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Original Research Article

Do Insecticide Formulations and Bottle Positions Influence Centers for Diseases Control and Prevention (CDC) Bottle Bioassay during Resistance Monitoring?

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Abstract

The current study was aimed at an investigation on the influence of insecticide formulations and bottle positions. Larvae and pupae of *Anopheles gambiae s.l.* mosquitoes were collected from breeding sites in Littoral department. CDC bottle bioassays were conducted on unfed females mosquitoes aged 2-5 days old with stock solutions of deltamethrin and bendiocarb (12.5µg per bottle) and fenitrothion (50µg per bottle) made from liquid insecticide or in powder. Test bottles were put vertical on their bottoms or on their sides on the lab bench of manipulation in the laboratory during the assessment of these susceptibility tests. The results obtained with deltamethrin, fenitrothion and bendiocarb when bottles coated with dilution from liquid insecticide were put vertical on their bottoms and those obtained when the bottles coated with dilution from insecticide in powder were put on their sides showed that insecticide formulations and bottle positions do not influence CDC bottle bioassay during resistance monitoring. Similar remark was made when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms and when the bottles coated with dilution from liquid insecticide were put on their sides. The current study clearly shows that the insecticide formulations and bottle positions do not influence the results obtained with 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.

Keywords

Benin
Bottle positions
CDC bottle bioassay
Insecticide formulations
Malaria vectors
Resistance

Introduction

In 2012, World Health Organization (WHO) estimated that 207 million case of malaria occurred worldwide with 627000 deaths. Most cases (80%) and deaths (90%) occurred in Africa, and most deaths (77%) were in children under 5years of age (WHO, 2013).

Malaria is caused by five species of parasite that affect humans, and all of these species belong to the genus *Plasmodium*. Malaria due to *Plasmodium falciparum* is the most deadly form, and it predominates in Africa. This disease is spread from one person to another by female mosquitoes of the genus *Anopheles*.

Malaria is an entirely preventable and treatable disease, provided the currently recommended interventions are properly implemented. These interventions include: vector control through the use of insecticide treated nets (ITNs), indoor residual spraying (IRS) and, in some specific settings, larval control.

Resistance monitoring should be seen as a critical element of any medium or large-scale deployment of an insecticidal intervention, and should be overseen and coordinated by National Malaria Control Programmes. This monitoring is usually done by using either World Health Organization (WHO) susceptibility test or the bottle bioassay developed by United States Centers for Diseases Control and Prevention (CDC). 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 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, 2014b). Thus, there is a need to investigate the influence of insecticide formulations and bottle positions during the assessment of bottle bioassay. The aim of this study was to investigate the influence of insecticide formulations and bottle positions during the assessment of bottle bioassay, an important resistance monitoring tool.

Materials and methods

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 Suru-lere location. Suru-lere is an urban location of Cotonou district. 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–July and September–November). The mean annual rainfall is over 1,500 mm.

Mosquito sampling

Anopheles gambiae s.l. mosquitoes were 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. 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 following CDC protocols on unfed female mosquitoes aged 2–5 days old, reared from the larval and pupal collections. All susceptibility tests were conducted in the CREC laboratory at 25±2°C and 70 to 80% relative humidity.

Testing insecticide susceptibility

CDC protocol

Principle: 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 of insecticides

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 *Anopheles gambiae s.l.* Kisumu susceptible reference strain before being applied to field populations. For *Anopheles 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 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 Ouemé department in southern Benin (2008-2010). We used fenitrothion, an organophosphate to assess cross-resistance with bendiocarb in locality surveyed. We used deltamethrin, because it is the insecticide used on PermaNets that are distributed free by the NMCP in the swampy areas of Ouemé (2008-2010). Ouemé department has a boundary with Littoral department.

