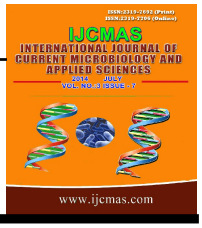
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Original Research Article

Determination of the days storage and re-use of a Wheaton coated bottle with DDT, fenitrothion, and bendiocarb in laboratory conditions

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Coated bottle, insecticide, days storage, re-use, laboratory conditions, Benin

A B S T R A C T

The definition of some strategies which would help to reduce or economize the amount of insecticide was useful in the assessment of insecticide vectors susceptibility tests in laboratory conditions. We investigated the days storage and the number of times that a Wheaton coated bottle maintained its efficacy. *An. gambiae s.l.* mosquitoes were collected in May 2013 during the rainy season in Allada district selected in southern Benin, in Dassa-Zoume district selected in the centre part of the country and in Parakou district selected in northern Benin. Efficacy tests were done using stock solutions of fenitrothion (50µg per bottle), DDT (100µg per bottle) and bendiocarb (12.5µg per bottle) following CDC protocol on unfed female mosquitoes aged 2–5 days old. These bioassays were repeated a certain number of times with the same coated bottle during four consecutive days. The temperature and relative humidity were monitored and recorded during the efficacy tests. The current study showed that a Wheaton coated bottle with fenitrothion, DDT and bendiocarb could be used four times during four consecutive days in laboratory conditions. The study of the efficacy of a Wheaton coated bottle with fenitrothion, DDT and bendiocarb in laboratory conditions was useful in the assessment of insecticide vectors susceptibility tests. Treated bottle storage and the ambient temperature and relative humidity recording in laboratory during susceptibility tests have also an important role in the efficacy study.

Introduction

There are major international efforts by donors such as the President's Malaria Initiative (PMI) underway in many parts of Africa to control malaria by scaling up of long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (Bhattarai *et al.*, 2007; Okiro *et al.*, 2007; Ceesy *et al.*, 2008). Organophosphates and carbamates are two alternatives to pyrethroids. Because of its negative environmental connotations, the Stockholm Convention on Persistent Organic Pollutants stipulates that, 'countries using DDT are encouraged to reduce and eliminate the use of DDT over time and

switch to alternative insecticides' (United Nations Environment Programme).

The shelf-life and the re-use of pre-prepared bottles are still not well documented or studied in laboratory conditions (Aïzoun *et al.*, 2013a). For this reason, a recent study was carried out to investigate the shelf-life and the re-use of a WHO impregnated paper with bendiocarb under field conditions and a Wheaton coated bottle with permethrin and deltamethrin under laboratory conditions (Aïzoun *et al.*, 2014). This study showed that a Wheaton treated bottle with 12.5 µg a.i deltamethrin per bottle and 21.5 µg a.i permethrin per bottle could be used at least three times during four consecutive days in laboratory conditions. After the fourth use, bottles have to be washed and re-coated before their re-use. Thus, there is a need to study the efficacy of a Wheaton coated bottle with fenitrothion, DDT and bendiocarb in laboratory conditions, as organophosphates, organochlorines and carbamates are three main insecticide classes used for vector control in public health in addition to pyrethroids.

The aim of this study was to determine the efficacy of a Wheaton coated bottle with fenitrothion, DDT and bendiocarb in laboratory conditions, in order to define some strategies which would help to reduce or economize the amount of insecticide useful in the assessment of insecticide vectors susceptibility tests in laboratory conditions.

Materials and Methods

Study area

The study was carried out in three districts selected for mosquito collection following a south-north transect Benin. The districts were: Dassa-Zoume located in the middle part of the country. Allada is located in the south part of the country whereas Parakou is located in the north of Benin. 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 southern region 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. The middle part of the country is characterized by a Sudano guinean climate with two rainy seasons (March–July and August) with an average rainfall of 1,000 mm per year. The northern zone is characterized by a Sudanian climate with only one rainy season per year (May to October) and one dry season (November-April). The temperature ranged from 22 to 33°C with the annual mean rainfall which is 1,300 mm.

