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Centre for Disease Control and Prevention (CDC) bottle bioassay: A real complementary method to World Health Organization (WHO) susceptibility test for the determination of insecticide susceptibility in malaria vectors

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In order to investigate the complementarities between both World Health Organization (WHO) and Centre for Disease Control and Prevention (CDC) methods and the specificities of each method useful for the determination of the insecticide susceptibility in malaria vectors, larvae and pupae of *Anopheles gambiae s.l.* mosquitoes were collected from the breeding sites in Littoral department. WHO susceptibility tests were conducted on unfed female mosquitoes aged 2 to 5 days old with impregnated-papers with deltamethrin (0.05%) and bendiocarb (0.1%), whereas CDC susceptibility tests were conducted with stock solutions of deltamethrin and bendiocarb (12.5 µg per bottle). WHO method and CDC method gave comparable results. There were complementarities between both methods. Both WHO and CDC methods are two complementary tools for the determination of insecticide susceptibility in malaria vectors. However, each method has also its own specificity.

Key words: Complementary tools, insecticide, resistance, malaria vectors.

INTRODUCTION

Monitoring of insecticide resistance is a necessary element of any medium-scale or large-scale deployment of an insecticidal intervention. In 2010, 78 countries reported that they were carrying out insecticide resistance monitoring (World Health Organization (WHO), 2011). Current methods of malaria control are highly dependent on a single class

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of insecticides, the pyrethroids, which is the only insecticide class used for insecticide treated nets (ITNs) and accounts for approximately 77% of indoor residual spraying (IRS) in terms of spray area covered. The widespread use of a single class of insecticide increases the risk that mosquitoes will develop resistance to it. This risk is of particular concern in sub-Saharan Africa, where insecticidal vector control is being deployed with unprecedented levels of coverage. Resistance to pyrethroids has been reported in 27 countries in sub-Saharan Africa; the point at which this reduces the effectiveness of vector control is still uncertain and may depend on the locally identified resistance mechanism (WHO, 2011).

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. A recent study was carried out to investigate the advantages and drawbacks of both Centre for Disease Control and Prevention (CDC) bottle bioassay and WHO susceptibility test (Aïzoun et al., 2013a).

The aim of this study was to investigate the complementarities between both WHO and CDC methods and the specificities of each method used for the determination of the insecticide susceptibility in malaria vectors.

METHODOLOGY

Study area

The study area is located in Republic of Benin (West Africa) and includes the department of Littoral in the Southern Benin. The study was carried out in Cotonou district more precisely in Ladji location. Ladji is a large village on the outskirts or on the periphery of Cotonou, the capital of Benin. This village floods annually during the rainy season, creating breeding sites for *Anopheles gambiae*. This location is crossed by the Nokoue Lake's streams. The choice of the study sites took into account the economic activities of populations, their usual protection practices against mosquito bites, and peasant practices to control farming pests. These factors have a direct impact on the development of insecticide resistance in the local mosquito vectors. Cotonou is characterized by a tropical coastal guinean climate with two rainy seasons (April to July and September to November). The mean annual rainfall is over 1,500 mm.

Mosquito sampling

A. gambiae s.l. mosquitoes were collected during the rainy seasons (April to July and September to November, 2011) across Cotonou district selected in southern Benin. Larvae and pupae were collected from breeding sites and kept in separated labeled bottles. The samples were reared to adults in the Centre de Recherche Entomologique de Cotonou, Benin (CREC) insectary. *A. 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 to 5 days old, reared from the larval and pupal collections. *A. 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 CREC laboratory 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 IRS campaign under the financial support of the President's Malaria Initiative (PMI) to control *A. gambiae s.l.* populations from Ouemé department in southern Benin (2008 to 2010). We used deltamethrin because it is the insecticide used on PermaNets that are distributed free by the National Malaria Control Programme (NMCP) in the swampy areas of Ouemé (2008 to 2010). Ouemé department has a boundary with Littoral department. An aspirator was used to introduce 20 to 25 unfed female mosquitoes aged 2 to 5 days old from batch 1 into five WHO holding tubes (four tests and one control) that contained untreated papers. WHO susceptibility test was done once without replicate. They were then gently blown into the exposure tubes containing the insecticide impregnated papers. After one-hour 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 min and mortalities at 24 h 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 relative 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 *A. gambiae* Kisumu susceptible reference strain before being applied to field populations. For *A. 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. 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 to 5

Table 1. Susceptibility of *Anopheles gambiae s.l.* populations to pyrethroids.

