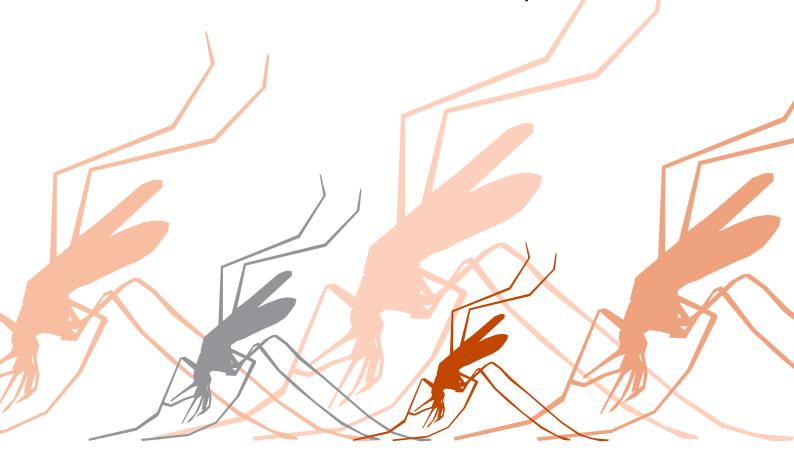


for insecticide resistance monitoring in malaria vector mosquitoes







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### List of abbreviations

CDC Centers for Disease Control and Prevention

DDT Dichloro-diphenyl-trichloroethane

ELISA Enzyme-linked immunosorbent assay

GMP Global Malaria Programme

GPIRM Global Plan for Insecticide Resistance Management in Malaria Vectors

IRM Insecticide resistance management

IRS Indoor residual spraying

ITNs Insecticide-treated nets

kdr knock-down resistance

LD50 Lethal dose 50% (i.e. the dosage expected to kill exactly 50% of exposed insects)

LLIN Long-lasting insecticidal net

NMCP National malaria control programme

PCR Polymerase chain reaction
WHO World Health Organization

WHOPES World Health Organization Pesticide Evaluation Scheme

### Glossary

Ace-1 A target site resistance gene for carbamate and organophosphate in-

secticides conferring insensitive acetylcholinesterase (AChE) caused by

a single mutation, G119S, of the Ace-1 gene.

Cross-resistance When resistance to one insecticide confers resistance to another in-

secticide, even where the insect has not been exposed to the latter

product.

F1 progeny Generally means "first generation offspring". In this context, however, it

refers to the use of adults raised from the eggs of wild-caught female mosquitoes in order to obtain an age-standardized sample of the wild

population for bioassay tests for resistance.

Insecticide combination The use of two or more insecticide applications within a building (e.g.

one insecticide on the walls and another on nets in the same household). Insecticide combinations differ from insecticide mixtures in that the same insect is likely, but not guaranteed, to come in contact with

both insecticides.

Insecticide mixture Two or more compounds mixed within a single product or formulation so

that the mosquito will contact both simultaneously.

Insecticide mosaic The spraying of compound A in one area and compound B in another

area (usually in a grid pattern), so that some mosquito populations are

exposed to A while others are exposed to B.

kdr Knockdown resistance is caused by a series of genes involving a mu-

tation in the sodium ion channel, the target site of pyrethroids and Dichloro-diphenyl-trichloroethane (DDT), and conferring resistance to

these insecticides.

Susceptibility tests Bioassays in which insects from a wild population are exposed to a

fixed dose of insecticide designed to reliably kill susceptible insects, so that any survivors may be assumed to be resistant. The WHO standard method is long-established, whereas the Centers for Disease Control

and Prevention (CDC) method is more recently developed.

Sympatry Species which occupy roughly the same area of land but do not inter-

breed are said to exist in sympatry.

Synergist A substance which does not itself have insecticidal properties, but

which, when mixed or applied with insecticides of a particular class, considerably enhances their potency by inhibiting an enzyme that nor-

mally acts to detoxify the insecticide.

### 1. Introduction

Global malaria control efforts have produced remarkable results over the past decade. In 2010, for example, there were an estimated 219 million episodes of malaria (range 154–289 million) and an estimated 660 000 deaths (range 490 000–836 000). These figures are significantly lower than in previous years. Similarly, an estimated 1.1 million malaria deaths were averted during the past decade and 58% of these lives were saved in ten countries with the highest malaria burden (1). Much of the recent decrease in the global malaria burden has been achieved through the scale-up of vector control interventions, in particular, the use of insecticides for indoor residual spraying (IRS) and for treating mosquito bed nets and other materials (2).

Although four classes of insecticide are recommended by WHO for use against adult mosquitoes in public health programmes,<sup>1</sup> in practice, modern-day malaria vector control has become highly dependent on just one class of insecticide – the pyrethroids. The pyrethroids offer several advantages over other insecticides in terms of cost, safety (less toxic to mammals) and duration of residual action. These insecticides are now widely used, both in agriculture and as household pesticides; their use as larvicides is limited because of their toxicity to non-target aquatic organisms including fish. Currently, pyrethroids are used on all approved long-lasting insecticidal nets (LLINs) and are the basis of the vast majority of IRS programmes worldwide (3).

The near ubiquitous use of just one class of insecticide has given rise to fresh concerns about the problem of resistance to insecticides in malaria vectors. Following an increase in entomological surveillance in malaria-affected regions in recent years, sufficient data have now been collected to confirm already strong suspicions that the wide-scale use of insecticide-based malaria control strategies over the past decade has been associated with the development of resistance in several important vector species, including *Anopheles gambiae*, *An. funestus* and *An arabiensis*. According to latest reports, resistance to at least one class of insecticide has been identified in 64 countries with ongoing malaria transmission, with resistance to the pyrethroids being the most common. Pyrethroid resistance has been reported in malaria vector mosquito populations in 27 countries in sub-Saharan Africa (4). The mechanisms responsible for the now widespread levels of resistance have also been identified; these tend to be of two main types, those mediated by changes at the target site of the insecticide (e.g. *kdr* mutations) and those caused by increases in the rate of insecticide metabolism. Resistance mechanisms and their implications for vector control strategies are explained further in Box 1.1.

The global malaria community is responding to the potential threat posed by emerging insecticide resistance; in May 2012, WHO launched the Global Plan for Insecticide Resistance Management in Malaria Vectors (GPIRM) (6), which sets out a comprehensive framework for action in five key areas (or "pillars"):

a) planning and implementing national insecticide resistance management strategies:

For a current list of WHO-recommended insecticides refer to the WHO Pesticide Evaluation Scheme web site at: http://www.who.int/whopes/en/

#### **BOX 1.1**

## Insecticide resistance mechanisms: implications for vector control programmes

The management of insecticide resistance is complicated by the fact that resistance takes a variety of forms. Broadly speaking, resistance mechanisms can be divided into two groups: *metabolic resistance* and *target-site resistance*.

