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Original Research Article

Importance of Two Tools for the Determination of Insecticide Susceptibility in Malaria Vectors: WHO Plastic Cylinder Tube Test and CDC Bottle Bioassay

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Abstract

The present study was aimed to investigate the complementarities between WHO and CDC bioassay methods and the specificities of each method. Larvae and pupae of *Anopheles gambiae s.l.* mosquitoes were collected from breeding sites in Atacora and Alibori departments. WHO susceptibility tests were conducted on unfed females mosquitoes aged 2-5 days old with impregnated-papers with deltamethrin (0.05%), fenitrothion (1%), and bendiocarb (0.1%) whereas CDC susceptibility tests were conducted with stock solutions of deltamethrin and bendiocarb (12.5µg per bottle) and fenitrothion (50µg per bottle). *Anopheles gambiae s.l.* populations from Malanville were resistant to deltamethrin and susceptible to bendiocarb according to both WHO and CDC methods. Regarding *Anopheles gambiae s.l.* populations from Tanguieta, they were resistant to bendiocarb and fenitrothion according to both methods. The current study clearly shows that even if each resistance monitoring tool has its own specificity, the WHO method and CDC method gave comparable results. Both WHO and CDC bioassays are two important tools for the determination of insecticide susceptibility in malaria vectors.

Keywords

Anopheles
Benin
Insecticide
Malaria vectors
Resistance monitoring tools

Introduction

In 2008, it was estimated that 243 million malaria cases occurred worldwide. The vast majority of those cases (85%) were in the African Region, followed by South-East Asia (10%) and Eastern Mediterranean Regions (4%). Malaria accounted for an estimated 863,000 deaths in 2008, of which 89% were in African Region, followed by Eastern Mediterranean (6%) and South-East Asia Regions (5%) (WHO, 2009).

Malaria vector control is intended to protect individuals against infected mosquito bites and, at the community level, to reduce the intensity of local malaria transmission. The two most

powerful and most broadly applied interventions are insecticide-treated nets (ITN) and indoor residual spraying (IRS) (WHO, 2009).

Bioassays with WHO diagnostic test kits were recommended in the assessment of insecticide susceptibility in malaria vectors. The protocol recommended by WHO in 1963 was revised in 1970, in 1981 and then in 1998 for research results reliability. 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 Aïzoun et al. (2013a) to investigate the advantages and drawbacks of both protocols. 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). Thus, there is a need to investigate the importance of both WHO and CDC bioassays, two tools for the determination of insecticide susceptibility in malaria vectors.

The aim of this study was to investigate the importance of both WHO and CDC bioassays, two tools used for the determination of insecticide susceptibility in malaria vectors.

Materials and methods

Study area

Study areas are located in the Republic of Benin (West Africa) and include two departments, the Atacora and Alibori departments. In the Atacora department located in North-Western Benin, the study was carried out in Tanguieta district under IRS with bendiocarb since 2011 and under IRS with pirimiphos-methyl since 2013 whereas in the Alibori department located in the far north of Benin, the study was carried out in Malanville district, a rice growing area located near the Niger River.

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) in progress in some of these localities 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 northern zone (Tanguieta and Malanville) is characterized by a Sudanian climate with only one rainy season per year (May to October) and one dry season (November to April). The temperature ranged from 22 to 33°C with the annual mean rainfall of 1,300 mm (Aïzoun et al., 2013b).

Mosquito sampling

Anopheles gambiae s.l. mosquitoes were collected during the rainy season (May to October 2012) across Tanguieta and Malanville districts selected in northern 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, three insecticides were tested: deltamethrin

(0.05%), 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. We used fenitrothion, an organophosphate to assess cross-resistance with bendiocarb in Tanguieta district surveyed. Deltamethrin was used to check if *Anopheles gambiae s.l.* populations from Malanville resistance level to this product was high considering the relatively low amount of insecticide use in this area. An aspirator was used to introduce 20 to 25 unfed female mosquitoes aged 2–5 days 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 hr post treatment were recorded following the WHO protocol (WHO, 1998).

