EXCELLENT PUBLISHERS

International Journal of Current Research in Biosciences and Plant Biology

ISSN: 2349-8080 Volume 1 Number 2 (September-2014) pp. 25-31

www.ijcrbp.com

Original Research Article

All the Inside of Wheaton Bottle is Coated and the Bottom and the Cap of WHO Plastic Cylinder Tube are not Impregnated: Does that Affect the Results of Susceptibility Tests?

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Abstract

In this study, we investigated the influence of no impregnated inside of the bottom and the cap of WHO plastic cylinder tube and of all the coated inside of Wheaton bottle on the results of susceptibility tests. WHO susceptibility tests were conducted on unfed *Anopheles gambiae s.l.* female mosquitoes aged 2-5 days with impregnated-papers with permethrin (0.75%), deltamethrin (0.05%), and bendiocarb (0.1%) whereas CDC susceptibility tests were conducted with stock solutions of deltamethrin and bendiocarb (12.5µg per bottle) and permethrin (21.5µg per bottle). *Anopheles gambiae* Houeyiho populations were resistant to permethrin but they were susceptible to deltamethrin in 2008 according to both methods. The resistance levels of *Anopheles gambiae s.l.* Houeyiho populations to permethrin and deltamethrin recorded with CDC bottle bioassay was slightly higher than those recorded with WHO susceptibility tests. In similar way, *Anopheles gambiae* Malanville populations were resistant to deltamethrin but they were susceptible to bendiocarb in 2012 according to both methods. The resistance levels of *Anopheles gambiae s.l.* Malanville populations to deltamethrin and bendiocarb recorded with CDC bottle bioassay was also slightly higher than those recorded with WHO susceptibility tests.

Keywords

Benin
CDC bottle bioassay
Coated bottle
Impregnated paper
Malaria vectors
Resistance
WHO susceptibility test

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). WHO recommends that, in areas targeted for malaria vector control, all

persons at risk should be protected by ITNs or IRS. The choice of ITNs or IRS depends on a number of entomological, epidemiological, and operational factors, including seasonality of transmission, housing density and distribution, and insecticide susceptibility of anopheline vectors (WHO, 2013a).

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 1981and 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, 2013b). 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).

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 on the influence of no impregnated inside of bottom and the cap of WHO plastic cylinder tube and of all the coated inside of Wheaton bottle on the results of susceptibility tests. The aim of this study was to investigate on the influence of no impregnated inside of bottom and the cap of WHO plastic cylinder tube and of all the coated inside of Wheaton bottle on the results of susceptibility tests.

Materials and methods

Study area

Study areas are located in Republic of Benin (West Africa) and include the departments of Alibori and Littoral. In Alibori department located in the far north of Benin, the study was carried out in Malanville district, a rice growing area of 100hectares located near the Niger River. In Littoral department located in the south of Benin, the study was carried out in Cotonou district more precisely in the vegetable area of Houeyiho. The vegetable area of Houeyiho is a big farm of 14 hectares in the town of Cotonou. In Houeyiho, more than 300 farmers are involved in the cultivation of a large variety of vegetables: cabbages, carrots, lettuces, amaranth, cucumber etc. Farming at Houeyiho is associated with the use of insecticides to fight pests (Akogbéto et al., 2006).

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. The northern zone (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. The southern region (Cotonou) is characterized by a tropical Guinean climate with two rainy seasons (April–July and September–November) with a mean annual rainfall over 1,500 mm.

Mosquito sampling

Anopheles gambiae s.l. mosquitoes were collected during the rainy season (May to October 2012) across Malanville district selected in northern Benin. *Anopheles gambiae s.l.* mosquitoes were also collected during the rainy season from April–July and September–November 2008 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 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 *Anopheles 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%), bendiocarb (0.1%) and permethrin (0.75%). 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 deltamethrin to check if *Anopheles gambiae s.l.* Malanville populations resistance level to this product was high considering the relatively low amount of insecticide use in this area. Deltamethrin was also used to assess cross-resistance with permethrin in the vegetable growing area of Houeyiho surveyed considering the relatively high amount of insecticide use in this area.

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 hrs 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 *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. whereas the diagnostic dose of 21.5 µg per bottle for pemethrin was used for the same diagnostic exposure time.

The solutions were prepared and the bottles coated according to the CDC protocol (Brogdon and Chan, 2010). Fifteen to 20 unfed female mosquitoes of 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 (10, 20, 30, 40, 50, 60 minutes) in 2008 and (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 minutes) in 2012. 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, 2013b) 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 three tested insecticides and assess the resistance levels of each tested *Anopheles 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

Comparison of resistance levels of *Anopheles gambiae s.l.* populations to permethrin in 2008

The results of 24 hrs mortality recording after mosquito exposure to WHO impregnated papers with permethrin (0.75%) were compared to those recorded with CDC bottles bioassays at the susceptibility threshold (30 min.). CDC bottles bioassays were performed with stock solutions of permethrin (2.15%) (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 Houeyiho were resistant to permethrin according to both WHO and CDC methods. The percent mortality recorded with WHO method was 72.64% (247/340), slightly higher than the percent mortality recorded with CDC method which was 62.70% (232/370) (Table 1).

Comparison of resistance levels of *Anopheles gambiae s.l.* populations to deltamethrin in 2008

The results of 24 hrs 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 with 100% mortality as a reference strain according to both WHO and CDC methods.

Anopheles gambiae s.l. populations from Houeyiho were susceptible to deltamethrin according to both WHO and CDC methods. The percent mortality recorded with WHO method was 100% (336/336), slightly higher than the percent mortality recorded with CDC method which was 98.70% (304/308) (Table 1).