Preparation of stock solutions

Obtaining stock solutions with insecticides in powder: The solutions were prepared according to the CDC protocol (Brogdon and Chan, 2010). The solutions which were used for CDC bottles coating were a mixing of stock solution and acetone. For example, to prepare the stock solution of deltamethrin 12.5µg per bottle, we weighed 12.5 mg of deltamethrin which were dissolved in 1 liter of acetone. Thus, 500 ml of acetone were put in a measuring test-piece which capacity is 1000 ml, and then 12.5 mg of deltamethrin well weighed were added. The solution was stirred up until complete dissolution of the powder and the acetone was progressively added up to the gauge line and the whole mixture homogenized. Once these solutions prepared, they were stocked in some no sensitized light bottles and put at refrigerator (4°C) until their use.

Obtaining stock solutions with liquid insecticides: Liquid insecticides of deltamethrin, fenitrothion and bendiocarb from CDC Atlanta, USA were contained in some vials. Each vial was dissolved in 100ml of acetone to obtain the stock solutions for bottle coating.

Wheaton bottles coating

The bottles coating was done following the protocol described by CDC (Brogdon and McAllister, 1998; Brogdon and Chan, 2010). After Wheaton bottles and caps had cleanly been washed and completely well sun-dried, each bottle and its cap were labeled (by the same number, the same insecticide name, and the same name of strain which has to be tested). Once the stock solutions prepared, they were stirred lightly to be homogenized before their use. 1ml of acetone was added to the control bottle and the cap was put back on tightly; and then 1ml of the stock solution of deltamethrin or fenitrothion or bendiocarb was added to four test bottles and the caps were also put back on tightly to avoid acetone evaporation. The contents inside the bottles were swirled so that the bottoms were coated, then the bottles were inverted and swirled to coat the inside of the caps. The bottles were placed on their sides for a moment to let the contents pool. Then the bottles were gently rotated while rocking so that all the sides around was coated. The caps were removed and bottles continued to be rolled on their sides until all visible signs of the liquid were gone from inside and the bottles were dry. Finally, the control and test bottles were left on their sides on the lab bench of manipulation in the laboratory, where they were protected from light until they were completely dried. Each cap was put in front of corresponding bottle and the inside of these caps facing the sky.

Performing CDC bottle bioassay

Coated bottles were put vertical on their bottoms or on their sides on the lab bench of manipulation in the laboratory during the assessment of the susceptibility tests. Fifteen to 20 unfed female mosquitoes aged 2–5 days old were introduced into four 250 ml Wheaton bottles coated with insecticide and one control bottle coated with acetone only. Bottles were not risen during mortality recording. The number of dead or alive mosquitoes was monitored at different time intervals (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 min.). This allowed us to determine the total percent mortality (Y axis) against time (X axis) for all replicates using a linear scale.

Statistic analysis

The resistance status of mosquito samples 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, 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 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 Suru-lere and from Kisumu to compare on the one hand for each of three tested insecticides, the resistance status when the bottles were put vertical on their bottoms and coated with dilution from liquid insecticide to when the bottles were put on their sides and coated with dilution from insecticide in powder. On the other hand, to compare for each of three tested insecticides, the resistance status when the bottles were put vertical on their bottoms and coated with dilution from insecticide in powder to when the bottles were put on their sides and coated with dilution from liquid insecticide. The software R-2.15.2. (R Development Core Team, 2011) was used for the 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

Comparison of resistance status to deltamethrin when bottles coated with dilution from liquid insecticide were put vertical on their bottoms to when bottles coated with dilution from insecticide in powder were put on their sides.

The results recorded after mosquito exposure to bottles coated with dilution from liquid insecticide and put vertical on their bottoms were compared to those recorded when bottles were coated with dilution from insecticide in powder and put on their sides at the susceptibility threshold (30min.).

CDC bottles bioassays were performed with stock solutions of deltamethrin (1.25%). Kisumu strain (control) confirmed its susceptibility status with 100% mortality as a reference strain with both deltamethrin formulations tested and with both bottle positions used in the current study.