Metabolic resistance arises because of changes in a mosquito's enzyme systems that result in a more rapid detoxification of the insecticide than normal, preventing the insecticide from reaching the intended site of action. In the case of malaria vectors, three enzyme systems are believed to be important: the esterases, the mono-oxygenases and the glutathione *S*-transferases.

Target-site resistance occurs when the protein receptor that the insecticide is designed to attack is altered by a mutation: when this happens the insecticide can no longer bind to the intended target site of the receptor and thus the insect is unaffected, or less affected, by the insecticide. In the case of DDT and the pyrethroids, the mutation occurs in the sodium channel receptors, conferring what is described as *kdr* or "knockdown resistance". In the case of the organophosphates and the carbamates, the mutation occurs in the protein, acetylcholinesterase (AChE, a neurotransmitter), conferring what is usually referred to as *Ace-1* resistance.

An added complication arises because of "cross-resistance" between different classes of insecticide that share the same mode of action. Thus vectors that are resistant to pyrethroids because they possess the *kdr*-resistant gene will probably also be resistant to DDT. Likewise, the *Ace-1* mutation can confer target site resistance to both carbamate and organophosphate insecticides. The existence of cross-resistance will restrict the choice of alternative insecticides in situations where resistance has been detected.

What impact the observed spread of resistance, and the increased presence of kdr genes in particular, will have on the effectiveness of current vector control programmes is however far from certain. Recent studies have provided conflicting evidence; some have shown that kdr resistance does not decrease the level of protection conferred by insecticide-treated nets (ITNs) while others have reported a fitness advantage for kdr-resistant phenotypes and there has been at least one report of decreased efficacy of ITNs in an area of pyrethroid resistance.

The consensus of opinion that has emerged is that it seems unlikely, on the basis of current evidence, that the presence of a resistant gene will render current vector control strategies ineffective overnight. Nevertheless, the possibility that emerging resistance will threaten the robustness of pyrethroid-based interventions (LLINs and IRS) in the longer term remains. The prudent course of action is therefore to adopt a proactive approach and modify current practices so as to delay the spread of resistance and preserve the effectiveness of current insecticides at least until new classes of insecticides and new tools can be developed.

Source: reference (5).

- b) ensuring entomological and susceptibility monitoring and effective data management;
- c) developing new, innovative vector control tools;
- d) filling knowledge gaps on resistance mechanisms and the impact of current insecticide resistance management approaches;
- e) enabling mechanisms to improve advocacy and build human and financial resources.

The GPIRM is unequivocal about the need for an intensification of the insecticide resistance monitoring effort, and calls for greater regularization of this function within national malaria control programmes (NMCPs). In particular, monitoring plans increasingly need to address the requirement for more detailed data on: vector species distributions and their relevant attributes (e.g. biting and resting preferences); the resistance status of each vector species to currently used insecticides; and the quality and efficacy of vector control interventions. Epidemiological studies which assess the operational implications of different types of resistance are also seen as a vital part of the expanded knowledge base that is now urgently needed to guide insecticide use and the development of strategies for managing insecticide resistance as part of malaria and other vector-borne disease control programmes (6).

WHO has a long tradition of providing support to countries in monitoring and managing insecticide resistance and this remains one of the core functions of its Global Malaria Programme (GMP). WHO has served as the global coordinator for information on vector resistance for more than 50 years, providing Member States with regularly updated advice and guidance on monitoring and managing insecticide resistance as it evolves. As part of this role, and to ensure comparability of insecticide resistance data from different countries and sources, WHO has developed standard test procedures and operational standards for detecting and monitoring insecticide resistance in a range of disease vectors, including mosquitoes. The supply of quality-assured susceptibility test kits for use in the field has also been a core component of WHO's work in this area (7).

In the case of the malaria vectors, a series of guidelines and instructions for testing for the presence of insecticide resistance using a standardized bioassay technique in adult mosquitoes have been published over the years (8, 9, 10). Successive updates of the guidelines have reflected developments in malaria control strategies, in particular, the introduction of new classes of insecticides in public health programmes. The latest version of these guidelines, published in 1998, covers all four of the main classes of insecticides in routine use, namely organochlorine, organophosphate, carbamate and pyrethroid insecticides (10). This document also includes guidance on assessing the biological efficacy of insecticides when applied on surfaces (e.g. walls of houses and insecticide-treated materials such as mosquito nets and curtains).

Given the mounting evidence of emerging resistance among malaria vectors, especially to pyrethroids, and the desire not to undermine recent gains made in the battle against malaria, attention has once more focused on the need for closer monitoring of insecticide resistance. This has prompted calls for an update of the 1998 guidelines, primarily to ensure that future monitoring efforts are better aligned to the information needs that are outlined in the GPIRM.

1. Introduction 3

## 2. Evolution of the WHO susceptibility test: background to current revision

The WHO insecticide susceptibility bioassay that is the subject of these revised guidelines is a simple direct response-to-exposure test. Mosquitoes are exposed to known concentrations of an insecticide for a fixed period of time at the end of which the number of fatalities is recorded. In its present form, the test is designed to distinguish between baseline susceptibility and resistance to insecticides in adult mosquitoes. As such, the test is intended to be used as a field and laboratory surveillance tool with the limitation that it gives little information on the underlying mode(s) or mechanism(s) conferring resistance where detected.

The test equipment and methodology has changed relatively little since WHO first recommended the use of a standard bioassay technique to detect insecticide resistance in the early 1960s (11). Any methodological changes that have been made over the years have been fairly minor, relating largely to the test conditions and the insect sampling protocols (8, 9, 10).

The impetus for the present revision stems from the recommendations of an informal consultation convened in May 2010 by WHO-GMP in order to review the current status of insecticide resistance in malaria vectors and to identify strategies for delaying the emergence of resistance. Recognizing the central role of insecticide resistance monitoring to the success of vector control, the informal consultation resulted in several recommendations regarding the future direction of insecticide resistance detection and monitoring. In addition to highlighting the need for an immediate scale-up in susceptibility testing, the experts participating in the consultation recommended the establishment of reporting mechanisms to ensure that resistance data are collected, collated and fed back into the decision-making process, thereby making vector control less "one-size-fits-all" and more responsive to the local situation (5).

It was recommended that the standard WHO susceptibility tests should continue to be the primary method by which resistance is detected and identified. However, it was considered necessary, as a matter of some urgency, to update the existing resistance monitoring guidelines (10) in order to reflect new priorities and information needs, and in particular, to highlight the need for accurate species identification of all test mosquitoes. Consequently, a working group, comprising a sub-set of meeting participants, was formed in order to undertake this task. Specific objectives for the working group charged with updating the insecticide resistance guidelines were identified as follows:

- to provide an update to the WHO test procedures for monitoring insecticide resistance in malaria vectors to align with new developments in vector resistance management;
- to provide an updated list of "discriminating dosages" for adult mosquitoes for all insecticides used either in malaria vector control or for research purposes;
- in the context of interpreting the results of the WHO susceptibility test, to refine the definition of "resistance" which triggers pre-emptive action by national control programmes to manage insecticide resistance;

to identify mechanisms for the process of reporting, collating and interpreting insecticide resistance data, which ensure that the data are used to inform resistance management plans and strategies.