Mosquito sampling

An. gambiae s.l. mosquitoes were collected in May 2013 during the rainy season in Allada district selected in southern Benin, in Dassa-Zoume district selected in the centre part of the country and in Parakou district selected in northern Benin. *Anopheles* pre-imaginal stages (L1 to L4 instars) were collected in these three districts within both padding and village using the dipping method on several breeding sites (brick pits, pools, marshes, streams, ditches, pits dug for plastering traditional huts, puddles of water, water pockets caused by the gutters). In these three districts, larvae and pupae were collected using the dipping on breeding sites and then kept in separated labeled bottles related to each locality. Otherwise, larvae collected from multiple breeding sites were pooled together related to each district then

re-distributed evenly in development trays containing tap water. Larvae were provided access to powdered TetraFin® fish food, and were reared to adults under insectary conditions of 25+/-2°C and 70 to 80% relative humidity at Centre de Recherche Entomologique de Cotonou (CREC) located in Akpakpa, in Cotonou district. The samples were reared up to adult emergence at the CREC insectary. *An. gambiae* Kisumu, a reference susceptible strain was used as a control for the bioassay tests. Efficacy tests were done following CDC protocol on unfed females mosquitoes aged 2-5 days old reared from larval and pupal collections. All efficacy tests were conducted in the CREC laboratory at 25+/-2°C and 70 to 80% relative humidity.

Diagnostic doses of insecticides

The diagnostic doses which were applied were the doses recommended by CDC (Brogdon W & Chan A, 2010). These doses were applied on *An. gambiae* Kisumu, reference susceptible strain before being applied on field populations (*An. gambiae* Parakou, *An. gambiae* Dassa-Zoume and *An. gambiae* Allada). For *An. gambiae s.l.*, the diagnostic dose 50µg of fenitrothion per bottle, 12.5µg of bendiocarb per bottle were applied for 120 minutes exposure period and for a diagnostic time of 30 minutes whereas the diagnostic dose 100µg of DDT per bottle were applied for 120 minutes exposure period and for a diagnostic time of 45 minutes. Fenitrothion, an organophosphate was used to assess cross-resistance with bendiocarb in use for Indoor Residual Spraying (IRS) in the northern Benin since 2011. Bendiocarb was used in order to check if the resistance detected to this product in the north of the country (Aïzoun *et al.*, 2013b; Aïkpon *et al.*, 2013) was already widespread in *Anopheles gambiae s.l.* from the central part of the country. DDT was tested because of its intensive use in the past for house-spraying applications in southern villages from 1953 to 1960 during WHO programmes of malaria eradication.

Preparation of stock solutions

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 bendiocarb 12.5µg per bottle, we weighed 12.5 mg of bendiocarb 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 bendiocarb 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 until their use.

Wheaton bottles coating

The bottles coating was done by following the protocol described by CDC (Brogdon WG & McAllister JC, 1998; Brogdon W & Chan A, 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 fenitrothion, DDT or bendiocarb was added to the test bottle and the cap was also put back on tightly to avoid acetone evaporation. The contents inside the bottle was swirled so that the bottom was coated,

then the bottle was inverted and swirled to coat the inside of the cap. The bottle was placed on its side for a moment to let the contents pool. Then the bottle was gently rotated while rocking so that all the sides around were coated. The cap was removed and bottle continued to be rolled on its side until all visible signs of the liquid were gone from inside and the bottle was 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 the efficacy tests

CDC bioassays were performed with a 250ml Wheaton coated bottle (new or clear bottle) with fenitrothion at diagnostic dose 50µg per bottle. One coated bottle with acetone only served as control. This dose was checked on the *An. gambiae* Kisumu susceptible reference strain before being applied to field populations (*An. gambiae* Parakou) reared from the larval and pupal collections to adults in the CREC (Centre de Recherche Entomologique de Cotonou, Benin) insectary. These bioassays were performed like this: an aspirator was used to introduce 15 to 20 unfed female mosquitoes from Parakou, aged 2–5 days into a 250 ml Wheaton coated bottle with fenitrothion. The number of dead or alive mosquitoes was monitored at different time intervals (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 minutes).