Table.1 Comparison of resistance status to deltamethrin when bottles coated with dilution from liquid insecticide were put vertical on their bottoms to when bottles coated with dilution from insecticide in powder were put on their sides

Populations	Insecticides	Bottle vertical coated with liquid insecticide			Bottle on its side coated with insecticide in powder		
		Number tested	% Mortality	Resistance status	Number tested	% Mortality	Resistance status
Kisumu	Deltamethrin	25	100	S	92	100	S
Suru-lere	Deltamethrin	79	86.07	R	93	93.54	R

Table.2 Comparison of resistance status to deltamethrin when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms to when the bottles coated with dilution from liquid insecticide were put on their sides

Populations	Insecticides	Bottle vertical coated with insecticide in powder			Bottle on its side coated with liquid insecticide		
		Number tested	% Mortality	Resistance status	Number tested	% Mortality	Resistance status
Kisumu	Deltamethrin	100	100	S	100	100	S
Suru-lere	Deltamethrin	40	85	R	19	89.47	R

Anopheles gambiae s.l. populations from Suru-lere were resistant to deltamethrin. When bottles were coated with dilution from liquid insecticide and put vertical on their bottoms, the percent mortality recorded was 86.07% (68/79) whereas when bottles were coated with dilution from insecticide in powder and put on their sides, the percent mortality was 93.54% (87/93) (Table 1).

Comparison of resistance status to deltamethrin when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms to when the bottles coated with dilution from liquid insecticide were put on their sides.

The results recorded after mosquito exposure to bottles coated with dilution from insecticide in powder and put vertical on their bottoms were compared to those recorded when the bottles were coated with dilution from liquid insecticide and put on their sides at the susceptibility threshold (30 min.).

The results obtained when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms and those obtained when the bottles coated with dilution from liquid insecticide were put on their sides also showed that *Anopheles gambiae s.l.* populations from Suru-lere were resistant to deltamethrin. The percent mortality recorded were 85% (34/40) and 89.47% (17/19) respectively (Table 2).

Comparison of resistance status to fenitrothion when bottles coated with dilution from liquid insecticide were put vertical on their bottoms to when bottles coated with dilution from insecticide in powder were put on their sides.

The results recorded after mosquito exposure to bottles coated with dilution from liquid insecticide and put vertical on their bottoms were compared to those recorded when bottles were coated with dilution from insecticide in powder and put on their sides at the susceptibility threshold (30min.). CDC bottles bioassays were performed with stock solutions of fenitrothion (5%).

Kisumu strain (control) confirmed its susceptibility status with 100% mortality as a reference strain with both fenitrothion formulations tested and with both bottle positions used in the current study.

Anopheles gambiae s.l. populations from Suru-lere were susceptible to fenitrothion. When bottles were coated with dilution from liquid insecticide and put vertical on their bottoms, the percent mortality recorded was 100% (67/67) whereas when bottles were coated with dilution from insecticide in powder and put on their sides, the percent mortality was 100% (99/99) (Table 3).

Comparison of resistance status to fenitrothion when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms to when the bottles coated with dilution from liquid insecticide were put on their sides.

The results recorded after mosquito exposure to bottles coated with dilution from insecticide in powder and put vertical on their bottoms were compared to those recorded when the bottles were coated with dilution from liquid insecticide and put on their sides at the susceptibility threshold (30 min.).