Table.1 Comparison of resistance levels of *Anopheles gambiae s.l.* populations to preshrink and deltamethrin in 2008

Populations	Insecticides	Number tested		% Mortality		Resistance status	
		WHO	CDC	WHO	CDC	WHO	CDC
Kisumu (Control)	Permethrin	200	200	100	100	S	S
Houeyiho	Permethrin	340	370	72.64	62.70	R	R
Kisumu (Control)	Deltamethrin	200	200	100	100	S	S
Houeyiho	Deltamethrin	336	308	100	98.70	S	S

Comparison of resistance levels of *Anopheles gambiae s.l.* populations to deltamethrin in 2012

The results of 24 hrs 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 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 Malanville were resistant to deltamethrin according to both WHO and CDC methods. The percent mortality recorded with WHO method was 13.09% (11/84), slightly higher than the percent mortality recorded with CDC method which was 0% (0/21) (Table 2).

Table.2 Comparison of resistance levels of *Anopheles gambiae s.l.* populations to deltamethrin and bendicarb in 2012

Populations	Insecticides	Number tested		% Mortality		Resistance status	
		WHO	CDC	WHO	CDC	WHO	CDC
Kisumu (Control)	Deltamethrin	92	25	100	100	S	S
Malanville	Deltamethrin	84	21	13.09	0	R	R
Kisumu (Control)	Bendiocarb	93	26	100	100	S	S
Malanville	Bendiocarb	93	48	98.92	97.91	S	S

Comparison of resistance levels of *Anopheles gambiae s.l.* populations to bendiocarb in 2012

The results of 24 hrs 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 Malanville were susceptible to bendiocarb according to both methods. The percentage of dead mosquitoes recorded with WHO method was 98.92% (92/93), slightly higher than the mortality rate recorded with CDC method which was 97.91% (47/48) (Table 2).

Discussion

The slight decrease of susceptibility obtained with *Anopheles gambiae s.l.* Houeyiho populations exposed to deltamethrin in 2008 with CDC bottle bioassay was not synonymous with resistance. A similar pattern was already observed with *Anopheles gambiae s.l.*Adjara and Dangbo populations exposed to bendiocarb in Ouemé department in southern Benin (Aïzoun et al., 2013a). *Anopheles gambiae s.l.* populations from Houeyiho were resistant to permethrin and susceptible to deltamethrin in 2008 according to both WHO and CDC methods. If *Anopheles* species collected in this vegetable growing area were susceptible to deltamethrin despite the use of insecticides in the locality against pests that could likely be explained by the eventual presence of other species different from *Anopheles gambiae s.s* such as *Anopheles arabiensis*. Similar pattern was already observed by Akogbeto et al. (2006) with *Anopheles gambiae s.l.* from the vegetable growing area of Parakou.

The resistance levels of *Anopheles gambiae s.l.* Houeyiho populations to permethrin and deltamethrin recorded in 2008 with CDC bottle bioassay were slightly higher than that recorded with WHO susceptibility tests. Mosquitoes sometimes avoided the impregnated papers by laying for a moment on the bottoms or on the caps of the test tubes during the assessment of WHO susceptibility tests whereas they could not avoid the insecticide when it was used for bottle coating

as all the inside of the test bottles was coated including the inside of their caps. So, that situation did not affect the results of susceptibility tests recorded with both methods. Similar pattern was already observed regarding the resistance levels of *Anopheles gambiae s.l.* populations from Oueme department to deltamethrin (Aïzoun et al., 2013a).

Anopheles gambiae s.l. populations from Malanville were resistant to deltamethrin in 2012 according to both WHO and CDC methods. Deltamethrin resistance in *Anopheles gambiae s.l.* Malanville was already observed by Aïzoun et al. (2014c) 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).

The resistance level of *Anopheles gambiae s.l.* Malanville populations to deltamethrin recorded in 2012 with CDC bottle bioassay was slightly higher than that recorded with WHO susceptibility tests. This result was also due to that mosquitoes sometimes avoided the impregnated papers by laying for a moment on the bottoms or on the caps of the test tubes during the assessment of WHO susceptibility tests whereas they could not avoid the insecticide when it was used for bottle coating as all the inside of the test bottles was coated including the inside of their caps. So, that situation did not affect the results of susceptibility tests recorded with both methods too. Similar pattern was already observed regarding the resistance levels of *Anopheles gambiae s.l.* populations from Oueme department to deltamethrin (Aïzoun et al., 2013a).

The slight decrease of susceptibility obtained with *Anopheles gambiae s.l.* Malanville populations exposed to bendiocarb in 2012 with CDC bottle bioassay was not synonymous with resistance. A similar pattern was already observed with *Anopheles gambiae s.l.* Adjara and Dangbo populations exposed to bendiocarb in Ouemé department in southern Benin (Aïzoun et al., 2013a). *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 Malanville district.

In similar way, the resistance level of *Anopheles gambiae s.l.* Malanville populations to bendiocarb recorded in 2012 with CDC bottle bioassay was slightly higher than that recorded with WHO susceptibility tests. So, that situation did not also affect the results of susceptibility tests recorded with both methods. Similar pattern was also already observed regarding the resistance levels of *Anopheles gambiae s.l.* populations from Oueme department to bendiocarb (Aïzoun et al., 2013a).

The current study clearly shows that even if all the inside of Wheaton bottle is coated and the bottom and the cap of WHO cylinder plastic tube are not impregnated, that does not affect the results of susceptibility tests recorded with both resistance monitoring tools. Susceptibility tests can be assessed either with WHO method or with CDC method.

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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