Because insecticide resistance also needs to be described in genetic terms, the experts at the informal consultation further recommended that routine susceptibility monitoring using the WHO bioassay be supplemented by additional genetic testing and, to a lesser extent, by biochemical testing. Supplementary test methods for determining the underlying mechanisms of resistance and tools for tracking the spread of resistance are important for decision-making to manage insecticide resistance at country level.

Note that guidelines and test procedures for laboratory and field testing of mosquito larvicides are also available. As the guidelines for larvicide testing are still considered valid, having been the subject of a more recent revision (in 2005), they are not repeated in the present document. Those interested in larvicides and their evaluation are advised to refer to the original documents which are available from WHO (12, 13). Also of note, brief mention is now made of a complementary method for field testing of insecticide resistance, the bottle assay developed by the United States Centers for Disease Control and Prevention (see section 6).

## 3. The WHO susceptibility test for adult mosquitoes

As mentioned above, the WHO susceptibility bioassay is a direct response-to-exposure test; it measures mosquito mortality to a known standard dose of a given insecticide (i.e. the diagnostic or discriminating concentration). The test procedure itself is summarized in Box 3.1.

#### 3.1 Discriminating concentrations

The concept of **discriminating** or **diagnostic concentrations** (or dosages) is now well established and has been widely adopted for the purposes of monitoring insecticide resistance in mosquitoes and other disease vectors (10, 14, 15). The use of discriminating concentrations in routine resistance monitoring is explained in more detail in Box 3.2.

Discriminating concentrations have been established under standardized laboratory conditions for all insecticides currently used in malaria control programmes; these have been reported in previous versions of these guidelines (8, 9, 10) and are updated again in this revision. Discriminating concentrations for a range of pyrethroid insecticides were included for the first time in the 1998 guidelines, having been the subject of a multi-centre study involving nine institutes (9). The anopheline species used in this study were An. aconites, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s, An. maculatus, An. minimus and An. stephensi.

Since then discriminating concentrations have been established for a further four insecticides, although as yet these are tentative pending confirmation by WHO's Pesticide Evaluation Scheme (WHOPES). For some of the newer slower-acting insecticides, such as chlorfenapyr, discriminating concentrations are likely to be based on longer holding periods (for instance, 48 hours or 72 hours, instead of 24 hours)(16).

Papers already impregnated with insecticide at the appropriate diagnostic concentrations are provided as part of the test kits supplied by WHO (see also section 3.2.3). In order to be certain that all susceptible mosquitoes are killed, WHO has traditionally defined its discriminating concentrations in one of two ways, that is, as either:

- twice the lowest concentration that gave systematically 100% mortality after 60 minutes exposure and a holding period of 24 hours on a susceptible strain or a susceptible population; or
- twice the LC<sub>99.9</sub> value as determined by baseline susceptibility testing of a susceptible strain or a susceptible population.

Table 3.1 lists the WHO recommended discriminating concentrations for insecticides used in malaria control and/or for research purposes (e.g. dieldrin) for adult malaria vectors.

#### 3.2 Equipment and supplies

#### 3.2.1 Procurement

Test kits and insecticide-impregnated papers are prepared on behalf of WHO by the Universiti Sains Malaysia, which is based in Penang, Malaysia. The procedures and conditions for procuring test kits

#### **BOX 3.1**

## Measuring susceptibility to insecticides in adult mosquitoes: the WHO bioassay test procedure

- Six sheets of clean white paper (12 x 15 cm), rolled into a cylinder shape, are inserted into six holding tubes (one per tube) and fastened into position with a steel spring-wire clip. The tubes are attached to slides.
- 2. At least 120–150 active female mosquitoes are aspirated (in batches) from a mosquito cage into the six holding tubes through the filling hole in the slide to give six replicate samples of 20–25 mosquitoes per tube.
- Once the mosquitoes have been transferred, the slide unit is closed and the holding tubes set in an upright position for one hour. At the end of this time, any damaged insects are removed.
- 4. Six exposure tubes are prepared in much the same way. Each of the 4 reddotted exposure tubes are lined with a sheet of insecticide-impregnated paper, while the 2 yellow-dotted control exposure tubes are lined with oil-impregnated papers; each is fastened into position with a copper spring-wire clip.
- 5. The empty exposure tubes are attached to the vacant position on the slides and with the slide unit open the mosquitoes are blown gently into the exposure tubes. Once all the mosquitoes are in the exposure tubes, the slide unit is closed and the holding tubes can be detached and set to one side.
- 6. Mosquitoes are kept in the exposure tubes, which are set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour (60 minutes).
- 7. At the end of the 1-hour exposure period, the mosquitoes are transferred back to the holding tubes by reversing the procedure outlined in step 5. The exposure tubes are detached from the slide units. A pad of a cotton-wool soaked in sugar water is placed on the mesh-screen end of the holding tubes.
- 8. Mosquitoes are maintained in the holding tubes for 24 hours (the recovery period). During this time, it is important to keep the holding tubes in a shady, sheltered place free from extremes of temperature (an insectary is ideal). Temperature and humidity should be recorded during the recovery period.
- 9. At the end of recovery period (i.e. 24 hours post-exposure), the number of dead mosquitoes is counted and recorded. An adult mosquito is considered to be alive if it is able to fly, regardless of the number of legs remaining. Any knocked-down mosquitoes, whether or not they have lost legs or wings, are considered moribund and are counted as dead.
- 10. On completion of the susceptibility test, mosquitoes may be transferred to individual, clearly labelled Eppendorf tubes (separating dead and live mosquitoes into separate tubes) for storage until such time that they can be transferred to suitable facilities for species identification and supplementary testing if necessary.

#### **BOX 3.2**

#### **Use of discriminating concentrations**

In order to investigate resistance in vector populations it is necessary to first obtain baseline susceptibility data for individual insecticides in a normal or "susceptible" population of a given species. (A susceptible population is one that has not been subjected to insecticidal pressure and in which the presence of resistant individuals is either absent or rare.) This is achieved by exposing non-resistant vectors to serial concentrations of a given insecticide (or to serial time exposures at a single concentration), and plotting the percentage mortality against exposure on logarithmic-probability paper in order to estimate the doses required to produce various levels of kill (alternatively this calculation can be done using a log-probit statistical model). By this means, it is possible to derive the concentration corresponding to 99.9% mortality (the  $LC_{99.9}$  value); at this concentration there is a very high probability that all individuals in a susceptible population will be killed. This concentration is conventionally known as the diagnostic or discriminating concentration.

Having established discriminating concentrations for individual insecticides under standardized laboratory conditions using known susceptible strains or populations of a range of mosquito vector species, it is then not necessary for routine monitoring purposes to conduct susceptibility tests at the full range of exposures. It is sufficient to conduct a standard bioassay resistance test using the diagnostic concentration only as any survivors at this concentration may be considered to be resistant. This approach has obvious advantages in terms of the cost and efficiency of testing.