The percent mortality at susceptibility threshold (30 minutes) was determined. These CDC bioassays were performed during several consecutive days under ambient temperature and relative humidity conditions in laboratory (which were each time recorded during each test at laboratory) until the coated bottle lost its efficacy. Bioassays were stopped as soon as the percent mortality recorded in the test bottle was inferior to 100% with *Anopheles gambiae* populations collected in the field, *Anopheles gambiae* Parakou populations and when there was significant difference between the mortality rates recorded in two consecutive tests or bioassays. Both field and reference populations susceptible to fenitrothion since the first CDC test were used in tests repetition. After each test, a line was marked on the label of the test bottle. This bottle was well kept aside under good temperature and relative humidity conditions in laboratory. As for the fenitrothion, the same procedure was followed in bioassays repetition with bendiocarb and DDT with *Anopheles gambiae* populations from Parakou and then from Dassa-Zoume and Allada.

Statistical analysis

The resistance status of mosquito samples from Parakou, Dassa-Zoume and Allada was determined according to the CDC criteria (Brogdon WG & McAllister JC, 1998; Brogdon W & Chan A, 2010). The susceptibility thresholds at the diagnostic time of 45 minutes for organochlorines and 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 WS, 1987).

Analysis using Fisher's exact test for equality of two proportions was performed

on the data sets gathered from the localities surveyed to compare for each of three tested insecticides, the mortality rate of *An. gambiae* populations obtained during the first susceptibility test to the one obtained during the repeated tests. Data are presented with 95% confidence limits.

Ethical approval

This study was approved by the Ministry of Health and the Center for Entomological Research of Cotonou, Benin.

Result and Discussion

Efficacy of a Wheaton coated bottle with fenitrothion in laboratory conditions

The analysis of table 1 shows that *Anopheles gambiae* Kisumu and *Anopheles gambiae* Parakou populations, after four susceptibility tests during four consecutive days in insecticide testing laboratory at CREC remained susceptible to fenitrothion with the mortality rate of 100%. After 30 minutes in CDC coated bottle with fenitrothion, which represents susceptibility threshold time or diagnostic time clearly defined by CDC protocol, these *Anopheles gambiae* populations were dead and none of them could fly. The mortality rate recorded during the fourth susceptibility test was 100% (P=1). There were no differences between dates (P>0.05).The temperature mean recorded during these susceptibility tests was 25°C. The relative humidity mean was 75% (Table 1).

Efficacy of a Wheaton coated bottle with bendiocarb in laboratory conditions

The analysis of table 2 shows that *Anopheles gambiae* Kisumu and *Anopheles gambiae* Dassa-Zoume populations, after four susceptibility tests during four consecutive days in insecticide testing laboratory at CREC remained susceptible to bendiocarb with the mortality rate of 100%. After 30 minutes in CDC coated bottle with bendiocarb, which represents susceptibility threshold time or diagnostic time clearly defined by CDC protocol, these *Anopheles gambiae* populations were dead and none of them could fly. The mortality rate recorded during the fourth susceptibility test was 100% (P=1). There were no differences between dates (P>0.05). The temperature mean recorded during these susceptibility tests was 25°C. The relative humidity mean was 75% (Table 2).

Efficacy of a Wheaton coated bottle with DDT in laboratory conditions

The analysis of table 3 shows that *Anopheles gambiae* Kisumu and *Anopheles gambiae* Allada populations, after four susceptibility tests during four consecutive days in insecticide testing laboratory at CREC, remained resistant to DDT with the mortality rate of 0%. After 45 minutes in CDC coated bottle with DDT, which represents susceptibility threshold time or diagnostic time clearly defined by CDC protocol, these *Anopheles gambiae* populations were knocked down. The mortality rate recorded during the fourth susceptibility test was 0% (P=1). There were no differences between dates (P>0.05). The temperature mean recorded during these susceptibility tests was 25°C. The relative humidity mean was 75% (Table 3).

Stock solution storage has an important role in the efficacy study. In the current study, after stock solutions had been prepared, they were stored in the refrigerator (4°C) in light-proof bottles. It is recommended to take the stock solutions out of the refrigerator at least 1hour before running the bioassay to allow them to come to room temperature before use (Brogdon W & Chan A, 2010).

