

Synergist bioassays: A simple method for initial metabolic resistance investigation of field *Anopheles gambiae* s.l. populations



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ABSTRACT

Metabolic resistance and the potential role of permeability-glycoprotein (P-gp) efflux pumps were investigated in a pyrethroid-resistant wild *Anopheles gambiae* s.l. Tiassalé population, using WHO susceptibility assays with deltamethrin (0.05%), with and without pre-exposure to synergists. The synergists used included an inhibitor of P-glycoprotein efflux pumps (verapamil), an inhibitor of esterases (EN 16-5), and an inhibitor of P450s and esterases (piperonyl butoxide). Pre-exposure to verapamil followed by deltamethrin led to a slight but non-significant ($P=0.59$) increase in mortality relative to exposure to deltamethrin alone (64.5% versus 69.2%). Similarly, pre-exposure to EN 16-5 yielded a non-significant increase in mortality (to 76.6%; $P=0.85$) but a significant increase in the knock down rate (from 48.3% to 78.7%; $P<0.01$). Pre-exposure with PBO caused a significant increase in mortality (to 93.1%; $P<0.001$) and knockdown rate (100%; $P<0.001$), which related to a 2.9 fold decrease in the resistance level. The results provide evidence that metabolic resistance mechanisms are present within the assessed mosquito population. The decrease in time to knock down of this population with deltamethrin following exposure to EN16-5 and PBO is of particular relevance to vector control, where quick knock down is a highly desired characteristic. The suspected resistance mechanisms present in this population merit further investigation through biochemical and molecular analyses for full resistance profile characterization. Bioassays with synergists can provide a quick and easy basis for initial characterization of resistant mosquito populations, without the need of preserved specimens, expensive equipment and substrates or specialized expertise.

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1. Introduction

The basic mechanisms underlying insecticide resistance include insecticide target-site mutations such as changes in the sodium channel that leads to so-called 'knockdown resistance', and increased metabolic detoxification of the insecticide through overproduction or elevated enzymatic activity (Hemingway and Ranson, 2000). Three enzyme families are primarily involved in insecticide detoxification: the carboxylesterases

(COEs), glutathione-S-transferases (GSTs) and cytochrome P450s (P450s). Even though the detoxification gene families involved in insecticide resistance have been identified, little is known about how many genes in each family are directly involved in conferring resistance. The molecular mechanisms involved in insecticide resistance in general, and the functioning of up-regulated P450 genes, GSTs, and esterases in particular, are not yet clear. Insects and other eukaryotes can also become resistant to xenobiotics through the activity of permeability glycoprotein (P-gp) that actively removes xenobiotics from the cell before it can cause harm. Also known as multidrug resistance protein or ATP-binding cassette, P-gp is an ATP-dependent transporter which transports a wide variety of substrates across extra- and intra-cellular membranes. The pump mechanism attaches a protein label to the xenobiotic and removes it by exocytosis (Sarkadi et al., 2006). Apart from its role in multidrug resistance in mammalian tumour cell lines, there is considerable evidence that insect P-gps may play an important role in insecticide resistance (Lanning et al., 1994; Buss and Callaghan, 2008). The pattern of cross-resistance observed in malaria vectors

Abbreviations: P450s, cytochrome P450 oxidases; P-gp, permeable-glycoprotein; PBO, piperonyl butoxide; COE, carboxylesterase; GST, glutathion S-transferase; KD, knock down; KDT, knock down time; MDR, multi drug resistance.

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is similar to multidrug resistance seen in tumour cell lines where insects develop resistance to structurally unrelated insecticides. This has stimulated our interest in investigating this mechanism in malaria vectors through the use of synergists. The use of microarrays has been crucial to detect up-regulated genes, including ATP-binding cassette transporters, but requires sophisticated equipment and training. Synergists can be used in bioassays as a relatively simple way of investigating the role of different enzymes in insecticide metabolism. Furthermore, we investigated the role of both P450s and COEs on a wild *Anopheles gambiae* s.l. population from Tiassalé (southern Côte d'Ivoire) where resistance to almost all the families of insecticides used in agriculture and public health has been described (Chouaïbou et al., 2012; Edi et al., 2012).