Table.3 Comparison of resistance status to fenitrothion when bottles coated with dilution from liquid insecticide were put vertical on their bottoms to when bottles coated with dilution from insecticide in powder were put on their sides

Populations	Insecticides	Bottle vertical coated with liquid insecticide			Bottle on its side coated with insecticide in powder		
		Number tested	% Mortality	Resistance status	Number tested	% Mortality	Resistance status
Kisumu	Fenitrothion	33	100	S	94	100	S
Suru-lere	Fenitrothion	67	100	S	99	100	S

Table.4 Comparison of resistance status to fenitrothion when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms to when the bottles coated with dilution from liquid insecticide were put on their sides

Populations	Insecticides	Bottle vertical coated with insecticide in powder			Bottle on its side coated with liquid insecticide		
		Number tested	% Mortality	Resistance status	Number tested	% Mortality	Resistance status
Kisumu	Fenitrothion	100	100	S	100	100	S
Suru-lere	Fenitrothion	67	100	S	99	100	S

The results obtained when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms and those obtained when the bottles coated with dilution from liquid insecticide were put on their sides also showed that *Anopheles gambiae s.l.* populations from Suru-lere were susceptible to fenitrothion. The percent mortality recorded were 100% (67/67) and 100% (99/99) respectively (Table 4).

Comparison of resistance status to bendiocarb when bottles coated with dilution from liquid insecticide were put vertical on their bottoms to when bottles coated with dilution from insecticide in powder were put on their sides.

The results recorded after mosquito exposure to bottles coated with dilution from liquid insecticide and put vertical on their bottoms were compared to those recorded when bottles were coated with dilution from insecticide in powder and put on their sides at the susceptibility threshold (30min.). CDC bottles bioassays were performed with stock solutions of bendiocarb (1.25%).

Kisumu strain (control) confirmed its susceptibility status with 100% mortality as a reference strain with both bendiocarb formulations tested and with both bottle positions used in the current study.

Anopheles gambiae s.l. populations from Suru-lere were susceptible to bendiocarb. When bottles were coated with dilution from liquid insecticide and put vertical on their bottoms, the percent mortality recorded was 100% (79/79) whereas when bottles were coated with dilution from insecticide in powder and put on their sides, the percent mortality was 100% (96/96) (Table 5).

Comparison of resistance status to bendiocarb when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms to when the bottles coated with dilution from liquid insecticide were put on their sides

The results recorded after mosquito exposure to bottles coated with dilution from insecticide in powder and put vertical on their bottoms were compared to those recorded when the bottles were coated with dilution from liquid insecticide and put on their sides at the susceptibility threshold (30 min.).

The results obtained when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms and those obtained when the bottles coated with dilution from liquid insecticide were put on their sides also showed that *Anopheles gambiae s.l.* populations from Suru-lere were susceptible to bendiocarb. The percent mortality recorded were 100% (79/79) and 100% (96/96) respectively (Table 6).

Discussion

A comparison of resistance status to deltamethrin when bottles coated with dilution from liquid insecticide were put vertical on their bottoms to when bottles coated with dilution from insecticide in powder were put on their sides was made. Another comparison of resistance status to deltamethrin when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms to when the bottles coated with dilution from liquid insecticide were put on their sides was also made. Both comparisons with deltamethrin showed that neither deltamethrin formulations nor bottle positions (on their bottoms

or on their sides) influence the results of Centers for Diseases Control and Prevention (CDC) bottle bioassay during resistance monitoring. So, bottles could be put in both positions during the assessment of bioassay. Even if the test bottles were put vertical on their bottoms or on their sides on the lab bench of manipulation during the assessment of CDC bottle bioassay, mosquitoes could not avoid the insecticide used for their coating as all the inside of these bottles was coated including their caps. In addition, either bottle coated with stock solutions from liquid insecticide or from insecticide in powder could be used. However, it would be important that the recording of the number of dead or alive mosquitoes during the assessment of bottle bioassay was done without rising the test bottles. In fact, when bottles were risen during the CDC susceptibility tests, mosquito movement could increase and that could not make this recording easy (Aïzoun et al., 2013a).