It is important to recognize that for mosquito species that are not routinely monitored and/or in novel situations where baseline data are not available, it is necessary to first establish the baseline susceptibility as described above (7).

and impregnated papers are specified in the WHO document, *Supplies for monitoring insecticide resistance in disease vectors. Procedures and conditions* (7). All items included as part of the test kits, including the impregnated papers, can be ordered separately. Full instructions for carrying out the susceptibility test are included as part of the kit, along with multiple copies of the recommended data recording forms. These are attached to this document as Annexes 1 and 2.

#### 3.2.2 Composition of the test kits

The composition of the kits supplied by Universiti Sains Malaysia has been modified slightly as a result of the present review of insecticide resistance monitoring procedures. Accordingly, the recommended test kit composition is as follows:

Table 3.1 Discriminating concentrations of insecticides for adult anopheline mosquitoes<sup>1</sup>

Insecticide class	Insecticide	Discriminating concentration (1-hour exposure period)	
Organochlorines	DDT	4%	
	Dieldrina	4%0.4%	
	Dielatilia	4%	
Organophosphates	Malathion	5%	
	Fenitrothion <sup>b</sup>	1%	
	Pirimiphos methyl <sup>c,d</sup>	0.25%	
Carbamates	Propoxur	0.1%	
	Bendiocarb	0.1%	
	Carbosulfan <sup>c,e</sup>	0.4%	
Pyrethroids	Permethrin	0.75%	
	Deltamethrin	0.05%	
	Lambda-cyhalothrin	0.05%	
	Cyfluthrin	0.15%	
	Etofenprox	0.5%	
Pyrroles	Chlorfenapyr <sup>c,f</sup>	5%	
Phenyl pyrazoles	Fipronil <sup>c,g</sup>	2%	

<sup>&</sup>lt;sup>a</sup> Exposure to dieldrin at 0.4% kills susceptible (ss) individuals but not resistant heterozygotes (Rs), while exposures to dieldrin at 4% kills heterozygotes (Rs) but not homozygous resistant (RR) individuals.

- 12 plastic tubes (125 mm in length and 44 mm in diameter) with each tube fitted at one end with 16-mesh gauze. The 12 tubes include:
  - four marked with a **red** dot for use as exposure tubes, i.e. for exposing mosquitoes to insecticide-impregnated papers;
  - two marked with a **yellow** dot for use as control tubes, for exposure of mosquitoes to the oiltreated control papers (i.e. without insecticide);

b Two-hour exposure.

<sup>&</sup>lt;sup>c</sup> Tentative; to be confirmed by WHOPES.

d Based on unpublished industry data, 2006.

<sup>&</sup>lt;sup>e</sup> Based on data published by N'Guessan et al. (2003) (17) and Ahoua Alou et al. (2010) (18).

Based on data published by Raghavendra et al. (2011) (16).
Based on data published by Kolaczinski & Curtis (2001) (19) and Brooke et al. (2000) (20).

Sources: based on references (10, 15), unless otherwise specified.

These are insecticides that need to be tested for resistance whenever possible but does not necessarily mean that they are recommended for use in malaria vector control.

- six marked with a green dot for use as holding tubes for pre-test sorting and post-exposure observation.
- Six slide units, each fitted with a screw-cap on both sides and a 15 mm filling hole.
- 40 sheets of clean paper (12 x 15 cm) for lining the holding tubes.
- 12 spring wire clips, 6 steel and 6 copper, to hold the paper in position against the walls of the tubes; the 6 steel clips are to be used with the green-dotted holding tubes and 6 copper clips are to be used with the 4 red-dotted exposure and the two-yellow-dotted control tubes.
- Two glass or plastic aspirator tubes of 12 mm internal diameter, together with 60 cm of tubing and mouthpieces.
- One roll of self-adhesive plastic tape.
- Instruction sheet and 20 copies of report forms.

#### 3.2.3 Insecticide-impregnated papers

The range of insecticides for which impregnated test papers are available remains unchanged at the present time, pending confirmation of discriminating concentrations for other pesticides; see section 3.1. The 11 different insecticide-impregnated papers which are currently available to order from WHO are listed in Table 3.2, together with details of the corresponding control papers to be used with each insecticide. Insecticide-impregnated papers are supplied in plastic boxes; each box contains 8 papers.

Papers impregnated with insecticides at other concentrations, i.e. at serial concentrations, are also available upon request from the Universiti Sains Malaysia. These are designed for use in situations where it is necessary to establish the baseline susceptibility of a mosquito species or population to a given insecticide.

#### 3.3 Sampling protocols

#### 3.3.1 Selection of test specimens

The age, physiological status and gender of mosquitoes are all factors that can influence the results of the susceptibility tests. 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. For this reason, susceptibility testing is conducted using only female mosquitos.

Studies using adult female mosquitoes have repeatedly shown that both age and physiological status (i.e. unfed or blood-fed, semi-gravid or gravid) have a marked effect on susceptibility to insecticides. For instance, it has been observed that older mosquitoes are sometimes less resistant to insecticides, especially when resistance is conferred by the presence of a detoxifying enzyme, the activity of

Table 3.2

Availability of insecticide-impregnated test papers for routine insecticide resistance monitoring, as at 31 December 2012

Insecticide class/insecticide	Discriminating concentration	Control paper
Organochlorines		
Dieldrin	4% and 0.4%	Risella oil
DDT	4%	Risella oil
Organophosphates		
Malathion	5%	Olive oil
Fenitrothion	1%	Olive oil
Carbamates		
Propoxur	0.1%	Olive oil
Bendiocarb	0.1%	Olive oil
Pyrethroids		
Permethrin	0.75%	Silicone oil
Deltamethrin	0.05%	Silicone oil
Lambdacyhalothrin	0.05%	Silicone oil
Cyfluthrin	0.15%	Silicone oil
Etofenprox	0.5%	Silicone oil

which tends to decline with age (4, 20). Consequently, it is recommended that susceptibility tests be performed on non-blood fed females, aged no more than 3–5 days post emergence.

In the interests of obtaining age-standardized results, it is recommended that susceptibility tests be performed using either adult females derived from larval collections (the preferred option) or, if larval collections are not possible, the F1 progeny of wild-caught female mosquitoes. If using larval collections, samples from the same place and the same type of breeding site may be pooled before testing in order to provide a sufficient number of test subjects. However, larval collections should ideally be made from a number of different breeding sites in order to avoid sampling individuals from single egg batches, which might otherwise result in a high proportion of siblings in the test population. Since the genotypic variability of the progeny of one adult female is likewise limited, wild-caught females should also ideally be collected from a number of different locations so as to ensure a broadly representative sample of the local population. In practice this means that at least 30 batches of eggs, more if there is a mixture of species, should be harvested from the wild-caught females and incubated.