Populations	Insecticides	Number tested		% Mortality		Resistance status		P-values
		WHO	CDC	WHO	CDC	WHO	CDC	
Kisumu (Control)	Deltamethrin	92	98	100	100	S	S	P=1
Ladji	Deltamethrin	72	64	100	100	S	S	P=1

days old from batch 2 were introduced into four 250 ml Wheaton bottles coated with insecticide and one control bottle coated with acetone only. CDC susceptibility test was also done once without replicate. The number of dead or alive mosquitoes was monitored at different time intervals (15, 30, 35, 40, 45, 60, 75, 90, 105 and 120 min). This allowed us to determine the total percent mortality (Y axis) against time (X axis) for all replicates using a linear scale.

Data analysis

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

1. Mortality rates between 98 to 100% indicate full susceptibility.
2. Mortality rates between 90 to 97% require further investigation.
3. 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 min for pyrethroids and carbamates are:

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

Abbott's modified 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 locality of Ladji surveyed and from Kisumu to compare each of two tested insecticides and assess the resistance status of each tested *A. gambiae* population using both WHO and CDC methods. The software R-2.15.2. (R Development Core Team, 2011) was used for the statistical analysis. The significance level was set at 5%.

RESULTS

Susceptibility of *A. gambiae s.l.* populations to pyrethroids

The results of 24 h mortality recording after mosquito exposure to WHO impregnated papers with deltamethrin (0.05%) were compared to those recorded with CDC bottles bioassays at the susceptibility threshold (30 min). CDC bottles bioassays were performed with stock solutions of deltamethrin (1.25%) (Table 1). Kisumu strain (control) confirmed its susceptibility status as a reference strain according to both WHO and CDC methods. The percentage of dead mosquitoes recorded with deltamethrin was 100% (92/92) with WHO method and 100% (98/98) with CDC method. *A. gambiae s.l.* populations from Ladji were also susceptible to deltamethrin according to both methods. The percentage of dead mosquitoes recorded was 100% (72/72) according to WHO method whereas the mortality rate recorded with CDC method was 100% (64/64).

Susceptibility of *A. gambiae s.l.* populations to carbamates

The results of 24 h 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 min). CDC bottles bioassays were performed with stock solutions of bendiocarb (1.25%) (Table 2). Kisumu strain (control) confirmed its susceptibility status as a reference strain according to both WHO and CDC methods. The percentage of dead mosquitoes recorded with bendiocarb was 100% (93/93) with WHO method and 100% (100/100) with CDC method. *A. gambiae s.l.* populations from Ladji were also susceptible to bendiocarb according to both methods. The percentage of dead mosquitoes recorded was 100% (56/56) according to WHO method whereas the mortality rate recorded with CDC method was 100% (56/56).

Complementarities between both WHO and CDC methods and specificities of each method

The complementarities and specificities of both methods are mentioned in Table 3.

DISCUSSION

A. gambiae s.l. Kisumu populations were susceptible to deltamethrin according to both WHO and CDC methods.

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

Populations	Insecticides	Number tested		% Mortality		Resistance status		P-values
		WHO	CDC	WHO	CDC	WHO	CDC	
Kisumu (Control)	Bendiocarb	93	100	100	100	S	S	P=1
Ladji	Bendiocarb	56	56	100	100	S	S	P=1

Table 3. Complementarities between both WHO and CDC methods and specificities of each method.

Parameter		Both WHO and CDC methods allow to determine insecticide susceptibility in malaria vectors
WHO and CDC methods	Complementarities	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 malara vectors
		The purchasse 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

Susceptibilirty tests done with these methods using deltamethrin with *A. gambiae s.l.* Ladji populations showed that these populations were also susceptible to this product. Aïzoun et al. (2013b) have recently shown that Ladji is crossed by the Nokoue Lake's streams which sweep and converge the environmental pollutants and pesticide residues in this locality, and these xenobiotics available in larval breeding sites in Ladji may be one of the possible factors selected for pyrethroid resistance in *A. gambiae* populations in this locality. However, *A. gambiae s.l.* Ladji populations were fully susceptible to deltamethrin in the current study. These results showed that *A. gambiae s.l.* populations resistance level varied from one year to another in a same location. So, the results obtained with both methods are comparable. A similar pattern was also obtained with bendiocarb. In fact,

both *A. gambiae s.l.* Kisumu and Ladji populations were fully susceptible to bendiocarb according to both methods. These results showed that *A. gambiae s.l.* populations from Southern Benin were still fully susceptible to bendiocarb. Simmilar results were also recently obtained by Aïzoun et al. (2013c) in Seme district in Ouemé Department.

