortality rate = 100%: the population is fully susceptible

– Mortality rate < 100%: the population is considered resistant to the tested insecticides.

Abbott's formula was not used in this study for the correction of mortality rates in either the test-tubes or test-bottles because the mortality rates in all controls was always less than 5% (Abbott, 1987). Analysis using Fisher's exact test and test of proportion was performed on the data sets gathered from the district surveyed and from Kisumu to compare each of two tested insecticides and assess the resistance status of each tested *An. gambiae s.l.* population using both WHO and CDC methods from 2008 to 2010. The software R-2.15.2. (R Development Core Team, 2011) was used for statistical analysis. The significance level was set at 5%.

**Ethical approval**

This study was approved by the Ministry of Health and the Center for Entomological Research of Cotonou, Benin.

## Results and Discussion

**Comparison of resistance status of *An. gambiae s.l.* populations to deltamethrin in 2008 and 2010**

The result of 24 hours post treatment mortality recorded after mosquitoes were exposed to the WHO impregnated papers with deltamethrin (0.05%) were compared to those recorded with CDC bottles bioassays at the susceptibility threshold (30 minutes). The CDC bottles bioassays were performed with stock solutions of deltamethrin (12.5%) (Table 1).

Kisumu strain (control) confirmed its susceptibility status with 100% mortality as a reference strain according to both WHO and CDC methods.

*Anopheles gambiae s.l.* populations from Dangbo were susceptible to deltamethrin in 2008, but they were resistant to this product in 2010 according to both WHO and CDC methods. The percentages of dead mosquitoes recorded with the WHO method were 100% (136/136), 73.8 % (62/84) respectively, whereas with the CDC method the mortality rates recorded were 100% (348/348), 50.76% (33/65) respectively (Table 1).

**Comparison of resistance status of *An. gambiae s.l.* populations to bendiocarb in 2008 and 2010**

The result of 24 hours post treatment mortality recorded after mosquitoes were exposed to the WHO impregnated papers with bendiocarb (0.1%) were compared to those recorded with CDC bottles bioassays at the susceptibility threshold (30 minutes). The CDC bottles bioassays were performed with stock solutions of bendiocarb (1.25%) (Table 1). Kisumu strain (control) confirmed its susceptibility status with 100% mortality as a reference strain according to both WHO and CDC methods.

*Anopheles gambiae s.l.* populations from Dangbo were susceptible to bendiocarb in 2008 and in 2010 according to both WHO

and CDC methods. The percentages of dead mosquitoes recorded with WHO method were 100% (198/198), 100% (45/45) respectively, whereas with the CDC method the mortality rates recorded were 100% (94/94), 98.33% (59/60) respectively (Table 1).

**Specificities of both WHO and CDC methods**

The specificities of both methods are mentioned in Table 2.

*Anopheles gambiae s.l.* Kisumu populations were susceptible to deltamethrin in 2008 and in 2010 according to both WHO and CDC methods. Susceptibility tests done with these methods using deltamethrin with *Anopheles gambiae s.l.* populations from Dangbo showed that these populations were also susceptible to this product in 2008, but they were resistant in 2010. These results showed that *Anopheles gambiae s.l.* populations resistance level to an insecticide, collected in the field, varied from one year to another in a same location. But these variations did not affect the results of WHO and CDC susceptibility tests. In fact, in the current study, the same resistance status were recorded with these resistance monitoring tools from one year to another in the same location.

The slight decrease of susceptibility obtained with *Anopheles gambiae s.l.* populations from Dangbo exposed to bendiocarb in 2010 with CDC bottle bioassay was not synonymous with resistance. A similar pattern was already observed with *Anopheles gambiae s.l.* populations from Adjara and Dangbo exposed to bendiocarb in Oueme department in South-Easthern Benin (Aïzoun *et al.*, 2013a). *Anopheles gambiae s.l.* populations from Dangbo were susceptible to bendiocarb in 2008 and 2010 according to both WHO and CDC methods. Similar results were already observed by Aïzoun *et al*. (2013b) with *Anopheles gambiae s.l.* from Seme in the same department as Dangbo. A recent study carried out by Aïzoun *et al.* (2014b) also showed fully susceptibility of *Anopheles gambiae s.l.* populations from the central part of Benin to carbamates. Howewer, the resistance level to bendiocarb of *Anopheles gambiae s.l.* populations from Dangbo need to be monitored because of the slight decrease of susceptibility obtained with these *Anopheles gambiae s.l.* populations exposed to bendiocarb in 2010 with the CDC bottle bioassay.