When relying on larval collections to provide young adult females for resistance monitoring, it is important to record the type of breeding site (e.g. rice field, rain water collection, irrigation channel, well) from which the larval collection was made. There are several reasons for this. Firstly, exposure to pesticide residues will vary with type of breeding site. Secondly, some taxa within the same species complex will preferentially discriminate between breeding sites. For example, the M molecular form of *Anopheles gambiae* is more likely to breed in rice fields whereas the S molecular form tends to predominate in rain water collections.

A third but least favoured option is to use wild-caught females directly. In this case, it is necessary to record the physiological status of the adults prior to testing (i.e. whether unfed or blood fed, semi-gravid or gravid). If necessary, females may be sustained with sugar-water until such time as the tests are carried out.

The main advantage of using wild-caught females directly is convenience. The main disadvantage is that their age is unknown, which may lead to greater variation in susceptibility test results (and most likely an underestimation of resistance) depending on the species distribution and the insecticide being tested (21). The relative merits of using F1 progeny of wild-caught females and wild-caught females directly are compared in more detail in Table 3.3.

#### 3.3.2 Frequency of susceptibility testing

Previous editions of these guidelines did not make specific recommendations regarding the timing and frequency of susceptibility testing, but noted that comparisons of test data from a single site over time are useful from the point of view of assessing temporal trends in resistance. Comparisons of test data from multiple sites provide helpful information about the geographical distribution of resistance.

The resistance frequencies, the prevalence of resistance mechanisms and the population distribution of different taxa in a single locality have been observed to vary markedly with time. As such, the current recommendation is that vector susceptibility to all four classes of insecticides approved by WHO be tested at several different times throughout the year, in accordance with the changing seasons and/or the calendar of agricultural crops. Considering the timing and frequency of susceptibility testing, the following are proposed as possible strategies:

- Insecticide resistance monitoring could be conducted across a network of sentinel sites, with these sites selected so as to represent the range of ecological zones and malaria transmission intensities that occur within a given country in order to determine the distribution of resistance.
- Testing could be repeated at the same sites in order to monitor changes in mosquito susceptibility over time, depending on the size of the vector population.
- Areas where the same insecticide is used for both vector control and for agricultural purposes may require a more intensive monitoring schedule because of the potential for additional selection pressure on vector populations from agricultural use.

Table 3.3
Advantages and disadvantages of using F1 progeny and wild-caught females for bioassays

Vector sample	Advantage	Disadvantage
F1 progeny	Age of vectors can be kept constant between tests, allowing results from different times and places to be compared.  In areas with low mosquito density, can be used even if it is not possible to catch sufficient numbers of adult wild female mosquitoes.	Requires better entomological facilities, which limits where the tests can be carried out.  Environmental conditions will differ from those within the insectary.  Since many eggs may be derived from just a few adult females, the number of genomes sampled from the wild population is likely to be less than the number of insects tested.
Wild-caught females	Fewer facilities are required, so can be carried out in a greater number of locations.  Changes in susceptibility will more closely reflect the changes in intervention efficacy seen in the field.  The age distribution of the vectors should be representative of the wild vector population at a given time and location.	Age distribution and physiological condition of vectors will vary between samples reducing the comparability or results.

#### 3.3.3 Sample size

Around 150 adult female mosquitoes are required to conduct a single set of WHO bioassay tests, 100 of which will be exposed to the insecticide that is being tested (in 4 replicates each of around 25 mosquitoes). The remaining 50 will serve as "controls" (i.e. 2 replicates each of around 25 mosquitoes). If testing more than one insecticide, additional batches of approximately 150 mosquitoes (per insecticide) will be required.

The control mosquitoes are exposed to papers impregnated with the appropriate carrier oil only, i.e. without insecticide (see Table 3.2). In all other respects, the control mosquitoes are treated in the same way as the exposed mosquitoes; they are tested in parallel and under the same conditions. The purpose of the inclusion of the controls is to provide an estimate of natural mortality during the test (see section 4.1) and to account for all variables that may induce mortality other than the insecticide being tested. In this revision, testing using a minimum of two controls (50 mosquitoes) is strongly recommended in order to improve the statistical significance of the results.

If it is not possible to collect enough mosquitoes on a single occasion (if working with wild-caught females for instance) then it is possible to store live mosquitoes until sufficient numbers have been collected. When relying on pooled samples, mosquitoes should be provided with access to a sugar meal until the bioassay can be carried out.

In the event that insecticide resistance is suspected (i.e. there are survivors at the diagnostic concentration: see section 4.1), it will be necessary to conduct further tests in order to identify the underlying mechanism(s) responsible for the resistance. This can be achieved using a combination of synergist, molecular and/or biochemical methods; these types of test are briefly described in section 5. As fresh subjects are required for the biochemical tests, it may be necessary to collect additional specimens or, if using larval collections, reserve a subsample of the emergent adults (see section 5.1).

#### 3.3.4 Species identification

In many malaria-endemic regions, several species of mosquito belonging to the same group or complex tend to occur in sympatry. For instance, the *Anopheles gambiae* species complex is comprised of seven cryptic species: *An. gambiae s.s., An. arabiensis, An. bwambe, An. melas, An. merus* and *An. quadriannulatus* A and B, some of which are sympatric. Other species complexes include *An. culicifacies* and *An. funestus.* Different members of the same species complex do not necessarily share the same resistance mechanisms, and nor do they necessarily exhibit the same insecticide resistance patterns. In situations where different mosquito species coexist, it is therefore recommended that samples collected from the field be identified to the species level wherever possible.

It may be possible to identify certain members of species complexes prior to conducting the bioassay on the basis of morphological characteristics. However, the development of molecular techniques has made it possible to distinguish individual members of mosquito species complexes relatively quickly and easily using simple PCR-based assays. With this technique, species identification can be carried out post-bioassay on dead specimens. Prior to identification by PCR, specimens should be stored on silica gel or in ethanol. If required, and in order to avoid DNA cross-contamination, individual mosquitoes can be placed in 0.5 ml Eppendorf tubes.

Suitable methods for identifying malaria vectors to species level are given in Methods in Anopheles Research. This document is available for download from the following web site:

http://www.mr4.org/Publications/MethodsinAnophelesResearch/tabid/336/ Default.aspx

It is strongly recommended that, as a minimum, all survivors and at least 20% of those killed in a bioassay test for any given insecticide should be identified to species level.

#### 3.4 Test conditions and protocols

The steps involved in conducting the WHO bioassay test have already been described (see Box 3.1). As previously mentioned, the basic procedure has remained more or less unchanged since the method was recommended for use as a standard test for susceptibility in 1976 (14). However, some minor modifications to the test protocols have been made over the years; for instance, the 1998 revision – when the pyrethroids were included for the first time – stipulated the need to maintain the holding tubes in a vertical position during the exposure and holding times (10).

In the present revision, attention is drawn to the following aspects of the test procedure where small changes to the recommended test conditions and protocols have been proposed. Note that a standard data form for recording information about the susceptibility test, including details of the study area, the test specimens (the collection method, age, physiological status, species), the insecticide(s) under test and the test conditions, is attached as Annex 1.