2. Material and methods

An. gambiae s.l. mosquitoes were collected as pre-imaginal stages (L1 to L4 instars) from the village of Tiassalé in Côte d'Ivoire in 2010. Larvae were fed with powdered TetraFin® fish food and reared to adults under insectary conditions of 25–28 °C and 70%–80% relative humidity. Unfed adult female *An. gambiae* s.l. at 1 to 3 days post-emergence were used in WHO susceptibility tests (WHO, 1998). Four different serials of susceptibility tests were performed. The first one was conducted with deltamethrin-treated papers alone (0.05%) in standard assays performed at 25 °C and 70%–80% relative humidity. The three other serials of tests were done by exposing mosquitoes to a synergist before exposure to deltamethrin. Three different synergists were used: (1) verapamil (0.01%), a calcium blocker known to be a potent inhibitor of P-gps (Podsiadlowski et al., 1998); (2) EN 16-5 (0.1%), an analogue of PBO known to inhibit esterases (Moore et al., 2009), and (3) PBO (0.1%), an inhibitor of P450s and esterases (Khot et al., 2008). For each of the three susceptibility tests, mosquitoes were pre-exposed to a specific synergist for 20 min prior to 60 min exposure to deltamethrin. Control batches were exposed to synergist only. During the 60 min exposure period, the knockdown rate (KD) was recorded at 5 min intervals. Unfed female adults of 1–3 days post-emergence from a susceptible *An. gambiae* s.s. (Kisumu) laboratory strain were used as a reference to validate the quality of treated papers. Following exposure, mosquitoes were supplied with 10% honey solution and kept overnight under insectary conditions with mortality noted at 24 h post-exposure. Results were analyzed using EpiInfo Version 6 to test for any significant difference in mortalities between the different groups via Mantel–Haenszel Chi square analyses. The time at which 50% of the test population were knocked down (KDT_{50}) was determined using PoloPlus software, via log-probit analysis. The resistance reduction was determined by dividing the KDT_{50} obtained for deltamethrin by the KDT_{50} obtained for deltamethrin plus the synergist.

3. Results and discussion

The *An. gambiae* s.s. Kisumu reference strain exhibited full susceptibility to deltamethrin (100% mortality) in standard WHO susceptibility tests, confirming bioefficacy of the treated papers used. For the wild Tiassalé strain, phenotypic resistance to deltamethrin was confirmed based on 64.37% mortality and 48.35% knock down (Fig. 1). A similar mortality (69.23%; $P=0.58$) and knockdown (52.87%; $P=0.59$) were observed when the population was pre-exposed to verapamil ($P=0.59$) (Fig. 1). Pre-exposure to EN 16-5 also yielded no significant increase in mortality (76.6%; $P=0.85$), although a significant increase in the knock down rate was observed (from 48.3% to 78.7%; $P<0.01$) (Fig. 1). The required time for 50% of the mosquitoes to be knocked down (KDT_{50}) shifted from 63.32 min for deltamethrin alone to 56.36

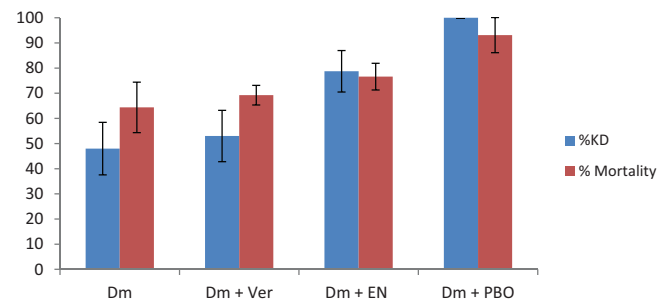


Fig. 1. Knock down time and mortality rates of a wild resistant *Anopheles gambiae* population from Tiassalé. Increase in mortality to deltamethrin was observed following 20 min pre-exposure to several synergists (verapamil = inhibitor of P-gps, EN 16-5 = COE inhibitor, PBO = P450 inhibitor).

