



Original Research Article

Similarity between WHO susceptibility test and CDC bottle bioassay, two important tools for the determination of insecticide susceptibility in malaria vectors

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ABSTRACT

Keywords

Resistance monitoring tools, Insecticide, Similarity, Malaria vectors, Benin

The Knowledge regarding the similitude between both WHO and CDC methods, was useful in the assessment of insecticide susceptibility tests in malaria vectors. The current study aimed at an investigation on the similitude between both WHO and CDC methods useful for the determination of the insecticide susceptibility in malaria vectors. Larvae and pupae of *Anopheles gambiae s.l.* mosquitoes were collected from breeding sites in Littoral and Alibori departments. WHO susceptibility tests were conducted on unfed females mosquitoes aged 2–5 days old with impregnated-papers with fenitrothion (1%) and bendiocarb (0.1%) whereas CDC susceptibility tests were conducted with stock solutions of bendiocarb (12.5µg per bottle) and fenitrothion (50µg per bottle). Both *An. gambiae* Suru-lere and Kandi populations were susceptible to fenitrothion and bendiocarb according to both methods. The current study clearly shows that both WHO susceptibility test and CDC bottle bioassay are two important tools for the monitoring of insecticide resistance, a necessary element of any medium-scale or large-scale deployment of an insecticidal intervention. There is similarity between both methods.

Introduction

Malaria is a severe public health problem, causing an estimated 225 million disease cases and 781,000 deaths per year (WHO, 2010). Most victims are children under five years old living in sub-Saharan Africa (WHO, 2010). Malaria is transmitted by *Anopheles* mosquitoes, and because there is currently no vaccine available, vector control is one of the most important means of malaria prevention.

This vector control is generally done with insecticides. In Benin, as across Africa, malaria control relies heavily on vector control through the use of insecticide-treated nets (ITN) and indoor residual spraying (IRS).

Bioassays with WHO diagnostic test kits were recommended in the assessment of insecticide susceptibility in malaria vectors.

The protocol recommended by WHO in 1970 was revised for research results reliability in 1981 and then in 1998. Recently, in 2013, WHO revised the protocol of 1998 as a new protocol for the determination of insecticide susceptibility in malaria vectors (WHO, 2013). Another protocol was invented by Brogdon and McAllister (1998) and then revised by Brogdon and Chan (2010) for the determination of insecticide susceptibility in malaria vectors.

A recent study was carried out by Aizoun *et al.* (2013a) to investigate the advantages and drawbacks of both protocols. Another recent study was carried out to investigate the self-life and 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 (Aizoun *et al.*, 2014b). Thus, there is a need to investigate the similitude between WHO susceptibility test and CDC bottle bioassay, two resistance monitoring tools.

The aim of this study was to investigate the similitude between WHO susceptibility test and CDC bottle bioassay, two resistance monitoring tools.

Materials and Methods

Study area

Study areas are located in Republic of Benin (West Africa) and include two departments, Littoral and Alibori departments. In Alibori department located in Northern Benin, the study was carried out in Kandi district, a cotton growing area whereas in Littoral department located in the south of Benin, the study was carried out in Cotonou district, more precisely in an urban location, Surulere.

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 in progress in the northern Benin since 2011 and with pyrimiphos-methyl since 2013 and IRS with bendiocarb recently implemented in the south of the country, and peasant practices to control farming pests. These factors have a direct impact on the development of insecticide resistance in the local mosquito vectors. The southern zone (Cotonou) is characterized by a tropical coastal guinean climate with two rainy seasons (April–July and September–November). The mean annual rainfall is over 1,500 mm. The northern zone (Kandi) is characterized by a Sudanian climate with only one rainy season per year (May–October) and one dry season (November–April). The temperature ranged from 22 to 33°C with the annual mean rainfall of 1,300 mm.

Mosquito sampling

Anopheles gambiae s.l. mosquitoes were collected during the rainy season (May–October 2012) across Kandi district selected in northern Benin. *Anopheles gambiae s.l.* mosquitoes were also collected during the rainy seasons (April–July and September–November 2012) across Cotonou district selected in southern Benin. Larvae and pupae were collected from breeding sites and kept in separated labeled bottles for each locality. The samples were reared to adults in the insectary of CREC (Centre de Recherche Entomologique de Cotonou, Benin).

Anopheles gambiae 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: fenitrothion (1%) 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) in progress in the north of the country since 2011.

This choice was also justified by the IRS with bendiocarb recently implemented in the south of the country thank to PMI. We used fenitrothion, an organophosphate to assess cross-resistance with bendiocarb in districts surveyed.

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 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 & 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 bendiocarb was used for a diagnostic exposure time of 30 minutes whereas the diagnostic dose of 50µg per bottle for fenitrothion was used for a diagnostic exposure time of 30minutes.

The solutions were prepared and the bottles coated according to the CDC protocol (Brogdon & 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 control bottle of 250 ml coated with acetone only.

The number of dead or alive mosquitoes was monitored at different time intervals (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 minutes). 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 & McAllister, 1998; Brogdon & Chan, 2010). The susceptibility thresholds at the diagnostic time of 30 minutes for organophosphates 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 districts surveyed and from Kisumu to compare each of two tested insecticides and assess the resistance status of each tested *An. gambiae* population using both WHO and CDC methods.