#### 3.4.1 Number of test subjects

As mentioned above, at least 100 mosquitoes should be tested for any insecticide at the diagnostic concentration, with at least 4 replicates of 20–25 mosquitoes per test. When it is not possible to test this number of mosquitoes on a single day, tests can be conducted over a few days until this number is reached. In this event, and to avoid multiple manipulations, impregnated papers can remain in the tubes, provided that they are wrapped in aluminium foil and kept at 4 °C between successive tests. Note that a minimum of two controls (50 mosquitoes) is specified in this revision in order to improve the statistical validity of the results.

#### 3.4.2 Ambient conditions

Research has established that ambient temperature can influence the toxicity of insecticides; similarly the relative humidity has been shown to affect the survival of mosquitoes during the holding period. It is therefore recommended, as previously, that temperature and humidity are controlled during the test and holding periods. If possible, tests should be carried out at 25 °C  $\pm$  2 °C and 80%  $\pm$  10% relative humidity. During the 1-hour exposure period and the subsequent 24-hour holding period, both the temperature and relative humidity should be monitored and the maximum and minimum values recorded at the start of the exposure period and again at the end of the 24-hour holding period. The holding period for chlorfenapyr may be potentially longer.

Throughout the test, the exposure and holding tubes should be held in a vertical position (even with those insecticides that have a knockdown effect). The temperature should never exceed 30 °C; in absence of an insectary or "field insectary" cool box, the tubes should be placed in a sheltered, shaded location and the mesh end covered with a piece of card. This will help limit mosquito contact with the mesh ends of the exposure and holding tubes.

#### 3.4.3 Multiple use of the impregnated papers

The efficacy of impregnated papers declines with the number of uses and the number of mosquitoes tested. This is especially true of the pyrethroid-impregnated papers. The current recommendation is that no insecticide-impregnated paper should be used more than 6 times, the equivalent of exposing around 150 mosquitoes. Previous versions of these guidelines allowed greater re-use of the non-pyrethroid impregnated papers (up to 20 times). Advice regarding the storage of papers between tests and time limits on use of papers once opened remains.

## Recording and reporting susceptibility test results

#### 4.1 Calculation of mortality and knock-down rates

Note that a standard form which can be used for recording and reporting the results of bioassays, both mortality and knock-down rates, is attached as Annex 2.

#### 4.1.1 Mortality

The assessment of mortality, i.e. a count of the number of dead mosquitoes in both the exposure and the control tubes, is made 24 hours post-exposure. A mosquito is classified as dead if it is immobile or unable to stand or fly in a coordinated way.

With some insecticides, most noticeably the pyrethroids, mosquitoes tend to lose their legs some hours after insecticide exposure. If at the end of the 24-hour post-exposure period mosquitoes are still able to fly, irrespective of the number of legs remaining, they should be counted as alive. If however the mosquito is "knocked-down" (i.e. is moribund), whether or not it is missing legs or wings, it is counted as dead. This may be justified on the grounds that in the wild a mosquito in this condition would likely be caught and eaten by predators and ants.

The mortality of test sample is calculated by summing the number of dead mosquitoes across all four exposure replicates and expressing this as a percentage of the total number of exposed mosquitoes:

A similar calculation should be made in order to obtain a value for the control mortality. If the control mortality is above 20%, the tests must be discarded. When control mortality is greater than 5% but less than 20%, then the observed mortality has to be corrected using Abbots formula, as follows:

If the control mortality is below 5%, it can be ignored and no correction is necessary.

When reporting mortality counts, the sample size should always be given, and preferably an estimate of the 95% confidence intervals.

#### 4.1.2 Knock-down rate

Pyrethroids and DDT are fast-acting insecticides which have a knock-down effect (see Box 1.1). When knock-down resistance (*kdr*) is involved, the rate of knock down (KD) has been shown to be a sensitive indicator for early detection of resistance.

Observations of the number of knocked-down mosquitoes are made during the hour-long exposure period. A mosquito is considered knocked down if it is unable to stand or fly in a coordinated way; it will usually fall to the bottom of the exposure tube. It is recommended that observations are made at regular intervals, usually after 10, 15, 20, 30, 40, 50 and 60 minutes into the exposure period, with the last observation just before transfer to the observation tube. If, after 60 minutes, the observed KD rate is less than 80%, another count at 80 minutes should be made of the mosquitoes in the observation tube. The holding container may be tapped a few times before this final determination is made. In very susceptible populations, the recording of knock down should be done more frequently, every 3 minutes.

From the observed KD counts, it is possible to calculate knock-down rates for 50%, as well as 95%, of mosquitoes (KD50 and KD95, respectively), either graphically using log-probit paper or by computer using a log time-probit statistical model. Although the calculation of KD50 and KD95 values is a relatively simple procedure, these measures are not widely used for routine monitoring of susceptibility for operational purposes.

#### 4.2 Interpretation of susceptibility test results

In light of new knowledge and the need for prompt action to counter the spread of resistance among vector populations, guidance on interpreting the results of the WHO bioassay has been revised. The current recommendations are as follows:

- A mortality in the range 98–100% indicates susceptibility.
- A mortality of less than 98% is suggestive of the existence of resistance and further investigation is needed.
- If the observed mortality (corrected if necessary) is between 90% and 97%, the presence of resistant genes in the vector population must be confirmed. The confirmation of resistance may be obtained by performing additional bioassay tests with the same insecticide on the same population or on the progeny of any surviving mosquitoes (reared under insectary conditions) and/or by conducting molecular assays for known resistance mechanisms. If at least two additional tests consistently show mortality below 98%, then resistance is confirmed.
- If mortality is less than 90%, confirmation of the existence of resistant genes in the test population with additional bioassays may not be necessary, as long as a minimum of 100 mosquitoes of EACH species was tested. However, further investigation of the mechanisms and distribution of resistance should be undertaken.
- When resistance is confirmed, pre-emptive action MUST be taken to manage insecticide resistance and to ensure that the effectiveness of insecticides used for malaria vector control is preserved.

The above criteria are recommended on the grounds that a greater than 2% survival at the diagnostic concentration is considered unlikely to be due to chance alone, provided that all the test conditions summarized below are met.

It can be considered that recording a mortality of less than 98% in tests that have been conducted under optimum conditions of temperature and humidity with a sample size of at least 100 mosquitoes, replicated two or three times using fresh impregnated papers (i.e. before the expiry date on the box) that have not been used more than six times and whose efficacy is confirmed with susceptible mosquitoes, is a strong suspicion of resistance and must be investigated further.

Caution must be exercised when interpreting the results of individual bioassays, especially when using wild-caught females. Sampling techniques may influence the results; for example, indoor catches may be biased towards insects that may have already been exposed to insecticides and have survived (i.e. include specimens that are more likely to be resistant). Mixed species samples may also produce inconclusive or misleading results as resistance gene frequency is highly likely to vary between species and even molecular forms of the same species. It is for this reason that it is important to identify test insects and to test each species separately for evidence of resistance.