CDC protocol

The principle of the 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 min. whereas the diagnostic dose of 50µg per bottle for fenitrothion was used for a diagnostic exposure time of 30 min. 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 (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 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, organophosphates, and carbamates are:

- Mortality rate = 100%: the population is fully susceptible.
- Mortality rate < 100%: the population is considered resistant to tested insecticides.

Analysis using Fisher's exact test and test of proportion was performed on data sets gathered from the districts surveyed and from Kisumu to compare each of three tested insecticides and assess the resistance status of each tested *An. gambiae s.l.* 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

Susceptibility of *An. gambiae s.l.* populations to deltamethrin, fenitrothion and bendiocarb

The result of 24 h post treatment mortality recorded after exposing mosquitoes to the WHO impregnated papers with deltamethrin (0.05%), fenitrothion (1%) and 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 deltamethrin (1.25%), fenitrothion (5%) and 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 Malanville were resistant to deltamethrin according to both WHO and CDC methods. The percent mortality recorded was 13.09% (11/84) with WHO method whereas with CDC method, the percent mortality was 0% (0/21).

Table.1 Susceptibility data recorded according to both WHO and CDC methods

Populations	Insecticides	Number tested		% Mortality		Resistance status	
		WHO	CDC	WHO	CDC	WHO	CDC
Kisumu (Control)	Deltamethrin	92	25	100	100	S	S
	Fenitrothion	94	33	100	100	S	S
	Bendiocarb	93	26	100	100	S	S
Malanville	Deltamethrin	84	21	13.09	0	R	R
	Bendiocarb	93	48	98.92	97.91	S	S
Tanguieta	Fenitrothion	32	116	84.37	76.72	R	R
	Bendiocarb	57	76	56.14	78.94	R	R

Table.2 Complementarities between both WHO and CDC methods and specificities of each method

WHO and CDC methods	Complementarities	- Both WHO and CDC methods allow to determine insecticide susceptibility in malaria vectors
		- CDC bottle bioassay can be assessed when the results obtained with WHO method require further investigation in order to clarify the resistance status of malaria vectors to insecticides
		- Both WHO and CDC protocols require the assessment of insecticide susceptibility tests in malaria vectors before any identification of mechanisms involved in malaria vector resistance to insecticides
		- Mosquitoes from the assessment of both WHO and CDC methods can be used for PCR test.
		- Both WHO and CDC protocols recommend to use female *Anopheles* mosquitoes in the assessment of insecticide susceptibility test in malaria vectors
		- The purchase of both WHO and CDC kits useful in the assessment of susceptibility tests are centralized.
	Specificities	- 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 - CDC bottle bioassay is more adaptable in the field conditions whereas there is logical complexity with WHO susceptibility test

Anopheles gambiae s.l. populations from Malanville were susceptible to bendiocarb according to both methods. The percentage of dead mosquitoes recorded was 98.92% (92/93) according to the WHO method whereas the mortality rate recorded with the CDC method was

97.91% (47/48). Regarding *Anopheles gambiae s.l.* populations from Tanguieta, they were resistant to bendiocarb according to both WHO and CDC methods with mortality rates of 56.14% (32/57) and 78.94% (60/76) respectively. *Anopheles gambiae s.l.* populations from Tanguieta were also resistant to fenitrothion according to both WHO and CDC methods with mortality rates of 84.37% (27/32) and 76.72% (89/116) respectively. The table 1 shows a similarity between both methods. In fact, WHO method and CDC method gave comparable results (Table 1). The complementarities and specificities of both methods are mentioned in Table 2.