Table.5 Comparison of resistance status to bendiocarb when bottles coated with dilution from liquid insecticide were put vertical on their bottoms to when bottles coated with dilution from insecticide in powder were put on their sides

Populations	Insecticides	Bottle vertical coated with liquid insecticide			Bottle on its side coated with insecticide in powder		
		Number tested	% Mortality	Resistance status	Number tested	% Mortality	Resistance status
Kisumu	Bendiocarb	26	100	S	93	100	S
Suru-lere	Bendiocarb	79	100	S	96	100	S

Table.6 Comparison of resistance status to bendiocarb when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms to when the bottles coated with dilution from liquid insecticide were put on their sides

Populations	Insecticides	Bottle vertical coated with insecticide in powder			Bottle on its side coated with liquid insecticide		
		Number tested	% Mortality	Resistance status	Number tested	% Mortality	Resistance status
Kisumu	Bendiocarb	100	100	S	100	100	S
Suru-lere	Bendiocarb	79	100	S	96	100	S

Deltamethrin resistance in *Anopheles gambiae s.l.* from Suru-lere in southern Benin may be explained by increased use of household insecticide and availability of xenobiotics for larval breeding sites in the urban. It was one of the possible factors selecting for pyrethroid resistance in *Anopheles gambiae s.l.* in urban areas (Aïzoun et al., 2014c).

A Comparison of resistance status to fenitrothion when bottles coated with dilution from liquid insecticide were put vertical on their bottoms to when bottles coated with dilution from insecticide in powder were put on their sides was made. Another comparison of resistance status to fenitrothion when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms to when the bottles coated with dilution from liquid insecticide were put on their sides was also made. Both comparisons with fenitrothion also showed that neither fenitrothion formulations nor bottle positions (on their bottoms or on their sides) influence the results of Centers for Diseases Control and Prevention (CDC) bottle bioassay during resistance monitoring.

The susceptibility to fenitrothion of *Anopheles gambiae s.l.* from Suru-lere confirmed that *Anopheles gambiae s.l.* populations in southern Benin were still susceptible to this product (Aïzoun et al., 2013b).

A Comparison of resistance status to bendiocarb when bottles coated with dilution from liquid insecticide were put vertical on their bottoms to when bottles coated with dilution from insecticide

in powder were put on their sides was made. Another comparison of resistance status to bendiocarb when the bottles coated with dilution from insecticide in powder were put vertical on their bottoms to when the bottles coated with dilution from liquid insecticide were put on their sides was also made. Both comparisons with bendiocarb also showed that neither bendiocarb formulations nor bottle positions (on their bottoms or on their sides) influence the results of Centers for Diseases Control and Prevention (CDC) bottle bioassay during resistance monitoring.

The susceptibility to bendiocarb of *Anopheles gambiae s.l.* from Suru-lere also confirmed that *Anopheles gambiae s.l.* populations in southern Benin were still susceptible to this product (Aïzoun et al., 2013b). A recent study was carried out by Aïzoun et al. (2014d) and showed that as *Anopheles gambiae s.l.* populations from southern Benin, those from the central part of the country were also susceptible to both carbamates and organophosphates. However, some resistance pockets to these products were recently reported in the northern Benin (Aïzoun et al., 2013b) and biochemical mechanisms such as the implication of esterases were also associated with carbamate resistance in *Anopheles gambiae s.l.* in this part of the country (Aïzoun et al., 2013c).

The temperature mean recording and the relative humidity recording during bioassays or susceptibility tests in laboratory were important (Aïzoun et al., 2014a) as they would play an important role in the influence of insecticide formulations and bottle positions during resistance monitoring using CDC bottle bioassay. In similar way, the storage of the stock solutions was also important in the current study as it could influence the obtained results. For this reason, after stock solutions were prepared, they were stocked in some no sensitized light bottles and put at refrigerator (4°C) until their use. It is recommended to take the stock solutions out of the refrigerator at least 1 hr. before running the bioassay to allow them to come to room temperature before use (Brogdon and Chan, 2010). It is also important to homogenize them before their use.

The current study clearly shows that the insecticide formulations and bottle positions 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.

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