#### 4.3 Reporting results of susceptibility testing

In line with WHO recommendations set out in the GPIRM, national malaria control programmes are encouraged to coordinate the timely collection, analysis, reporting and sharing of insecticide resistance data. If one does not already exist, a national database with the relevant capacity should be established for this purpose (6). Ideally, the results of susceptibility testing should be submitted to the central coordinating body within three months of their collection.

Results of bioassay testing should also be submitted to WHO for inclusion in an aggregated global database on insecticide resistance which is currently under development by WHO. This database will build on, and be linked with, regional databases and networks (see Box 4.1).

#### **BOX 4.1**

#### Managing insecticide resistance: regional data-sharing initiatives

- Resource support networks for monitoring insecticide resistance in malaria vectors in Africa, initiated by the Multilateral Initiative for Malaria (MIM) network on insecticide resistance in Southern Africa, now cover the whole continent in the form of the African Network on Vector Resistance (ANVR). Similar networks are being developed for other regions. These will then be linked to a global database as outlined in the GPIRM (6).
- A similar network for Mekong region (South East Asia and the West Pacific Regions of WHO) was established to coordinate and strengthen capacity for monitoring insecticide resistance for both malaria and dengue vectors.
- Other regions of WHO (e.g. the Eastern Mediterranean) have focused on strengthening national capacities for monitoring insecticide resistance and results and experiences are regularly shared during regional annual meetings of control programme managers.

## 5. Further investigations: identification of resistance mechanisms

As noted above (section 4), if on the diagnostic concentrations a significant number of survivors are found (more than 2%), it will be necessary to conduct further tests in order to determine the underlying genetic mechanisms responsible for the observed resistance. These investigations should include identification of the survivors, and at least 20% of dead insects, in order to identify in which species of mosquito the signs of resistance are present. This information will not only assist in assessing the likelihood of cross-resistance between insecticide classes, but will also provide valuable information about the potential for spread of resistance in vector populations. For instance, if evidence of the presence of *kdr* mutations (which confers resistance against the pyrethroids) were found in a given vector population, it is likely that the same population would also be resistant to DDT.

Although the observed pattern of cross-resistance between different insecticides may be suggestive of a common mechanism (for example, evidence of resistance to both DDT and the pyrethroids suggests the presence of target site resistance mechanisms), the nature of the resistance mechanism should always be confirmed by appropriate tests. In any given mosquito population, both metabolic and target site mechanisms can be present and thus both possibilities should be investigated.

As understanding of the mechanisms of insecticide resistance in mosquitoes and other insect vectors has evolved, significant progress has been made in developing new diagnostic tests for the detection of resistance mechanisms. A range of biochemical enzyme assay techniques, which detect the presence of metabolic resistance mechanisms, are now available, as are a number of molecular assays which can be used to test for target site mutations (*kdr* for pyrethroids and DDT and *Ace-1* for the organophosphates and carbamates). Despite these advances, this type of work remains relatively resource intensive in that it requires specialized equipment and expertise. In settings where resources and facilities are limited, it is recommended that help with analysing representative samples be sought from external institutions.

Detailed descriptions of the recommended techniques and methods for biochemical and molecular analysis are beyond the scope of these guidelines; for this type of information, users are advised to consult *Methods in Anopheles Research*, which is available via the following link: <a href="http://www.mr4.">http://www.mr4.</a>
<a href="http://www.mr4.">org/Publications/MethodsinAnophelesResearch/tabid/336/Default.aspx</a>

#### 5.1 Biochemical enzyme assays

Mosquitoes which are to be subject to biochemical enzyme assays for metabolic resistance should preferably be fresh. Alternatively, specimens can be stored at -70 °C or in liquid nitrogen for later use.

Specimens for biochemical analysis should not have been exposed to an insecticide, which is another reason why it is often preferable to use the F1 progeny of wild-caught females rather than wild-caught females directly for susceptibility testing. Moreover, wild-caught adult females should not be biochemically assayed because ingested blood meals contain proteins that are reactive to the substrates used in the assays, causing significant shifts in the amount of enzyme detected per

mosquito. This is especially true of the monooxygenase assay. When relying on larval collections, a subsample (n = 100) of the adults emerging from the larvae can be reserved and stored for biochemical analysis as suggested. If the collections of wild females are very small, then it will be necessary to rear individual egg batches. However, each egg batch will also need to be tested on the appropriate insecticide so that the resistance profile of the progeny is known. This is a large amount of work and is best carried out by laboratories that have the specialized equipment and skills. These tests should be carried out for each new focus of resistance.

#### 5.2 Molecular (biological) tests

Molecular (biological) tests for resistance (target site resistance mechanisms) can be performed post-bioassay. However, in order to carry out the molecular techniques successfully, it is important to store the mosquitoes appropriately. Once tested on the insecticides, mosquitoes can be stored in plastic tubes containing silica gel (with blue indicator to show when the gel is dry/wet), ethanol, or solutions designed specifically for this purpose (e.g. RNA-LATER®) and maintained at  $-20\,^{\circ}\text{C}$ . Mosquitoes should be placed individually into 0.5 ml Eppendorf tubes. The tubes must be labelled according to insecticide tested and whether the individual was dead or alive after 24 hours.

As a rough rule of thumb, all surviving mosquitoes and a representative sample of the dead mosquitoes at the diagnostic concentration should be investigated.

Although mosquitoes should be stored in separate tubes to avoid DNA cross-contamination, in places where only one species is present, and mosquitoes are only tested for resistance mechanisms, several mosquitoes can be put in the same tube. It is important to process both alive and dead mosquitoes in order to have a representative sample of the population. Some resistance mechanisms, such as the *kdr* mutations, are recessive and therefore it is recommended that, if checking a population for target site mutations, a large number of mosquitoes are tested even if the population appeared susceptible on the diagnostic tests.

#### 5.3 Synergist assays

Insight into possible resistance mechanisms can also be provided by synergist assays. These are bioassay-type experiments that are designed to assess the extent to which detoxifying enzymes contribute toward the production of resistant phenotypes. They are based on the use of synergists which are non-insecticidal compounds that can attenuate the expression of insecticide resistance by providing alternative substrates for particular detoxifying enzyme classes.

At present, these assays are used as research tools and interpretation of the results requires considerable expertise. Guidelines for use of synergists will be covered in a forthcoming WHO publication.

## 6. Complementary susceptibility tests: the CDC bottle bioassay

The CDC bottle bioassay provides a complementary method for detecting insecticide resistance in malaria vector populations and is widely used for routine, day-to-day monitoring of mosquito populations. In contrast to the WHO bioassay, which measures mortality rates in mosquitoes exposed to a high concentration of insecticide for a fixed period of time, the CDC bottle assay takes as its measure the length of time it takes to kill a sample of adult mosquitoes exposed to a known concentration of insecticide.

The CDC bottle test can be performed on adult females collected from the field or on those reared in an insectary from larval collections. Like the WHO bioassay, the test can be standardized by determining diagnostic doses and exposure times for individual insecticides and each main vector species using populations known to be susceptible. Once these have been determined, subsequent testing can be done at the diagnostic dose and time only. The test involves recording the number of mosquitoes surviving after the diagnostic exposure time (i.e. the time that reliably killed 100% of the original test population).