Discussion

Anopheles gambiae s.l. populations from Malanville were resistant to deltamethrin according to both the WHO and CDC methods. Deltamethrin resistance in *Anopheles gambiae* s.l. populations from Malanville was already observed by Aïzoun et al. (2014b) and maybe explained by the presence of higher *kdr* mutation frequency but also by the presence of detoxifying enzymes such as higher oxidase activity (Djègbé et al., 2011). *Anopheles gambiae s.l.* populations from Malanville were fully susceptible to bendiocarb according to both methods. Similar results were already observed by Aïzoun et al. (2013b) and maybe explained by the absence of selection pressure of *Ace-1* gene as there was no IRS campaign in Alibori department more precisely in the Malanville district.

Anopheles gambiae s.l. populations from Tanguieta were resistant to bendiocarb and fenitrothion according to both WHO and CDC methods. Fenitrothion and bendiocarb resistance in *Anopheles gambiae s.l.* populations from Tanguieta may be explained by the presence of selection pressure of *Ace-1* gene. In fact, Tanguieta is a district under IRS with bendiocarb. It is also a district where high amounts of insecticides were used in cotton growing areas. Similar results were already observed by Aïkpon et al. (2013). In addition, Aïzoun et al. (2013c) showed that esterases might also play a little role in *Anopheles gambiae s.l.* populations from Tanguieta resistant to bendiocarb.

Insecticide susceptibility in malaria vectors can be detected either with the WHO method or with the CDC method. In addition, when the results obtained with the WHO method required further investigation, the CDC method can be assessed to clarify the real vector resistance status regarding the used insecticide. Both WHO and CDC protocols required the use of diagnostic doses or concentrations already pre-established for numerous insecticides used in public health against malaria vectors in the assessment of insecticide susceptibility tests in malaria vectors. But, in case where these diagnostic doses did not correspond to those of some local vectors, the CDC protocol required to establish its own diagnostic doses for these local vectors.

Regarding the new PCR-based molecular tests used to detect the presence of *kdr* mutations in *An. gambiae* from western parts of Africa, this is not a generally recommended practice and the WHO susceptibility tests (or the CDC bottle bioassays) should always be carried out in addition to the molecular assays (WHO, 2013). In fact, after the assessment of insecticide susceptibility tests with either the WHO method or CDC method, mosquitoes from these tests can be used for PCR-based molecular tests. Both the WHO and CDC protocols required the use of female *Anopheles* mosquitoes only in the assessment of insecticide susceptibility tests in malaria vectors. The use of males is not recommended for resistance monitoring as they are usually smaller and more fragile than females, and therefore tend to have higher control mortalities. For this reason, susceptibility testing is conducted using only female mosquitoes (Brogdon and Chan, 2010; WHO, 2013).

In addition, Aïzoun et al. (2014c) showed that male *Anopheles* mosquitoes were not able to transmit malaria. Some resistance mechanisms are also sex- linked, and one can be misled by using males in the control (Brogdon and Chan, 2010). WHO assay requires the purchase of all components (WHO kit) from a centralized source and that allows easy comparison of results from one year to another and from one study site to another (Aïzoun et al., 2013a). Test kits and insecticide-impregnated papers are prepared on behalf of WHO by the Vector Control Research

Unit of the Universiti Sains Malaysia, which is based in Penang, Malaysia for this purpose. In similar way, CDC assay requires the procurement of all components (CDC kit) from a centralized source (CDC Atlanta, USA). Test kits and test insecticides should be procured from the CDC for this purpose (WHO, 2013). According to Perea et al. (2009), the bottle assay is a simple, flexible and robust resistance monitoring tool that was able to discriminate pyrethroid (deltamethrin) resistance in mosquito populations as effectively as the WHO assay.

Regarding the specificities of each method, it is important to mention that 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 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). 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 (Aïzoun et al., 2014d). 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 (Aïzoun et al., 2014e).

The current study clearly shows that even if each resistance monitoring tool has its own specificity, the WHO method and CDC method gave comparable results. Both WHO and CDC bioassays are two important tools for the determination of insecticide susceptibility in malaria vectors.

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