and 36.31 min where there had been pre-exposure to verapamil or EN16-5, respectively (Figs. 2–3, Table 1). PBO pre-exposure followed by deltamethrin resulted in 93.10% mortality and 100% knock down, which was significantly higher than for deltamethrin alone, deltamethrin + verapamil and deltamethrin + EN16-5 ($P<0.001$, for all). Mortality in all control groups was consistently below 5%, and thus no correction was required.

A reduction in the time to knock down houseflies, using PBO with pyrethrins, has previously been observed at Rothamsted (Khambay, pers comm). This has been attributed to the enhancement of pyrethroid penetration due to the surfactant properties of synergists such as PBO and EN16-5 (Unpublished internal report, Dept. University of Florence, 2007). Although the pre-exposure in this study was of a short duration (20 min) instead of 1 h (Nwane et al., 2013), the level of resistance was reduced by around 3-fold with PBO which was accompanied by a shift in KDT_{50} from 63.32 min to 21.86 min (Fig. 4), and for 1.7 fold with EN 16-5 (Table 1). This suggests that both P450s and COEs play an important role in the deltamethrin resistance observed in this vector population. Temporal synergism in this population should be further explored, using various pre-exposure times to verify if this can affect outcomes (Moore et al., 2009).

PBO is primarily referred as a synergist of cytochrome P450s, which belong to a superfamily of enzymes (Feyereisen, 1999) that are well known for their ability to metabolize a wide range of compounds (David et al., 2013). Elevated levels of P450 activity have been observed in pyrethroid-resistant malaria vectors in Africa, particularly in *Anopheles funestus* from southern Africa (Brooke et al., 2001; Wondji et al., 2009, 2012). The role of PBO in COE inhibition has also been demonstrated (Khot et al., 2008). COEs can confer resistance to organophosphates, carbamates and pyrethroids which are rich with ester-bonds. Elevated COEs have been detected in *An. culicifacies* resistant to malathion in India (Malcolm and Boddington, 1989). GSTs are dimeric multifunctional enzymes that play a role in detoxification of a large range of xenobiotics, and are likely important in detoxification of DDT. Although we did not investigate the role of GSTs,

Table 1

Time necessary for 50% of wild resistant *An. gambiae* mosquitoes from Tiassalé to be knocked down and magnitude of resistance reduction following exposure to deltamethrin-treated papers in standard WHO susceptibility tests (WHO, 1998) with and without pre-exposure to synergist-treated papers.

	KDT_{50} (min)	IC95 (%)	Resistance reduction fold
Delta	63.32	[57.26–75.31]	–
Delta + ver	56.36	[53.43–61.02]	1.1
Delta + EN	36.31	[34.87–38.34]	1.7
Delta + PBO	21.86	[21.21–22.64]	2.9