Regarding the specificities of each method, the CDC bottle bioassay is more adaptable in the field conditions whereas there is logical complexity with WHO susceptibility test. The CDC bottle bioassay determines the time it takes an insecticide to penetrate an arthropod, traverse its intervening tissues, get to the target site, and act on that site relative to a susceptible control. The WHO plastic cylinder tube test determines the percent mortality or mortality rates of malaria vectors to insecticide. The WHO bioassays utilize plastic cylinder tubes whereas the CDC bottles bioassays use 250 ml Wheaton bottles which are made from glass. The WHO papers do not need to be treated by oneself before their utilization because they are ordered in the impregnated form. Conversely, the CDC bottles need to be coated with insecticide by oneself before each bioassay. In fact, the shelf-life and the re-use of pre-prepared bottles are still not well documented or studied in laboratory conditions (Aïzoun *et al.*, 2013a). But a recent study was carried out for this purpose (Aïzoun *et al.*, 2014a).

**Table.1** Susceptibility data recorded according to both WHO and CDC methods in 2008 and in 2010

| | | | Number tested | | %Mortality | | Resistance status | |
|---|---|---|---|---|---|---|---|---|
| Populations | Years | Insecticides | WHO | CDC | WHO | CDC | WHO | CDC |
| Kisumu(Control) | 2008 | Deltamethrin | 200 | 200 | 100 | 100 | S | S |
| | 2010 | Deltamethrin | 103 | 110 | 100 | 100 | S | S |
| | 2008 | Bendiocarb | 200 | 200 | 100 | 100 | S | S |
| | 2010 | Bendiocarb | 99 | 111 | 100 | 100 | S | S |
| Dangbo | 2008 | Deltamethrin | 136 | 348 | 100 | 100 | S | S |
| | 2010 | Deltamethrin | 84 | 65 | 73.8 | 50.76 | R | R |
| | 2008 | Bendiocarb | 198 | 94 | 100 | 100 | S | S |
| | 2010 | Bendiocarb | 45 | 60 | 100 | 98.33 | S | S |

**Table.2** Specificities of both WHO and CDC methods

| |
|---|
| -CDC bottle bioassay is more adaptable in the field conditions whereas there is logical complexity with WHO susceptibility test |
| -The insecticide formulations (liquid or powder) and bottle positions (on the bottom or on the side) do not influence the results of Centers for Diseases Control and Prevention (CDC) bottle bioassay during resistance monitoring using this tool |
| -Even if the bottom and the cap of WHO cylinder plastic tube are not impregnated, that does not affect the results recorded with WHO resistance monitoring tool |
| -WHO bioassays utilize cylinder plastic tubes whereas CDC bottles bioassays use 250 ml Wheaton bottles which are made from glass |
| -WHO susceptibility test uses impregnated-papers with insecticide whereas CDC bottle need to be coated with insecticide by oneself before each bioassay |
| -WHO cylinder plastic tube test determines directly the percent mortality or mortality rate of malaria vectors to insecticide |
| -CDC bottle bioassay determines the time it takes an insecticide to penetrate an arthropod, traverse its intervening tissues, get to the target site, and act on that site relative to a susceptible control |

In addition, another recent study was also carried out to investigate the advantages and drawbacks of both the WHO and CDC methods (Aïzoun *et al.*, 2013a). The insecticide formulations (liquid or powder) and bottle positions (on the bottom or on the side) do not influence the results obtained with Centers for Diseases Control and Prevention (CDC) bottle bioassay during resistance monitoring using this tool. However, it would be useful to maintain test bottles intact on the lab bench of manipulation in the laboratory without moving them during mortality recording. Even if the bottom and the cap of WHO plastic cylinder tube are not impregnated, that does not affect the results recorded with WHO resistance monitoring tool. Recently, in 2013, WHO revised the protocol of 1998 as a new

protocol for the determination of insecticide susceptibility in malaria vectors (WHO, 2013) whereas a new protocol invention by Brogdon is in progress and is called" Intensity bottle bioassay".

The current study clearly shows that even if each resistance monitoring tool has its own specificity, the dynamic of insecticide resistance does not affect the results obtained with WHO and CDC susceptibility tests. Susceptibility tests can be assessed either with WHO method or with CDC method from one year to another with *Anopheles* mosquitoes from the same area or location under laboratory conditions.

## Acknowledgements

We are grateful to the Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (MESRS) of Benin which financially supported this study and the doctoral training of Nazaire. We are also grateful to the President's Malaria Initiative (PMI) of the U.S. Government through USAID which financially supported certain of research activities of Nazaire in the framework of his doctoral training. We would like to thank Dr William G. BROGDON from CDC Atlanta, USA who supplied us the reagents used for CDC bioassays. The authors would also like to thank Frederic OKE-AGBO for statistical analysis and Damien TODJINOU for providing technical assistance. Tel: (229) 95317939.

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