Insecticide susceptibility in malaria vectors can be detected either with WHO method or with CDC method. In addition, when the obtained results with WHO method require further investigation, 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, CDC protocol required its own diagnostic doses establishment for these local vectors. Regarding the new PCR-based molecular tests used to detect the presence of *kdr* mutations in *A. 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 WHO method or CDC method, mosquitoes from these tests can be used for polymerase chain reaction (PCR)-based molecular tests. In addition, both 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 (WHO, 2013; Brogdon and Chan, 2010). In addition, male *Anopheles* mosquitoes were not able to transmit malaria. For this reason, susceptibility testing is conducted using only female mosquitoes (WHO, 2013; Brogdon and Chan, 2010). In addition, some resistance mechanisms are 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 Universiti Sains Malaysia, which is based in Penang, Malaysia for this purpose. In a similar way, CDC assay requires the purchase 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. Even if there were complementarities between both methods, there were also some specificities between them.

Regarding the specificities of each method, it is important to mention that 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. WHO cylinder plastic tube test determines the percent mortality or mortality rates of malaria vectors to insecticide. WHO bioassays utilize cylinder plastic tubes whereas CDC bottles bioassays use 250 ml Wheaton bottles which are made from glass. WHO papers do not need to be treated by oneself before their utilization because they are always ordered in the impregnated form. Conversely, 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).

Conclusion

The current study clearly shows that both WHO and CDC methods are two complementary tools for the determination of insecticide susceptibility in malaria vectors. However, each method has also its own specificity. Insecticide susceptibility in malaria vectors can be assessed either with WHO cylinder plastic tube test or CDC bottle bioassay.

Conflict of Interest

The authors declare that they have no conflict of interests.

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REFERENCES

Abbott WS (1987). A method of computing the effectiveness of an insecticide. J. Am. Mosq. Control Assoc. 3(2):302-303.

Aïzoun N, Ossè R, Azondekon R, Alia R, Oussou O, Gnanguenon V, Aikpon R, Padonou GG, Akogbéto M (2013a). Comparison of the standard WHO susceptibility tests and the CDC bottle bioassay for the determination of insecticide susceptibility in malaria vectors and their correlation with biochemical and molecular biology assays in Benin, West Africa. Parasit. Vectors 6:147.

Aïzoun N, Aïkpon R, Gnanguenon V, Azondekon R, Oké-Agbo F, Padonou GG, Akogbéto M (2013b). Dynamics of insecticide resistance and effect of synergists piperonyl butoxide (PBO), S.S.S-tributylphosphorotrithioate (DEF) and ethacrynic acid (ETAA or EA) on permethrin, deltamethrin and dichlorodiphenyltrichloroethane (DDT) resistance in two *Anopheles gambiae s.l.* populations from southern Benin, West Africa. J. Parasitol. Vector Biol. 6(1):1-10.

Aïzoun N, Aïkpon R, Gnanguenon V, Oussou O, Agossa F, Padonou GG, Akogbéto M (2013c). Status of organophosphate and carbamate resistance in *Anopheles gambiae sensu lato* from the south and north Benin, West Africa. Parasit. Vectors 6:274.

Brogdon WG, McAllister JC (1998). Simplification of adult mosquito bioassays through use of time-mortality determinations in glass bottles. J. Am. Mosq. Control Assoc. 14(2):159-164.

Brogdon W, Chan A (2010). Guidelines for Evaluating Insecticide Resistance in Vectors using the CDC Bottle Bioassay/ Methods in anopheles research. Second edition. CDC Atlanta USA: CDC technical report. P 343.

Perea EZ, León RB, Salcedo MP, Brogdon WG, Devine GJ (2009). Adaptation and evaluation of the bottle assay for monitoring insecticide resistance in disease vector mosquitoes in the Peruvian Amazon. Malar. J. 8:208.

World Health Organization (WHO) (1998). Report of the WHO Informal Consultation: Tests procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces. Geneva: World Health Organization: Parasitic Diseases and Vector Control (PVC)/Communicable Disease Control, Prevention and Eradication (CPE). p. 43.

World Health Organization WHO/CDS/CPC/MAL/98.12.

World Health Organization (WHO) (2011). World Malaria Report. Geneva, World Health Organization.

World Health Organization (WHO) (2013). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva, World Health Organization.