A detailed description of the CDC bottle bioassay, including the methodology was published by the United States Centers for Disease Control and Prevention, based in Atlanta, GA, in October 2010 as *Guideline for evaluating insecticide resistance in arthropod vectors using the CDC bottle bioassay* (available in English and Spanish). An on-line version of the guidelines, complete with animation is available at <a href="http://www.cdc.gov/ncidod/wbt/resistance/assay/bottle/index.htm">http://www.cdc.gov/ncidod/wbt/resistance/assay/bottle/index.htm</a>

A pdf version with photographs is available at <a href="http://www.mr4.org/AnophelesProgram/TrainingMethods.aspx">http://www.mr4.org/AnophelesProgram/TrainingMethods.aspx</a>

If the CDC bottle bioassay is to be used for routine insecticide susceptibility surveillance, then the following guidance should be noted:

- procedures detailed in the CDC guidelines should be strictly adhered to, in particular those relating to the use of the recommended insecticide solvents (ethanol/acetone) and the bottle treatment protocols;
- test insecticides should be procured from the CDC;
- insecticide exposure times and concentrations as listed in Table 6.1 should be adhered to.

The CDC bottle bioassay offers a number of advantages over the WHO susceptibility tube tests. These may be summarized as follows:

- the use of pre-prepared test kits and insecticide-impregnated papers (which have to be sourced from the WHO Collaborating Centre in Malaysia) is avoided, allowing greater flexibility in the type and concentration of insecticide that can be evaluated;
- the procedure is relatively simple and quick to carry out (for instance, a 24-hour holding period is not required);

Table 6.1
Insecticide concentrations and diagnostic time (in minutes) for mosquitoes

Insecticide	Insecticide concent (microgran	Diagnostic time (minutes)		
	Anopheles	Aedes		
Bendiocarb	12.5	12.5	30	
Cyfluthrin	12.5	10	30	
Cypermethrin	12.5	10	30	
Deltamethrin	12.5	10	30	
Lambdacyhalothrin	12.5	10	30	
Permethrin	21.5	15	30	
DDT	100	75	45	
Malathion	50	50	30	
Fenitrothion	50	50	30	
Primiphos-methyl	20	_	30	

Source: reference (22).

- the procedure may also be performed with various synergists, providing a rapid and inexpensive alternative to more complex biochemical and molecular methods for testing for the presence of metabolic resistance mechanisms:
- owing to the use of lower discriminating dosages, resistance may be detected earlier.

#### Disadvantages include:

- difficulties associated with maintaining a high level of quality assurance and control (the preparation of the glass bottles is especially susceptible to control assurance problems as different laboratories are likely to differ in the way they go about preparing the equipment before and after testing);
- the need to transport glass bottles in the field, particularly over extended periods when access to laboratories is not possible;
- the requirement to separate dead and live mosquitoes after the required exposure period for storage for subsequent species identification and mechanistic investigations.

Note that although both methods report percentage mortalities, the results obtained from the CDC bottle bioassay are **not** directly comparable with those obtained from the WHO susceptibility tube test. However, both methods have been shown to reliably identify insecticide resistance where it occurs (23).

### 7. Additional recommendations

There are a number of general recommendations regarding insecticide resistance monitoring that merit a mention; these are as follows:

- Efficient resistance monitoring programmes are dependent on adequately trained personnel. This is still a major challenge in most countries and is being addressed by a number of institutions and implementing partners. Training needs at country level should be reassessed and addressed.
- Simple, rapid through-put PCR-based molecular tests have been developed in recent years and are currently being used to detect the presence of *kdr* mutations. In some cases, monitoring the frequency of the *kdr* mutations in *An. gambiae* in western parts of Africa using such methods has been used as a proxy for estimating the presence of DDT or pyrethroid resistance. 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. Of course, knowledge of the mechanisms involved in resistance is always advantageous since cross-resistance between classes of insecticides can be inferred and bioassays then used to test for resistant phenotypes. This type of information is very useful for planning resistance management strategies.
- Where laboratory facilities are available, resistance data can be linked to parasite infection data by processing the same wild mosquito adults used in bioassays for sporozoite detection. Positive samples detected with the ELISA test should be confirmed by a second ELISA that has been heated (24) or by PCR.
- For novel insecticides that are not acting primarily through lethal effects but that disturb the insect physiology, such as blood feeding behaviour or fecundity/fertility, it is recommended that other guidelines be developed for testing these products.

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# Annex 1. Form for recording susceptibility test data in the field

Village Code 🔲 🔲 🔲	Test Number 🔲 🔲 📗		Date	(dd-mm-yy) 🔲 🔲 / 📗 🧎 / [	
Investigator name:			Code	investigator 🔲 🔲	
Area information					
Country:			Provir	nce:	
District:	Commune :		Villag	e:	
GPS position <b>UTM_X</b> 🔲 🔲 [			UTM	_Y	
On what is forward an					
Sample information		0			
Species tested:					
Sex:		Age (days)	:	(only if known: colony	/ & F1)
Collection method					
Human Landing Indoor	Resting night Indoor	[		Resting morning Indoor	
Cattle Collect	Human Landing Outdoor	[		Resting night Outdoor	
Other: specify	. Larval collection	[		Progeny F1	
Colony	Name of colony strain:				
Physiological stage					
Non-blood fed B	lood fed Semi-	gravid		Gravid	
Test insecticide information					
Insecticide tested:		. Date o	of exp	iry: 🔲 🖂 / 🖂 🖂 .	
Impregnated papers prepared by				st open:	П
Concentration:				times this paper is used:	
Storage conditions: Room temp					
Test conditions					
Ex	posure period: Start	E	nd te	st	
Temperature °C					
Relative humidity (%)				]	

# Annex 2. Form for recording results of susceptibility testing in the field

	Replic	cate 1	Kepli	cate 2	Replic	cate 3	Keplic	cate 4	Cont	trol 1	Cont	rol 2	
No. exposed													
Number of	knocked	d down (	(KD) mos	squitoes	after exp	osure fo	or minute	es					
	Replic	ate 1	Repli	cate 2	Replic	ate 3	ate 3 Replicate 4 Control 1			trol 1	Control 2		
	Time	No.	Time	No.	Time	No.	Time	No.	Time	No.	Time	No.	
START													
10'													
15′													
20′													
30′													
40′													
50'													
60'													
Number of	1		1										
<u> </u>	Repli	cate 1	Repli	icate 2	Repli	cate 3	Repli	cate 4	Cont	trol 1	Cont	trol 2	
No. dead													
No. alive													
To be com				t the end	l of the to	est							
Comments													
I confirm th Date: [] [ Name	/[	/											
Signature													
To be com	pleted by	y data e	ntry cle	rks durir	ıg data e	ntry							
Data ent	ry clerk '	1	-			Data	entry cl	erk 2					
Date [	_/[	/				Date//							
0:						Ciana							



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