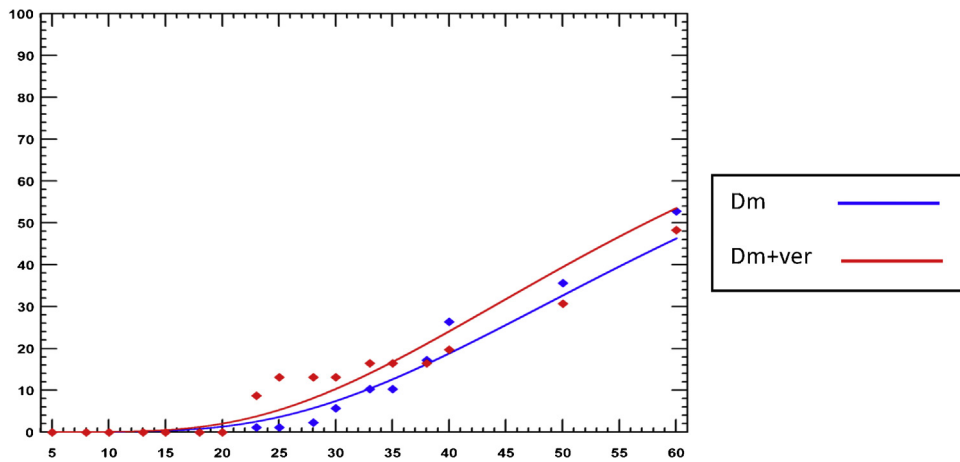


Fig. 2. Knock down time responses of a resistant *An. gambiae* population from Tiassalé to deltamethrin. A slight reduction in KDT was observed after 20 min pre-exposure to the inhibitor of P-gps (verapamil).

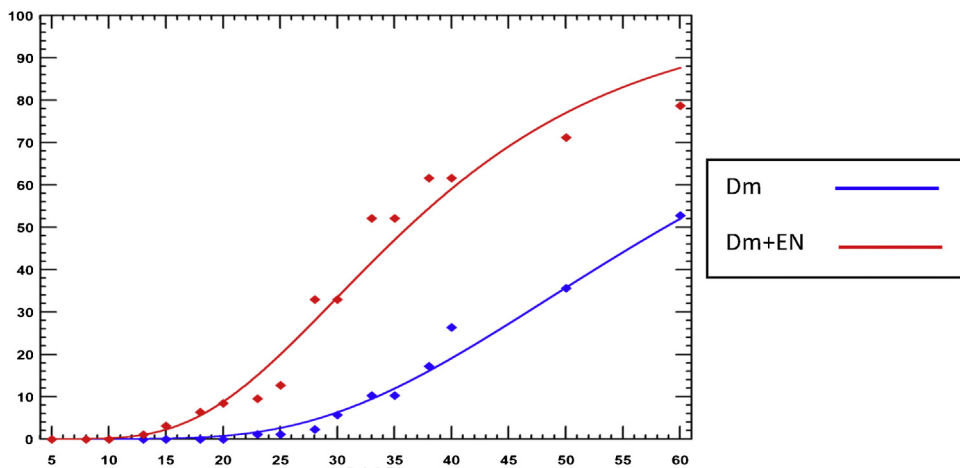


Fig. 3. Knock down time responses of a resistant *An. gambiae* population from Tiassalé to deltamethrin. A reduction in KDT was observed after 20 min pre-exposure to the inhibitor of COE (EN 16-5). KDT_{50} shifted from 63.32 min for deltamethrin alone to 36.31 min for deltamethrin + EN 16-5.