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

Susceptibility of *An. gambiae s.l.* populations to fenitrothion

The results of 24 hours mortality recording after mosquito exposure to WHO impregnated papers with fenitrothion (1%) were compared to those recorded with CDC bottles bioassays at the susceptibility threshold (30 minutes). CDC bottles bioassays were performed with stock solutions of fenitrothion (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 Suru-lere and Kandi were susceptible to fenitrothion according to both WHO and CDC methods. The percentages of dead mosquitoes recorded were 100% (99/99) and 100% (100/100) respectively with WHO method whereas with CDC method, these percentages were 100% (67/67) and 100% (50/50) respectively (Table 1).

The table 1 shows a similitude between both methods. In fact, WHO method and CDC method gave comparable results regarding the susceptibility of *An. gambiae s.l.* populations to fenitrothion.

Susceptibility of *An. gambiae s.l.* populations to bendiocarb

The results of 24 hours mortality recording after mosquito exposure to WHO impregnated papers with bendiocarb (0.1%)

were compared to those recorded with CDC bottles bioassays at the susceptibility threshold (30 minutes). CDC bottles bioassays were performed with stock solutions of bendiocarb (1.25%) (Table 2).

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 Suru-lere and Kandi were susceptible to bendiocarb according to both methods. The percentages of dead mosquitoes recorded were 100% (96/96) and 100% (99/99) respectively according to WHO method whereas the mortality rates recorded with CDC method were 100% (79/79) and 100% (42/42) respectively (Table 2).

The table 2 shows a similitude between both methods. In fact, WHO method and, CDC method gave comparable results regarding the susceptibility of *An. gambiae s.l.* populations to bendiocarb.

Anopheles gambiae s.l. populations from Suru-lere and Kandi were fully susceptible to both fenitrothion and bendiocarb according to both WHO and CDC methods. Similar results were already observed by Aïzoun *et al.* (2013b) with *Anopheles gambiae s.l.* from Seme and Kandi and maybe explained by the absence of selection oppression of *Ace-1* gene as there was no IRS campaign in Alibori and Littoral departments. But, as Kandi is a cotton growing area, the resistance levels to these products of these *Anopheles gambiae s.l.* populations need to be monitored. A recent study carried out by Aïzoun *et al.* (2014a) also showed fully susceptibility of *Anopheles gambiae s.l.* populations from the central part of Benin to organophosphates and carbamates.

Given the importance of insecticide use in malaria vector control, and the continuous development of insecticide resistance in malaria mosquitoes, monitoring the susceptibility of vectors to organophosphates and carbamates is essential as they were two insecticide classes useful for Indoor Residual Spraying (IRS) implementation.

Routine monitoring of insecticide resistance in the natural populations of vectors helps us to detect early resistance and improve effectiveness of operational control strategies (Aïzoun *et al.*, 2013a). Insecticide resistance in a vector population is initially detected and characterized by using some sort of bioassay to determine whether a particular insecticide is able to control a vector at a given time. Ideally, this fundamental question should be answered before a particular insecticide is chosen and procured for vector control (Brogdon and Chan, 2010). So, insecticide susceptibility in malaria vectors is the first important step in insecticide resistance surveillance.

In 2013, World Health Organization (WHO) released new guidance about recommended test procedures for insecticide resistance (WHO, 2013), including recommended equipment and supplies, and a detail description of test conditions and protocols. The document contains recommendations on how susceptibility test results should be recorded and reported, including how mortality and knock-down rates should be calculated, how susceptibility test results should be interpreted, and how susceptibility test results should be reported.

Current testing procedures also include the bottle bioassay developed by the United States Centers for Disease control and prevention (CDC). Regarding the bottle bioassay procedures, the protocol describes the material and reagents needed how to

determine the diagnostic dose and diagnostic time, how to prepare the stock solutions, how to mark the clean and dried bottles, how to coat the bottles, how to perform the bottle bioassay and how the bottle bioassay can be used with synergists. This document also contains recommendations on how susceptibility test results should be recorded and reported, including how mortality and knock-down rates should be calculated, how susceptibility test results should be interpreted, and how susceptibility test results should be reported (Brogdon and Chan, 2010).

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 (Aïzoun *et al.*, in press). 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 (Aïzoun *et al.*, in press). 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 (Aïzoun *et al.* in press). A recent study carried out by Aïzoun *et al.* (in press) showed 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. A recent study was also carried out by Aïzoun *et al.*, (in press) and showed that a Wheaton treated bottle with 100µg a.i DDT per bottle, 12.5µg a.i bendiocarb per bottle and 50µg a.i fenitrothion per bottle could be used four times during four consecutive days in laboratory conditions. Another recent study was also carried out to investigate the complementarities and the specificities of both WHO plastic cylinder tube test and CDC bottle bioassay (Aïzoun *et al.*, 2014c).

The current study clearly shows that both WHO susceptibility test and CDC bottle bioassay are two important tools for the monitoring of insecticide resistance, a necessary element of any medium-scale or large-scale deployment of an insecticidal intervention. There is similarity between both methods.

Table.1 Susceptibility of *An. gambiae s.l.* populations to organophosphates

Populations	Insecticides	Number tested		% Mortality		Resistance status	
		WHO	CDC	WHO	CDC	WHO	CDC
Kisumu (Control)	Fenitrothion	102	33	100	100	S	S
Suru-lere	Fenitrothion	99	67	100	100	S	S
Kandi	Fenitrothion	100	50	100	100	S	S

Table.2 Susceptibility of *An. gambiae s.l.* populations to carbamates

Populations	Insecticides	Number tested		% Mortality		Resistance status	
		WHO	CDC	WHO	CDC	WHO	CDC
Kisumu (Control)	Bendiocarb	101	26	100	100	S	S
Suru-lere	Bendiocarb	96	79	100	100	S	S
Kandi	Bendiocarb	99	42	100	100	S	S

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