Table.1 Efficacy of a Wheaton coated bottle with fenitrothion 50 µg/bottle in laboratory conditions

Temperature: 25°C					Relative humidity : 75%			
Population	Number tested	% Mortality 1st test	Number tested	% Mortality 2nd test	Number tested	% Mortality third test	Number tested	% Mortality fourth test
Kisumu	22	100	11	100	20	100	16	100
Parakou	18	100	16	100	14	100	19	100

Table.2 Efficacy of a Wheaton coated bottle with bendiocarb 12.5µg/bottle in laboratory conditions

Temperature: 25°C					Relative humidity : 75%			
Population	Number tested	% Mortality 1st test	Number tested	% Mortality 2nd test	Number tested	% Mortality third test	Number tested	% Mortality fourth test
Kisumu	14	100	12	100	16	100	18	100
Dassa-Zoume	16	100	17	100	18	100	15	100

Table.3 Efficacy of a Wheaton coated bottle with DDT 100µg/bottle in laboratory conditions

Temperature: 25°C					Relative humidity: 75 %			
Population	Number tested	% Mortality 1st test	Number tested	% Mortality 2nd test	Number tested	% Mortality third test	Number tested	% Mortality fourth test
Kisumu	19	100	16	100	18	100	15	100
Allada	19	0	15	0	14	0	16	0

It is also important to homogenize them before their use. Treated bottle storage also has an important role in the efficacy study. In this study, after the assessment of each susceptibility test in laboratory, bottles were capped and stored in a cardboard and put in a safety place in order to prevent them from breakages before the next test. The ambient temperature and relative humidity recording in laboratory and in field during insecticide vectors susceptibility tests were necessary (Aïzoun *et al.*, 2014).

Anopheles gambiae Kisumu populations (control) after four CDC susceptibility tests during four consecutive days in insecticide testing laboratory at CREC remained susceptible to fenitrothion, DDT and bendiocarb. These results showed that *Anopheles gambiae* Kisumu populations arrived at the CREC insectary in 1999 were well reared up and stored in good conditions in Benin.

Anopheles gambiae Parakou populations, after four susceptibility tests during four consecutive days in insecticide testing laboratory at CREC remained susceptible to fenitrothion. A Wheaton treated bottle with 50µg a.i fenitrothion per bottle could be used four times during four consecutive days in laboratory conditions. On the fifth use, the bottle still maintained its efficacy (data not shown). In similar way, *Anopheles gambiae* Dassa-Zoume populations, after four susceptibility tests during four consecutive days in insecticide testing laboratory at CREC remained susceptible to bendiocarb. A Wheaton treated bottle with 12.5µg a.i bendiocarb per bottle could be used four times during four consecutive days in laboratory conditions. On the fifth use, the bottle still maintained its efficacy too (data not shown). Regarding *Anopheles gambiae* Allada populations, after four susceptibility tests during four consecutive days in insecticide testing laboratory at CREC, these *Anopheles gambiae* populations remained resistant to DDT. There was no knocked down mosquito on the fifth use (data not shown). A Wheaton treated bottle with 100µg a.i DDT per bottle could be used four times during four consecutive days in laboratory conditions. In laboratory conditions, the study by Aïzoun *et al.* (2014) showed that a Wheaton treated bottle with 12.5 µg a.i deltamethrin per bottle and 21.5 µg a.i permethrin per bottle could be used at least three times during four consecutive days. After the fourth use, bottles have to be washed and re-coated before their re-use. In field conditions, the same study showed that a WHO impregnated paper with bendiocarb could be used four times during four consecutive days. On the fifth use, the paper still maintained its efficacy. In field conditions, another study by Perea *et al.* (2009) showed that treated bottles with 10 µg a.i deltamethrin per bottle could be stored for at least 14 days and re-used on three occasions if these bottles had been capped and stored in the dark at ambient temperatures in the field. These authors showed that after three uses, they appear to lose effect. That was presumably due to the redistribution of insecticide caused by contact with mosquitoes, aspirators and moisture from the air or from mouth aspiration. These authors also showed that wherever bottles are to be pre-prepared and stored, similar tests should be used to define their effective shelf-lifes.

The study of the efficacy of a Wheaton coated bottle with fenitrothion, DDT and bendiocarb in laboratory conditions was useful in the assessment of insecticide vectors susceptibility tests**.** Treated bottle storage and the ambient temperature and relative humidity recording in laboratory during susceptibility tests have also an important role in the efficacy study.

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