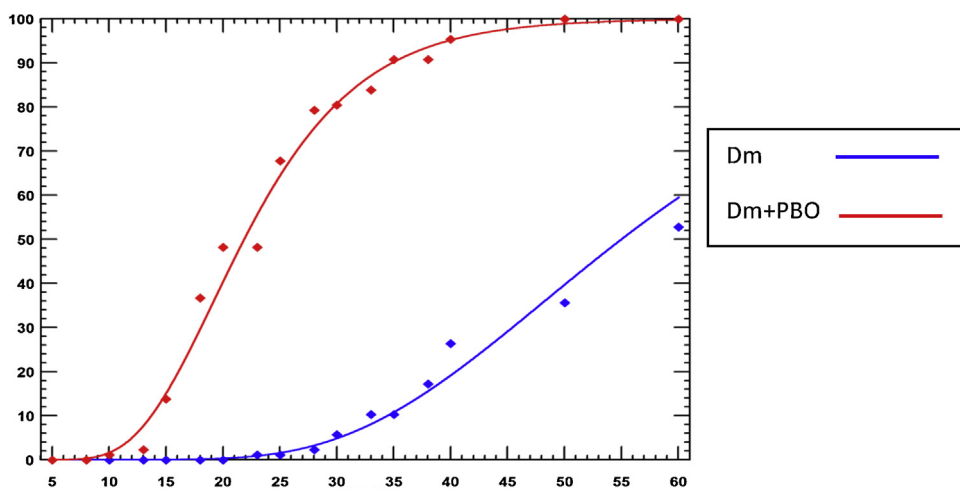


Fig. 4. Knock down time responses of a resistant *An. gambiae* population from Tiassalé to deltamethrin. A significant reduction in knockdown time was observed after 20 min pre-exposure to the P450 inhibitor (PBO). KDT_{50} shifted from 63.32 min for deltamethrin alone to 21.86 min for deltamethrin + PBO.

their involvement merits further investigation using the synergist diethyl maleate (Nwane et al., 2013) as these could also contribute to the high level of DDT resistance previously observed in Tiassalé *An. gambiae* s.s. population (Chouaibou et al., 2012; Edi et al., 2012). The target site mutations that confer cross-resistance to DDT and pyrethroids (*kdr*), and those implicated in resistance to carbamates and organophosphates (modified acetylcholinesterase, *Ace-IR*) have also been previously detected in this population (Chouaibou et al., 2012; Edi et al., 2012; Alou et al., 2010).

Only a low and non-significant activity of P-gps was seen in this study, although it is important to note that recent reports indicate that verapamil can inhibit some P450s. Our observations may be due to short pre-exposure time insufficient to allow full inhibition to occur (20 min). Furthermore, since only a single pyrethroid (deltamethrin) was used in this study, there was a reduced probability of observing any possible role of efflux pumps that may have an effect against other types or classes of insecticides. There is substantial evidence to indicate that the presence of the *kdr* mutation may not always produce a resistance phenotype that is of significance to operational control (Brooke, 2008). A number of studies have reported high-level insecticide resistance associated with the *kdr* mutation in populations from farm fields in West Africa (Diabaté et al., 2002; Tripet et al., 2007; Chouaibou et al., 2012; Edi et al., 2012). For these examples, it is highly likely that the resistance phenotype may be influenced by metabolic mechanisms that were not tested for because of the lack of common diagnostic tools.

Microarray technology has provided evidence of several putative genes involvement in metabolic resistance. However, the implementation of this technology is very expensive and its use is technically complex requiring specialized expertise. Furthermore, the process of handling and conservation of mosquito specimens following field collections can also affect the quality of results. Therefore, given the speed with which resistance phenomena occur and their geographic spread, microarray technology is currently not suitable for prompt and timely data to enable evidence-based decision making to guide vector control interventions. Bioassays remain the simplest and quickest method to assess metabolic resistance in field mosquitoes. The use of synergists in bioassays should be implemented along with standard bioassay monitoring procedures and investments in promoting understanding of how to read and report results from these tests is needed. Ideally, synergist-impregnated papers should be made available and accessible through the same channel as the WHO insecticide-treated papers. Only then can we understand which insecticides will likely work with resistant populations and appropriately design and deploy new tools. From this stage, a more complete characterization including biochemical and molecular analyzes can be undertaken ultimately to build up specific rapid diagnostic tools better adapted for the sub-Saharan African context.

The effort to overcome the insecticide resistance problem in malaria vectors should take into account all putative sources of resistance and mechanisms involved. The results herein provide evidence that metabolic resistance mechanisms are present within the mosquito population investigated. These mechanisms warrant further investigation through biochemical and molecular analyses, in order to fully characterise the resistance profile of the test population. The reduction in time to knock down following pre-exposure to EN16-5 and PBO for the Tiassalé population is an interesting finding and of particular relevance to vector control tools, where quick knock down is a highly desirable characteristic. Bioassays with synergists can provide a quick and easy basis for initial characterization of resistant mosquito populations, without the need

for correctly-preserved specimens, sophisticated equipment and specialized training of laboratory personnel.

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